Abrogation of MAP4K4 protein function causes congenital anomalies in humans and zebrafish

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HUMAN GENETICS

Abrogation of MAP4K4 protein function causes congenital anomalies in humans and zebrafish


We report 21 families displaying neurodevelopmental differences and multiple congenital anomalies while bearing a series of rare variants in mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4). MAP4K4 has been implicated in many signaling pathways including c-Jun N-terminal and RAS kinases and is currently under investigation as a druggable target for multiple disorders. Using several zebrafish models, we demonstrate that these human variants are either loss-of-function or dominant-negative alleles and show that decreasing Map4k4 activity causes developmental defects. Furthermore, MAP4K4 can repress hyperactive RAS signaling in early embryonic stages. Together, our data demonstrate that MAP4K4 negatively regulates RAS signaling in the early embryo and that variants identified in affected humans abrogate its function, establishing MAP4K4 as a causal locus for individuals with syndromic neurodevelopmental differences.

INTRODUCTION

The RAS signaling pathway is one of many signaling cascades used throughout development to sculpt the growing embryo. The RAS pathway is activated by ligand binding to a receptor tyrosine kinase (RTK), which ultimately leads to a signaling cascade involving the sequential phosphorylation of RAS, rapidly accelerated fibrosarcoma, mitogen-activated protein kinase (MAPK) kinase (MEK) and extracellular signal–regulated kinase (ERK). Many proteins are directly involved in the transduction of signals, and many more act to positively or negatively modulate pathway activity (1).

While RAS signaling helps direct cell survival, growth, and differentiation in the healthy state, aberrations in signaling can cause disease. Loss of signaling is embryonically lethal, but variants in RAS, or other pathway components, that lead to hyperactive RAS signaling are a common cause of cancer (2, 3). These activating variants generally arise in somatic cells, but when such variants are inherited through the germ line, they cause a group of developmental syndromes collectively known as RASopathies (4, 5). Although each RASopathy is clinically distinct, there is substantial overlap in the symptoms observed in affected individuals (6, 7). Particularly common are craniofacial anomalies (CFAs), congenital heart defects (CHDs), developmental delay (DD), short stature, and a predisposition to developing cancer. Although some RASopathy syndromes are rare, others are more common and collectively RASopathies occur in one of 1000 births (5).

Many RASopathy-associated genes have been identified, and the precise pathway disruption dictates the clinical presentation of the disease (6, 7). For example, disease-causing variants in MEK cause cardiofaciocutaneous syndrome, while disease-causing variants in NRAS, RAF1, and others are associated with Noonan syndrome. Still, many affected individuals lack a molecular diagnosis following exome or genome sequencing, and many presumably carry variants in genes not previously associated with human disease. Identifying and validating pathogenic variants in candidate genes causing RASopathy phenotypes in a multicenter cohort provides an opportunity to discover regulators of RAS signaling while potentially providing targets for therapeutic intervention in both the cases under study, and larger RAS-related conditions.

MAPK kinase kinase kinase 4 (MAP4K4) encodes a Ste20 family serine/threonine protein kinase, which contains a kinase domain, an interdomain, and a citrulline homology (CNH) domain (8, 9). The interdomain and CNH domain mediate binding to interaction partners, with the CNH domain having an additional role in regulating kinase activity (10, 11). MAP4K4 is expressed in all tissues, although expression is highest in brain and testes, and five isoforms have been isolated (12). Loss of MAP4K4 disrupts embryonic development (13–16), whereas elevated protein levels have been implicated in diseases including cancer, atherosclerosis, and diabetes (12, 17–28). While MAP4K4 has been implicated in several signaling pathways, the protein has been reported to have a role in regulating RAS signaling (13, 29–31).

Here, we report a cohort of 26 affected individuals from 21 unrelated families with neurodevelopmental differences, cardiac issues, and CFAs who share a phenotype overlap with RASopathies and harbor a series of rare variants in MAP4K4. We use a zebrafish model to demonstrate that these variants reduce MAP4K4 function.
Some of the variants work through either a loss of function (LOF) or dominant negative (DN) in nature. We show that increasing or decreasing MAP4K4 activity causes similar developmental defects in zebrafish embryos. Last, we demonstrate that MAP4K4 can restrain hyperactive RAS signaling in early zebrafish embryos. Together, our data demonstrate that MAP4K4 is a negative regulator of RAS signaling in the context of the early embryo and that variants identified in affected humans abrogate its function, releasing inhibition of RAS signaling. This work establishes an association of variants in MAP4K4 with features that overlap other RASopathies.

RESULTS

Individuals with rare variants in MAP4K4 display neurodevelopmental differences and congenital anomalies

Exome sequencing of an affected female diagnosed with Noonan-like syndrome identified a c.2591T>A;p.Leu864Ter variant in MAP4K4 (GenBank identifier: NM_001242559.1), which was absent from ~123,350 individuals in the genome aggregation database [The Genome Aggregation Database (gnomAD) v2.1.1] and had a probability of LOF intolerance of 1 and missense z score of 4.74. The affected individual displayed dysmorphic craniofacial features, limb abnormalities, pulmonary artery stenosis, intellectual disability (ID) and DD, and behavioral disorders (table S1). Sequencing of family members revealed that the variant was present in the proband’s mother and sibling. Both mother and sibling presented with mild ID/DD, and the sibling displayed CFA. There were no other MAP4K4 variants of interest identified in the remaining affected individuals in this original discovery cohort.

A LOF de novo variant in MAP4K4 (p.Gln1157Ter) was previously reported in a fetus with a complex CHD and congenital abnormalities of the kidneys and urinary tract. The functional impact of this change was not assayd, but the locus was considered a candidate disease-causing gene (32). Through international collaboration, including via GeneMatcher (33), we identified 26 individuals from 21 unrelated families with heterozygous variants in MAP4K4 (Table 1 and table S1). Two missense variants (p.Gly173Asp and p.Arg152Trp) are recurrent and have arisen de novo in two families each, yielding an allelic series of 19 variants; eight truncating, nine missense, and two intronic splice site variants (Fig. 1, A and B). These variants were not detected in gnomAD, and either arose de novo, or were inherited from an affected parent. Some parents were unavailable for testing to determine inheritance. Individuals harboring MAP4K4 variants display a high incidence of DD (80%), ID (42%), and short stature (40%). Variable symptoms include CFA (up to 29%), CHD (27%), hypotonia (25%), limb abnormalities (29%), microgastria (29%), posteriorly rotated ears (29%), attention deficit disorder (30%), central nervous system structural defects (31%), and fetal finger pads (19%) (Fig. 2 and Table 1). Although the clinical presentation of affected individuals is varied, most display symptoms of neurodevelopmental conditions with a constellation of features overlapping those observed in patients with RASopathies. These data are consistent with the previously reported role for MAP4K4 in regulating RAS signaling.

Truncation of MAP4K4 abrogates protein function

To determine the effect of MAP4K4 variants on protein function, we used zebrafish to conduct rapid screening. We generated an expression construct encoding human MAP4K4 and performed site-directed mutagenesis to introduce seven randomly selected variants identified in cases; five truncating and two missense variants. The truncation of MAP4K4 abrogates protein function (Fig. 1A).

Zebrafish embryos were injected at the one-cell stage with mRNA encoding either wild-type (WT) or variant MAP4K4 at equivalent doses across conditions. MAP4K4 expression causes a range of developmental defects by 3 days post fertilization (dpf). Larvae had a shorter body length compared to vehicle-injected controls and displayed CFA, cardiac edema indicative of heart defects, hydrocephaly, small or malformed eyes, yolk extension defects, and curved body axes (Fig. 3A). These phenotypes occurred in any and all combinations. Notably, ectopic human MAP4K4 mRNA is introduced to a WT background, in which endogenous MAP4K4 is expressed starting from zygotic stages and continuing...
Table 1. Clinical features in 27 individuals with heterozygous MAP4K4 variants. The most common features are developmental delay (DD), intellectual disability (ID), attention deficit disorder, abnormal brain magnetic resonance imaging (MRI), and short stature. M, male; F, female; Unk, unknown.

<table>
<thead>
<tr>
<th>Individual gender</th>
<th>13 M/12 F/1 Unk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range</td>
<td>0–40 years</td>
</tr>
<tr>
<td>Clinical findings:</td>
<td></td>
</tr>
<tr>
<td>Short stature</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>Frontal bossing</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Cranial abnormality</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Ocular hypertelorism</td>
<td>3/21 (14%)</td>
</tr>
<tr>
<td>Arched eyebrows</td>
<td>3/21 (14%)</td>
</tr>
<tr>
<td>High/broad nasal bridge</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Antverted nares</td>
<td>5/21 (24%)</td>
</tr>
<tr>
<td>Long philtrum</td>
<td>6/21 (29%)</td>
</tr>
<tr>
<td>Micrognathia</td>
<td>6/21 (29%)</td>
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<tr>
<td>Down-slanted palpebral fissures</td>
<td>6/21 (29%)</td>
</tr>
<tr>
<td>Median nose tip groove</td>
<td>2/21 (10%)</td>
</tr>
<tr>
<td>Posteriorly rotated ears</td>
<td>6/21 (29%)</td>
</tr>
<tr>
<td>Preauricular pits/tags</td>
<td>2/21 (10%)</td>
</tr>
<tr>
<td>Cleft lip</td>
<td></td>
</tr>
<tr>
<td>Cleft palate</td>
<td>1/21 (5%)</td>
</tr>
<tr>
<td>Webbed palate</td>
<td>1/21 (5%)</td>
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<tr>
<td>Limb abnormalities</td>
<td>6/21 (29%)</td>
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<tr>
<td>Wide nipples</td>
<td>6/21 (27%)</td>
</tr>
<tr>
<td>Pectus excavatum</td>
<td>3/21 (14%)</td>
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<tr>
<td>Hemangiomas</td>
<td>2/21 (10%)</td>
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<tr>
<td>Congenital heart defect</td>
<td>6/22 (27%)</td>
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<tr>
<td>Umbilical hernia/omphalocele</td>
<td>1/21 (5%)</td>
</tr>
<tr>
<td>Anorectal malformations</td>
<td>1/21 (5%)</td>
</tr>
<tr>
<td>Small broad hands</td>
<td>3/21 (14%)</td>
</tr>
<tr>
<td>Clinodactyly</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Fetal finger pads</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Renal anomaly</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>Abnormal brain MRI</td>
<td>5/16 (31%)</td>
</tr>
<tr>
<td>ID</td>
<td>8/21 (42%)</td>
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<tr>
<td>Age first sitting</td>
<td>Range: 6 months to 12 years</td>
</tr>
<tr>
<td>Age first walking</td>
<td>Range: 10 months to 3 years</td>
</tr>
<tr>
<td>Age first words</td>
<td>Range: 12 months to 5 years</td>
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<tr>
<td>Autism</td>
<td>3/18 (17%)</td>
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<tr>
<td>Attention deficit disorder</td>
<td>6/20 (30%)</td>
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<tr>
<td>Seizures</td>
<td>6/22 (27%)</td>
</tr>
<tr>
<td>Hypotonia</td>
<td>5/20 (25%)</td>
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</table>

At least through larval day 5 (Expression Atlas: www.ebi.ac.uk/gxa/experiments/E-ERAD-475). Hence, this assay does not recapitulate affected human phenotype; rather, it allows assessment of the effect of WT protein on development for comparison to variant protein.

We assessed the impact of MAP4K4 variants on morphology. An equivalent dose of mRNA bearing case-associated variants affected the same tissues as MAP4K4 WT, albeit with varying severity (Fig. 3A). Larvae were scored for body defects (Fig. 3B), including truncations, and lateral or dorsoventral curvatures. All variants except MAP4K4P232X significantly increased the incidence of body defects, with the greatest effects observed for MAP4K4R152W and the least effect measured for MAP4K4L889X. A similar trend was observed for the other three classes of defects; eye defects, including small eyes and malformations such as coloboma; heart defects, evidenced by pericardial edema; and yolk defects, observed as anteroposterior shortening or dorsoventral thickening of the yolk extension or absence of the yolk constriction (Fig. 3, C to E). We also measured body length (Fig. 3F) and detected a similar trend, with some variants having little effect on body length, while others greatly reduced the length of embryos, as does ectopic expression of MAP4K4 WT.

A subset of larvae expressing MAP4K4 variants appeared to have CFA at 3 dpf, with the eyes appearing more anteriorly positioned compared to controls. Because CFA was observed in our human cohort with MAP4K4 variants, we next examined craniofacial development at 5 dpf, using Alcian blue stain to visualize the cartilage (Fig. 4A). We measured the distance between the Meckel’s and ceratohyal cartilages at the midline as a proxy for jaw length. Expression of MAP4K4 WT reduced the length of the jaw (Fig. 4B), as did most variants, although to varying extents. The incidence of CFA was also increased, with expression of MAP4K4 variants causing misshapen or misplaced cartilages (Fig. 4C). In extreme cases, the cartilage developed bilaterally instead of medially, possibly due to aberrant neural crest cell migration.

For all the developmental structures assayed, the five truncation variants had the least effect upon development. The missense variants, MAP4K4R152W and MAP4K4G173D, were the most disruptive. Because MAP4K4 WT disrupted larval development, this indicates that the truncation variants reduce MAP4K4 function, such that it no longer hinders development in the same way as ectopic expression of MAP4K4 WT does. While this finding supports the fact that loss of MAP4K4 function underlies disease in cases with frameshift variants, it does not explain how missense variants may lead to disease in the remainder of our cohort.

**Missense variants have DN effects**

To address whether missense variants may confer a DN effect, we examined the effect of introducing a known DN version of the protein, MAP4K4D153N (Figs. 3 and 4). This variant is a kinase-dead variant that is capable of interfering with the endogenously expressed Map4k4 protein, effectively knocking down protein function (10). Expression of MAP4K4D153N caused an increase in embryonic abnormalities of all classes and reduced the length of both body and jaw. In most cases, the measured effect is similar to or greater than that of expressing MAP4K4 WT, suggesting that decreasing Map4k4 function has a similar impact to increasing it by supplying exogenous MAP4K4. We therefore hypothesized that a subset of the missense variants that we observed to have
similar effects to MAP4K4\textsuperscript{WT} in our assay may represent DN hypomorphic alleles.

Because our patient cohort was composed of individuals displaying overlap with the clinical features of RASopathy syndromes, we expect that disease etiology will arise from modulation of the RAS signaling pathway. Previous work has demonstrated that modulating the RAS pathway in zebrafish affects the aspect ratio of embryos at 11 hours post fertilization (hpf) (35–44). Pathway activation by expressing hyperactive RASopathy-associated MEK or BRAF variants causes embryo elongation, while pharmacological inhibition of MEK shortens the embryo (35). Furthermore, the extent of elongation or shortening correlates with the strength of pathway activation (44). We predicted that if MAP4K4 modulates RAS signaling, then this assay would allow us to distinguish between four allele types; compared to MAP4K4\textsuperscript{WT}, DN variants should have an opposing effect on the aspect ratio, LOF variants should have no effect, hypomorphic alleles should have a similar effect but to a lesser degree, and gain-of-function variants should cause an effect greater than MAP4K4\textsuperscript{WT}.

Overexpression of MAP4K4\textsuperscript{WT} significantly reduced the aspect ratio at 11 hpf (Fig. 5A). This indicates that the signaling pathway is inhibited upon increasing the amount of functional protein. Introducing MAP4K4\textsuperscript{D153N} leads to elongation of the embryo, consistent with hyperactivation of the signaling pathway as a result of losing Map4k4 function. Together, these data demonstrate that MAP4K4 acts as a negative regulator of RAS signaling in this context.

We next assessed the impact of variants on the aspect ratio. Ectopic expression of variant MAP4K4 mRNA either had little effect or caused embryo elongation (Fig. 5A), allowing us to group variants as either DN or LOF. DN variants—MAP4K4\textsuperscript{R152W}, MAP4K4\textsuperscript{G173D}, and MAP4K4\textsuperscript{V1033X}—increased the aspect ratio, although not as strongly as the MAP4K4\textsuperscript{D153N} variant. The remaining variants—MAP4K4\textsuperscript{S17X}, MAP4K4\textsuperscript{P232X}, MAP4K4\textsuperscript{L889X}, and MAP4K4\textsuperscript{V1093X}—had no effect on the aspect ratio. These variants were designated as LOF, since they neither exerted an additive effect like combining MAP4K4\textsuperscript{WT} and endogenous Map4k4 nor interfered with the function of endogenous Map4k4, as does MAP4K4\textsuperscript{D153N}. Thus, all the variants detected in patients reduce protein function, with a subset capable of further interfering with the function of WT alleles.

We also tested a variant that is not present in humans, MAP4K4\textsuperscript{I841S}, but resulted from plasmid mutagenesis. This variant retained enough function to reduce the aspect ratio compared to vehicle-injected controls, but not to the same extent as MAP4K4\textsuperscript{WT}. Thus, we concluded that p.Ile841Ser is a hypomorphic allele. Although not directly relevant to our human cohort, this serendipitous result demonstrates that we can distinguish between hypomorphic and LOF alleles with this assay. The expression of hypomorphic MAP4K4 represents an intermediate state between overexpression of WT and LOF variants, and analysis of the later
phenotypes show a phenotype that is similar to but weaker than that caused by overexpression of MAP4K4<sub>WT</sub> (Figs. 3 and 4). This supports our earlier conclusion that either increasing or decreasing endogenous protein function disrupts development similarly, with LOF alleles producing modest phenotypic consequences.

**Both increasing and decreasing MAP4K4 function causes similar phenotypes**

To compare the phenotypic output induced by ectopic expression of MAP4K4 across variants, we performed hierarchical clustering (Fig. 5B). Clustering analysis ranked the DN MAP4K4<sup>D153N</sup> and MAP4K4<sup>R152W</sup> and the hypomorphic MAP4K4<sup>I815S</sup> as being more similar to MAP4K4<sub>WT</sub> than to the weaker DN variants or LOF alleles. Thus, these aggregate data suggest that either increasing or decreasing the amount of functional MAP4K4 leads to similar phenotypic output in a developmental context.

Consistent with this hypothesis, treating zebrafish embryos with the MAP4K4 inhibitor PF06260933 to deplete endogenous Map4k4 caused developmental defects in a dose-responsive manner. Larvae exhibited cardiac edema, small eyes, and yolk malformations at 3 dpf as well as CFA at 5 dpf (Fig. 6, A and B). The incidence of abnormal embryos is increased upon exposure to higher doses of inhibitor (Fig. 6C), while both body and jaw lengths are shortened by
drug treatment (Fig. 6, D and E). These phenotypes are highly reminiscent of those observed for embryos that overexpress MAP4K4.

To corroborate the observations from MAP4K4 pharmacological inhibition, we performed ablation of the endogenous map4k4 transcript. We obtained a splice blocking morpholino (MO) targeting the exon 13 donor site, validated exon skipping by reverse transcription polymerase chain reaction (RT-PCR) of embryos injected with either 6 or 9 ng of MO, and performed cloning and sequencing to confirm the excision of 73 base pairs to induce a frameshift and premature truncation (fig. S1, A to C). Next, we assessed the mandibular phenotype of larvae injected with increasing doses of MO at 3 dpf using live automated imaging of the -1.4col1a1:egfp transgene (45). Consistent with our observations with the MAP4K4 inhibitor compound, we noted a dose-dependent exacerbation of CFA, as indicated by broadening of the ceratohyal angle and shortening of the mandible, while body length was decreased significantly only at the...
highest dose (Fig. 7, A to C; and fig. S1, D to H). Furthermore, we injected MO in cmlc2:gfp cardiac reporter (46) embryos and noted a significant increase at atrium and ventricle chamber size at maximum diastole at 2 dpf, a direct proxy for cardiac defects observed in human cases. Both the cardiac chamber size and jaw phenotypes were rescued significantly by coinjection of MAP4K4WT mRNA (Fig. 7).

**MAP4K4 is a negative regulator of RAS signaling in early zebrafish embryo**

Loss of endogenous Map4k4 function affects similar tissues to those affected in affected individuals, supporting the model that loss of MAP4K4 function causes RASopathy-like disease. Accordingly, MAP4K4 acts as a negative regulator of RAS signaling in the early zebrafish embryo (Fig. 5A). To confirm this, we tested the ability of MAP4K4WT to restrain hyperactive RAS signaling, which can be induced by ectopic expression of MEK1F53L mRNA (44), a cancer-associated variant (Fig. 8A). Increased signaling then causes embryo elongation at 11 hpf. Coexpression of MAP4K4WT rescues this morphological defect significantly, but by contrast, the LOF variant MAP4K4L889X only partially ameliorates the aspect ratio elongation. Thus, increasing the levels of MAP4K4 in the embryo can counter the morphological effects of increasing RAS pathway activity, consistent with a role as a negative pathway regulator.

We also tested whether the embryo elongation observed upon expression of MAP4K4D153N is caused by hyperactive RAS signaling. We treated injected embryos with either PD0325901, a MEK inhibitor, or SU5402, a fibroblast growth factor receptor (FGFR) inhibitor (Fig. 8B). Treatment with either drug reduced the aspect ratio of vehicle-injected embryos and those expressing MAP4K4D153N.
Together, these data suggest that embryo elongation in the context of MAP4K4 ablation is mediated through elevated RAS signaling.

We further tested this hypothesis that MAP4K4 affects RAS signaling at the molecular level. Embryos were injected with MAP4K4WT, and protein was extracted at 50% epiboly, a time point at which we have previously shown that hyperactivation of the RAS pathway causes increased levels of dually phosphorylated ERK (dpERK) at the blastoderm margin. We performed Western blotting, quantified band intensity using ImageJ, and normalized the level of dpERK to the amount of total ERK (Fig. 8C).

Overexpression of MEKF53L, a variant of MEK associated with cancer and shown to increase RAS signaling, caused an increase in the amount of dpERK consistent with hyperactivation of the pathway. Conversely, overexpression of MAP4K4WT caused a statistically significant decrease in the amount of dpERK compared to uninjected siblings, suggesting that increasing MAP4K4 levels inhibits the RAS signaling pathway at the molecular level. This decrease is smaller than the extent of the change caused by overexpression of MEKF53L but is consistent with the fact that we observe a smaller effect on aspect ratio at 11 hpf for MAP4K4WT overexpression than observed for MEKF53L overexpression (Fig. 8B). This is likely due to the fact that MEKF53L is associated with cancer rather than RASopathies and cancer-associated mutations disrupt the pathway more severely. Thus, increasing the levels of MAP4K4 in the embryo can counter the morphological effects of increasing RAS pathway activity, consistent with a role as a negative pathway regulator.

***DISCUSSION***

Here, we report MAP4K4 as the cause of a previously undescribed syndrome with phenotypes overlapping with RASopathy syndromes. These heterozygous pathogenic variants are reported here in 26 individuals from 21 pedigrees. Individuals display overlapping clinical features affecting development of the brain, heart, and face. Affected individuals with rare variants in MAP4K4 should be clinically assessed, as they are at risk of DD, cardiac disease, renal disease, hearing loss, short stature, and neurobehavioral differences.

Functional studies in zebrafish showed that MAP4K4 variants caused hypomorphic, LOF, or DN effects. The missense variants identified in our cohort were mostly clustered within the kinase domain (Fig. 1A). Arg152 and Asp153 are both found within the arginine-glycine-aspartic acid (RGD) motif of the catalytic loop, while Gly173 is located within the activation loop, close to the adenosine 5′-triphosphate (ATP) binding region. The variants at these residues all acted as DN alleles, consistent with a reduction in kinase activity with no effect on protein interaction.

It is expected that the truncation variants would abolish protein function, but previous studies into the role of the CNH domain have been contradictory (10, 11). In our studies, truncating MAP4K4 at p.Ser17, p.Pro232, p.Leu864, or p.Val1068 all caused loss of protein
function. p.Val1093Ter contains the sequence for the full kinase domain and intradomain, and half of the CNH domain, while p.Ser17Ter contains no functional domains. This may indicate either that the truncated protein does not fold, no matter its length, or that the complete CNH domain is necessary for protein function.

A subset of identified variants disrupts the canonical splice site sequences (Fig. 1B), but specimens were unavailable for experimental testing of mRNA splicing. A splice acceptor site is disrupted by c.695-3del, and we predict that this will cause skipping of the subsequent exon. Loss of exon 9 would be predicted to cause a frameshift (p.Pro232ArgfsTer8), which we modeled using the p.Pro232Ter variant and showed to be a LOF variant. A splice donor site is disrupted by c.639+1G>C, and we predict that this variant will cause skipping of the preceding exon, exon 7, during splicing. Loss of exon 7 would be predicted to cause a frameshift of the protein (p.Leu169fsTer2), which would likely be a LOF allele.

By extrapolating from our functional data for a subset of variants, we can make predictions about the allelic series (Table 2). We cannot exclude the possibility of nonsense-mediated decay causing loss of the mRNA before translation in patients, such that the protein would never be expressed regardless of the predicted variant. Furthermore, we engineered the truncation variants to introduce stop codons instead of frameshifts. It is therefore possible that frameshift variants act slightly differently in patients than in our overexpression assays. However, our data suggest that all variants reduce protein function, even if they do so by reducing the amount of mRNA expressed.

MAP4K4 was previously shown to be required for embryonic development. In Drosophila, the MAP4K4 homolog misshapen is required for dorsal closure during embryogenesis (14). MO-mediated knockdown in zebrafish was reported to cause mild gastrulation defects, but these were not characterized (15). Complete loss of Map4k4 is embryonic lethal in mice, causing posterior truncation, while endothelial cell–specific deletion causes vascular defects and early postnatal lethality (13, 16). Deleting Map4k4 from adult mice, however, had little effect on tissue homeostasis (47).

The precise function of MAP4K4 in regulating the RAS pathway remains unclear. MAP4K4 was shown to associate with FGFR1, an RTK that signals via RAS, and to be required for mediating an FGF-induced signal (31). Furthermore, MAP4K4 can inhibit protein phosphatase 2A (PP2A), which itself can dephosphorylate ERK to inactivate signaling (30). Treating MAP4K4<sup>D153N</sup>-injected embryos with a MEK inhibitor prevents elongation of the embryonic axis, suggesting that MAP4K4 may inhibit RAS signaling upstream of MEK. This would exclude the possibility of MAP4K4 modulating the pathway via PP2A, which exerts its effects on ERK (30). Accordingly, the actions of MAP4K4 upon PP2A were reported to positively regulate the pathway, while we found that MAP4K4 was an inhibitor of signaling.

MAP4K4 has also been reported to be necessary to inhibit RAS signaling and maintain endothelial cell fate, potentially by activating Ras p21 protein activator 1 (RASA1), a guanosine triphosphatase (GTPase)–activating protein that promotes inactivation of RAS (13). Because RASA1 is upstream of MEK and inhibits signaling by enhancing the GTPase activity of RAS, it represents a plausible candidate for affecting the role of MAP4K4 in the pathway. RASA1 was shown to interact with MAP4K4 to maintain endothelial fate in mouse by inhibiting signaling, although it has roles beyond
angiogenesis (13). Furthermore, RASA1 is involved in the pathogenesis of diverse conditions, including CHD and cancer (48), while MAP4K4 has also been implicated in cancer (12, 17, 20, 25, 26), and we link it to CHD in our work. Thus, the effects of MAP4K4 on RAS signaling may be mediated through RASA1, although it is possible that additional targets are involved. MAP4K4 is also known to act through c-Jun N-terminal signaling, which would be of interest to test in further experiments.

MAP4K4 is likely haplosensitive. This is consistent with our finding that the levels of MAP4K4 must be maintained within a window of activity to promote proper embryonic development. This conclusion may be explained by the fact that variants that
Increase RAS signaling can ultimately lead to reduced pathway activity (42). Considering the similarity of the phenotypic consequences of increasing and decreasing MAP4K4 function, it is possible that many of the developmental defects observed here are caused by down-regulation of signaling. If that is the case, then increasing WT MAP4K4 levels would cause inhibition of signaling, while reducing protein function by supplying DN MAP4K4 would initially lead to activation of the pathway, followed by down-regulation in response to negative feedback. The fact that elevated amounts of MAP4K4 are detected in conditions such as cancer, atherosclerosis, and diabetes and that reducing MAP4K4 activity can rescue the disease state lends further support to the critical importance of maintaining MAP4K4 at a suitable level (12, 19, 23, 26–28).

In summary, we have added MAP4K4 to the repertoire of genes that may be associated with RASopathy-like developmental disease. Targeted reduction of MAP4K4 activity has been touted as a possible therapeutic approach for diverse conditions (23, 27), yet we demonstrate that abrogation of MAP4K4 function is also associated with disease. The implications of our work, therefore, suggest that

<table>
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<tr>
<th>Variant</th>
<th>Location</th>
<th>Predicted impact on gene and protein</th>
<th>Allele type</th>
</tr>
</thead>
<tbody>
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<td>c.46-47dup p.Ser17ProfsTer55</td>
<td>N terminus</td>
<td>Frameshift: loss of kinase domain, interdomain, and CNH</td>
<td>LOF demonstrated</td>
</tr>
<tr>
<td>c.52delC p.Leu18CysfsTer53</td>
<td>N terminus</td>
<td>Frameshift: loss of kinase domain, interdomain, and CNH</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.116 T&gt;G p.Val39Gly</td>
<td>P loop, kinase domain</td>
<td>Loss of kinase activity: Val^{39} is critical for ATP binding within the P loop</td>
<td>DN predicted</td>
</tr>
<tr>
<td>c.328G&gt;A p.Ala110Thr</td>
<td>Kinase domain</td>
<td>Loss of kinase activity: not a known critical residue but lies close to ATP binding site and likely reduce kinase function</td>
<td>DN predicted</td>
</tr>
<tr>
<td>c.391G&gt;A p.Ala131Thr</td>
<td>Kinase domain</td>
<td>Loss of kinase activity: not a known critical residue but lies close to ATP binding site and likely reduce kinase function</td>
<td>DN predicted</td>
</tr>
<tr>
<td>c.454C&gt;T p.Arg152Trp</td>
<td>RGD motif, kinase domain</td>
<td>Loss of kinase activity: Arg^{152} is part of the RGD motif of the catalytic loop</td>
<td>DN demonstrated</td>
</tr>
<tr>
<td>c.639+1G&gt;C p.Leu169fsTer2</td>
<td>Splice donor site</td>
<td>Excision of exon 7 from the transcript. Frameshift: truncation of kinase domain, loss of interdomain and CNH</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.518G&gt;A p.Gly173Asp</td>
<td>Activation loop, kinase domain</td>
<td>Loss of kinase activity: Gly^{173} is the activation loop, close to the ATP binding region</td>
<td>DN demonstrated</td>
</tr>
<tr>
<td>c.560C&gt;G p.Thr187Arg</td>
<td>Activation loop, kinase domain</td>
<td>Loss of kinase activity: Thr^{187} lies in the activation loop</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.695-3delC p.Pro232LfsTer8</td>
<td>Splice acceptor site</td>
<td>Excision of exon 9 from transcript. Frameshift: truncation of the kinase domain, loss interdomain and CNH</td>
<td>LOF demonstrated</td>
</tr>
<tr>
<td>c.736C&gt;T p.Pro246Ser</td>
<td>Kinase domain</td>
<td>Loss of kinase activity: Pro^{246} is not a known critical residue, but variants likely reduce kinase function</td>
<td>DN predicted</td>
</tr>
<tr>
<td>c.1489C&gt;T p.Arg497Ter</td>
<td>Interdomain</td>
<td>Premature stop codon: truncation of the interdomain and loss of CNH</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.2426T&gt;G p.Val864Ter</td>
<td>Interdomain</td>
<td>Reduced function or disrupted regulation: The interdomain harbors protein interaction sites and phosphorylation sites</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.2591T&gt;A p.Leu864Ter</td>
<td>Interdomain</td>
<td>Premature stop codon: truncation of the interdomain, loss of CNH</td>
<td>LOF demonstrated</td>
</tr>
<tr>
<td>c.3023-42del p.Val1008GlyfsTer19</td>
<td>CNH domain</td>
<td>Frameshift: truncation of CNH. DN properties may suggest possible autoinhibitory region within CNH</td>
<td>DN demonstrated</td>
</tr>
<tr>
<td>c.3146C&gt;T p.Ala1049Val</td>
<td>CNH domain</td>
<td>Reduced function: CNH domain regulates kinase function and contains protein interaction sites that may be disrupted</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.3197-3200dup p.Val1068IlefsTer4</td>
<td>CNH domain</td>
<td>Frameshift: truncation of the CNH</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.3218-9del p.Tyr1147TrpfsTer61</td>
<td>CNH domain</td>
<td>Frameshift: truncation of the CNH</td>
<td>LOF predicted</td>
</tr>
<tr>
<td>c.3460C&gt;T p.Gln1157Ter</td>
<td>CNH domain</td>
<td>Premature stop codon: truncation of the CNH</td>
<td>LOF predicted</td>
</tr>
</tbody>
</table>
care must be taken not to reduce activity too far at the risk of exacerbating disease. Despite this caveat, MAP4K4 remains an attractive target for pharmacological modulation of RAS signaling in humans.

MATERIALS AND METHODS
This study was approved by the Institutional Review Board (IRB) of the Children’s Hospital of Philadelphia. Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included.

Exome and genome sequencing
Genomic DNA was extracted from whole blood from the affected children and their parents. The original patients were enrolled under an approved IRB from the Children’s Hospital of Philadelphia. Exome or genome sequencing was performed with a variety of standard capture kits and the general assertion criteria for variant classification following American College of Medical Genetics and Genomics, Association of Molecular Pathology (ACMG/AMP) guidelines. There were no other variants in these patients who survived filtration and analysis using either dominant or recessive models and could explain the phenotypes.

Zebrafish strains and husbandry
Zebrafish were maintained, and all experiments conducted according to Princeton University or Northwestern University Institutional Animal Care and Use Committee–approved protocols. The PWT strain was used for injection of mRNA: -1.4col1a1:gfp and cmnc2:gfp transgenic lines were used for mapk4 knockdown studies. Embryos were raised at 28°C until phenotypic end point.

Pharmacological treatment of zebrafish embryos
PF06260933 (Sigma-Aldrich) was added immediately following embryo collection to a final concentration of 1, 5, or 10 μM. Water was changed on the second day, with fresh drug applied. For inhibition of MEK, PD0325901 (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) was added to a final concentration of 1 μM, while the FGFR inhibitor SU5402 (Sigma-Aldrich) in DMSO was used at a concentration of 5 μM. These concentrations were previously shown to rescue embryo elongation caused by MEK. Control embryos were treated with an equivalent amount of DMSO. Both drugs were applied from 4.5 hpf until imaging.

Cloning and site-directed mutagenesis
pDONR223-MAp4K4 was a gift from W. Hahn and D. Root (Addgene, plasmid no. 23486). This construct contains human MAP4K4 transcript variant 13 (NM_001384485.1) with a deletion of six nucleotides (Δ2078–2083). The human MAP4K4 sequence was subcloned into pCS2+ and tagged with mCherry and a p2A termination sequence. Plasmids were transfected into 5-alpha competent cells (New England Biolabs) and purified using the QIAprep Spin Miniprep Kit (Qiagen) or the Quantum Prep Plasmid Miniprep Kit (Bio-Rad). Overlapping primers (Integrated DNA Technologies) were designed to contain base changes corresponding to variants identified in affected individuals, obtained from Integrated DNA Technologies, and used in a PCR to introduce changes to the plasmid sequence. Sequence changes were verified by Sanger sequencing (GeneWiz).

mRNA transcription and microinjection in zebrafish
mRNA was generated using the mMESSAGE mMACHINE Sp6 kit (Invitrogen) according to the manufacturer’s instructions and quantified using a NanoDrop One (Thermo Fisher Scientific). Embryos were injected as described (51) using a PV820 Pneumatic PicoPump (World Precision Instruments). Embryos at the one- to four-cell stages were injected with 150 pg of MAP4K4 mRNA and/or 55 pg of MEK mRNA suspended in 500 pl of phenol red (phenolsulfophthalein; 5 mg/ml)/0.2 M KCl. Vehicle-injected embryos received 500 pl of phenol red (5 mg/ml)/0.2 M KCl. For each variant, at least three clutches of at least 30 embryos each were injected, with mRNA generated on at least two separate occasions.

Transient suppression of mapk4 in zebrafish
A splice-blocking MO was designed to target the exon 13 splice donor site of Danio rerio mapk4 (ENSDART00000165230.2; e13113; Gene Tools). We injected 1 nl of MO at different doses (3, 6, and 9 ng) into one- to four-cell–staged zebrafish embryos. MO efficiency was validated by extracting total RNA from pools of 2 dpf embryos (30 animals per condition; controls and MO injected) using TRIzol (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA was synthesized with the QuantiTect RT kit (Qiagen). We performed RT-PCR of the MO target using flanking primers, and resulting PCR product was migrated on a 1% agarose gel. Bands were excised, and PCR product was purified with the QIAquick Gel Extraction Kit (Qiagen) and cloned in TOPO-TA vector (Thermo Fisher Scientific). Purified plasmid from resulting colonies (n = 4 per PCR product) were sequenced using BigDye 3.1 terminator chemistry according to standard protocols. Rescue experiments were carried out with 9 ng of mapk4 MO and 150 pg of WT human MAP4K4 mRNA.

Alcian blue staining for zebrafish craniofacial analysis
Larvae were euthanized at 5 dpf and fixed in 4% paraformaldehyde/ phosphate-buffered saline (PBS) overnight. Larvae were washed in PBS, dehydrated through an ethanol series to 70% EtOH, and incubated in 0.04% Alcian blue 8GX/50 mM MgCl2/70% EtOH overnight at 4°C. After several rinses in 70% EtOH, embryos were rehydrated through an ethanol series to 0.1% Tween 20/PBS, washed in 2% KOH, bleached in 1% H2O2/2% KOH, washed in 2% KOH, and then processed through a glycerol series to 80% glycerol/2% KOH for imaging.

Zebrafish imaging and analysis
mRNA-injected embryos were imaged using a Leica M205 FA stereoscope with a DFC365FX or DFC450C camera attachment. For imaging at 3 dpf, embryos were immobilized in tricaine (ethyl-3-amino benzoate methanesulfonate salt; 0.08 mg/ml; Sigma-Aldrich), which was removed after image acquisition. Phenotypic abnormalities were manually scored for each embryo, and prevalence was calculated as a percentage of all embryos injected with that condition.

MO-injected larvae were tricaine-anesthetized at 3 dpf, and craniofacial features were imaged using the Vertebrate Automated Screening Technology (VAST) BioImager (Union Biometrica) mounted to an Axio Imager.M2m microscope (Zeiss) with a 10× objective lens. Larvae were passed sequentially through a 600-mm capillary on the detection platform. Each larva was detected and oriented automatically for ventral [green fluorescent protein (GFP)] or

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lateral (brightfield) images with a preprovided template setting in the VAST software (version 1.2.6.7) operated in automatic imaging mode with a 70% minimum similarity threshold, as described (52). We captured fluorescent images from ventrally positioned larvae with an AxioCam 503 monochrome camera (Zeiss) and ZenPro software (Zeiss). For heart phenotyping, we captured GFP signal of the ventral aspect of live embryos with a Nikon AZ100 microscope facilitated by a Nikon camera controlled by Nikon NIS-Elements Software at 2 dpf. For aspect ratio phenotyping of gastrulating embryos, we captured brightfield images at 11 hpf with a Nikon SMZ745 stereoscope and Nikon digital sight camera.

ImageJ was used to measure the lengths of the major and minor axes of the yolk at 11 hpf, the length of the embryo at 3 dpf, the ceratohyal angle and the distance between the ceratohyal and Meckel’s cartilages at 3 and 5 dpf, and the area of heart chambers at 2 dpf. Measurements were performed with the investigator masked to experimental condition and normalized to the mean of the vehicle-injected control group to allow combination of embryos from multiple clutches. A one-way analysis of variance (ANOVA) with Bonferroni correction was performed to compare the whole group, while pairwise Student’s t tests were used to compare variant-injected embryos to vehicle-injected controls. Hierarchical clustering was performed using RStudio.

Protein extraction and Western blotting
A total of 100 injected embryos were incubated in pronase (1.3 mg/ml; Sigma-Aldrich) at 28°C for 5 min, and then chorions were removed by agitation at 50% epiboly. Embryos were rinsed three times in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄), then resuspended in 200 μl of E3 buffer, and homogenized by pipetting. One milliliter of 0.5x Ringer’s solution (50 mM NaCl, 10 mM KCl, and 1.25 μM NaHCO₃) was added, and samples were vortexed before cells were precipitated by centrifugation [300 relative centrifugal force (rcf), 1 min, 4°C]. Cells were resuspended in 50 μl of lysis buffer [20 mM tris (pH 8.0), 50 mM NaCl, 2 mM EDTA, 1% Igepal CA-630, and 200 μM phenylmethylsulfonyl fluoride] with protease inhibitor cocktail (Sigma-Aldrich), and debris was pelleted (14,000 rcf, 10 min, 4°C). Equal amounts of protein lysate were prepared by adding NuPAGE LDS sample buffer and reducing agent (Thermo Fisher Scientific) and boiling at 100°C for 10 min. SDS–polyacrylamide gel electrophoresis was performed using 4 to 12% Tris–Bis NuPAGE gels and the XCell SureLock Mini-Cell Electrophoresis system (Thermo Fisher Scientific) according to the manufacturer’s instructions. Proteins were transferred to nitrocellulose (Li-COR) and blocked with 5% milk powder in 1% Tween 20/PBS (PBT), and primary antibody (Cell Signaling Technology) was applied at 1:1000 dilution in 5% bovine serum albumin (BSA; Sigma-Aldrich)/PBT. Membranes were incubated with shaking overnight at 4°C and then washed with PBT. Fluorescently labeled secondary antibody (Li-COR, Invi- trogen) was applied at 1:5000 dilution in 5% BSA/PBT, and membranes were incubated with shaking for 1 hour at room temperature and then washed with PBT. Membranes were imaged using the Azure 600 imager, and band intensity was quantified using ImageJ.

Supplementary Materials
This PDF file includes:
Legend for table S1
Other Supplementary Material for this manuscript includes the following:
Table S1

View/request a protocol for this paper from Bio-protocol.

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