Exploiting codon usage identifies intensity-specific modifiers of Ras/MAPK signaling in vivo

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Abstract

Signal transduction pathways are intricately fine-tuned to accomplish diverse biological processes. An example is the conserved Ras/mitogen-activated-protein-kinase (MAPK) pathway, which exhibits context-dependent signaling output dynamics and regulation. Here, by altering codon usage as a novel platform to control signaling output, we screened the Drosophila genome for modifiers specific to either weak or strong Ras-driven eye phenotypes. Our screen enriched for regions of the genome not previously connected with Ras phenotypic modification. We mapped the underlying gene from one modifier to the ribosomal gene RpS21. In multiple contexts, we show that RpS21 preferentially influences weak Ras/MAPK signaling outputs. These data show that codon usage manipulation can identify new, output-specific signaling regulators, and identify RpS21 as an in vivo Ras/MAPK phenotypic regulator.

Author summary

Cellular communication is critical in controlling the growth of organs and must be carefully regulated to prevent disease. The Ras signaling pathway is frequently used for cellular communication of tissue growth regulation but can operate at different signaling strengths. Here, we used a novel strategy to identify genes that specifically tune weak or strong Ras signaling states. We find that the gene RpS21 preferentially tunes weak Ras signaling states.

Introduction

Conserved signal transduction pathways are employed throughout nature during diverse processes such as cell fate decisions and tissue growth. These same pathways can be aberrantly...
regulated in disease. Large numbers of molecular regulators of these pathways have been identified using high-throughput genetic screening. Additionally, quantitative imaging approaches have revealed intricate signaling regulation. This regulation includes feedback control of the duration or strength of a downstream biochemical signaling output (e.g., weak or strong activation of a target gene). A current challenge is to place the numerous identified signaling pathway regulators in the context of complex signaling dynamics, and to relate such regulation to *in vivo* signal-dependent processes.

An example of the complexity of signaling regulation is the evolutionarily conserved Ras/mitogen activated protein kinase (MAPK) pathway [1]. In canonical MAPK signaling, receptor tyrosine kinase stimulation converts the Ras GTPase to an active GTP-bound conformation. Ras-GTP then activates the MAPK pathway, comprised of Raf kinases, which are activated by Ras and phosphorylate/active Mek kinases, which do the same to Erk kinases. Through highly successful modifier screen approaches in models such as the *Drosophila* eye [2–9] and *C. elegans* vulva [10–14], regulators of this core pathway have been identified. Additional *Drosophila* cell-based screens using a biochemical MAPK output (Erk phosphorylation) have identified many other Ras/MAPK regulators [15–17].

These numerous molecular regulators contribute to a diversity in Ras/MAPK signaling dynamics. Using an optogenetics-driven MAPK activation approach in cultured mammalian cells, it was revealed that distinct Ras/MAPK regulation (such as a paracrine STAT3 circuit) can distinguish between biochemical signaling outputs, namely sustained (strong) or transient (weak) Erk activation by Ras [18]. These biochemical outputs are regulated by negative feedback on Erk [19]. Importantly, *in vivo* context plays a role in whether a given strength of signaling output leads to a phenotypic output. Specifically, taking a similar optogenetic approach in the early developing fly embryo, it was shown that manipulating Erk activation strength has minimal effects on cells at the poles of the embryo, but has a profound impact on development of cells in the middle of the embryo [20]. Further, expressing an activating mutant of Mek in either *Drosophila* or zebrafish was recently shown to either activate or repress Erk phosphorylation depending on the cell type and gene expression environment [21]. The degree of Ras/MAPK signaling also plays a critical role in disease states. For example, altering the amount of Ras protein influences whether tumors develop in a carcinogenesis mouse model of lung cancer, or whether cancer cells mount a successful resistance response to chemotherapeutics [22,23]. These observations suggest that distinct Ras/MAPK regulation operates in distinct cellular contexts, and that this has biological consequences. Taken together, these studies highlight the need to better understand how distinct Ras/MAPK signaling states (e.g., strong or weak) are controlled by distinct sets of Ras/MAPK molecular regulators, in the context of an *in vivo* phenotype.

Here, we introduce a novel approach to genetically screen for signal output-specific regulators of Ras/MAPK signaling. This approach, which should be applicable to any signaling pathway, involves controlling the amount of active Ras protein produced by changing codon usage in the single *Drosophila* Ras gene [24] (FlyBase: *Ras85D*, hereafter *Ras*). Rare codons are well-associated with poor mRNA translation [25]. Manipulating codon usage has been successfully employed in bacteria, for example as a means to tightly control the fatty acid synthesis pathway [26], and we previously demonstrated that changing rare codons in the mammalian Ras isoform *KRAS* to their common counterparts leads to elevated translation, protein, signaling, and transformation [27]. In this study, we report the generation and characterization of transgenic flies and cell lines whereby the amount of active Ras protein produced, the resultant level of Erk activation, and resultant rough-eye phenotype is dictated solely by the codon usage engineered into a given *Ras* transgene. We then report the use of such transgenic flies to screen a whole genome deficiency (termed *Df* for convenience) kit for genetic modifiers of eye...
phenotypes that are specific to only strong or only weak Ras/MAPK signaling. Our screen specifically looked for modifiers unique to specific Ras signaling states, by leveraging the differential signaling phenotypic output driven by rare versus common codons in the Ras gene. Importantly, the Ras gene enriched in rare codons used in our screen models more closely the rare codon-enriched sequence of human KRAS [27], which is the most frequently mutated RAS family member in human cancers [28].

Our screen enriched for genomic regions not previously ascribed to Ras phenotypic modification. Of the 15 Dfs identified, we successfully mapped the modification of Df(2L)BSC692, an enhancer of the rough-eye phenotype driven only by weak Ras/MAPK signaling, to the ribosomal protein S21 gene (RpS21). We show that RpS21 negatively regulates Ras protein levels in several contexts, the effect of which is preferentially manifested at low levels of MAPK signaling. This approach highlights the usefulness of codon manipulation as a viable approach to identify signal output-specific signaling regulation and introduces new genetic reagents to explore weak Ras signaling regulation in Drosophila. Our uniquely identified modifiers include those specific to Ras with rare codons, like that of human KRAS.

Results

Exploiting codon usage to control MAPK signaling output

To identify Ras/MAPK molecular regulators that differentially impact strong or weak signaling outputs, we required a platform to tightly control the strength of MAPK signaling. To activate the pathway, we expressed a highly conserved, mutant active (G12V) Drosophila Ras transgene (termed RasV12 here for convenience). To control MAPK signaling strength during fly development, we opted for the new approach of simply changing the codon usage of a RasV12 transgene. Codons that occur infrequently in a given genome (rare codons) are known to impede translation, including in Drosophila [29–35]. By engineering a gene enriched in rare codons for each given amino acid, it is possible to create an mRNA that is poorly translated without altering the amino acid sequence of the encoded protein [36,37]. This has the distinct advantage that control of protein expression is embedded in the DNA and requires no additional factors or experimental variables. We used established data on Drosophila codon usage (see Methods) and created four distinct versions of Drosophila Ras transgenes: 1) we altered none of the codons (RasV12Native), 2) we made all codons the most commonly occurring in the genome (RasV12Common), 3) we made all codons the most rare in the genome (RasV12Rare), and 4) we created a control wild-type version lacking the V12 mutation and also lacking codon alteration (RasWTNative). To monitor expression, all four transgenes were epitope-tagged at the N-terminus with a 3XFLAG-tag sequence and expressed under the control of a Gal4-inducible UAS promoter (Fig 1A, see Methods). We note that RasV12Native has primarily common codons and a similar Codon Adaptation Index (CAI [38]) to RasV12Common [24], while the CAI for RasV12Rare is much lower (S1A and S1B Fig). To control for position effects, all transgenes were integrated at the same site in the genome (see Methods). Our altering of the codon sequence yielded a Drosophila RasV12Rare transgene that has a closer nucleic acid identity to the human KRASB isoform than the endogenous Drosophila Ras85D sequence (S1C–S1E Fig).

To measure signaling output strength of our transgenes, we first chose to use an in vivo phenotypic readout rather than a biochemical readout, an approach validated by quantitative studies of MAPK activation in Drosophila embryos [20,21]. For genetic screening of Ras/MAPK phenotypic regulators, the Drosophila eye is a highly accessible model. Driving expression of RasV12 in the developing eye with an eye-specific promoter such as sevenless (sev) dysregulates the proper differentiation of the R7 photoreceptor cell, leading to an easily scored ‘rough-eye’
Fig 1. Exploiting codon usage to control MAPK signaling output. (a) Schematic representation of the FLAG epitope-tagged Ras\textsuperscript{V12} transgenes encoded by rare, common, or native codons. (b) Images representing the eye phenotypes assessed and the scoring system. Scale bars = 0.5mm. (c) The mean ± SEM eye severity score of the indicated Ras\textsuperscript{V12} transgenes from three replicate experiments at 25˚C. Tukey’s multiple comparisons test was used for statistical comparisons. (d) Serial dilution western blot comparing protein levels of FLAG-Ras\textsuperscript{V12} common versus rare.
phenotype [39,40]. This phenotype relies on Ras action through the conserved MAPK pathway [2,41].

We assayed the phenotypic output of each Ras transgene in vivo by driving their expression in the developing fly eye using sevenless (sev)-Gal4. As expected [39], expression of Ras\(^{WT}\) Native in this manner does not result in a rough-eye phenotype (S2A Fig). However, when we expressed the constitutive-active versions of Ras (Ras\(^{V12}\)), we found a range of rough-eye phenotypes (Fig 1B). We binned these phenotypes into one of three classes: severe, moderate, or mild. Each class was assigned an increasing numeric score, based on the incidence and severity of eye phenotypes such as necrotic spots and discoloration (Fig 1B, see Methods). We then calculated an average severity score for each Ras transgene. Ras\(^{V12}\)Native and Ras\(^{V12}\)Common animals exhibit a similar phenotypic score, reflecting their similar CAI. Further, this phenotypic score is, on average, approximately 2-fold more severe than that of Ras\(^{V12}\)Rare (Fig 1C). To determine whether Ras protein levels track with the difference in Ras-driven rough-eye phenotype, we isolated heads from flies encoding common and rare Ras\(^{V12}\) transgenes and performed serial dilution immunoblotting with an anti-FLAG antibody (Figs 1D and S2D). Separately, flies expressing all three active Ras transgenes were again immunoblotted with an anti-FLAG antibody, and protein levels were normalized to a loading control (S2B and S2C Fig). In both experiments, we found Ras\(^{V12}\)Common flies express roughly 1.5 to 1.9-fold more Ras protein than flies expressing Ras\(^{V12}\)Rare (Figs 1D, and S2B–S2D). Additionally, Ras\(^{V12}\) protein levels are similar between Ras\(^{V12}\)Native and Ras\(^{V12}\)Common flies (S2B and S2C Fig), which is consistent with the similar codon content between these transgenes (S1 Fig). These experiments established that codon usage can be manipulated to examine an in vivo, Ras signal-driven output (eye phenotype), and identified both weak (Ras\(^{V12}\)Rare) and strong (Ras\(^{V12}\)Common) versions of this output. Further, these results are consistent with Ras\(^{V12}\)Rare serving as a model of the rare codon bias of human KRAS, the most commonly mutated RAS family member in human cancers.

We next assessed the impact of codon content in the Ras gene on Ras signaling and Ras GTPase activity. To examine the effect of expression of Ras\(^{V12}\)Rare versus Ras\(^{V12}\)Common transgenes on MAPK signaling, we measured the level of phosphorylated Mek (p-Mek, FlyBase: Dsor) and Erk (p-Erk, FlyBase: rolled) compared to the total level of these proteins by immunoblot analysis. Ras\(^{V12}\)Common animals exhibit elevated levels of p-Erk and p-MEK compared to Ras\(^{V12}\)Rare fly heads (Figs 1E- see figure legend for quantitation and S2E). We independently verified this difference in cultured S2 and KC insect cells (see Methods), again finding that Ras\(^{V12}\)Common is expressed higher and more robustly activates the MAPK pathway compared to Ras\(^{V12}\)Rare (S1F Fig). Further, using a Ras binding domain (RDB) pull-down assay [42], we found that S2 cells expressing Ras\(^{V12}\)Common contain a higher total level
of active Ras than cells expressing RasV12 Rare (S1G Fig). In sum, our findings establish RasV12 Rare and RasV12 Common as two distinct transgenes that either weakly or strongly activate Ras/MAPK signaling output (as measured by RasV12 protein expression, Ras activity, and MAPK activation), and that transgene-driven signal strength tracks with an observable difference in phenotypic output.

Codon bias has been shown to impact not only translation fidelity and efficiency [43–47] but also pre-translational processes, including transcription [48,49] and mRNA stability [50–53]. To assess whether our codon-altered Ras transgenes impact Ras protein levels and Erk signaling through pre-translational processes, we performed quantitative RT-PCR (Methods). Paralleling our findings with Ras protein, RasV12 Common mRNA is significantly higher than RasV12 Rare in adult heads (Fig 1F). These findings are consistent with the model that altering codon usage of Drosophila Ras impacts Ras RNA, Ras protein, and Erk signaling.

We next examined the impact of RasV12 Rare and RasV12 Common at single cell resolution. Using the FLP-out system [54], we generated mosaic clones of cells throughout developing larvae that expressed these transgenes under a ubiquitous Tubulin-Gal4 driver. Clones generated in this system are marked with GFP. We used heat shock to control the frequency of FLP-out events, and used a level of heat shock that resulted in 1–2 discrete clones in leg imaginal discs (see Methods). In these animals, RasV12 Common, but not RasV12 Rare or RasWT Native, mosaic expression leads to animal lethality (Fig 1G). We note that RasV12 expression is connected to animal lethality in other contexts, including when induced transiently or in somatic clones [55–57]. In surviving animals, RasV12 Rare significantly increases clone size relative to controls (Fig 1H and 1I), which is consistent with the well-known role of Ras/Erk signaling in promoting cell proliferation. Interestingly, in surviving RasV12 Common animals, clones are no bigger than in RasNative controls (Fig 1H and 1I). Taken together with the organisinal death and frequent necrotic spots seen in the eyes of sev-Gal4, UAS-RasV12 Common animals, we interpret this result to likely reflect the increased apoptosis or cellular senescence that can result from increased Ras expression [58,59]. Given that RasV12 Rare and RasV12 Common have such differing effects on cell proliferation in leg discs, our results underscore the critical importance of signal output levels on Ras-driven phenotypes and highlight that lower Ras levels can actually drive more cell proliferation in specific contexts.

A genome-wide screen uncovers differential phenotypic regulation between strong and weak Ras/MAPK signaling states

We next sought to use our codon alteration system to gain insight into how the Ras/MAPK pathway can be differentially regulated in different signal-strength states. To do so, we screened for molecular regulators that modify Ras/MAPK phenotypes driven only by strong or only by weak signaling states. We first confirmed that RasV12 Common and RasV12 Rare rough-eye phenotypes were both in the range that can be modified. Specifically, two different heterozygous loss-of-function mutations known to suppress active Ras phenotypes, namely the S-627 allele of kinase suppressor of ras, (FlyBase: ksr) [9], and the S-2554 allele of beta subunit of type I geranylgeranyl transferase, (FlyBase: betaggt-I) [3]. As with previous work, we find these mutations suppress the rough-eye phenotype for RasV12 Common and RasV12 Rare (S3A Fig). Next, we examined heterozygous mutants of the yan-XE18 allele of anterior open, or aop, which is known to enhance the active Ras phenotype [60,61]. Although we did not observe clear eye enhancement for aopyan-XE18/+ , we did observe a marked decrease in another phenotypic readout- animal survival. As for our FLP-out experiments with Tubulin-Gal4, sev-Gal4 expression of RasV12 Common leads to considerably more organisinal death than with RasV12 Rare (S2B Fig). This sev-Gal4-driven lethality likely reflects the expression of
sevenless-Gal4 in other tissues [62]. Survival is lower for aop^yan-XE18/+ animals expressing both Ras^{V12}^Common and Ras^{V12}^Rare transgenes (S3B Fig). These results establish that codon-altered Ras^{V12} transgenes are subject to phenotypic modification, including by dose-sensitive heterozygous mutations.

Previous modifier screens, including in the eye, employed the native Ras cDNA to express activated Ras [2,4,8,40,63]. This sequence has a strong common-codon bias (S1B Fig) and is similar to Ras^{V12}^Common in terms of MAPK biochemical and phenotypic outputs (Fig 1). To find unidentified modifiers that may be specific to weaker (or stronger) Ras/MAPK-driven phenotypes, we conducted a genome-wide unbiased heterozygous mutant screen to specifically identify modifiers of the rough-eye phenotype driven by only Ras^{V12}^Rare, (or only Ras^{V12}^Common), (Fig 2A). We used the Bloomington Deficiency (Df) Kit, which covers 98.3% of the euchromatic genome [64]. In a primary screen (Fig 2B and S1 Table), we crossed 470 Dfs representing 99.1% of the Df collection to animals with Ras^{V12}^Rare or Ras^{V12}^Common expressed in the eye by sev-Gal4, and scored the resulting eye severity in an average of 30 (Ras^{V12}^Common) or 60 (Ras^{V12}^Rare) progeny animals per cross. We also factored animal lethality into our scoring (see Methods).

As expected, we found general Ras modifiers that either enhance or suppress eye phenotypes driven by both Ras^{V12} transgenes (Fig 2C and 2D and S1 Table). Interestingly, we identified more enhancers than suppressors (16% versus 7%, Fig 2C). The reason for this remains to be determined, but we note that our calculation of phenotypic modification (see Methods) included scoring animal lethality, which may identify strong enhancers of Ras^{V12}^Common not identified in previous screens based solely on a rough-eye phenotype. Of great interest, we also identified Dfs whereby Ras^{V12}^Common and Ras^{V12}^Rare are differentially modified (Fig 2A), meaning they scored as only modifying the eye phenotype driven by a single signaling state (Ras^{V12}^Common or Ras^{V12}^Rare, not both). Using a low-stringency cutoff score (see Methods), we identified 178 putative differential modifier Dfs in our primary screen (Fig 2B and S1 Table). To filter our hits to those that were the most robust, these Dfs were then re-tested in a secondary screen (Fig 2B) by crossing them a second time to sev-Ras^{V12}^Common and sev-Ras^{V12}^Rare. In this screen, we used a more stringent cutoff score to ensure repeatability to define a robust differential modifier (see Methods). This scoring and replicate analysis reduced the number of candidates to 15 Dfs, or 3% of the tested Dfs (Fig 2E and 2D and S1 Table), that reproducibly differentially modify either only Ras^{V12}^Common or only Ras^{V12}^Rare (Fig 3A). Among these differential modifiers, we again recovered more enhancers than suppressors, although importantly we recovered both enhancers of Ras^{V12}^Common and suppressors of Ras^{V12}^Rare, arguing that our screen had the dynamic range to modify both strong (Ras^{V12}^Common) and weak (Ras^{V12}^Rare) Ras/MAPK signaling outputs (Fig 3B).

We next queried both the general (signal output-independent) and differential (signal output-dependent) modifiers against a FlyBase database of all reported Ras genetic enhancers and suppressors (see Methods). 56% of our general modifier Dfs covered regions of the genome containing reported Ras enhancers or suppressors. These data support the idea that our approach can identify Ras eye modifiers. Additionally, we note that among our identified differential modifier Dfs, most (73%) do not encompass known Ras modifiers, supporting the idea that our signal strength-specific modifier hits are enriched in new Ras enhancers and suppressors (Fig 3C). To explore possible relationships amongst these 15 differential modifier Dfs, we queried the genes within differential versus enhancer and suppressor Dfs against the established list of FlyBase Gene Groups (FBGG). Interestingly, the gene groups enriched in the differential Dfs do not overlap with those in the general enhancer/suppressor Dfs (Fig 3D), suggesting that the differential modifiers may represent a distinct class of Ras modifiers. Unlike the general modifier Dfs, differential modifier regions are enriched for basic Helix
Fig 2. A genome-wide screen uncovers differential phenotypic regulation between strong and weak Ras/MAPK signaling states. (a) Schematic of the Ras modifiers types scored in the Df screen. (b) Schematic of screening approach. (c) Pie chart showing the number of Df with the indicated types of Ras modifiers. (d) Genome map of deficiencies color coded as in c for the class of Ras modifier.

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### Table: Deficiency, Location, Chromosome, Number of Genes, Type of Differential

| Deficiency                  | Location Coordinates | Chromosome | Number of Genes | Type of Differential      |
|-----------------------------|----------------------|------------|-----------------|---------------------------|
| *Df(2L)BSC454              | 271351..307085       | 2L         | 16              | Enhancer of Common        |
| *Df(2L)BSC692              | 2830265..2868633     | 2L         | 12              | Enhancer of Rare          |
| *Df(2L)BSC188              | 6612189..6742726     | 2L         | 27              | Enhancer of Common        |
| *Df(2L)BSC278              | 16025369..16289284   | 2L         | 25              | Enhancer of Rare          |
| Df(2L)ED1102               | 16350236..16684883   | 2L         | 32              | Enhancer of Rare          |
| *Df(2L)BSC781              | 16325113..16417726   | 2L         | 13              | Enhancer of Rare          |
| *Df(2R)BSC598              | 22641779..22678681   | 2R         | 25              | Enhancer of Common        |
| Df(2R)BSC780               | 24203216..24685191   | 2R         | 91              | Enhancer of Rare          |
| *Df(3L)M21                 | 2674593..3336860     | 3L         | 114             | Suppressor of Common      |
| Df(3R)BSC47                | 5632351..5861298     | 3R         | 52              | Enhancer of Rare          |
| *Df(3R)BSC633              | 7080388..7123376     | 3R         | 9               | Enhancer of Common        |
| *Df(3R)ED10642             | 16453757..16625271   | 3R         | 30              | Enhancer of Common        |
| *Df(3R)BSC887              | 16158034..16442926   | 3R         | 51              | Suppressor of Rare        |
| *Df(3R)BSC321              | 25637827..25680519   | 3R         | 13              | Enhancer of Rare          |
| Df(3R)BSC140               | 25735650..26055884   | 3R         | 77              | Suppressor of Common      |

### Figure b: Number of Dis

- Enhancer of Rare: 8
- Enhancer of Common: 6
- Suppressor of Rare: 4
- Suppressor of Common: 2

### Figure c: Percentage of Enhancers and Suppressors

- Enhancers: 44% (Total=108)
- Suppressors: 73% (Total=15)

### Figure d: Differential Enrichment Analysis

- Basic helix-loop-helix transcription factors: 9/59 genes
- Ras FlyBase groups: 1/36 genes
- Divergent ionotropic receptors: 28/42 genes
- Cytoplasmic lysine-CTT transfer RNAs: 12/14 genes
- Ras FlyBase groups: 11/36 genes
Loop Helix (bHLH) transcription factors, potentially reinforcing their distinct regulation of Ras/MAPK signaling. In summary, by controlling Ras/MAPK signal output strength through codon usage and using a phenotypic output screen, we successfully identified Dfs that alter a Ras/MAPK phenotype in a signaling output-specific fashion.

**RpS21 negatively regulates Ras/MAPK signaling in a signal strength-specific manner**

To identify a differential modifier from our screen at the single gene level, we focused on Df(2L)BSC692 as it was one of the smallest deficiencies, encompassing only 12 genes, that specifically enhanced Ras\(^{V12\text{Rare}}\) (Figs 3A and S4A). Of these 12 genes, Ribosomal protein S21, or RpS21 (also known as overgrown hematopoietic organs 23B/oho23B), represented a plausible candidate modifier. RpS21 stands out among small ribosomal subunits for its reported negative regulation of hematopoietic and imaginal disc hyperplasia [65]. To determine if RpS21 is a responsible gene in Df(2L)BSC692 for specifically enhancing Ras\(^{V12\text{Rare}}\), we assessed the rough-eye phenotype of Ras\(^{V12\text{Common}}\) and Ras\(^{V12\text{Rare}}\) in the background of the mutant RpS21\(^{03575}\). Indeed, only the sev-Ras\(^{V12\text{Rare}}\) rough-eye phenotype is enhanced in the RpS21\(^{03575/+}\) background (Fig 4A). RpS21\(^{03575/+}\) did not score as a hit by our animal lethality criteria (see Methods), suggesting our comparison of eye phenotypes between control and mutant animals was not impacted by animal viability. We also note that the RpS21\(^{03575}\) chromosome also carries a mutation in *cinnabar* (cn). However, cn mutations were also present in 4 other Dfs in our screen, only one of which was a hit. Therefore, RpS21 and not cn is the likely modifier on the RpS21\(^{03575}\) mutant chromosome. Similar to our findings in the eye, RpS21\(^{03575/+}\) preferentially impacts the phenotype of Ras\(^{V12\text{Rare}}\) leg imaginal disc clones. We observe smaller average clone sizes in RpS21\(^{03575/+}\), Ras\(^{V12\text{Rare}}\) animals relative to Ras\(^{V12\text{Rare}}\) alone, whereas RpS21\(^{03575/+}\) does not impact clone size in Ras\(^{V12\text{Common}}\) animals (Fig 4B and 4C). Together, these findings identify RpS21 as a responsible modifier of Ras\(^{V12\text{Rare}}\) in one Df from our Ras/MAPK signal strength-specific screen.

From our genome-wide screen and follow-up mapping efforts, we were able to identify both an RpS21 mutant allele and a small deficiency encompassing this gene (Df(2L)BSC692) as differential Ras\(^{V12\text{eye}}\) phenotype modifiers. We next examined the molecular alterations of Ras signaling that underlie this signal intensity-specific modification. To this end, we assessed Ras\(^{V12}\) levels and/or MAPK pathway activation by immunoblot analysis in three distinct cellular and signal output settings: ectopic Ras activation in adult fly heads, ectopic Ras activation in cultured S2 cells, and endogenous MAPK signaling in ovaries. Our results overall show that while RpS21 reduction impacts Ras/MAPK in numerous settings, there is a more pronounced effect in cases where signaling output is weaker.

In the heads of Ras\(^{V12\text{Rare}}\) flies, transgenic Ras protein levels increase in RpS21\(^{03575/+}\) animals relative to wild type. This result is consistent with the enhanced Ras\(^{V12\text{Rare}}\) eye phenotype in RpS21\(^{03575/+}\) animals. However, unlike our lack of an observable phenotypic enhancement of Ras\(^{V12\text{Common}}\) in the eye, at the biochemical output level we also observe an increase in the level of Ras\(^{V12\text{Common}}\) in the RpS21\(^{03575/+}\) background (Figs 4D and S4B). This result shows that RpS21\(^{03575}\) modifies both sevenless-driven Ras\(^{V12\text{Rare}}\) and
Fig 4. RpS21 negatively regulates Ras/MAPK signaling in a signal strength-specific manner. (a) The mean ± SEM eye severity score of the genotypes from three replicate experiments at 25°C. (b) The mean ± SEM leg imaginal disc clone size in pixels for each genotype (3 replicate experiments, N = 10–33 total animals. One-way ANOVA test. (c) Images representing the leg imaginal disc Tubulin-Gal4 FLP-out clone sizes generated in the indicated genotype backgrounds. Scale bars = 20 um. (d) Immunoblot detection of transgenic RasV12 (with an anti-FLAG antibody), and actin as a loading control from lysates derived from the heads of flies with the indicated versions of transgenic RasV12 in either the wild-type (+/+ ) or mutant (RpS2103575/+ ) RpS21 backgrounds. (e) Quantitative RT-PCR, measured using 2^ΔΔCt, of animals expressing the indicated versions of transgenic RasV12 in either the wild-type (+/+ ) or mutant
Ras\textsuperscript{V12}Common protein levels in the adult fly head, but only Ras\textsuperscript{V12}Rare modification leads to an observable phenotypic output in this setting. This difference between eye phenotype and protein level effects could suggest that a large difference in Ras protein change is needed to cause a detectable change at the eye phenotype level. Alternatively, our adult head assay focuses on Ras levels in the adult animal, whereas our eye assay focuses on the effect of RpS21 reduction during eye development. \textit{RpS21\textsuperscript{103575/+}} does not impact Ras\textsuperscript{V12}Rare or Ras\textsuperscript{V12}Common RNA levels in adult heads, suggesting \textit{RpS21} acts at the translational level to impact Ras signaling (Fig 4E).

Next, we examined the impact of RpS21 on Ras signaling in additional cellular contexts. We first transduced S2 cells with an expression vector encoding either Ras\textsuperscript{V12}Common or Ras\textsuperscript{V12}Rare, and then used RNAi to reduce RpS21 levels. As in the fly head, RpS21\textsuperscript{RNAi} elevates Ras\textsuperscript{V12}Rare protein levels. However, unlike in the head, Ras\textsuperscript{V12}Common protein levels in S2 cells are unaffected by RpS21\textsuperscript{RNAi} (Figs 4F and S4C). We note that, in these cells, our expression system led to particularly robust expression of the Ras\textsuperscript{V12}Common protein (Figs 4F and S4C). We also examined MAPK activation in S2 cells. Whereas p-Mek and p-Erk are noticeably increased in RpS21\textsuperscript{RNAi} S2 cells expressing Ras\textsuperscript{V12}Rare, we see no overt increase in these MAPK activation readouts upon RpS21\textsuperscript{RNAi} in S2 cells expressing Ras\textsuperscript{V12}Common (Figs 4F and S4C). Taken together, our results in the head and in S2 cells suggest that when Ras signaling is above a particular threshold (e.g., Ras\textsuperscript{V12}Common expression in S2 cells), RpS21 reduction does not impact pathway output.

We also assessed whether endogenous MAPK signaling can be regulated by RpS21 \textit{in vivo}. To do so, we examined the effect of disrupting one allele of the \textit{RpS21} gene on endogenous MAPK signaling in the ovaries of flies, a tissue where EGFR/Erk signaling has a well-defined role \cite{66,67} and where phosphorylated Mek and Erk are readily detected (Fig 4G). Of note, \textit{RpS21\textsuperscript{103575/+}} animals have no obvious female fertility defects. In this tissue, endogenous p-Mek and p-Erk levels increase in both \textit{Df(2L)BSC692/+} and \textit{RpS21\textsuperscript{103575/+}} animals relative to control \textit{w\textsuperscript{118}B} animals (Fig 4G). Although we were not able to successfully determine endogenous Ras levels in the ovary with existing reagents (not shown), our overall findings are consistent with RpS21 negatively regulating endogenous Ras/MAPK signaling in this tissue.

Collectively, we find that loss of RpS21 elevates Ras/MAPK signaling in multiple contexts. Our immunoblot analysis validates our genetic screen finding that RpS21 can negatively regulate Ras and/or MAPK signaling, in a manner that potentially depends on the strength of Ras/MAPK signaling. One interpretation of these data is that RpS21 has a minimal effect on MAPK signaling output above a certain threshold of MAPK signaling. Such a model would predict that experimentally reducing the amount of Ras\textsuperscript{V12}Common expression should render fly eye development sensitive to the \textit{RpS21\textsuperscript{103575/+}} mutant background. To experimentally test this threshold model, we took advantage of the well-known fact that expression of transgenes using the Gal4-UAS system is responsive to temperature, with higher temperature resulting in higher expression over the physiological range of 18° C-29° C. We thus evaluated the rough-eye phenotype of \textit{sev-Ras\textsuperscript{V12}Common} versus \textit{sev-Ras\textsuperscript{V12}Rare} flies in a wild-type versus RpS21\textsuperscript{103575/+} mutant background, only this time at 18° C. At this lower temperature, RpS21\textsuperscript{103575/+} now...
acts as an enhancer of Ras$^{V12\text{Common}}$ (Fig 4H). Interestingly, RpS21$^{03575/+}$ no longer enhances Ras$^{V12\text{Rare}}$, underscoring the sensitivity of RpS21/+ to Ras/MAPK signaling strength. Therefore, RpS21 regulation of the Ras pathway appears to be signal-strength dependent, rather than codon-dependent. Collectively, these results demonstrate that while RpS21 negatively regulates Ras-MAPK signaling in diverse contexts, at the phenotypic level this regulation preferentially impacts weak Ras/MAPK signaling. These findings are consistent with a model whereby above a certain signaling intensity threshold, regulators that impact Ras signaling at weaker intensity levels are no longer effective (Fig 4I).

**RpS21 downregulation does not alter expression of a codon-altered GFP reporter**

Our above results suggested that it is Ras/MAPK signaling strength, and not codon manipulation specifically, that determine whether RpS21 heterozygosity impacts protein expression. To test this idea further, we generated an additional pair of transgenes with identical protein sequence but distinct codon usage. Specifically, we generated two GFP transgenes- one with GFP containing 100% common codons, and one where the same GFP had 50% synonymous substitutions of rare codons dispersed throughout the protein. Both transgenes were expressed under a ubiquitin promoter and were integrated into the same site in the genome (Figs 5A and S5, see Methods). Consistent with our results for altering codon content of the Ras gene, GFPCommon protein is expressed at a higher level in adult animals than GFPRare protein (Fig 5B and 5C). Given this, we next tested whether RpS21 downregulation alters GFP protein expression in a codon-dependent manner. RpS21$^{03575/+}$ animals exhibit similar GFPRare protein expression as wild type animals (Fig 5D and 5E). Additionally, RpS21$^{03575/+}$ animals exhibit similar GFPCommon protein expression as wild type animals (Fig 5F and 5G). These results indicate that RpS21 downregulation does not impact translation of at least one other tested transgene pair, suggesting that RpS21 may, to some degree, act specifically to regulate the Ras/MAPK pathway at specific signaling intensity levels. Overall, our findings highlight the ability of our approach to reveal new Ras/MAPK regulators that preferentially impact specific signaling outputs.

**Discussion**

Here, we revisit a well-proven strategy to identify Ras/MAPK modifiers (a heterozygous mutant screen in the *Drosophila* eye) but do so with the new angle of altering codon usage in a core signaling component to find signal strength-dependent regulators. We show here that changing codon usage in a signaling pathway component can be an effective strategy to find signal strength-dependent modifiers, as evidenced by our identification of 15 Df from a whole-genome screen that only modify the rough-eye phenotype driven by either a common or rare codon-enriched Ras$^{V12}$ transgene, but not both. From these efforts, we identify the RpS21 gene as a negative regulator of a weak or low-level Ras phenotype in the *in vivo* context of eye development. These findings are further supported by our finding that RpS21 reduction in other contexts also impacts (low) endogenous Ras signaling in the ovary, but not higher Ras signaling in S2 cells.

Our results show that altering codon usage can serve as a valuable platform to stably alter protein production to undertake signal strength-specific screens. Clearly, there are other ways that one can modulate signal output strength, such as modulating gene expression strength as we also do here, or through use of an allelic series [68]. However, an advantage of altered codon usage is that it can be hard-wired into the genome, and thus no additional (and potentially confounding) experimental parameters such as altering temperature, inducing genes
Fig 5. Rps21 downregulation does not impact codon-dependent GFP protein expression. (a) Schematic representation of the GFP transgenes encoded by rare or common codons. (b) Immunoblot detection of GFP protein and α-Tubulin as a loading control from lysates derived from adult flies with the indicated versions of transgenic GFP. (c) Quantification of protein levels for blot in b. a.u. = arbitrary units. Data represent mean ± SEM, 3 replicates, One-way ANOVA and Tukey’s multiple comparisons test. (d,f) Immunoblot detection of GFP protein and α-Tubulin as a loading control from lysates derived from adult flies with the indicated versions of transgenic GFP, and in the indicated genetic backgrounds. (e,g) Quantification of protein levels for blot in d, f respectively. a.u. = arbitrary units. Data represent mean ± SEM, 3 replicates, One-way ANOVA and Tukey’s multiple comparisons test. *** (p<0.001), N.S. = not significant.

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with drugs, and so forth are required. Our approach should be applicable to any signal transduction pathway. The utility of our approach is underscored in the fact that signal strength-specific modifiers found in our screen appear to be enriched for genome regions not previously linked to Ras genetic modification. The causative genes contained within 14 of these differential Df hits remain to be mapped and represent a potentially rich source of new genes modulating Ras/MAPK signaling. Previous work found that different levels of MAPK activity impact different biological processes [68]. Intriguingly, our differential hits appear to be enriched in bHLH transcription factors. Of note, the bHLH transcription factor Myc is a well-known Erk target [69–72], and it will be interesting to explore whether specific bHLH transcription factors are preferentially targeted by this pathway in signal strength-dependent contexts.

Given the importance of Ras/MAPK signaling in many settings across evolution, our identified modifiers may shed insight into how this pathway is controlled at different signal strengths. While our focus here is on Drosophila eye development, signal strength dependencies of the Ras/MAPK pathway are appreciated to play a role in human disease. Activating mutations in the MAPK pathway of humans underlie a class of human diseases termed RASopathies [73]. Further, relevant to our approach here, of the three human RAS genes, KRAS, is the most enriched in rare codons [27] and is the most commonly mutated RAS isoform in human cancers [28,74]. Changing the rare codons to more common codons in a single exon of the mouse KRAS gene leads to fewer tumors following carcinogen exposure [22], which is in line with current thinking on a “sweet spot” level of Ras/MAPK signaling required to initiate tumorigenesis [28]. We argue that the larger clone size that we observe in leg imaginal discs of animals expressing RasV12 Rare vs. RasV12 Common reflects this same concept. As such, the new tools we report here may provide valuable reagents to more accurately model KRAS-relevant regulation in Drosophila and ultimately in KRAS-driven disease.

Our approach found that RpS21 functions as a negative regulator of weak Ras/MAPK signaling. While one might expect that a codon-based approach would pull out ribosomes as hits, we show here that codon-independent manipulation of Ras signaling, through temperature change, confirms that RpS21 is responding to specific signaling levels rather than specific codons. As Ras/MAPK signaling is known to drive tissue growth in diverse settings, this may suggest that RpS21 can function as a negative regulator of tissue or tumor growth. Interestingly, downregulation of RpS21 was previously shown to cause excessive hyperplasia in hematopoietic organs and imaginal disc overgrowth during larval development, suggesting RpS21 acts as tumor suppressor in Drosophila [65]. Although this finding may seem paradoxical given that ribosomal mutants in flies are well-known to cause minute phenotypes, characterized by short bristles, small body size, and delayed growth [75–78], a subset of ribosomal proteins including RpS21 have been identified to have a growth suppressive role [65,79–83]. Further, heterozygosity of many ribosomal proteins is reported to be tumorigenic in zebrafish [84], and heterozygous inactivating mutations of ribosomal proteins have been described in human cancers [85,86]. Several mechanisms have been proposed to account for this apparent tumor suppressor activity of ribosome protein downregulation, including activation of p53 [87–89], inhibition of NF-KB [90], E2F [91], MYC [92], and CDK8 [93]. Thus, RpS21 joins the ranks of an emerging number of ribosomal proteins with roles in growth suppression, although whether RpS21 acts as a tumor suppressor in mammals awaits investigation.

The mechanism underlying the negative regulation of Ras/MAPK signaling by RpS21 remains to be determined. Future work can explore how direct the regulation is, and whether RpS21 acts in a cell autonomous or, has been shown for a subset ribosomal subunits, a non-autonomous manner to regulate tissue growth [83]. Future work can also explore whether other signaling pathways connected to eye development are also impacted by RpS21 reduction.
In our work, we found that RpS21 downregulation promotes elevated levels of RasV12 protein in multiple settings. The effect of RpS21 on RasV12 protein level could potentially be through RpS21’s canonical ribosomal function or through an extra-ribosomal function. Dose-dependent ribosome dysfunction is linked to the human disease Diamond-Blackfan anemia, where heterozygous mutations in specific ribosomal subunits are linked, at least in part, to compromised ribosome biogenesis and translation [94–97]. A defect in RpS21 ribosomal function may trigger ribosomal biogenesis defects that alter translational fidelity or promote generation of oncoribosomes to preferentially express subset of mRNA pools [98,99]. Alternatively, RpS21 might participate in other cellular processes independent of its canonical ribosomal function, as has been shown for other ribosomal subunits [100–103].

We note that RpS21 has been connected to positive regulation of Ras/MAPK in other contexts. While this manuscript was in review, a recent study revealed that downregulation of human RPS21 inhibits metastatic behavior of osteosarcoma cells in a MAPK-dependent manner [104], underscoring the potential human relevance of our findings here. Further, in contrast to our screen results revealing negative regulation by RpS21 in multiple contexts, numerous ribosomal proteins (RpS21 included) were found among 1,162 genes to positively regulate Erk phosphorylation in a previous primary screen in cultured Drosophila S2R+ cells [15]. Unlike this Erk activation screen, we note that our RasV12 eye modifier screen hits were not preferentially enriched for ribosomal subunits, and that ribosomes in general are not enriched among known FlyBase Ras genetic enhancers/suppressors. We hypothesize that the addition of insulin to the growth media, required for Erk activation in the context of the S2R+ cell screen, revealed a dependency for cell growth, which is dependent on both ribosomes and Erk activation. S2R+ cells have known differences from S2 cells in response to external signaling, and this could reflect differences in MAPK regulation in this context as well [105], underscoring the need to understand signaling dynamics and regulation in a given biological context.

Another question for future investigation is why RpS21 regulation of Ras signaling is non-functional in contexts of heightened Ras/MAPK signaling, as we observed in S2 cells with strong Ras/MAPK biochemical output, as well as at the phenotypic output level where Rps21/+ failed to noticeably modify the eye phenotype of RasV12Common. One possible explanation is that different MAPK signaling strengths activate a different host of MAPK targets, and this impacts the degree of negative regulation by RpS21. To that end, it will be important to further mine our screen to identify single gene modifiers in the other 14 Dfs, which may similarly yield new regulatory insight into the Ras/MAPK pathway.

In summary, we show here the value of manipulating codon usage of one component of a pathway to modulate the corresponding signaling output, and the use thereof to screen for modifiers of specific signaling intensities. This approach proved successful, identifying a novel regulator of the Ras/MAPK pathway, RpS21. As such, this approach may find value in similarly interrogating other signaling pathways.

Methods

Generation of codon-altered genes in Drosophila

Codon-altered exon sequences for RasV12Common, RasV12Rare, GFPRare, and GFPCommon were created using the Kazusa codon usage database (https://www.kazusa.or.jp/codon/) and subsequently generated by Gene Synthesis (ThermoFisher Scientific, Invitrogen GeneArt). A cDNA clone (LD17536, Drosophila Genomics Resource Center) was used as a template to generate the non-altered Ras85D sequence. To generate RasV12Native, the QuikChange II Site-Directed Mutagenesis Kit (Agilent) was used to change codon 12 in Ras85D from GGA
(glycine) to GTA (valine). Subsequently, primers (sequences available upon request) were designed to amplify Ras sequences and the Invitrogen Gateway BP Clonase II Enzyme Mix (ThermoFisher Scientific) was used to insert these sequences into the Gateway entry vector pDONOR221 (ThermoFisher Scientific). Subsequently, the Invitrogen LR Clonase Enzyme Mix (ThermoFisher Scientific) was used to insert the Ras WT, and Ras V12 Native, Common, and Rare sequences into the Gateway destination vector pBID-UASC-FG (Addgene Plasmid #3520 [106]), which has a N-terminal FLAG tag and a PhiC31 site for site-directed genomic insertion. pBID-UASC-FG-Ras plasmids were prepared with a ZymoPURE II Plasmid Midiprep Kit (Zymo Research) and sent to Model System Injections (Durham, NC, USA) for injection into attP40 (2L) flies. GFP sequences were cloned into a pBID plasmid (modified from Addgene Plasmid #3520), and DNA and transgenic flies were prepared as for Ras transgenes. For cell culture, Ras V12 Common and Ras V12 Rare transgenes were cloned into pMKInt-Hyg vectors, which were sequenced to confirm the correct sequence.

**Fly stocks.** All flies were raised at 25°C on standard media unless noted otherwise (Archon Scientific, Durham NC). FlyBase (http://FlyBase.org) describes full genotypes for all stocks used in this study. See S1 Table for Df stock information. All other stocks were the following genotypes (Bloomington Drosophila Stock Center numbers in parentheses when available): ksr S-627/TM3,Sb (#5683), aop am- N[8]/CyO (#8777), betagtl P[2554] (#5681), RpS2103575/CyO (#11339), hsflp: UAS-GFP, tubulin-FRT-STOP-FRT-Gal4 [54], and the Bloomington Deficiency(Df) kit. The following stocks were generated for this study: UAS-FLAG-Ras, UAS-FLAG-Ras V12 Native, UAS-FLAG-Ras V12 Common, and UAS-FLAG-Ras V12 Rare, ubi-GFP Rare, ubi-GFP Common.

**Fly genetics and deficiency screen.** To examine mitotic clones in leg imaginal discs and associated animal survival in such experiments, flies containing UAS Ras transgenes were crossed to hsflp: UAS-GFP, tubulin-FRT-STOP-FRT-Gal4 animals. F1 larvae were collected 96 hours after egg laying and heat shocked at 37 degrees for 20 minutes. After 24 hours, leg imaginal discs were dissected from living larvae. Discs were fixed as done previously for imaginal discs [107] and probed with DAPI for DNA. Images were taken on a Nikon A1 confocal microscope. Clone sizes were determined using FIJI’s Tracing and measuring tools.

To examine eye phenotypes and associated animal survival in such experiments, the Ras transgenes were combined with a sev Gal4 driver and subsequently crossed to Df/Balancer flies. After 16–18 days after egg laying, the rough eye phenotype of the resulting progeny was scored (both males and females). The scoring system was as follows (category = numerical score, qualitative description): Mild = 1, no discoloration or necrotic tissue; Moderate = 3, discoloration and no necrotic tissue; Severe = 5, discoloration and necrotic tissue (see Fig 1B and 1C). Severity scores for each genotype was calculated as follows: (#Mildx1+#Moderatex3 +#Severex5)/Total # of flies. To determine if heterozygosity for a subset of genes altered the rough eye phenotype the following two genotypes for each deficiency (Df) were compared: Ras transgene only and Ras transgene + Df (used as an internal comparison to control for background effects). Then, we calculated a fold change score for both Ras V12 Common and Ras V12 Rare for each deficiency: Ras transgene + deficiency/Ras transgene. We note that none of the Df animals on their own had detectable eye phenotypes. For the primary screen, the fold change score was defined as follows: enhancer (fold change ≥1.35 or 5X less flies eclosed); suppressor (fold change ≤0.65 or 5X more flies eclosed). For the secondary screen, the fold change score was defined as follows: enhancer (fold change ≥1.95 or 5X more flies eclosed); suppressor (fold change ≤0.50 or 5X less flies eclosed). The final phenotype for a deficiency was defined as follows: not a modifier (neither Ras V12 Common or Ras V12 Rare + Df were modified); enhancer (both Ras V12 Common and Ras V12 Rare + Df were enhanced); suppressor (both Ras V12 Common and Ras V12 Rare + Df were enhanced); differential (only Ras V12 Common or Ras V12 Rare + Df were modified). We note that overall eye size was relatively unaffected by
different Ras transgenes. Images of fly eyes were obtained using a Leica MZ10F microscope with a PlanApo 1.6X objective, Pixel Shift Camera DMC6200, and LASX software.

**Protein preparation and analysis.** All protein samples were prepared by homogenizing tissue on ice. For Figs 5, S1B and S5 samples were processed in Laemmli buffer and then boiled for 5 min. Samples were separated by 12% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and transferred to an Odyssey nitrocellulose membrane (LI-COR Biosciences) for immunoblotting. The following antibodies were used: anti-FLAG M2 (1:500, Sigma, anti-mouse), anti-α-tubulin (1:20,000, Sigma, anti-mouse), rabbit anti-GFP (Life Technologies, #A11122), IRDye 800CW (1:20,000, LI-COR Biosciences, anti-mouse or anti-donkey), and Alexa Fluor 680 goat anti-mouse IgG (H+L) (Invitrogen, #A21058). Signal was detected using LI-COR Odyssey CLx and analyzed using Image Studio (LI-COR Biosciences). For all other immunoblots, samples were processed in RIPA buffer containing 1% IGE-PAL, 50 mM NaCl, 2 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.1% Glycerol, 50 mM NaF, 10 mM Na3VO4, and protease inhibitors (Roche). Drosophila heads and ovaries were collected and transferred to cold lyses to be homogenized with a pellet pestle. Lysates were incubated at 4 °C for 30 min on end-to-end rotator and then centrifuged at 21,000 x g for 10 min. The supernatant was transferred to a new tube. Total protein was quantified using a BCA kit (Bio-Rad) and 10 to 50 micrograms of protein was used for separation on either 12.5% or 15% gradient SDS-PAGE gels. Proteins on SDS gels were transferred onto polyvinylidene difluoride membranes. These membranes were probed with anti-Flag (Sigma, anti-mouse 1:1000), anti-β-actin (Cell Signaling, 1:1000), anti-p-MEK1/2 (Cell Signaling, 1:500), anti-MEK1/2 (Cell Signaling, 1:500), anti-p-ERK1/2 (Cell Signaling, 1:1000), anti-ERK1/2 (Cell Signaling, 1:1000), and anti-RpS21 (Abcam, 1:2000) primary antibodies in blocking buffer containing 5% milk goat anti-mouse IgG (H+L) HRP (Life Technologies, 1:10000) or goat anti-rabbit IgG (H+L) HRP (Thermo Fisher Scientific, 1:10000). Immunoblots were visualized using Clarity Max ECL Western Blotting Detection Reagent (Bio-Rad) followed by exposure to digital acquisition using Chemi Doc Imager (Bio-Rad). For all blots, the contrast and/or brightness were altered equally across the entire image and then images were cropped for displaying as figures. Flag band intensity was measured using Image Lab v6.0.1 software and then each band was normalized to the lowest intensity band. The active Ras detection kit (Cell Signaling, #8821) was used to detect GTP-bound RasV12, both rare and common.

**RT-PCR.** Animals were aged 3–7 days at 25 °C on standard fly medium. RNA was extracted from adult fly heads using TRIzol™ reagent (ThermoFisher, cat#15596026) according to the manufacturer’s protocol (10–40 heads per sample in 500 ul TRIzol™ reagent). Purified RNA was resuspended in molecular grade water. RNA was DNase treated with DNase I at room temp for 15 minutes, then the reaction was terminated by adding 25 mM EDTA and incubating at 65 °C for 10 minutes. DNase efficiency was confirmed using a positive control. DNase treated RNA was reverse transcribed into cDNA using iScript cDNA synthesis kit (BIO-RAD, cat#170–8891) according to the manufacturer’s protocol. Subsequent cDNA was treated with RNase H prior to use in qPCR reactions. The concentration of the RNA was quantified on a NanoDrop spectrophotometer and samples were diluted with molecular grade water to match the concentration of the lowest concentration sample. Luna Universal qPCR Master Mix (NEB #M3003) was used to run the qPCR reaction according to the manufacturer’s specifications. Primers for the detection of Ras constructs were designed against an identical region containing the 3xFLAG sequence shared by both RasV12Common and RasV12Rare transcripts. Primers were designed against Drosophila Beta Tubulin 56D as a reference gene. Ras qPCR FW primer: TGGACTACAAAGACCATGACGGT, Ras qPCR RV primer: ACTTGTATACCGGTGCTTGTCA T, Tubulin qPCR FW primer: GGACGAGACCTACTG CATCG, Tubulin qPCR RV primer: GGTCACCGTATGTGGGTGTC.
**Cell culture.** KC and S2 cell lines were obtained from Bloomington (Indiana University DGRC Bloomington) and as a gift from Dr. David MacAlpine (Duke University) respectively. These cells were cultured in Schneider’s *Drosophila* medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin–L Glutamine (Invitrogen) at 25°C. FBS was heated for 60 minutes in 58°C and then cooled down before being added to the medium. These cells were confirmed to be free of mycoplasma infection, as measured by the Duke Cell Culture Facility using MycoAlert PLUS test (Lonza). S2 and KC cell lines were stably transduced with the pMKInt-Hyg vector encoding *Ras*\(^{V12\text{Common}}\) and *Ras*\(^{V12\text{Rare}}\) cDNAs using 1000 ng of DNA in 6 well plates per manufacturer instructions (Effectene transfection reagent, Qiagen). The following day, Schneider’s media was changed, and cells were seeded in a coated culture dish (100x20 mm). Four days later, cells were passaged with fresh Schneider’s medium and 200 µg/ml hygromycin (Invitrogen) was added. The stably transfected cells were selected within a month growing in media containing hygromycin. Three days prior to any experiment, these cells were grown in media without hygromycin. Four million S2 cells that were stably transduced with *Ras*\(^{V12\text{Common}}\) and *Ras*\(^{V12\text{Rare}}\) plasmids were seeded into coated tissue culture dishes (60x15mm, VWR) with 2 ml of Schneider’s media (without FBS). Sixty micrograms of RpS21 dsRNA were added on top of these cells. One hour later, two ml Schneider’s media containing 20% FBS were added on top of 2 ml Schneider’s media without FBS resulting in medium with 10% FBS concentration in total media of this culture. Within 16–24 hours after RNAi treatment, expression of *Ras*\(^{V12\text{Common}}\) and *Ras*\(^{V12\text{Rare}}\) transgenes were induced by CuSO4 for another 12 hours. Finally, these cells were collected 30–36 hours after dsRNA treatment.

**dsRNA synthesis.** S2 cell DNA was used to produce a PCR template for RpS21 dsRNA production using the forward primer “TAATACGACTCTATAGGGTTACTGACCAGC GCCGATACCC” and reverse primer “TAATACGACTCACTATAGGGCCA CGCTTAGAAGTTCCCTGC”. Next, 500 ng of RpS21 PCR template was used for an *in vitro* production of dsRNA as instructed in the MEGAscript T7 transcription kit (ThermoFisher). The dsRNA solution was cleared using MegaClear kit (ThermoFisher). Finally, the concentration of RpS21 dsRNA was measured and stored in -80°C for future use.

**Gene enrichment analysis and statistical analyses.** To determine the Codon Adaptation Index (CAI), sequences were entered at the CAIcal web-server (http://genomes.urv.es/CAIcal [108]). For gene enrichment, deficiency sequence boundaries were defined using coordinates available through FlyBase [109] and the Bloomington *Drosophila* Stock Center website. Deficiencies were then uploaded as a custom BED track to the UCSC Genome Browser (Reference Assembly ID: dm6). Genes overlapping the deficiency coordinates were then extracted using BEDtools for additional analysis [110]. A deficiency was determined to contain known *Ras* modifiers if any of the deficiency covered genes known as *Drosophila Ras85D* genetic interactors (332 interactors, FlyBase). Enhancers and suppressor deficiencies were analyzed using the same metric against known *Ras85D* interactors of the same respective modifier type. Statistical analysis (chi-square) was performed using Graphpad Prism v8.1. FlyBase Gene Group Enrichment analysis was performed by comparing deficiency covered genes with pre-defined FlyBase Gene Groups. Analysis and statistical tests were performed in R using Gene Overlap package (https://rdrr.io/bioc/GeneOverlap/) and results are reported as adjusted p-values (False Discovery Rate [111], using Benjamini Hochberg correction). Graphs and statistical analyses were generated using GraphPad Prism 7. Statistical tests and adjusted P-values are detailed in figure legends. For all tests, adjusted P-value reporting is as follows: (P>0.05, n.s.; P<0.05,*; P<0.01,**; P<0.001,***, P<0.0001,****).
Supporting information

S1 Fig. Drosophila Ras\textsuperscript{V12} Rare more closely resembles Human KRasB than other Drosophila Ras transgenes. (a) Alignments of Ras transgenes. Nucleotide changes highlighted for Ras\textsuperscript{V12} Common (red) and Ras\textsuperscript{V12} Rare (blue). Table with overall CAI score and GC content for Ras transgenes. (b) Codon Adaptation Index (CAI) plot. Transparent circles, squares, and triangles are individual CAIs per codon. Solid lines represent a best-fit line of individual points for each transgene. (c) Amino acid alignment of endogenous Drosophila Ras85D with human KRASA and KRASB, over a region of sequence divergence between KRASA and KRASB. The percent identity is noted. (d) Nucleic acid alignment of the four transgenes used in this study with human KRASB. (e) Nucleic acid phylogenetic tree of human KRASB and the four transgenes used in this study, with the percent identity of each gene/transgene to Drosophila Ras\textsuperscript{WT}. Native indicated.

S2 Fig. Codon manipulation of Ras\textsuperscript{V12} promotes differential MAPK signal strength levels in Drosophila. (a) Representative image of adult eye from animal expressing sevGal4>\textit{Ras\textsuperscript{WT}} (b) Immunoblot detection of transgenic Ras\textsuperscript{V12} protein (with an anti-FLAG antibody) and \textalpha Tubulin as a loading control from lysates derived from the head of flies with the indicated versions of transgenic Ras\textsuperscript{V12}. (c) Quantification of protein levels at 25\textdegree C for blot in S1B Fig. a. u. = arbitrary units. Data represent mean ± SEM, 3 replicates, Tukey's multiple comparisons test. (d) Biological replicate of serial dilution of Ras\textsuperscript{V12} common versus rare. 10, 20, and 30 ug of lysates derived from the heads of flies expressing the indicated versions of transgenic Ras\textsuperscript{V12} were immunoblotted with an anti-FLAG antibody, demonstrating differential expression of Ras\textsuperscript{V12} common and rare. Bottom: quantification and protein loaded. (d) Immunoblot detection of transgenic Ras\textsuperscript{V12} (with an anti-FLAG antibody), phosphorylated (p-) and total Mek and Erk, and actin as a loading control from lysates derived from (e) the head of flies with the indicated versions of transgenic Ras\textsuperscript{V12} or (f) S2 and KC cells stably transduced with expression vectors expressing the indicated Ras\textsuperscript{V12} transgenes. First lane is S2 cells without any transfection. (g) Levels of GTP-bound Ras\textsuperscript{V12} common versus rare. GTP-bound Ras from lysates derived from S2 cells stably expressing Ras\textsuperscript{V12} common versus rare (or no transgene as a control) were affinity captured with a Ras Binding Domain (RBD IP) and immunoblotted with an anti-FLAG antibody to detect the ectopic active portion of the expressed Ras\textsuperscript{V12} protein. Whole cell lysates (WCL) were immunoblotted with an anti-FLAG antibody to detect total ectopic Ras\textsuperscript{V12} protein and Actin as a loading control. One representative blot from multiple replicates is shown.

S3 Fig. Known Ras modifiers alter phenotypes driven by codon-altered Ras transgenes. (a) Quantification of eye severity scores for Ras transgenes that are also heterozygous for known Ras modifiers. Data represent mean ± SEM, multiple replicates (using Dennett’s multiple comparison test). (b) The average number of flies eclosed per experiment for Rare and Common transgenes in a known Ras modifier background.

S4 Fig. RpS21 negatively regulates Ras/MAPK signaling in settings of low signal output. (a) Genome map of Df(2L)BSC692. RpS21 is highlighted in green. (b) Immunoblot detection of transgenic Ras\textsuperscript{V12} (with an anti-FLAG antibody), and actin as a loading control from lysates derived from the head of flies with the indicated versions of transgenic Ras\textsuperscript{V12} in either the wild-type (+/+) or mutant (RpS21\textsuperscript{D5577/7/5577/+}) backgrounds. (c) Immunoblot detection of transgenic Ras\textsuperscript{V12} (with an anti-FLAG antibody), phosphorylated (p-) and total Mek and/or Erk.
RpS21, and actin as a loading control from lysates derived from S2 cells stably transduced with expression vectors expressing the indicated RasV12 transgenes in the absence (-) and presence (+) of RpS21 RNAi (Data represent two independent replicates.)

S5 Fig. Sequence of codon-altered GFP transgenes. (a) Alignments of GFP transgenes. GFP Common contains all common codons. Nucleotide changes to generate rare codons in GFP Rare are highlighted in (blue). Table with overall CAI score and GC content for GFP transgenes. (b) Codon Adaptation Index (CAI) plot. Triangles are individual CAIs per codon. Solid lines represent a best-fit line of individual points for each transgene.

S1 Table. Results of a genome-wide deficiency screen for modifiers of RasV12 common and RasV12 Rare eye phenotypes. See text for details.

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Author Contributions

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