Mutations in the TGF-β repressor SKI cause Shprintzen-Goldberg syndrome with aortic aneurysm

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Elevated transforming growth factor (TGF)-β signaling has been implicated in the pathogenesis of syndromic presentations of aortic aneurysm, including Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS)1–4. However, the location and character of many of the causal mutations in LDS intuitively imply diminished TGF-β signaling5. Taken together, these data have engendered controversy regarding the specific role of TGF-β in disease pathogenesis. Shprintzen-Goldberg syndrome (SGS) has considerable phenotypic overlap with MFS and LDS, including aortic aneurysms6–8. We identified causative variation in ten individuals with SGS in the proto-oncogene SKI, a known repressor of TGF-β activity9,10. Cultured dermal fibroblasts from affected individuals showed enhanced activation of TGF-β signaling cascades and higher expression of TGF-β-responsive genes relative to control cells. Morpholin-induced silencing of SKI paralogs in zebrafish recapitulated abnormalities seen in humans with SGS. These data support the conclusions that increased TGF-β signaling is the mechanism underlying SGS and that high signaling contributes to multiple syndromic presentations of aortic aneurysm.

The TGF-β family of cytokines influences a diverse repertoire of cellular processes, including cell proliferation, differentiation, survival and synthetic activity. One of three TGF-β ligand isoforms (TGF-β1, TGF-β2 or TGF-β3) initiates signaling by binding to the TGF-β receptor complex, which is composed of type 1 and type 2 receptor subunits (TβR1 and TβR2, respectively)11. The complex then transmits the signal through the canonical (SMAD-dependent) pathway or the non-canonical mitogen-activated protein kinase (MAPK) cascades, including extracellular signal–regulated kinase (ERK).

The ability of the nuclear SMAD proteins to precisely regulate gene transcription is further modulated by the recruitment of transcriptional coactivators, such as p300 and CREB-binding protein (CBP), and corepressors, such as the Sloan-Kettering Institute proto-oncoprotein, SKI12. The SKI family of proteins, which also includes the SKI-like protein SKIL, negatively regulate SMAD-dependent TGF-β signaling by impeding SMAD2 and SMAD3 (SMAD2/3) activation, preventing nuclear translocation of the receptor-activated SMAD (R-SMAD)-SMAD4 complex and inhibiting TGF-β target gene output by competing with p300/CBP for SMAD binding and recruiting transcriptional repressor proteins, such as mSin3A and HDAC1 (Supplementary Fig. 1)13–15. A role for SKI in the regulation of MAPK cascades has not been described16,17.

Dysregulated TGF-β signaling has been implicated in the pathogenesis of syndromic presentations of aortic aneurysm. Excessive canonical and non-canonical TGF-β signaling is observed in the aortic wall and other diseased tissues in mouse models of MFS, a systemic connective tissue disorder caused by mutations in the FBN1 gene encoding the extracellular matrix protein fibrillin-1 (refs. 1,3,18–20). Furthermore, pharmacological antagonism of TGF-β and/or ERK signaling in mouse models of MFS has been shown to ameliorate multiple disease manifestations, including aortic aneurysm, suggesting that high TGF-β signaling drives disease progression3,4.
An excess of canonical TGF-β signaling has also been shown in the aortic wall of individuals with LDS. However, this finding has been difficult to reconcile with the character of the underlying mutations in this syndrome. LDS is predominantly caused by heterozygous missense substitutions affecting the kinase domain of either TβR1 or TβR2 (encoded by the TGFBR1 and TGFBR2 genes, respectively)21. When mutant proteins are expressed in cells that are naïve for the corresponding receptor subunit, the cells are unable to propagate SMAD-dependent TGF-β signaling22. More rarely, LDS-like phenotypes can be caused by haploinsufficiency for SMAD3 or the TGFBR2 gene encoding the TGFβ2 ligand, positive effectors of TGF-β signaling22,23. Notably, heterozygous loss-of-function mutation in SMAD3 or TGFBR2 associates with high TGF-β signaling in the aortic wall of both affected humans and mouse models22,23. Taken together, these seemingly contradictory data have engendered considerable controversy regarding the precise role of TGF-β in the pathogenesis of aortic aneurysm.

SGS is a systemic connective tissue disorder of unknown etiology that includes virtually all the craniofacial, skeletal, skin and cardiovascular manifestations of MFS and LDS, with the additional findings of mental retardation and severe skeletal muscle hypotonia6,8. We hypothesized that aberrant TGF-β activity also underlies SGS and that identification of the genetic basis of this syndrome would inform our understanding of other syndromic presentations of aneurysm5. We performed whole-exome sequencing for a single trio comprising a child with SGS and the unaffected parents. An average of 6.9 Gb of sequence was generated per individual as paired-end 75-bp reads, out of which >98.9% mapped to the human reference genome (UCSC hg19). This analysis identified only one variant, a heterozygous missense change in exon 1 of the SKI gene (c.347G>A, p.Gly116Glu; NM_003036) that was not present in SNP databases, was predicted to be damaging (PolyPhen-2 score of 0.999, SIFT score of 0.05), was not present in either parent and was a strong functional candidate because of a described relationship to TGF-β signaling (Supplementary Fig. 2)24,25. We subsequently sequenced SKI in 11 other sporadic cases of SGS (Fig. 1a and Table 1) and identified heterozygous variants in 9 of these individuals, including 8 missense mutations and a 9-bp deletion (Supplementary Fig. 3). These mutations were absent from dbSNP134, the 1000 Genomes Project database and over 10,000 exomes reported on the National Heart, Lung, and Blood Institute (NHLBI) Exome Variant Server, and they were confirmed to be de novo when testing of the unaffected parents was possible (for 5 of 9 affected individuals). Collectively, ten mutations in ten individuals with SGS were identified in SKI by a combination of whole-exome and Sanger sequencing, including a recurrent mutation in two unrelated probands. Each mutation resulted in the substitution or deletion of amino acids that show complete evolutionary conservation in members of the SKI gene family (Supplementary Fig. 4)26. No mutations were identified in sequencing of SKIL (NM_005414) in the two remaining affected individuals. The alterations in SKI clustered in two distinct N-terminal regions of the protein (Fig. 1b). The first region is located in the SMAD2/3-binding domain of SKI (residues 17–45), and

**Figure 1 SKI mutations in individuals with SGS.** (a) Clinical features and mutations seen in individuals with SGS. Features shown involve the craniofacial (abnormal head shape due to craniosynostosis, widely spaced eyes, small and receding chin, high-arched palate), skeletal (long fingers, joint contractures, chest wall deformity, spine curvature, foot deformity) and cardiovascular (aortic root aneurysm, open arrowheads; mitral valve prolapse, white arrowhead) systems. Cardiovascular imaging was performed using either computerized tomography (1.II:1 and 2.II:1) or echocardiography (10.II:1). Pedigrees indicate that all cases were sporadic (affected, filled symbols; unaffected, open symbols). Mutation status is indicated below each individual (−/−, mutation negative; +/−, heterozygous; blank, not available for testing) along with the SKI protein alteration. Permission to publish photographs was obtained from the affected individuals or their parents. (b) Location of alterations with respect to known binding sites (highlighted in yellow) of SKI-binding partners. The position of the DHD is also indicated.
the second region localizes to a portion of the Dachshund-homology domain (DHD) of the SKI protein that mediates binding to SNW1 and N-CoR, proteins that are essential for the transforming activity of SKI and for the recruitment of transcriptional corepressors, such as histone deacetylases, respectively. One of the alterations (p.Leu21Arg) results in the substitution of an amino acid that was previously shown to be essential for SKI-SMAD3 interaction. Two of the alterations (p.Gly116Glu and p.Gly117Arg) result in substitutions at immediately adjacent glycine residues at positions that contribute to an exposed β hairpin loop in the DHD. In silico analysis suggests that both glycine substitutions maintain the β-turn; whereas there is variation in where the I-TASSER structural prediction algorithm defines the margins of the adjacent β sheets, the overall structures of the DHD are fully overlapping for the reference and mutant sequences.

### Table 1 Clinical manifestations in SGS and related disorders

| Subject ID | SGS7 | SGS8 | Pt.1 | Pt.2 | Pt.3 | Pt.4 | Pt.5 | Pt.6 | Pt.7 | Pt.8 | Pt.9 | Pt.10 | SGS (SKI-negative) | Classic MFS | Classic LDS |
|------------|------|------|------|------|------|------|------|------|------|------|------|------|-----------------|-------------|-------------|
| Age (years) | 43   | 6    | 16   | 12   | 22   | 21   | 2    | 6    | 5    | 4    |      |      |                 |              |              |
| Sex        | M    | M    | M    | F    | M    | M    | M    | M    |      |      |      |      |                 |              |              |
| Features typical of SGS |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Craniofacial |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Craniosynostosis | 7/7  | 4/7  | +    | +    | +    | +    | +    | +    | +    | +    | +    | 1/2  | –    | –    |              |              |
| Dolichocephaly | 16/17 | 11/14 | +    | +    | +    | +    | +    | +    | –    | +    | +    | +    | +    | +    |              |              |
| Hypertelorism | 15/16 | 8/13  | +    | +    | +    | +    | +    | +    | +    | +    | +    | 0/2  | –    | –    |              |              |
| Down-slanting eyes | 14/16 | 11/14 | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Proposis | 13/17 | 11/12  | –    | +    | +    | –    | +    | +    | +    | +    | 1/2  | –    | –    |              |              |
| Malar hypoplasia | 15/15 | 7/8   | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |              | +    | +    |
| High/narrow palate | 17/17 | 12/12 | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Micrognathia | 16/17 | 13/14 | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Low-set ears | 13/13 | 12/14 | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | –    | –    |              |              |
| Skeletal |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Arachnodactyly | 15/17 | 14/14 | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Camptodactyly | 10/17 | 6/14  | +    | +    | +    | +    | +    | –    | +    | +    | +    | +    | +    | +    |              |              |
| Scoliosis | 7/14  | 11/13  | +    | +    | +    | +    | +    | –    | –    | +    | +    | 2/2  | +    | +    |              |              |
| Pectus deformity | 15/17 | 13/13 | +    | +    | +    | +    | +    | –    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Joint hypermobility | 9/14  | 10/10 | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | +    | +    |              |              |
| Joint contracture | 8/17  | 2/10  | +    | +    | +    | +    | +    | –    | +    | +    | +    | 1/2  | –    | –    |              |              |
| C1/2 spine malformation | 4/16  | –    | –    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |              |              |
| Neuromuscular |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Hypotonia | 14/16 | 11/12  | +    | +    | +    | +    | +    | +    | +    | +    | 1/2  | –    | –    |              |              |
| Developmental delay | 15/17 | 12/14 | +    | +    | +    | +    | +    | +    | +    | +    | +    | 2/2  | –    | –    |              |              |
| Cardiovascular |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Aortic root dilatationa | 5/17  | 3/11  | +    | +    | +    | +    | +    | –    | +    | +    | 0/2  | +    | +    |              |              |
| Other features of MFS or LDS |      |      |      |      |      |      |      |      |      |      |      |      |                 |              |              |
| Dural ectasia | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    | +    |              |              |
| Ectopia lenta | 1/15b | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | 0/2  | +    | –    |              |              |
| Cleft palate | 2/11  | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | 0/2  | –    | +    |              |              |
| Broad/Bifid uvula | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | 1/2  | –    | –    |              |              |
| Club foot deformity | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | 0/2  | –    | +    |              |              |
| Arterial tortuosity | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | +    | –    | –    |              |              |
| Other aneurysma | +    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    | –    |              |              |

Table 1 Clinical manifestations in SGS and related disorders

*Empty cells, not determined. M, male; F, female.

*aAtypical affected individual with FBN1 mutation. bSplenic artery aneurysm. cSplenic artery aneurysm with spontaneous rupture.

To assess the functional consequences of SKI mutations identified in SGS, we monitored TGF-β signaling in primary dermal fibroblasts derived from two individuals with SGS and two control individuals. Protein blot analysis showed excessive SMAD2/3 and ERK1 and ERK2 (ERK1/2) phosphorylation in the cells of the affected individuals compared to controls, both at baseline and after acute (30-min) stimulation with exogenous TGF-β2 (Fig. 2a). This implies loss of suppression of the TGF-β–dependent signaling cascades in SGS cells. In contrast, there was no difference in the activation of c-Jun N-terminal kinase (JNK) or p38 between SGS and control cells at baseline or in response to TGF-β2. The alterations in canonical TGF-β and ERK signaling directly parallel previous findings in the aortas of both affected humans and mouse models of MFS and LDS. We also observed a significant increase in mRNA expression for TGF-β–dependent genes in SGS fibroblasts relative to controls, including COL1A1, COL3A1, FBN1, VIM and CDKNIA (encoding collagen type 1, collagen type 3, fibronectin, vimentin and p21, respectively) (Fig. 2b). This is in keeping with previous work showing that transcription of these genes is normally suppressed by SKI. TGF-β also induces transcription of genes encoding negative regulators of the TGF-β pathway, including SKI, SKIL and SMAD7, as part of an autoregulatory loop. We observed higher mRNA expression of each of these genes in SGS cells compared to controls, suggesting that SKI normally suppresses the expression of itself as well as other negative regulators of TGF-β signaling. Notably, the TGF-β–regulated target genes CTGF and SERPINE1...
showed equivalent expression in SGS and control cells, suggesting either gene-specific differences in their sensitivity to attenuation of SKI function or to the regulatory influence of other transcriptional repressors such as SKIL or SMAD7.

Despite the near-complete phenotypic overlap between LDS and SGS, it is notable that the aneurysm phenotype in SGS is less penetrant, less diffuse (generally restricted to the aortic root) and less severe than that seen in LDS. We reasoned that this might relate to the temporal and regional expression pattern of SKI. To test this hypothesis, we performed a developmental survey of SKI expression in wild-type mice (Fig. 3a). At embryonic day (E) 13.5, SKI protein was robustly expressed throughout the vessel wall in the proximal ascending aorta with less expression in the descending segment and was localized to both the cytoplasm and nucleus. At birth (postnatal day 0, P0), aortic expression was somewhat reduced, and SKI was predominantly localized to the cytoplasm. In adult mice (P90), SKI expression was further reduced in the medial layer of the aortic root, with exclusive cytoplasmic localization; in the more distal ascending aorta, SKI expression was largely excluded from the central zone of the aortic media, despite some residual expression in the intimal and adventitial layers. Taken together, these data support the hypothesis that SKI may predominantly be required in the very proximal aorta at early stages of development for the proper regulation of TGF-β signaling within the arterial media.

We show that the multisystem manifestations of SGS are caused by primary mutations in a prototypical repressor of TGF-β signaling and that these mutations associate with a cell-autonomous increase in SMAD2/3 activation at steady state and in the acute phase response to TGF-β ligand, as well as with increased transcriptional output of TGF-β-responsive genes. It remains to be determined whether the increased ERK1/2 activation seen in SGS cells represents loss of a previously unrecognized direct function of SKI or indirect cellular events. The conclusion that release from the suppressive effects of SKI on TGF-β signaling underlies SGS is consistent with previous observation of central nervous system (CNS) patterning alterations, skeletal muscle hypoplasia and craniofacial defects (all cardinal manifestations of SGS) in homoygous Ski-targeted mice. It is further strengthened by our ability to replicate SGS-like defects after morpholino-based knockdown of the two paralogs of mammalian SKI (ski and skib) in zebrafish. ski- and skib-morphant zebrafish embryos showed marked craniofacial cartilage deficits, including shortened and flat Meckel’s cartilage, irregular lengths of palatoquadrates, shortened ceratohyales and depleted ceratobranchial arches (Fig. 3b and Supplementary Table 1). These deficits manifest in larval fish as maxillary hypoplasia, malformed ethmoid plate, micrognathia and microcephaly and are frequently accompanied by ocular hypertelorism and spinal malformations. Furthermore, ski- and skib-morphant embryos showed severe cardiac anomalies, characterized by partial-to-complete failure in cardiac looping and malformations of the outflow tract (Fig. 3b and Supplementary Table 1). Thus, in comparison to Ski-null mice, the zebrafish morphants more closely recapitulate the human SGS craniofacial phenotype and uniquely imply that there is a requirement for SKI in early cardiovascular development. Previous findings of a prominent role for SKI in both neurogenesis and myogenesis reconcile the highly penetrant developmental delay and hypotonia seen...
in SGS, respectively 35,36. It is also notable that a recurrent mutation in SMAD4 that strongly impairs TGF-β transcriptional responses by the R-Smad–Smad4 complex has recently been shown to cause Myhre syndrome, a condition characterized by short bones and aortic stenosis, in marked contrast to the bone overgrowth and aortic dilatation seen in SGS 37. Persons with the recurrent 1p36 deletion syndrome are haploinsufficient for SKI and show some phenotypic overlap with SGS, including hypotonia, developmental delay, craniofacial dysmorphism, vertebral abnormalities and structural heart disease 38,39. Possible reasons for the features that distinguish these two conditions include the involvement of contiguous genes in the 1p36 deletion syndrome and/or the putative dominant-negative potential of mutant forms of SKI in SGS, which might retain their ability to form homodimeric complexes due to structural preservation of the SKI-interacting domain at the C terminus, with functional deficits imposed by N-terminal alterations that selectively perturb R-Smad and/or N-CoR interactions. Substitution of neighboring cysteine residues (p.Cys1223Tyr and p.Cys1221Tyr) in an epidermal growth factor (EGF)-like domain of fibrillin-1 have been reported in association with an SGS-like phenotype, including all features of MFS 38,39. Although it remains to be determined whether this tentative genotype-phenotype correlation is primarily a consequence of FBNI genotype, modifier loci or chance, it seems notable that both fibrillin-1 and SKI are recognized regulators of TGF-β signaling.

In conclusion, this manuscript provides evidence that mutations predicted and observed to enhance TGF-β signaling are sufficient to cause human phenotypes that have variably been associated with low TGF-β signaling states, including craniosynostosis, altered palatogenesis and aortic aneurysm. The development of individualized treatment strategies will require further work to determine whether low and high signaling states achieve the same phenotypic consequence or, as we propose, complex compensatory events during tissue morphogenesis and homeostasis culminate in a common pathogenic mechanism for seemingly disparate disease etiologies 42.

URLs. Picard, http://picard.sourceforge.net/.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.C.D., B.L.L., G.M., T.H., A.D., R.C.H., P.H.A. and C.J.C. recruited participants for the study. H.C.D., A.J.D., J.J.D. and M.E.L. were instrumental in the experimental design and interpretation of the data. A.J.D. performed bioinformatics analysis, with the assistance of D.W.M. and A.F.S., and carried out DNA sequencing and analysis, receiving guidance and supervision from M.J.C. and H.C.D. J.J.D. performed all cell culture experiments and protein blot and quantitative PCR analyses, with the assistance of N.D.H. S.L.B. and S.M. performed all zebrafish experiments under the supervision of N.D.H. S.L.B. and S.M. performed all zebrafish experiments under the supervision of A.S.M. B.L.L. and L.V.L. supervised D.S. and E.G., who performed the study. H.C.D., A.J.D., J.J.D. and M.E.L. were instrumental in the experimental design and interpretation of the data. A.J.D. performed bioinformatics analysis, with the assistance of D.W.M. and A.F.S., and carried out DNA sequencing and analysis, receiving guidance and supervision from M.J.C. and H.C.D.

J.J.D. performed all cell culture experiments and protein blot and quantitative PCR analyses, with the assistance of N.D.H. S.L.B. and S.M. performed all zebrafish experiments under the supervision of A.S.M. B.L.L. and L.V.L. supervised D.S. and E.G., who performed DNA sequencing and analysis. K.S. and R.A.N. performed the mouse developmental studies. D.L. performed structural prediction analyses. A.J.D. and J.J.D. drafted and were co-lead authors for the manuscript. H.C.D., B.L.L. and L.V.L. critically revised and gave final approval for the manuscript to be published.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study participants. Affected individuals were recruited from the Connective Tissue Clinic at Johns Hopkins Hospital (H.C.D.), Radboud University Hospital/Antwerp University Hospital (B.L.L. and G.M.), The Nemours Children’s Clinic (P.H.A.) and Genetic Medicine Central California (C.J.C.). All skin biopsies and research protocols were collected in compliance with the Institutional Review Board at each respective institution after informed consent was obtained. The diagnosis of SGS was made after exclusion of other known congenital syndromes on the basis of distinguishing phenotypic features. Echocardiograms were performed and interpreted as previously described. Aortic root aneurysm was defined by a maximal aortic root z score of ≥2.0.

Whole-exome sequencing. Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. DNA fragmentation was performed using a Covaris S2 system, exon capture was performed using the Agilent SureSelect 38 Mb Human All Exon Target Enrichment system and DNA sequencing was performed on an Illumina Genome Analyzer Ix instrument, with all using standard protocols for 75-bp paired-end runs.

Bioinformatics analysis. Reads were mapped to the human reference genome (UCSC hg19) using the Burrows-Wheeler Aligner (BWA) and a variant list created using SAMtools and were annotated using ANNOVAR. Local realignment and recalibration of base call quality scores was performed using the Genome Analysis Toolkit (GATK). Duplicates were identified using Picard. We selected for novel variants (absent from dbSNP134 or 1000 Genomes Project databases), focusing only on exonic non-synonymous, splice-site and insertion and/or deletion (indel) variants. Variants were viewed directly using the Integrated Genome Viewer (IGV) and excluded if reads were only present in one direction, if ambiguously mapped reads were present or if an indel occurred within 3 bp of the end of the read.

Mutation validation and Sanger sequencing of candidate genes. PCR was performed using a DNA Engine Dyad thermal cycler (Bio-Rad). Phusion Flash High Fidelity PCR Master Mix was used in accordance with the manufacturer’s instructions for each primer set (Dharmacon). Primer sequences and reaction conditions are listed in Supplementary Table 2. Cycle sequencing was performed using the BigDye Terminator v3.1 kit and an ABI 3730 xl DNA Analyzer in accordance with the manufacturer’s instructions (Life Technologies). Samples were purified using the QIAquick PCR Purification kit (Qiagen).

Cell culture. Primary human dermal fibroblasts were derived from forearm skin biopsies from two control individuals and two individuals with SGS. They were cultured in DMEM supplemented with 10% FBS in the presence of antibiotics and were passaged at confluence. All cell experiments were conducted to standard methods. Embryos were injected with previously published skia (12 ng) or skib (14 ng) morpholino antisense oligonucleotides at the 1- to 2-cell stage and were analyzed at 2, 3, 5, 6 and 8 d.p.f. for G-RCFP expression via fluorescence microscopy. Morpholino oligonucleotide sequences are listed in Supplementary Table 2. Previous work established the specificity of these morpholinos for their stated targets via concomitant use of random morpholinos and mRNA rescue experiments. Cartilage staining was performed as previously described on zebrafish embryos fixed in 4% paraformaldehyde at 5, 6 and 8 d.p.f. (ref. 54). All experiments were performed in accordance with ethical permits by the Johns Hopkins Animal Care and Use Committee.

Statistical analysis. All quantitative data are shown as box and whisker plots produced using the R statistical package. The upper and lower margins of the box define the 75th and 25th percentiles, respectively; the internal line defines the median, and the whiskers define the range. Statistical analysis was performed using two-tailed t tests in Excel (Microsoft). P values of <0.05 were considered statistically significant.

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