Noonan syndrome gain-of-function mutations in \textit{NRAS}
cause zebrafish gastrulation defects

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\textbf{SUMMARY}

Noonan syndrome is a relatively common dominantly inherited genetic disorder characterized by congenital heart defects, reduced growth, facial dysmorphism and variable congenital defects (Gelb et al., 2001). The syndrome is caused by activating mutations in genes encoding upstream factors of the Ras-MAPK pathway, including \textit{PTPN11} (Shp2) (Tartaglia et al., 2001) and \textit{SOS1} (Roberts et al., 2007; Tartaglia et al., 2007), as well as \textit{KRAS} (Pandit et al., 2007; Schubbert et al., 2006), \textit{SHOC2} (Cordeddu et al., 2009) and the more downstream signal transducers \textit{RAFI} and \textit{BRAF} (Pandit et al., 2007; Razzaque et al., 2007; Tartaglia et al., 2007). The most recent addition to the group of genes that are associated with Noonan syndrome is \textit{NRAS}. In a cohort of 917 individuals with typical features of Noonan syndrome, who were negative for mutations in previously known Noonan-syndrome-associated genes, two distinct mutations, T50I and G60E, were identified in \textit{NRAS} in four individuals. Mutant N-Ras-G60E and, to a lesser extent, N-Ras-T50I activated MAPK signaling in cells (Cirstea et al., 2010). Activation of N-Ras was previously found to be associated with acute myeloid leukemia (AML) (Bos et al., 1985; Bos et al., 1987) and melanoma (van ’t Veer et al., 1989), and a germline activating mutation in \textit{NRAS} causes autoimmune lymphoproliferative syndrome (Oliveira et al., 2007).

Here, we report the identification of a new mutation in \textit{NRAS} in an individual with Noonan syndrome that results in an amino acid substitution, I24N. Mutant N-Ras-I24N activates downstream MAPK signaling. We used zebrafish embryos to assess the in vivo effects of dominant mutations in genes that are associated with Noonan syndrome, as we and others have done previously (Anastasaki et al., 2009; Jopling et al., 2007; Razzaque et al., 2007; Stewart et al., 2010). Expression of N-Ras-I24N or two other Noonan-syndrome-associated N-Ras mutants, T50I or G60E, in zebrafish embryos resulted in severe developmental defects during epiboly and gastrulation that resemble the defects observed in Noonan syndrome.

\textbf{INTRODUCTION}

Activating mutations in genes encoding components of the Ras-MAPK pathway have not only been identified in various tumor types, but also in developmental disorders (Aoki et al., 2008; Karnoub and Weinberg, 2008; Schubbert et al., 2007). Germline mutations in genes of the Ras-MAPK pathway have been found in a group of genetic syndromes that are collectively called \textbf{RASopathies}, including Noonan syndrome (OMIM 163950), LEOPARD syndrome (OMIM 151100), Costello syndrome (OMIM 218040) and cardio-facio-cutaneous (CFC) syndrome (OMIM 115150) (Denayer et al., 2008; Tidyman and Rauen, 2009). These syndromes are characterized by partially overlapping symptoms, including distinctive craniofacial features and cardiovascular anomalies, and they are genetically heterogeneous, with mutations in known disease genes accounting for 70–80% of the cases.

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activate H-Ras was not provided, other patients with similar Q22 mutations have been described in three patients diagnosed with RASopathies, specifically Noonan syndrome (Q22R), CFC syndrome (Q22E) and Costello syndrome (Q22K) (van der Burgt et al., 2007; Zenker et al., 2007). Although biochemical data that the Q22 mutations activate H-Ras, suggesting an important regulatory role for the region between the P-loop and Switch I, which includes N-Ras I24 (Fig. 1B).

Two mutations in NRAS were previously identified in Noonan syndrome patients, one of which (G60E) is characterized as highly GTP bound, whereas the other (T50I) showed normal GTP loading (Cirstea et al., 2010). To assess the activation state of N-Ras-I24N, GTP-bound Ras was selectively precipitated from cells stimulated with serum. We found that a higher proportion of N-Ras-I24N was precipitated relative to wild-type N-Ras (Fig. 2A). A similar effect was observed for N-Ras-G12V and N-Ras-G60E, but not for N-Ras-T50I. In the absence of serum, out of these mutants only oncogenic N-Ras-G12V was highly GTP bound. These results group N-Ras-I24N in the class of ‘mild activating’ mutations, the activation of which is dependent on upstream signaling. Mild activating mutations are commonly associated with Noonan and Noonan-related syndromes (Tidyman and Rauen, 2009). The side chain of Ile24 (I24) in the α1-helix points towards the β2-strand (Fig. 1C) and mutation to Asn (N) would probably result in repulsing forces between the α1-helix and the β2-strand, thereby destabilizing the fold of the G protein. Hence, the nucleotide affinity might be weakened, resulting in an increased exchange rate and therefore higher GTP loading under physiological conditions, because the GTP:GDP ratio is ~9:1 in cells. This would be in agreement with the ‘mild activation’ character of the I24N mutant.

We next investigated MAPK activation, a downstream consequence of N-Ras activation, in 293T cells expressing N-Ras-I24N and found robust MAPK phosphorylation in the presence of serum in comparison with wild-type N-Ras (Fig. 2B). N-Ras-G60E and the positive control, N-Ras-G12V, also enhanced MAPK activation, but N-Ras-T50I did not, which is in agreement with a weak or no increase in Ras-binding domain (RBD) binding (Fig. 2A). Together, these results demonstrate that the I24N amino acid substitution increases the fraction of active N-Ras and enhances MAPK activation, which might be due to enhanced GTP loading from subtle changes in the conformation of the GTP-binding pocket.

Fig. 1. Identification and localization of an I24N amino acid substitution in N-Ras in an individual with typical features of Noonan syndrome. (A) The heterozygous c.71T>A nucleotide substitution in exon 2 that was first identified in the patient (a) was not detected in the father (b) or the mother (c) and was detected in DNA isolated from fibroblasts (d) and sputum (e) of the patient. (B) Genomic organization (top), protein structure (middle) and sequence of N-Ras (HVR) are also indicated in the protein structure. (C) Ribbon representation of N-Ras-GDP (Protein Data Bank code: 3con), with GDP (blue) and residues Ile24, Thr50 and Gly60 highlighted as ball and stick representation. Missing connectivity for residues 61 to 71 is indicated by a dotted line.
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To evaluate the functional consequences of activating mutations in Noonan-syndrome-associated genes at the organismal level, we and others have used zebrafish as a model system (Anastasiaki et al., 2009; Jopling et al., 2007; Razzaque et al., 2007; Stewart et al., 2010). Mutant N-Ras was expressed in zebrafish embryos at the one-cell stage. Embryos were lysed at 24 hpf and loaded onto SDS-PAGE gels, blotted and probed with antibodies against phospho-MAPK, or against Tubulin as a loading control. (C) Synthetic RNA (5 pg) encoding wild-type or mutant N-Ras was injected into zebrafish embryos at the one-cell stage. Embryos were lysed at 24 hpf and loaded onto SDS-PAGE gels, blotted and probed with antibodies against phospho-MAPK, MAPK and, as a loading control, against actin.

N-Ras-G60E from Noonan patients both strongly affected the major:minor axis ratio, whereas N-Ras-T50I only had mild effects (Fig. 3C). Activating mutants in B-Raf were recently reported to induce defects in epiboly (Anastasiaki et al., 2009); thus, we included the robustly active B-Raf-V600E mutant as a positive control in our assay. As expected, this mutant exerted a strong effect on the major:minor axis ratio, whereas wild-type B-Raf had no effect (Fig. 3C).

The effects of the N-Ras mutants on early zebrafish development were probably due to activation of the MAPK pathway. Using the MEK inhibitor CI-1040 to suppress downstream MAPK activation, we found that the deleterious effects of N-Ras-I24N on epiboly and later development were completely rescued by inhibition of MEK (Fig. 3A). Quantification of the epiboly defects confirmed the complete rescue of the N-Ras-I24N, N-Ras-G12V, N-Ras-G60E and N-Ras-T50I mutants, as well as B-Raf-V600E (Fig. 3C). Taken together, our data demonstrate that N-Ras-I24N is a functionally activated mutant of N-Ras that induces developmental defects, similar to those induced by N-Ras-G12V and N-Ras-G60E in a pathway requiring N-Ras-mediated activation of MAPK. It should be noted that the rescues were mediated by a pulse of MEK inhibitor between 4.5 and 5.5 hpf, whereas a more prolonged exposure to the inhibitor induced developmental defects on its own, consistent with a previous report (Anastasiaki et al., 2009).

N-Ras-I24N is a mildly activating mutant of N-Ras, which requires serum to be present for activation of the Ras-MAPK pathway in 293T cells (Fig. 2B). Fibroblast growth factor receptor (FGFR) signaling is an upstream factor in convergence and extension cell movements during gastrulation. We assessed the effect of the FGFR inhibitor SU5402 on N-Ras-I24N-induced developmental defects. Interestingly, we observed that SU5402 partially rescued the gastrulation defects that were induced by N-Ras-I24N, whereas SU5402 at these concentrations had no effect on non-injected control embryos (Fig. 3D). We conclude that upstream FGFR signaling is required for the developmental defects that are induced by N-Ras-I24N.

To further characterize the activated N-Ras-induced epiboly and gastrulation cell-movement defects, we used classical molecular markers for in situ hybridization. Convergence and extension cell movements during gastrulation shape the embryos, making the embryo narrower (convergence) and longer (extension) (Solnica-Krezel, 2006). A well-known marker for convergence and extension cell movements during gastrulation is the ratio between the width of the krox20-positive rhombomeres 3 and 5 and the distance between somites 1 and 8, visualized by myoD staining at the eight-somite stage (12 hpf) (Li et al., 2008). Injection of N-Ras-I24N led to widening of the embryos at 12 hpf (Fig. 4A). N-Ras-G12V and N-Ras-G60E had extreme effects, whereas N-Ras-T50I resembled wild-type N-Ras (Fig. 4A). Quantification of the ratio of the width to the length revealed significant increases in this ratio in embryos injected with RNA encoding mutant N-Ras-G12V, N-Ras-I24N and N-Ras-G60E, compared with wild-type nras-injected and non-injected controls (Fig. 4B). N-Ras-T50I also induced a significant albeit small increase in the ratio, relative to wild-type N-Ras. Another marker for convergence and extension cell movements is dlx3 and hgg1 staining at the one-somite stage (10 hpf) (Jopling and den Hertog, 2005; van Eekelen et al., 2010). These markers indicate that N-Ras-G12V, N-Ras-I24N and N-Ras-G60E induced...
significant defects in convergence and extension cell movements as well (supplementary material Fig. S2). Taken together, these results demonstrate that activating N-Ras mutants induce gastrulation cell-movement defects at severities that parallel the capacity to activate MAPK.

To monitor gastrulation cell movements directly, we have developed time-lapse fluorescent microscopy of zebrafish embryos injected with fluorescent Histone H1 protein, which labels all nuclei (van Eekelen et al., 2010). Using this cell tracking technology, we found that expression of N-Ras-I24N resulted in a 10% reduction in convergence and 6% reduction in extension of paraxial mesendodermal cells, compared with embryos injected with RNA encoding wild-type N-Ras. These results are statistically significant (P<0.001) because they are based on more than 1000 cell tracks each. One of the underlying mechanisms of convergence and extension is cell intercalation and mediolateral elongation (Concha and Adams, 1998; Myers et al., 2002). By cell membrane labeling and ratiometric analysis of cell shape and orientation, we found that cell polarization is reduced upon N-Ras-I24N expression (supplementary material Fig. S3), which is consistent with the observed convergence and extension cell-movement defects.

One of the hallmarks of Noonan syndrome is hypertelorism and we have demonstrated before that expression of mutant Shp2 leads to craniofacial defects, including wide-set eyes, in zebrafish embryos (Jopling et al., 2007). We assessed defects in craniofacial cartilage structures using the transgenic Tg(-4.9sox10:EGFP)ba2 zebrafish line, which expresses GFP in all neural crest cells (Carney et al., 2006). The angle of the ceratohyal is a direct measure for the width and the bluntness of the head (Fig. 4C), and is an excellent marker for hypertelorism. Expression of N-Ras-I24N led to a significant increase in the angle of the ceratohyal (P<0.05; Student’s t-test) (Fig. 4C,D) and hence we conclude that N-Ras-I24N expression induced craniofacial defects in zebrafish embryos that are reminiscent of the symptoms in individuals with Noonan syndrome.

In conclusion, we report an activating mutation in N-Ras, I24N, that is associated with Noonan syndrome. Interestingly, our in vivo data in zebrafish embryos (Figs 3, 4) demonstrate for the first time that the activating N-Ras mutants, I24N and G60E, are sufficient for inducing developmental defects that resemble the defects induced by active mutants of downstream factors, B-Raf, Raf-1 and MEK, from Noonan and related syndromes (Anastasaki et al., 2009), which are consistent with the observed convergence and extension cell-movement defects.
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and hence firmly establish NRAS as a gene that causes Noonan syndrome upon activation. The observed craniofacial defects in zebrafish embryos (Fig. 4C,D) actually resemble symptoms in individuals with Noonan syndrome. Taken together, our in vivo data demonstrate that NRAS is a bona fide Noonan-associated gene that has a causal role in the syndrome.

Ras mutations found in human cancer are thought to contribute to disease by activating at least three major downstream signaling pathways, i.e. the Raf-MAPK pathway, the PI3K-PKB/AKT pathway and the RalGDS-Ral pathway. In the case of Noonan N-Ras mutants, it becomes apparent that Raf-MAPK activation is sufficient. Mutational activation of B-Raf causes developmental defects in zebrafish that are identical to those in the activating N-Ras mutants, and pharmacological inhibition of MEK using CI-1040 completely rescues these developmental defects. MEK and/or MAPK inhibitors are currently in clinical trials for the treatment of cancers harboring active RAS mutations. This raises the interesting possibility that these inhibitors might also be appropriate for the treatment of developmental disorders that are caused by RAS mutations that depend exclusively on Raf-MAPK activation to convey their pathology.

METHODS

Experimental subjects

Written informed consent was obtained from all subjects and/or their legal representatives, and the work was done according to the guidelines of the medical ethical committee of Radboud University, Nijmegen, The Netherlands.

All procedures involving experimental animals were performed in compliance with local animal welfare laws, guidelines and policies.

DNA isolation and sequencing

DNA was isolated from blood, fibroblasts or sputum. Mutational screening of NRAS (ENSG00000213281) was performed by PCR of all four exons, using the following primers: NRAS-1-F 5'-CGCCCAATTAACCCGTATTAC-3'; NRAS-1-R 5'-AGAGAC-AGGATCAGGTCAAGC-3'; NRAS-2-F 5'-ATAGCATTGCATA-TCCCTGTCG-3'; NRAS-2-R 5'-CACAAGATCATCTCTC-TCAGA-3'; NRAS-3-F 5'-CCACTGTACCCACCGCTTAATC-3'; NRAS-3-R 5'-AAAAGCTTGGACAAATGTGTCTG-3'; NRAS-4-F 5'-ATTTG-GATTGTGTCGCTG-3'.

Bidirectional resequencing was performed using a commercial sequencing kit (ABI BigDye Terminator Sequencing kit V2.1; Applied Biosystems) and an automated capillary sequencer (ABI 3730; Applied Biosystems).

RBD assays and MAPK phosphorylation

Ras activation was determined with the minimal RBD of Raf-1 as an activation-specific probe, as described previously (de Rooij and Bos, 1997). Briefly, 293T cells [American Type Culture Collection (ATCC)] were transfected with CMV-promoter-driven expression vectors for N-Ras and mutants. The cells were lysed and active Ras was precipitated with glutathione-agarose beads coupled to GST-RBD. Precipitated N-Ras was detected by western blotting using an N-Ras-specific antibody (Santa Cruz). Zebrafish embryos (24 hpf) were lysed in boiling sample buffer and equal amounts of lysate were loaded onto SDS-PAGE gels. MAPK phosphorylation was established by western blotting using phospho-MAPK-specific mouse monoclonal antibody (mAb; Cell
**TRANSLATIONAL IMPACT**

**Clinical issue**
Noonan syndrome is a relatively common, dominantly inherited genetic disorder characterized by congenital heart defects, reduced growth, facial dysmorphism and other variable congenital defects. The syndrome is caused by activating mutations in genes encoding factors of the RAS-MAPK signaling pathway and hence belongs to a group of genetic syndromes that are collectively called RASopathies. Mutations in known disease-associated genes are found in 70-80% of patients with Noonan syndrome, and ~50% of patients have activating mutations in a gene encoding an upstream factor in the RAS-MAPK pathway [protein-tyrosine phosphatase, non-receptor type 11 (SHP2), encoded by PTPN11]. The most recent addition to the group of genes that are associated with Noonan syndrome is NRAS, which encodes a member of the Ras protein family.

**Results**
In this study, the authors report on a mutation in NRAS resulting in an I24N amino acid substitution that they identified in an individual bearing typical Noonan syndrome features. The I24N mutation mildly activates N-Ras, resulting in enhanced downstream signaling, similar to other activating N-Ras mutations that are associated with Noonan syndrome. By using zebrafish embryos as a model, they go on to show that expression of I24N or other disease-associated activating N-Ras mutants causes developmental defects, including cell migration defects during gastrulation. The craniofacial defects observed in the zebrafish embryos carrying disease-associated mutations in N-Ras resemble the defects observed in individuals with Noonan syndrome. Furthermore, developmental defects induced by activated N-Ras phenocopy the developmental defects that are induced by the expression of other genes known to be associated with Noonan syndrome, including PTPN11, in zebrafish embryos. Finally, they show that pharmacological inhibition of MEK, a downstream factor in the RAS-MAPK pathway, rescues the phenotype induced by activating N-Ras mutations, demonstrating that the observed defects are mediated exclusively by enhanced activation of the RAS-MAPK signaling pathway.

**Implications and future directions**
This study provides new evidence that NRAS is a bona fide Noonan-syndrome-associated gene and, moreover, that activation of N-Ras can cause the clinical phenotype. Hence, NRAS should be included in diagnostic screening for mutations in individuals with symptoms of Noonan syndrome. Notably, pharmacological inhibition of MEK rescues the developmental defects that are induced by activated N-Ras. Given that the RAS-MAPK pathway is also implicated in cancer, developing inhibitors of this pathway has been a major focus of many pharmaceutical companies, and clinical trials to test them in a subset of cancer patients are currently underway. Such inhibitors might also be effective for treating some RASopathies, including Noonan syndrome, that are caused by hyperactivation of the RAS-MAPK pathway.

Zebrafish, injections and in situ hybridization
Zebrafish were kept and embryos were raised under standard conditions. Zebrafish were staged as described before (Westerfield, 1995). Constructs encoding human N-Ras and mutants were derived by PCR and verified by sequencing. The wild-type and mutant B-Raf-encoding constructs (Anastasaki et al., 2009) were a gift from Liz Patton. 5’-capped sense RNAs were synthesized using mMessage mMACHINE kit (Ambion). Synthetic RNA was injected at the one-cell stage. The amount of RNA encoding wild-type N-Ras was titrated down to an amount that did not induce morphological defects by itself (5 pg). Subsequently, 5 pg of all mutants was injected at the one-cell stage. 30 pg RNA encoding wild-type or mutant B-Raf was injected at the one-cell stage (Anastasaki et al., 2009). Phenotypes were assessed at the indicated stages. At 11 hpf, batches of embryos were photographed and the ratio of major:minor axis of each embryo was determined individually using ImageJ software. Pharmacological inhibitors were added directly to the embryo medium at 4.5 hpf. CI-1040 (Selleck Chemicals) was removed at 5.5 hpf to avoid inadvertent side effects of prolonged treatment, whereas SU5402 (Merck) was left on the embryos for the duration of the experiment. In situ hybridizations were performed essentially as described (Thisset et al., 1993) using probes specific for myod and krox20 at the eight-somite stage (~12 hpf) (Li et al., 2008). Quantification of convergence and extension cell-movement defects by analysis of the ratio of the width of krox20 staining and the length from somite-1 to somite-8 was done using ImageJ software as described before (van Eekelen et al., 2010).

Analysis of cell movement and polarization
Mesendodermal cell tracking was performed exactly as described before (van Eekelen et al., 2010). Briefly, embryos were injected at the one-cell stage with Histone-H1 tagged with Alexa Fluor 488 from Molecular Probes (H1). The embryos were dechorionated and mounted at shield stage in 1% low-melting-point agarose. A Leica SP2 confocal microscope with a 40× objective was used for live imaging and time points were recorded every 2 minutes from shield stage until the one-somite stage. Time-lapse images were analyzed using ImageJ (http://rsweb.nih.gov/ij/). Following image processing, uniform objects were readily traced, and convergence and extension cell movements quantified. Statistical analysis was performed by the Microsoft Excel Student t-test assuming unequal variances with alpha=0.05.

Craniofacial defects
Craniofacial defects were determined by measurement of the ceratohyal angle at 5 dpf. The Tg(–4.9sox10:EGFP)hau2 transgenic line, in which neural crest cells are marked(Carney et al., 2006), was used, facilitating detection of the ceratohyal. The angle was defined by two lines, one parallel to the left part of the ceratohyal and the other parallel to the right part (cf. Fig. 4C).

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
V.R., M.v.E., B.B. and J.d.H. conceived and designed the experiments. V.R., M.v.E., J.O. and B.B. performed the experiments. V.R., M.v.E., H.R., B.B. and J.d.H. analyzed the data. H.G.Y., W.M.N., A.v.H. and I.v.d.B. provided samples. V.R., M.v.E. and J.d.H. wrote the paper.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/dmm.007112/-/DC1

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