The American Journal of Human Genetics
Supplemental Data

Mutations in the Endothelin Receptor Type A Cause Mandibulofacial Dysostosis with Alopecia

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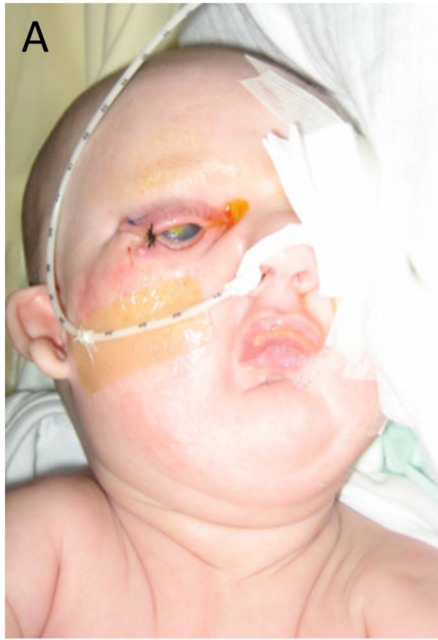
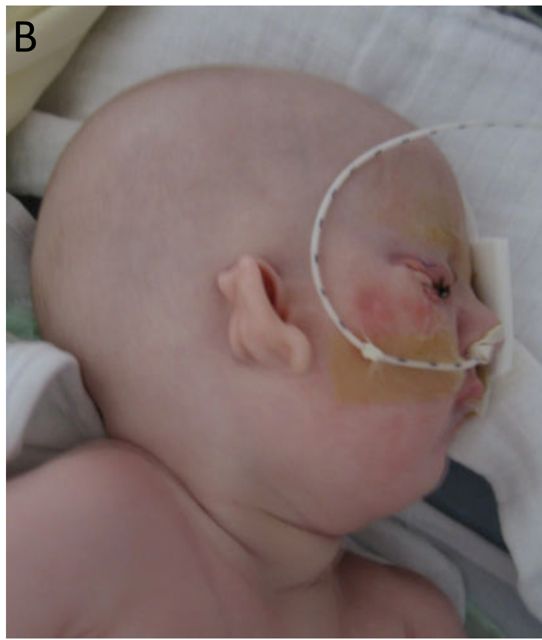

A

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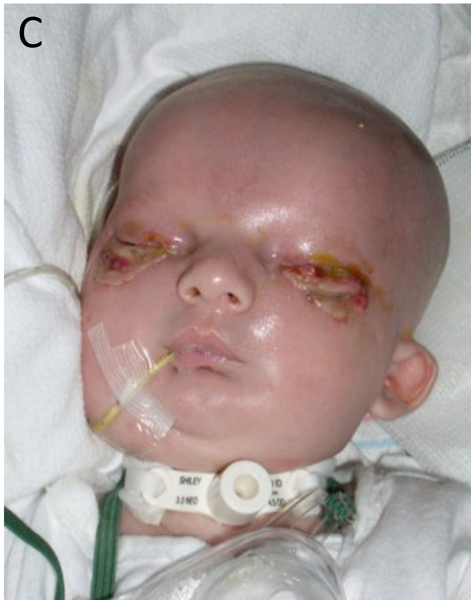
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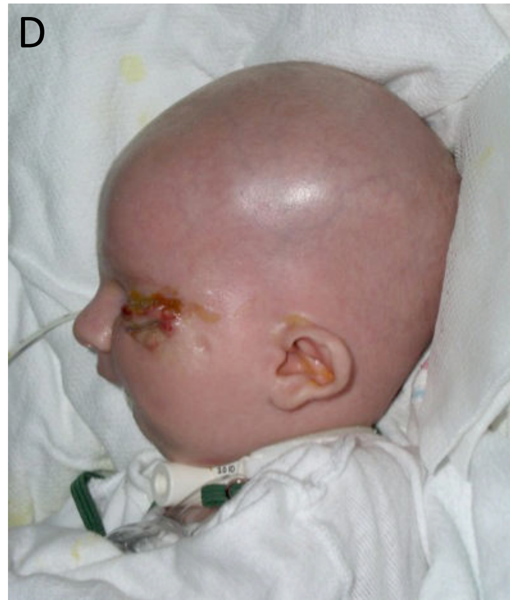
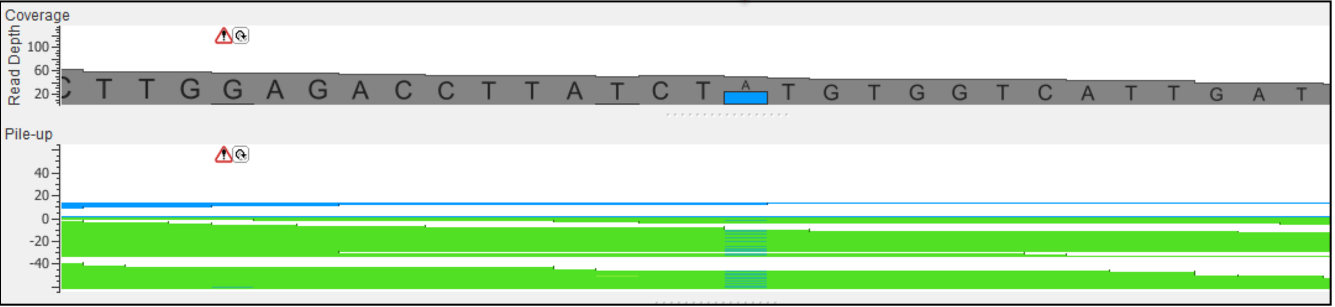
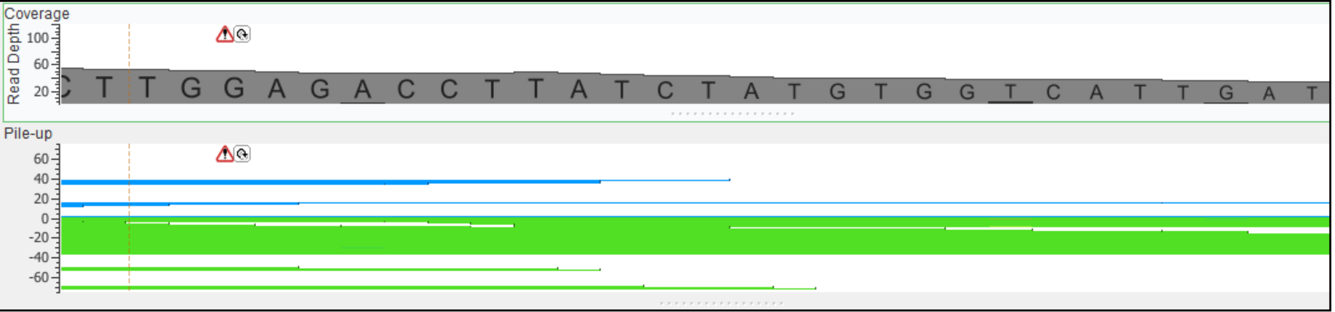
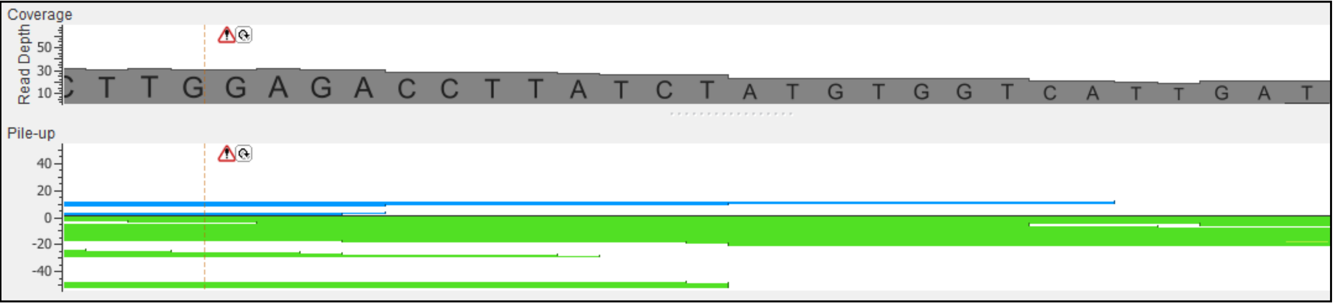

D

Figure S1. MFDA facial features of individual 1 and individual 3 as infants. A, B: individual 1. C, D: individual 3. Note ectopic post-auricular tissue in B.

A







B

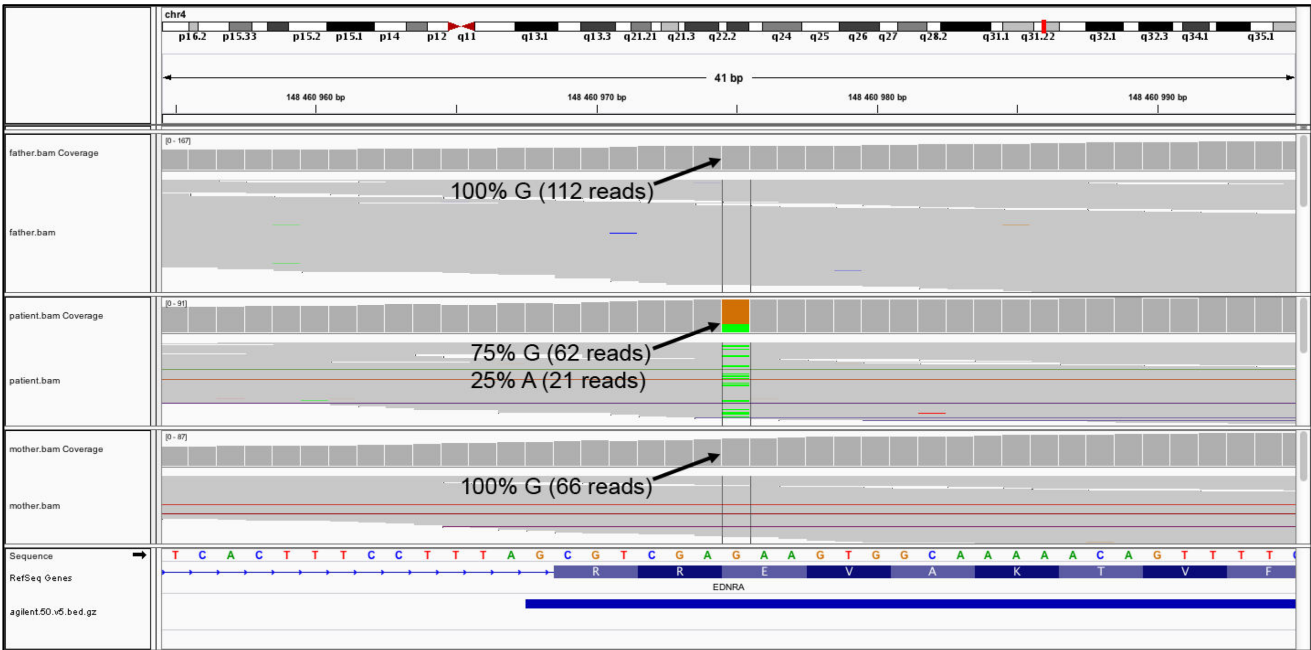
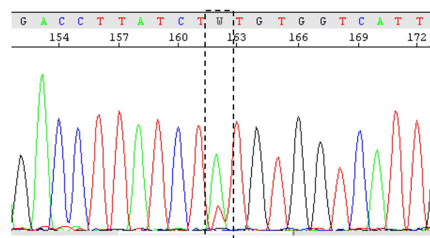
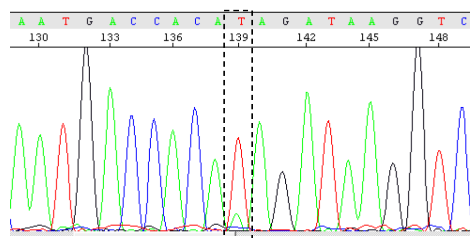
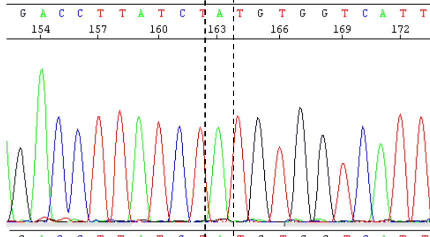
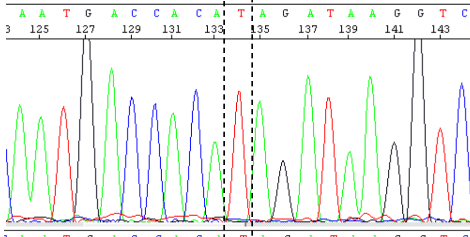
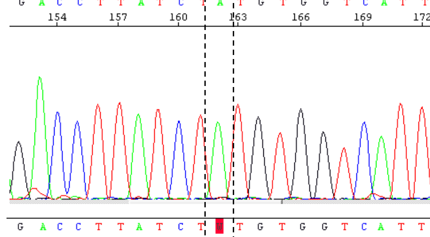
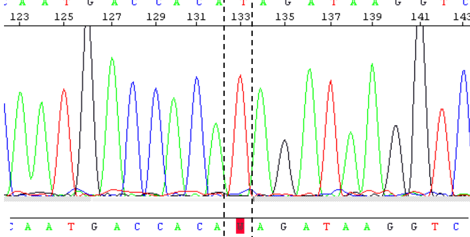


Figure S2. Next generation sequencing reads depicting the *EDNRA* mutations identified by trio exome sequencing. A: individual 3. B: individual 4. A red arrow indicates the position of the mutation in A.

forward
reverse

Individ. 1



Individ. 1 mother



Individ. 1 father



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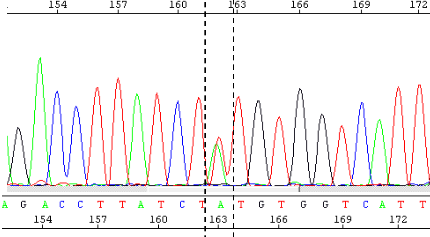
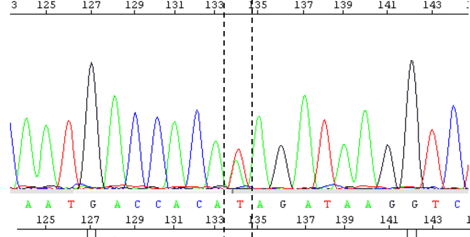
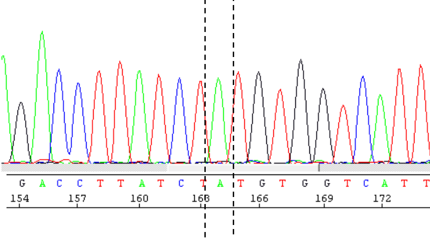
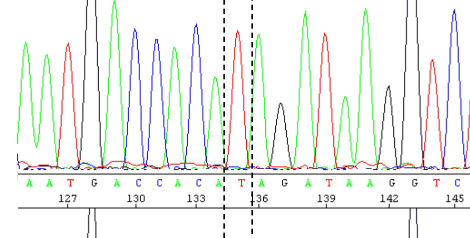
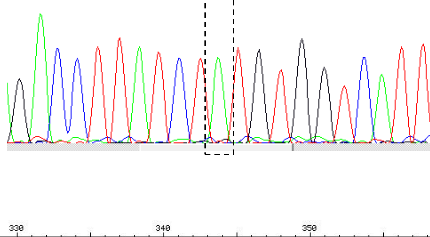
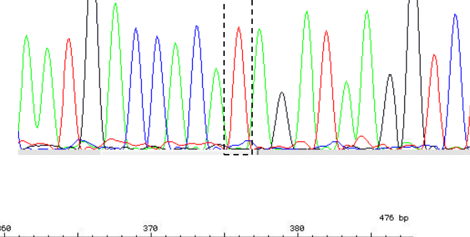
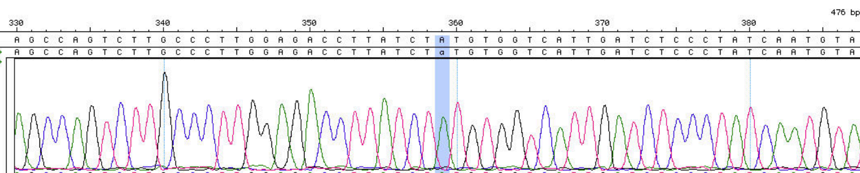
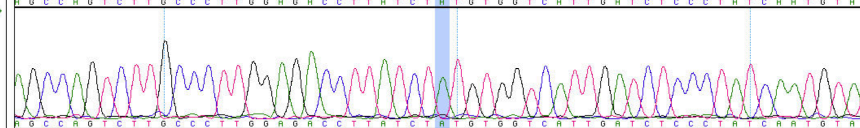



Individ. 2 mother



Individ. 2 father



Individ. 3 father


Individ. 3 mother


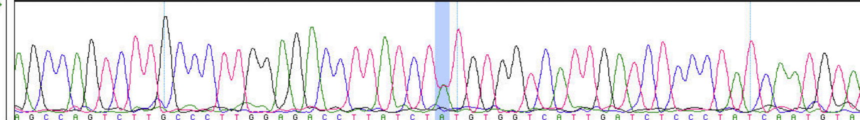
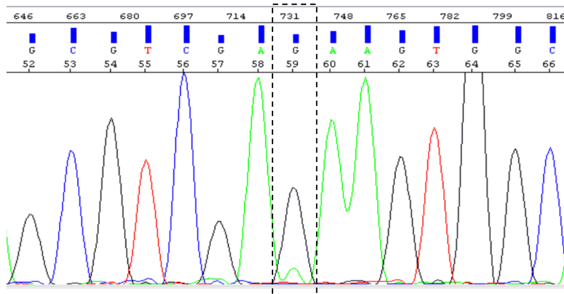
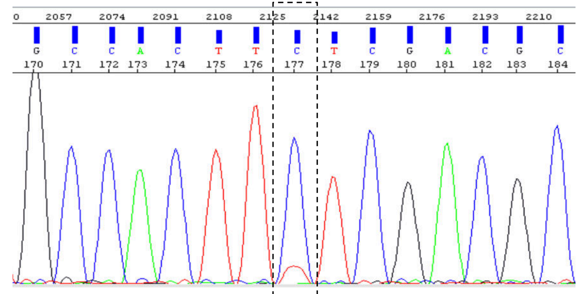
Individ. 3


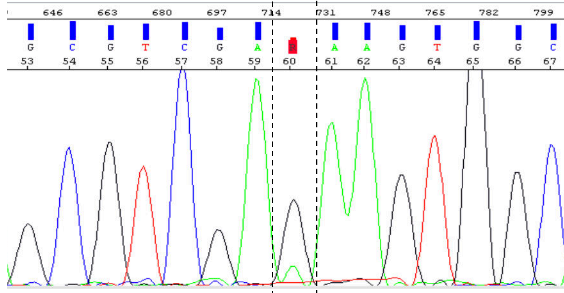
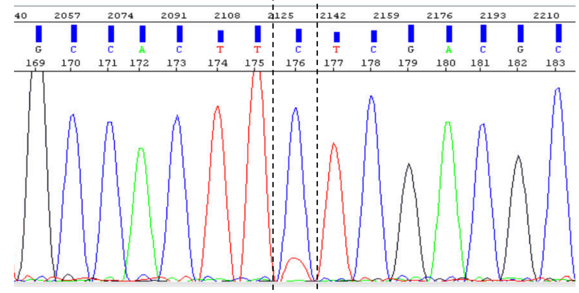
Figure S3. Sanger sequencing chromatograms of the *de novo* mutation identified in *EDNRA* (c.386A>T, p.Tyr129Phe) in individuals 1-3. Sanger sequencing was performed on genomic DNA extracted from blood in all cases, by standard techniques. Primer sequences are available on request. The position of the mutation is indicated by a dashed box (individuals 1 and 2) or is highlighted in blue (individual 3). The mutation is mosaic in individual 1.

forward reverse

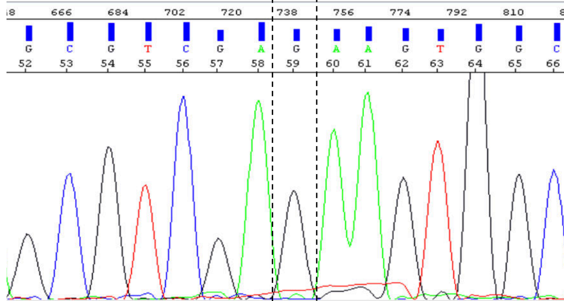
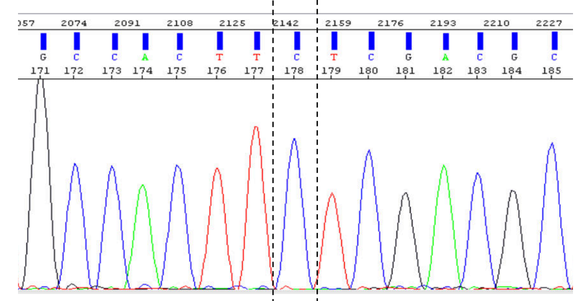
Individual 4 (1st blood sample)

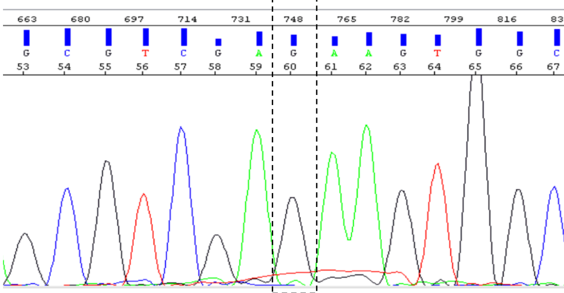
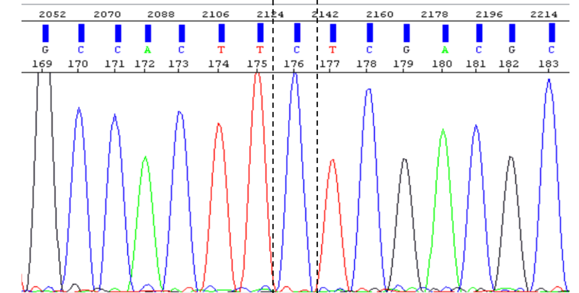
Individual 4 (2nd blood sample)

Individual 4 father

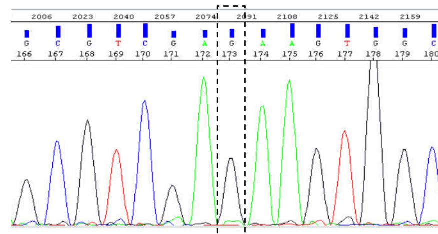



Individual 4 mother

Cloning of PCR products, individual 4, *EDNRA*, c.907G>A:

Wildtype allele (12 bacterial colonies):



Mutant allele (3 bacterial colonies):

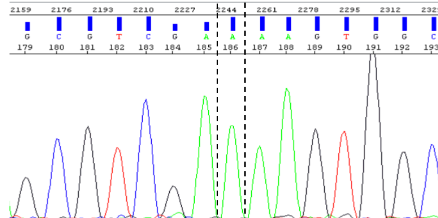


Figure S4. Sanger sequencing chromatograms of the *de novo* mutation identified in *EDNRA* (c.907G>A, p.Glu303Lys) in individual 4. Sanger sequencing was performed on genomic DNA extracted from blood in all cases, by standard techniques. Primer sequences are available on request. A dashed box indicates the position of the mosaic mutation. The lower part of the Figure depicts representative chromatograms from sequencing of bacterial colonies transformed with PCR products, amplified from individual 4 genomic DNA (encompassing the *EDNRA* c.907G>A mutation) and cloned into pCRII-TOPO (Invitrogen).

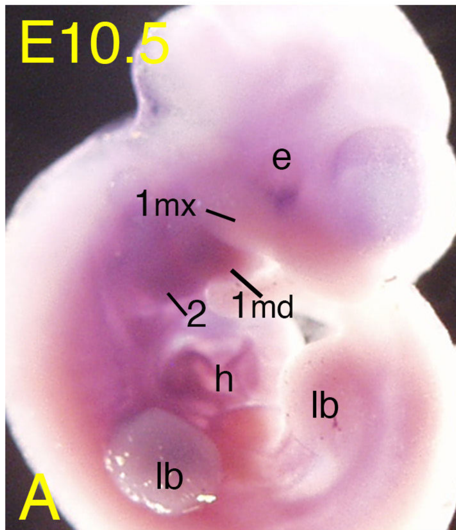
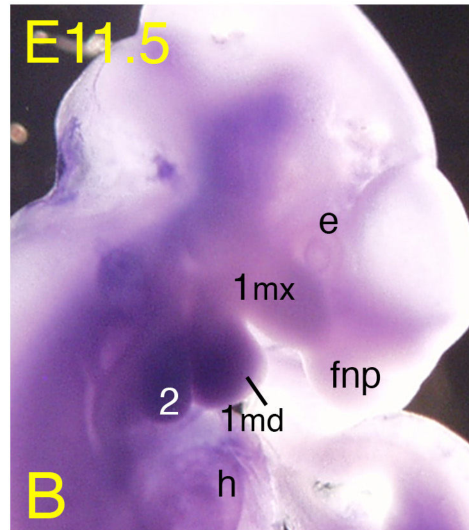
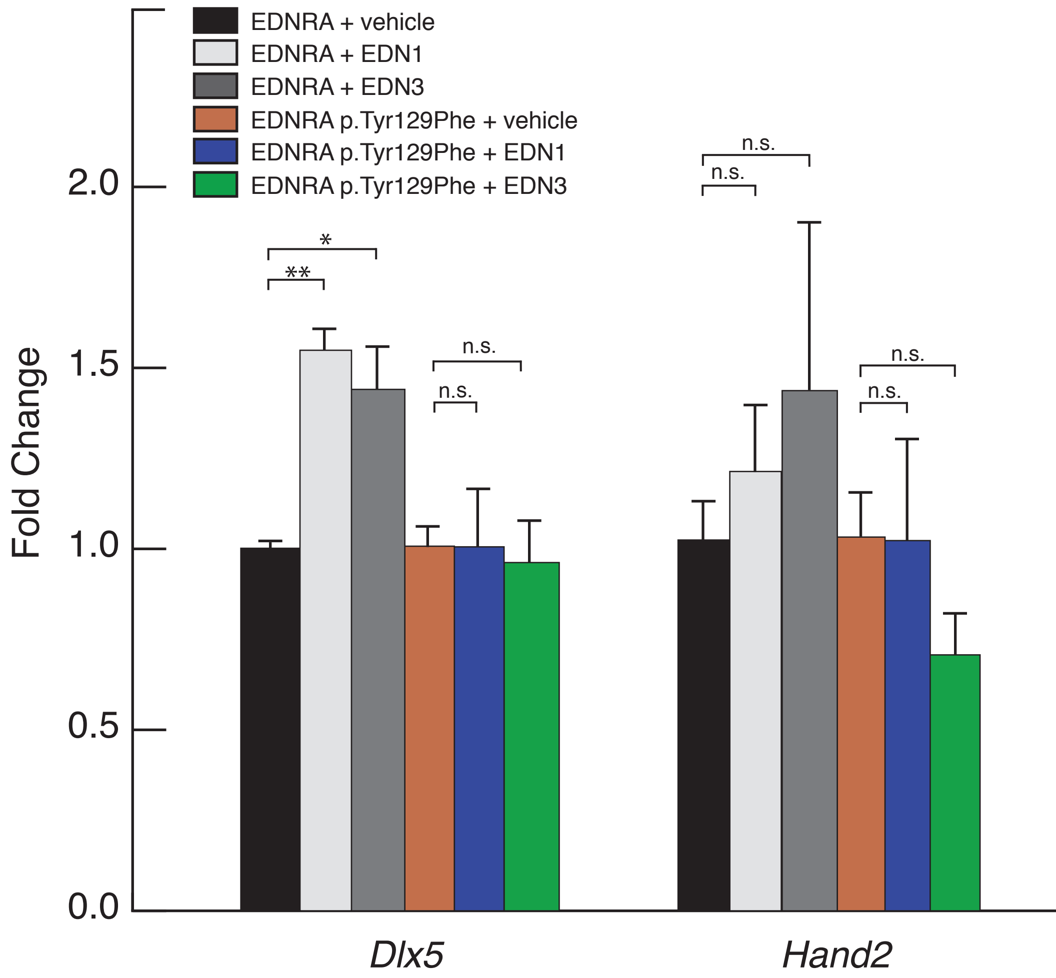
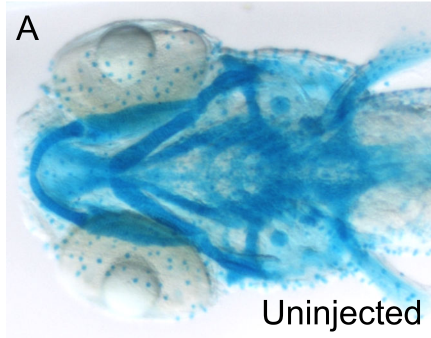

E10.5
e
1mx
2
1md
h
lb
lb
A

E11.5
e
1mx
fnp
2
1md
h
B

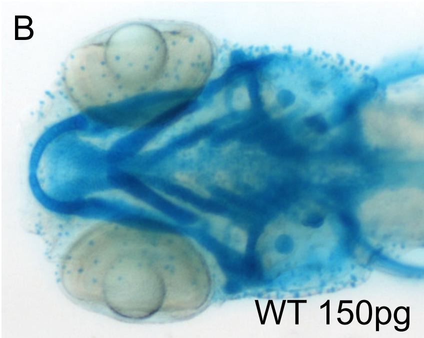
Figure S5. *Edn3* expression during mouse embryogenesis. In situ hybridization analysis of *Edn3* expression in E10.5 (A) and E11.5 (B) wild type mouse embryos. Expression at E10.5 (purple color) is present in the mandibular portion of the first pharyngeal arch (1md) and in the second arch (2), with expression in both arches stronger at E11.5. 1mx, maxillary prominence of the first pharyngeal arch; e, eye; h, heart; lb, limb bud; fnp, frontonasal process.



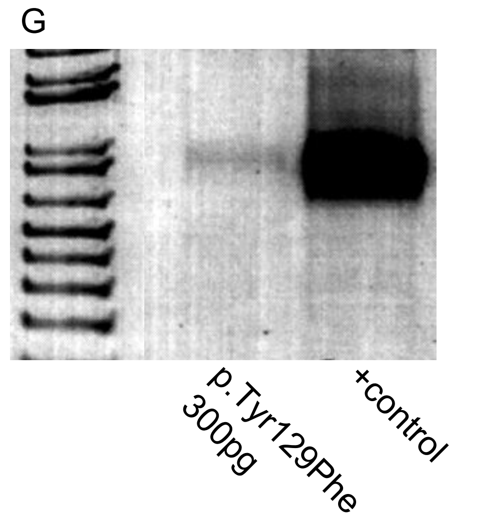
EDNRA + vehicle
EDNRA + EDN1
EDNRA + EDN3
EDNRA p.Tyr129Phe + vehicle
EDNRA p.Tyr129Phe + EDN1
EDNRA p.Tyr129Phe + EDN3
Fold Change
2.0
1.5
1.0
0.5
0.0
*
**
n.s.
n.s.
n.s.
n.s.
n.s.
n.s.
Dlx5
Hand2

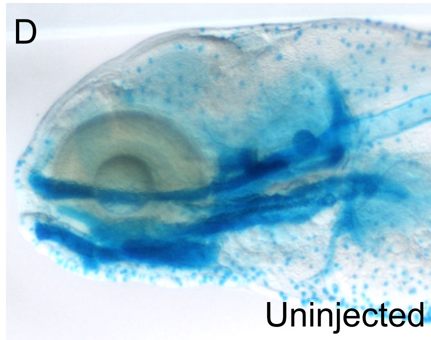
Figure S6. Response of EDNRA wild type and EDNRA p.Tyr129Phe to EDN1 and EDN3 *in vitro* is blunted in the absence of the EDNRB antagonist BQ788. Following transfection of MC3T3-E1 cells with an expression vector encoding either EDNRA wild type or EDNRA p.Tyr129Phe, cells were treated with vehicle, EDN1 or EDN3 and then RNA collected for qRT-PCR analysis of *Dlx5* and *Hand2* expression. The EDNRB antagonist BQ788 was not used for these experiments, so the influence of endogenous EDNRB signaling is possible. Compared to results of experiments performed in the presence of BQ788, upregulation of both *Dlx5* and *Hand2* was less robust or no longer significantly changed. *, $p<0.05$; **, $p<0.001$; n.s., not statistically significant ($p>0.05$). Statistical test was a two-tailed t-test (assuming equal variance). Each condition represents an average of results from three separate transfection experiments. Error bars represent standard error of the mean.

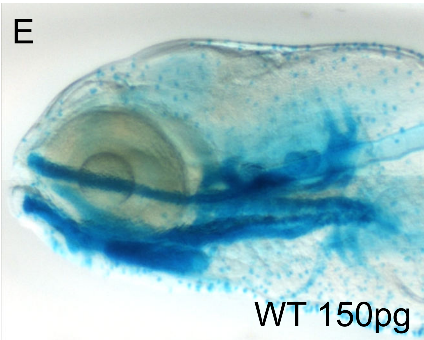

A
Uninjected


B
WT 150pg

C
p.Tyr129Phe 300pg


G
p.Tyr129Phe
300pg
+control


D
Uninjected


E
WT 150pg

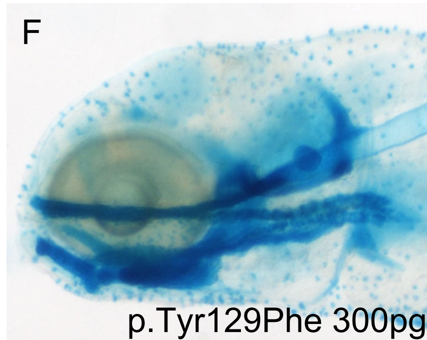
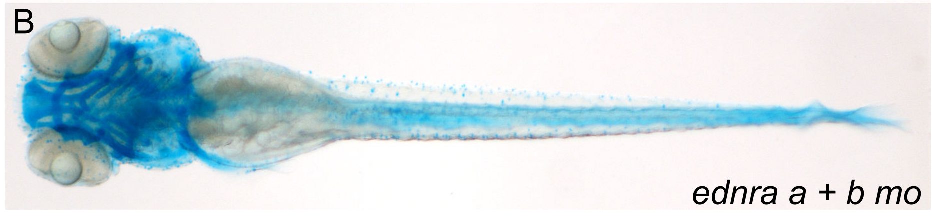
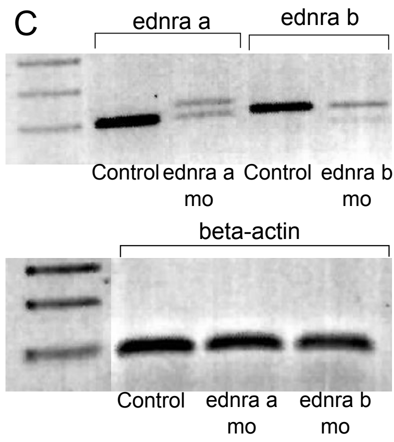
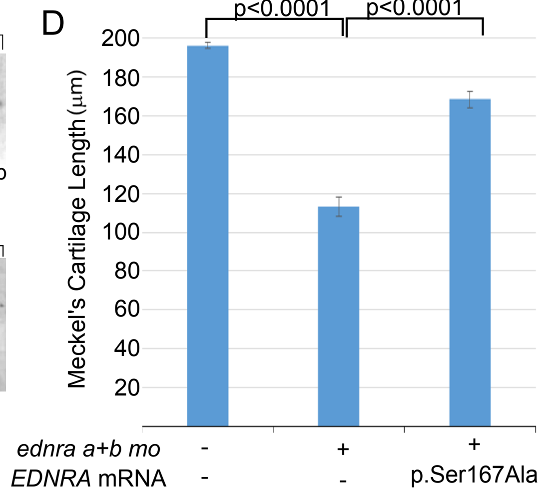

F
p.Tyr129Phe 300pg

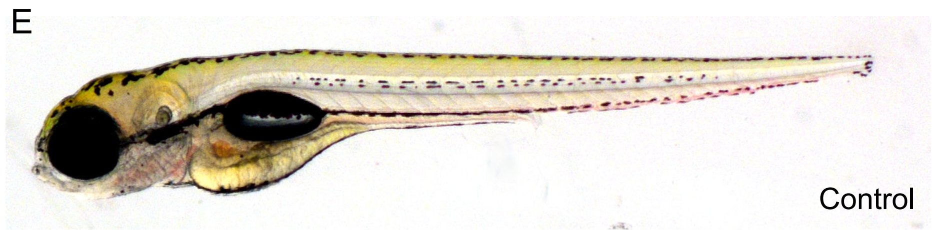
Figure S7. Injection of high doses of mRNA encoding EDNRA wild type or p.Tyr129Phe does not result in any discernable phenotype in zebrafish embryos. Ventral (A-C) and lateral (D-F) views of control embryos and embryos injected with mRNAs encoding EDNRA wild-type (WT) or p.Tyr129Phe and stained with alcian blue at 5 dpf. Uninjected control embryos (A,D) are indistinguishable from embryos injected with 150 pg of WT-encoding mRNA (B, E) or 300 pg of mRNA encoding p.Tyr129Phe (C,F). (G) At 50 hours post-fertilization (hpf), by which time developing cartilages are present within the pharyngeal arches, embryos injected with 300 pg of mRNA encoding p.Tyr129Phe were harvested, RT-PCR was performed, and *EDNRA* was amplified with human-specific primers. The stock mRNA used for injection was also reverse-transcribed and used as a positive control. Injected mRNA was detectable at 50 hpf.

A
Control


B
ednra a + b mo


C
ednra a
ednra b
Control
ednra a mo
Control
ednra b mo
beta-actin
Control
ednra a mo
ednra b mo


D
p<0.0001
p<0.0001
Meckel's Cartilage Length (μm)
200
180
160
140
120
100
80
60
40
20
ednra a+b mo - + +
EDNRA mRNA - - p.Ser167Ala


E
Control

F
EDN1 + EDNRA WT

Figure S8. Injection of *ednra* morpholinos (MOs) or co-injection of mRNAs encoding endothelins and EDNRA does not result in generalized dysmorphology. (A) Uninjected control and (B) embryos injected with *ednra a* and *b* MOs at 5 days post-fertilization (dpf) stained with alcian blue. Injected embryos display hypoplasia of the ventral cartilages (Meckel's and ceratohyal) with no body length defect or cardiac/yolk edema. (C) RT-PCR showing that the *ednraa* and *ednrab* MOs induce aberrant splicing and decreased levels of the wild-type transcripts. Controls are from uninjected embryos. Beta-actin was used as a cDNA loading control. (D) Meckel's cartilage was measured as shown in Figure 5 in embryos injected with *ednra a* and *b* MOs alone or *ednra a* and *b* MOs and 100 pg of mRNA encoding EDNRA p.Ser167Ala. The first column represents uninjected embryos. Error bars represent standard error of the mean. EDNRA p.Ser167Ala partially rescued the Meckel's hypoplasia phenotype induced by the *ednra a* and *b* MOs. Representative images of uninjected control embryos (E) and embryos co-injected with 5 pg each of *EDN1* and wild type (WT) *EDNRA* mRNAs (F) at 5 dpf, the latter displaying no major morphological defects, with the exception of the rostral head phenotype.

FILTER	VARIANTS/GENES		
Total variants, filtered for quality	80,866 variants		
Coding, non-synonymous	13,832 variants		
Minor allele frequency <0.01 (1kG, ESP6500)	1463 variants		
	De novo model = 3 variants	Recessive model (homozygous) = 1 variant	Recessive model (compound heterozygous) = 43 variants
Removed variants in highly exonically variable genes[a]; removed variants predicted benign	*EDNRA* *WNT4* *ITGAE*	-	-

Table S1. Variant filtering strategies following trio exome sequencing for individual 3.

[a]includes certain *MUC* genes and genes encoding extremely long proteins.

FILTER	VARIANTS/GENES		
Total substitutions, deletions, insertions	106,404 variants		
Frequency <1% in dbSNP, EVS, 1KG, in-house exomes[a]	1,053 variants		
	De novo model = 20 variants (15 genes)	Recessive model (homozygous) = 17 variants (20 genes)	Recessive model (compound heterozygous) = 42 variants (21 genes)
Essential splicing, non-synonymous, frameshift and stop variants	*EDNRA, HLA-DQA2*	*SGK110*	*PAXIP1, SYNE2, ZAN*
Predicted damaging by Polyphen and Sift	*EDNRA* (NM_001957.3; p.Glu303Lys)	-	*PAXIP1* (NM_007349.3; p.Val28Ala and p.Ser322Cys)

Table S2. Variant filtering strategies following trio exome sequencing for individual 4.

[a]SNP databases: dbSNP (build 135), Exome Variant Server (release ESP6500SI-V2), 1000 Genomes (release date May 21, 2011) and over 4,000 in-house exomes performed at the Institut Imagine.