Supplementary Information

In vivo severity ranking of Ras pathway mutations associated with developmental disorders

Authors: Granton A. Jindal[a,b,c,1], Yogesh Goyal[a,b,1], Kei Yamaya[b,c], Alan S. Futran[a,b], Iason Kountouridis[b], Courtney A. Balgobin[c], Trudi Schüpbach[c], Rebecca D. Burdine[c], and Stanislav Y. Shvartsman[a,b,c,2]

Affiliations:

[a]Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ 08544

[b]The Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544

[c]Department of Molecular Biology, Princeton University, Princeton, NJ 08544

[1]G.A.J. and Y.G. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: stas@princeton.edu.

SI Materials and Methods

Fly stocks: Three different Gal4 driver lines (MTD, Bloomington stock # 31777; Tub, Bloomington stock number # 5138; GMR, Bloomington stock # 9146) were used for tissue-specific expression of MEK variants. Flies were raised under standard conditions and crosses were performed at 25^{o}C unless otherwise specified.

RNA synthesis for microinjection: In pCS2(+) (Invitrogen), the EcoRI site was switched to EcoRV using site-directed mutagenesis (SDM), and human MEK1 (a gift from Rony Seger, Weizmann Institute of Science, Rehovot, IL) was subsequently cloned between the BamHI and EcoRV restriction sites, while mCherry was cloned between the EcoRV and XhoI restriction sites. In the resulting plasmid, the MEK1 gene is connected to the mCherry gene by a GCGATATCC linker sequence. The set of 16 mutations (Table S1) were introduced into this construct with SDM using the Phusion enzyme (NEB). The plasmid was linearized using Not1 (NEB), and synthetic capped mRNA was synthesized using the SP6 RNA polymerase mMessage mMachine kit (Ambion) and purified using the TRIzol reagent (Ambion).

Drug treatments: Zebrafish embryos were switched from methylene blue water to E3 medium (5 mL) with varying concentrations of PD0325901 (Biotang Inc.) for one hour from 4.5 to 5.5 hpf with 0.1% DMSO as the vehicle. The media of the embryos was switched back to methylene blue water at 5.5 hpf after three washes of E3 medium in order to prevent effects from residual MEK inhibitor seen with only one wash. Subsequently, the oval shape of the embryos was imaged at 11 hpf and the lethality percentage was recorded at 48 hpf. For each measurement at a given drug dose, there was also a measurement of a set of siblings treated with DMSO only.

Protein blotting: Standard Western blotting protocols were used to detect MEK1-mCherry as well as dually-phosphorylated ERK (dpERK). Primary antibodies to the mCherry tag (1:1000, Life Technologies, M11217), dpERK (1:2000, Cell Signaling Technologies (CST), 4370S), and the loading control α-tubulin (1:1000, CST, 3873S) were used. Alexa fluor secondary antibodies were used at 1:2000. The Bio-Rad ChemiDoc MP Imaging System was used to capture images and Bio-Rad Image Lab software was used for analysis.

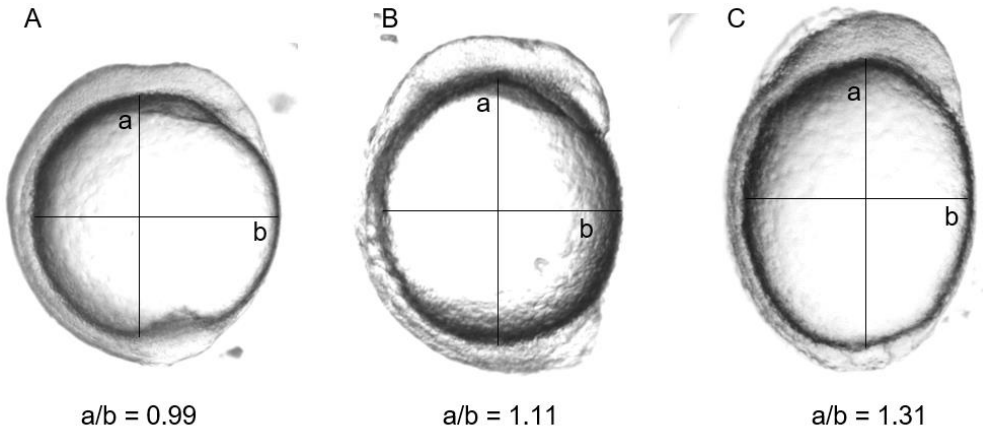

Figure S1. Measurement of oval shape dimensions of the zebrafish embryo at 11 hpf. Prior to imaging, each embryo was manually positioned so that it was lying laterally. Embryos that died or developed improperly were not considered for measurement. The major (a) and minor (b) axes of the yolk of the embryo were measured on ImageJ, using the Measure feature after drawing a line between the two points demarcating the start and the stop of the axis. The line for measurements were drawn so that the maximum length between the two ends is captured. An example (A) WT-MEK injected embryo, (B) G128V-MEK injected embryo, and (C) F53L-MEK injected embryo are shown.

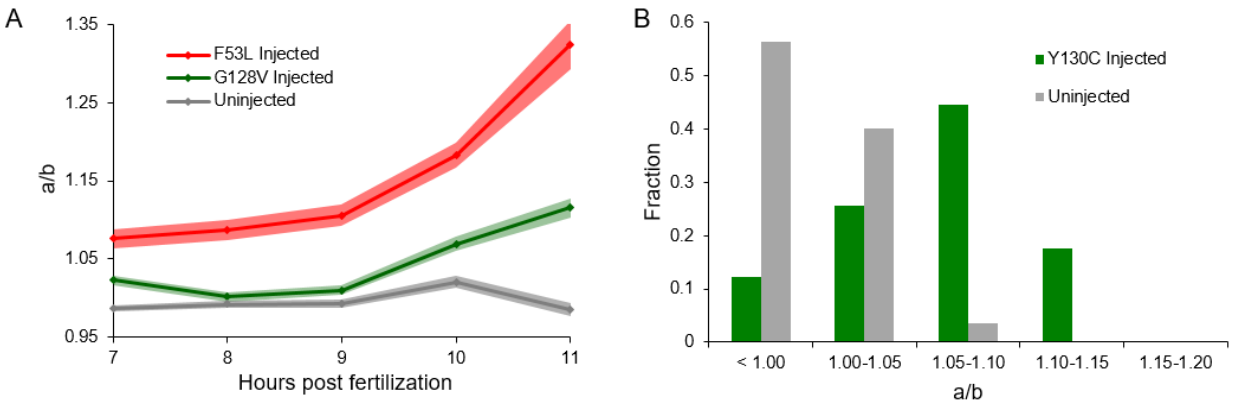

Figure S2. Zebrafish oval shape evolution from 7 to 11 hpf and distribution at 11 hpf. (A) Embryo shape over time from 7 hpf (60% epiboly) until 11 hpf (3-somite stage) after injection with MEK variants. The plot shows the average with standard error of the mean (s.e.m.) indicated for MEK1-F53L-injected embryos (N_{7hpf} = 34, N_{8hpf} = 34, N_{9hpf} = 32, N_{10hpf} = 27, N_{11hpf} = 16), MEK1-G128V-injected embryos (N_{7hpf} = 26, N_{8hpf} = 29, N_{9hpf} = 29, N_{10hpf} = 29, N_{11hpf} = 22), and uninjected embryos (N_{7hpf} = 18, N_{8hpf} = 20, N_{9hpf} = 20, N_{10hpf} = 20, N_{11hpf} = 17). The 11 hpf time point was selected to provide the most dynamic range with minimal lethality. (B) Distribution of major to minor axis ratios for uninjected embryos and MEK1-Y130C-injected embryos from three technical replicates. An average from three such distributions is taken for both conditions with $N_{Uninjected}$ = 55 and N_{Y130C} = 74.

MEK Mutation	Mutant codon used
L42F	TTT
E44G	GGA
F53L	TTG
F53S	AGC
T55P	CCC
D67N	AAC
P124L	CTG
P124Q	CAG
G128N	AAC
G128V	GTG
Y130C	TGC
Y130H	CAC
Y130N	AAC
Q164K	AAA
E203K	AAG
E203Q	CAG

Table S1. Codons used for MEK1 variant expression in zebrafish. In the zebrafish mRNA injection experiment, 16 MEK1 variants were tested. The codons of each of the amino acid variants are included here.

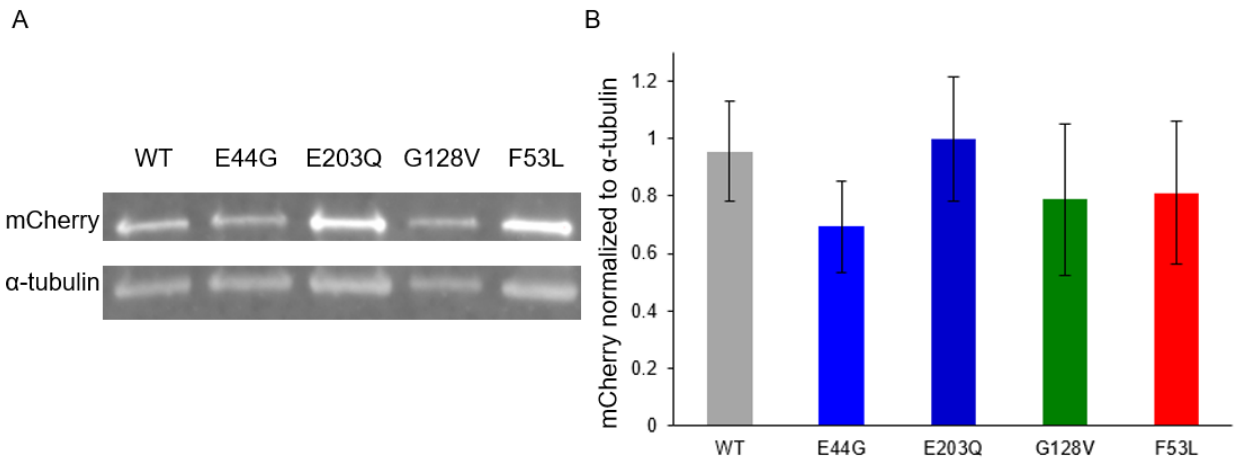

Figure S3. MEK1-mCherry expression levels in zebrafish across the three classes of mutations. mRNA injection induced similar amounts of protein in each embryo. (A) A sample Western blot showing the mCherry channel, which detects MEK1-mCherry and the α-tubulin channel, as a loading control. (B) The average of the ratios of mCherry fluorescence to α-tubulin fluorescence for 4 mutations and WT-MEK across the three classes of mutations is plotted, with the s.e.m. indicated. The ratios were normalized so that the highest ratio for a Western blot was 1. The differences between each pair of the five readings are not statistically significant. RASopathies (blue), RASopathies and cancer (green), and cancer only (red). Here, each value is an average from 3 Western blots, except for E44G, which is an average from 2. One-way analysis of variance (ANOVA) with Bonferroni correction was used for statistical analysis: *P < 0.10, **P < 0.01, ***P < 0.001.

	UN	WT	E44G	G128N	T55P	Y130N	Q164K	L42F	E203Q	D67N	Y130C	P124L	Y130H	P124Q	F53S	G128V	F53L	E203K
UN		1	1	1	1	1	0.11	0.0029	0.00066	6.05E-06	5.20E-10	1.42E-11	5.00E-16	5.48E-12	8.87E-22	2.22E-22	8.70E-53	4.89E-55
WT			1	1	1	1	0.17	0.0049	0.0011	1.27E-05	1.34E-09	5.09E-11	2.40E-15	1.90E-11	5.31E-21	1.36E-21	3.93E-51	2.14E-53
E44G				1	1	1	0.06	0.0016	0.00038	2.54E-06	1.66E-10	2.88E-12	6.73E-17	1.16E-12	8.95E-23	2.17E-23	4.80E-55	2.86E-57
G128N					1	1	1	0.61	0.12	0.0073	2.97E-06	4.96E-07	9.11E-11	1.71E-07	5.32E-16	1.50E-16	1.34E-43	7.03E-46
T55P						1	1	1	1	0.30	0.00036	0.00014	7.53E-08	4.95E-05	9.97E-13	3.03E-13	1.58E-38	8.23E-40
Y130N							1	1	1	0.42	0.00055	0.00023	1.19E-07	7.74E-05	1.53E-12	4.64E-13	1.32E-38	6.89E-41
Q164K								1	1	0.97	0.00097	0.00027	5.91E-08	9.19E-05	2.87E-13	7.84E-14	7.84E-44	4.71E-46
L42F									1	1	0.76	1	0.0091	0.46	1.83E-06	7.17E-07	2.99E-26	1.91E-28
E203Q										1	1	1	1	1	0.0031	0.0016	6.99E-17	9.33E-19
D67N											1	1	0.54	1	0.00038	0.00016	3.20E-22	2.23E-24
Y130C												1	1	1	1	0.76	1.85E-12	2.74E-14
P124L													1	1	0.05	0.02	1.27E-19	8.27E-22
Y130H														1	1	1	2.69E-13	2.62E-15
P124Q															0.29	0.15	1.27E-16	1.03E-18
F53S																1	1.62E-05	4.86E-07
G128V																	5.61E-05	1.89E-06
F53L																		1
E203K																		

Table S2. Pairwise comparisons of mutation strength by zebrafish oval shape. The Kruskal-Wallis test with the Bonferroni correction was used for statistical analysis of the data in Figure 1B: gray - not significant, orange - $P < 0.10$, yellow - $P < 0.01$, green - $P < 0.001$. Here, $N_{Uninjected} = 83$, $N_{WT} = 80$, $N_{E44G} = 88$, $N_{G128N} = 85$, $N_{T55P} = 90$, $N_{Y130N} = 94$, $N_{Q164K} = 144$, $N_{L42F} = 83$, $N_{E203Q} = 55$, $N_{D67N} = 92$, $N_{Y130C} = 74$, $N_{P124L} = 135$, $N_{Y130H} = 125$, $N_{P124Q} = 115$, $N_{F53S} = 93$, $N_{G128V} = 91$, $N_{F53L} = 109$, $N_{E203K} = 97$.

	UN	WT	E44G	G128N	T55P	Y130N	Q164K	L42F	E203Q	D67N	Y130C	P124L	Y130H	P124Q	F53S	G128V	F53L	E203K
UN		1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.27	0.13	0.058
WT			1	1	1	1	1	1	1	1	1	1	1	1	1	0.27	0.13	0.058
E44G				1	1	1	1	1	1	1	1	1	1	1	1	0.47	0.23	0.10
G128N					1	1	1	1	1	1	1	1	1	1	1	1	1	1
T55P						1	1	1	1	1	1	1	1	1	1	0.58	0.28	0.14
Y130N							1	1	1	1	1	1	1	1	1	1	1	1
Q164K								1	1	1	1	1	1	1	1	1	1	1
L42F									1	1	1	1	1	1	1	1	1	0.72
E203Q										1	1	1	1	1	1	1	1	1
D67N											1	1	1	1	1	1	1	1
Y130C												1	1	1	1	1	1	1
P124L													1	1	1	1	1	1
Y130H														1	1	1	1	1
P124Q															1	1	1	1
F53S																1	1	1
G128V																	1	1
F53L																		1
E203K																		

Table S3. Pairwise comparisons of mutation strength by zebrafish lethality. The Kruskal-Wallis test with the Bonferroni correction was used for statistical analysis of the data in Figure 1C: gray - not significant, orange - $P < 0.10$, yellow - $P < 0.01$, green - $P < 0.001$. Here, $N_{Uninjected} = 83$, $N_{WT} = 80$, $N_{E44G} = 88$, $N_{G128N} = 85$, $N_{T55P} = 90$, $N_{Y130N} = 94$, $N_{Q164K} = 144$, $N_{L42F} = 83$, $N_{E203Q} = 55$, $N_{D67N} = 92$, $N_{Y130C} = 74$, $N_{P124L} = 135$, $N_{Y130H} = 125$, $N_{P124Q} = 115$, $N_{F53S} = 93$, $N_{G128V} = 91$, $N_{F53L} = 109$, $N_{E203K} = 97$.

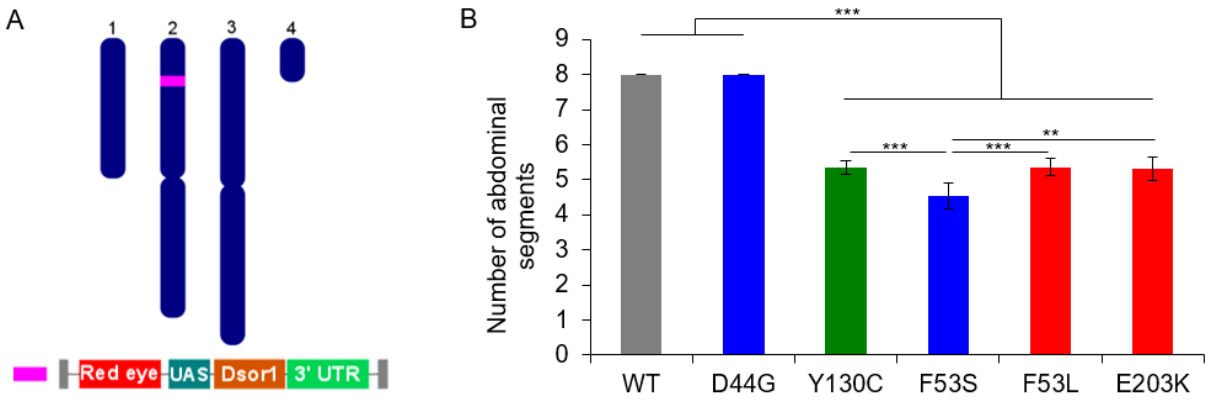

Figure S4. Transgenic *Drosophila* creation and cuticle quantification. (A) Creation of transgenic *Drosophila* using site-directed insertions. (B) Quantification of cuticle abdominal segments in transgenic *Drosophila*. The average of the number of abdominal segments (dead embryos) across the three classes of mutations is plotted, with the s.e.m. indicated. RASopathies (blue), RASopathies and cancer (green), and cancer only (red). Here, N values are as follows: N_{WT} = 20; N_{D44G} = 21; N_{Y130C} = 102; N_{F53S} = 52; N_{F53L} = 103; N_{E203K} = 68. One-way analysis of variance (ANOVA) with Bonferroni correction was used for statistical analysis: *P < 0.10, **P < 0.01, ***P < 0.001. The differences of the following pairs were not statistically significant: WT, D44G; Y130C, F53L; Y130C, E203K; F53L, E203K.

	0 µM	0.25 µM	0.5 µM	1 µM	5 µM	10 µM
F53L & Mock	1.80E-15	1.03E-05	4.06E-09	9.04E-12	8.15E-05	5.41E-03
F53L & G128V	1.35E-08	6.81E-03	2.04E-05	4.52E-13	7.88E-03	1
F53L & E203Q	4.78E-14	0.000158	3.25E-09	9.95E-11	0.0002267	0.1586341
G128V & Mock	0.0007438	0.2131801	0.0188466	1	1	0.0107679
G128V & E203Q	8.78E-03	0.976422	0.0220637	1	1	0.2401772
E203Q & Mock	1	1	1	1	1	0.9518654

Table S4. Pairwise comparisons of zebrafish oval shapes at different MEK inhibitor doses. One-way analysis of variance (ANOVA) with Bonferroni correction was used for statistical analysis of the data in Figure 5B: gray - not significant, orange - $P < 0.10$, green - $P < 0.01$. For each set of embryos treated with a MEK inhibitor dose, there was a control set of embryos treated with just DMSO. Here, the 0 µM dose is a pooled average of the control set(s) of embryos. $N_{Mock,\ 0} = 36$, $N_{E203Q,\ 0} = 40$, $N_{G128V,\ 0} = 78$, $N_{F53L,\ 0} = 70$; $N_{Mock,\ 0.25} = 17$, $N_{E203Q,\ 0.25} = 16$, $N_{G128V,\ 0.25} = 21$, $N_{F53L,\ 0.25} = 16$; $N_{Mock,\ 0.5} = 17$, $N_{E203Q,\ 0.5} = 19$, $N_{G128V,\ 0.5} = 33$, $N_{F53L,\ 0.5} = 17$; $N_{Mock,\ 1} = 17$, $N_{E203Q,\ 1} = 16$, $N_{G128V,\ 1} = 35$, $N_{F53L,\ 1} = 16$; $N_{Mock,\ 5} = 17$, $N_{E203Q,\ 5} = 22$, $N_{G128V,\ 5} = 15$, $N_{F53L,\ 5} = 20$; $N_{Mock,\ 10} = 18$, $N_{E203Q,\ 10} = 23$, $N_{G128V,\ 10} = 14$, $N_{F53L,\ 10} = 17$.

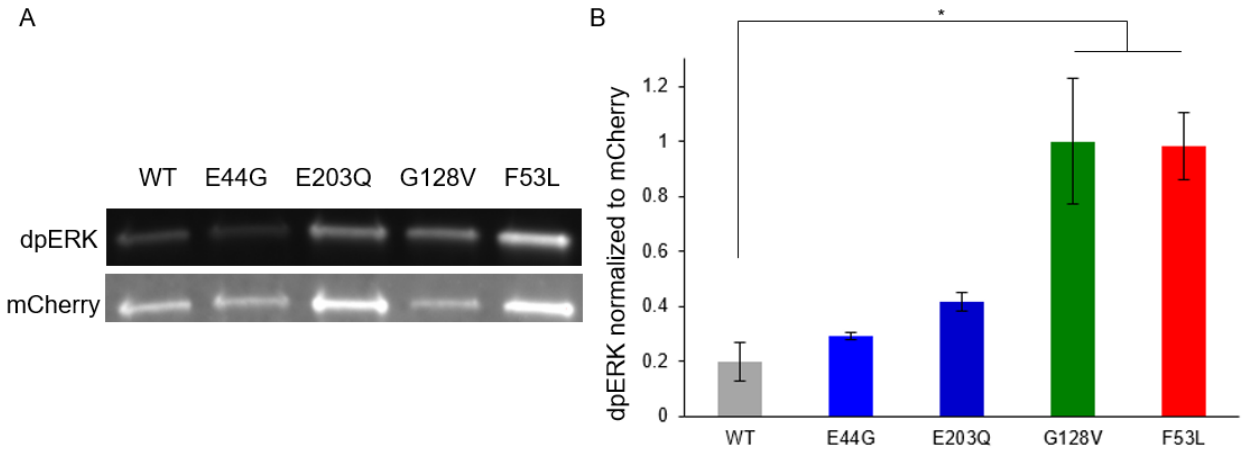

Figure S5. dpERK levels in zebrafish across the three classes of mutations. (A) A sample Western blot showing the dpERK channel and the mCherry channel, which detects MEK1-mCherry. (B) The average of the ratios of dpERK fluorescence to mCherry fluorescence for 4 mutations and MEK-WT across the three classes of mutations is plotted, with the s.e.m. indicated. The ratios were normalized so that the highest ratio for a Western blot was 1. Statistically significant differences were observed for the following pairs: MEK-WT/MEK-G128V and MEK-WT/MEK-F53L. RASopathies (blue), RASopathies and cancer (green), and cancer only (red). Here, each value is an average from 3 Western blots, except for E44G, which is an average from 2. One-way analysis of variance (ANOVA) with Bonferroni correction was used for statistical analysis: *P < 0.10, **P < 0.01, ***P < 0.001.

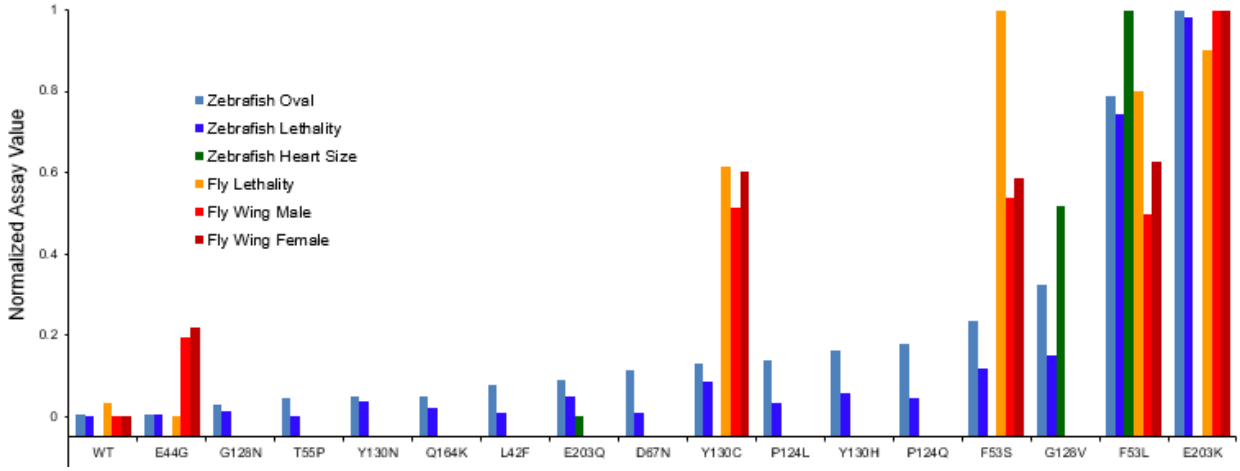

Figure S6. Meta-analysis of the strength of mutations across cellular contexts in zebrafish and *Drosophila*. The metrics used to quantify the phenotypes, normalized to have values from 0 to 1, are graphed together. While the ranking is largely consistent, the response varies with cellular context.

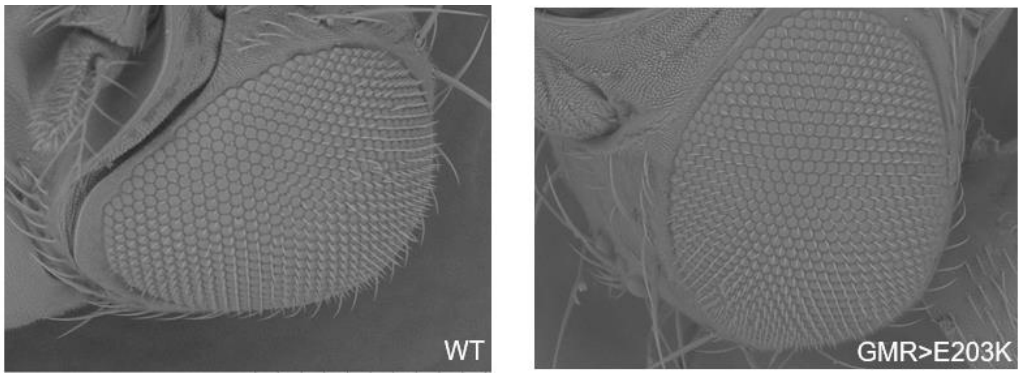

Figure S7. Eye phenotype of transgenic *Drosophila*. Images of eye phenotype in wild-type flies and those with overexpressed Dsor1-E203K using an eye-specific driver (GMR-Gal4). Eye development is not affected, consistent with reports for GOF Csw (the *Drosophila* ortholog of Shp2) mutations.

	Rodriguez-Viciana et al., Science 2006		Dentici et al., EJHG 2009		
Number of phenotypes examined	14		46		
Mutation	F53S	Y130C	L42F	Y130C	Y130C
Number of phenotypes observed	13	9	22	22	26
Percentage of phenotypes observed	92.86	64.29	47.83	47.83	56.52
Average percentage of phenotypes	92.86	64.29	47.83	52.17	

Table S5. Compilation of phenotypic analysis of individuals with MEK1 mutations over the age of 5 from the literature. Judging from the percentage of phenotypes assayed in each study, the individual with the F53S mutation had a higher percentage of phenotypes than the individual with Y130C, and the averaged percentage of phenotypes of individuals with Y130C mutations was higher than that of the individual with the L42F mutation.