Neuroinflammatory signals drive spinal curve formation in zebrafish models of idiopathic scoliosis

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The etiopathogenesis of idiopathic scoliosis (IS), a highly prevalent spinal deformity that occurs in the absence of obvious congenital or physiological abnormalities, is poorly understood. Although recent zebrafish genetic studies have linked cilia motility and cerebrospinal fluid (CSF) flow defects with scoliosis progression, underlying mechanisms were not identified. Here, we use next-generation sequencing and conditional genetic methodologies to define the spatial and biological origins of spinal curve formation in ptk7 mutant zebrafish, a faithful IS model. We demonstrate that focal activation of proinflammatory signals within the spinal cord is associated with, and sufficient for, induction of spinal curvatures. Furthermore, administration of acetylsalicylic acid (aspirin) or N-acetylcysteine (NAC) to juvenile ptk7 mutants significantly reduces the incidence and/or severity of scoliosis phenotypes. Together, our results implicate neuroinflammation, downstream of CSF defects, in spinal curve formation and provide intriguing evidence that simple immunomodulating therapies might prove effective in managing idiopathic-like spinal deformities.

INTRODUCTION

Idiopathic scoliosis (IS) is characterized by rotational deformities of the spine that arise in the absence of congenital vertebral malformations or other obvious neuromuscular or physiological defects. IS afflicts ~4% of the population, although curve severity, rotational complexity, and age of onset differ among individuals (1). Genetic studies have linked multiple loci with broad physiological implications to IS progression, including rare variants in genes associated with centriole (POC5) (2) and musculoskeletal collagen function (3), as well as noncoding mutations associated with GPR126, PAX1, LBX1, and BNC2, genes that play varied roles in bone, cartilage, muscle, and nervous system development (4–7). Nevertheless, biological mechanisms underlying IS are not understood and, as a result, treatment options remain limited to antiquated measures such as restrictive brace wear and invasive surgical correction (1).

Recently, zebrafish have emerged as a powerful model for probing the genetic and biological mechanisms underlying IS-like spinal deformities (8). Notably, zebrafish protein tyrosine kinase 7 (ptk7) mutants, deficient in a key regulator of Wnt signal transduction (9), develop late-onset spinal curvatures that faithfully recapitulate defining aspects of human IS (10). Wnt signals control the biogenesis and polarized architecture of motile cilia (11), and analysis of ptk7 mutants, as well as additional zebrafish models of IS, revealed a surprising link between motile cilia–driven cerebrospinal fluid (CSF) flow defects and scoliosis (12). Reintroduction of Ptk7 specifically within motile ciliated cell lineages of ptk7 mutant animals (using a foxj1a::ptk7 transgene) rescued ependymal cell cilia function, restored CSF flow through adult brain ventricles, and fully suppressed spinal curve formation. As human congenital defects that disrupt CSF flow (Chiari malformation, syringomyelia, and spina bifida) (13–15) also associate with high incidences of developmental scoliosis, zebrafish IS studies predict an evolutionarily conserved function for CSF flow and/or homeostasis in normal spine development (12). However, pathobiological mechanisms underlying spinal curve formation remain unknown.

Using conditional genetic methodologies, we now demonstrate that normal spine morphogenesis requires Ptk7 function specifically within motile ciliated cell lineages of the brain, consistent with a role for CSF flow and/or homeostasis defects in IS pathogenesis. We further demonstrate that focal activation of proinflammatory signals within the spinal cord is associated with, and sufficient for, induction of idiopathic-like spinal curvatures. Together, our results implicate neuroinflammation, downstream of CSF defects, in spinal curve formation and provide intriguing evidence that simple immunomodulating therapies might prove effective in managing both the incidence and severity of IS-like spinal deformities.

RESULTS

Defining tissue-specific requirements for Ptk7 in normal spine morphogenesis

The suppression of scoliosis in ptk7 + Tg(foxj1a::ptk7) zebrafish indicated an essential role for Ptk7 within foxj1a+ motile ciliated cell lineages, which include the laterality organ, proenephric duct, and midline cells of the central nervous system (CNS), as previously scored by Tg(foxj1a::eGFP) reporter activity (12). However, additional foxj1a+ cell populations (i.e., in vertebrae or supporting tissue) may have been overlooked in Tg(foxj1a::eGFP) transgenic animals because of weak or transient enhanced green fluorescent protein (eGFP) reporter expression. To comprehensively determine where Ptk7 may function in spine development, we traced the fates of all foxj1a-expressing cells. Transgenic fish expressing Cre recombinase under the foxj1a promoter (foxj1a::iCre) were generated and crossed to a ubiquitous ubi::loxP-eGFP-loxP-mCherry (ubi::Switch) reporter line (16) such that all foxj1a+ cells are permanently labeled by mCherry expression. In larval- and juvenile-staged animals, mCherry was largely confined to the CNS and we failed to detect mCherry+ cells within the bone, muscle, or cartilage around or within the spinal column (fig. S1). However, novel foxj1a+ expression domains were observed within the spinal cord, lateral line, and motor neuron cell populations (fig. S1, B, C, and E′), and movie S1). Therefore, neurosensory or neuromuscular origins of scoliosis in ptk7 models cannot be discounted.
To further define the spatial requirements for Ptk7 in spine development, we developed a conditional genetic approach in which scoliosis in ptk7 mutants is suppressed by a Cre-excisable loxP:foxj1a::ptk7-loxP (floxed-foxj1a::ptk7) transgene (fig. S2A). Early and ubiquitous Cre expression from a heat shock–inducible hsp:Cre transgene (17) can restore scoliosis in these mutant animals, indicating that the Tg(floxed-foxj1a::ptk7) cassette can be efficiently recombined in vivo (fig. S2B and C). To restrict the temporal and spatial domains of Cre expression, we used a modified soldering iron strategy (18) and performed localized heat shocks on ptk7 + Tg(floxed-foxj1a::ptk7) + Tg(hsp:Cre) + Tg(ubi:Switch) mutant embryos at 2.5 days post-fertilization (dpf), imaged for Cre recombination activity at 5 dpf, and grew animals with successful recombination to adulthood (Fig. 1A to D, and fig. S2D). Trunk- and tail-specific recombination of Tg(floxed-foxj1a::ptk7), as reported by Tg(ubi:Switch) mCherry expression, failed to restore scoliosis phenotypes in all trials (Fig. 1B and F; n = 38 of 38). In contrast, head-specific recombination of Tg(floxed-foxj1a::ptk7) restored IS phenotypes in ptk7 mutants (Fig. 1C and G; n = 14 of 49). Notably, ptk7/+ sibling controls that were locally heat shocked did not develop spinal curvatures (Fig. 1H; n = 82 of 82), indicating that scoliosis observed in experimental cohorts was specific to Tg(floxed-foxj1a::ptk7) recombination. Together, with lineage tracing experiments, our results indicate that Ptk7 is not required in the trunk peripheral nervous system, spinal cord, vertebrae, cartilage, or muscle for normal spine development. Rather, loss of Ptk7 specifically within foxj1a+ lineages of the brain is sufficient to induce scoliosis.

As the brain and spine are spatially and functionally distinct, the pathogenesis of scoliosis in ptk7 mutants likely involves secondary signals. Notably, foxj1a+ lineage tracing broadly labels ependymal and choroid plexus cells within the brain, which function in the production and movement of CSF (fig. S1E) (19), and CSF flow defects have been demonstrated within adult ptk7 brain ventricles (12). To investigate whether CSF flow dynamics are abnormal in ptk7 mutants at developmental stages relevant to scoliosis phenotypes, we performed live imaging of soluble dye injected into the brain ventricles of juvenile-staged zebrafish. Notably, severe defects in the movement of CSF into the spinal cord of ptk7 mutants were observed before scoliosis onset (Fig. 1, I and J; n = 12 of 12), consistent with abnormalities of CSF flow and/or homeostasis contributing to the pathogenesis of IS.

**Abnormal immune responses, downstream of CSF defects, associate with scoliosis**

To identify molecular mechanisms underlying spine curvature, downstream of brain and/or CSF defects, we performed next-generation RNA sequencing (RNA-seq) on ptk7 mutant versus ptk7/+ sibling fish at a stage correlating with severe IS progression (~1 cm in length; fig. S3B). Heads were dissected at the level of the gill to remove upstream...
Fig. 2. Spinal curve progression is associated with immune and neuroinflammatory responses. (A) and (B) Top 20 significantly (*P < 0.05) up-regulated (A) and down-regulated (B) genes (sorted by log; fold change (LFC)) identified in *ptk7* mutant fish with severe spinal curvatures versus *ptk7/+* control siblings, padj, Benjamini-Hochberg adjusted P value. (C) Gene Ontology (GO) term enrichment analysis of biological processes on all significantly up-regulated genes. Teal, complement response genes; yellow, other immune responses; blue, *ptk7*. (D) Heatmap depicting expression of immune and inflammatory response genes significantly (*P < 0.05) up-regulated in *ptk7* mutants at curve onset or during mild and severe curve progression versus *ptk7 + Tg(foxj1a::ptk7)* control siblings. (E) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) verifying differential expression of selected genes in *ptk7* mutants with severe curves versus *ptk7 + Tg(foxj1a::ptk7)* siblings. *P < 0.05, **P < 0.01. n.s., not significant.
signals associated with abnormal Ptk7 function in the brain, and because downstream cellular and biological origins of scoliosis remained unknown, RNA-seq was performed on bulk RNA samples collected from the entire trunk and tail. As expected, differential gene expression analysis revealed that ptk7 mutant transcripts, which are subject to nonsense-mediated decay (9), are significantly reduced in scoliotic animals (Fig. 2B). Genes associated with bone, muscle, or connective tissue development were not significantly dysregulated in scoliotic fish (Fig. S3, D to F). Rather, most of the transcripts significantly up-regulated in ptk7 mutants encoded proteins involved in complement activation and innate immune response (Fig. 2, A to C; fig. S3C; and data file S1).

Because Ptk7 and Wnt signals control diverse biological activities across numerous tissues, abnormal immune responses observed in ptk7 mutant fish might be unrelated to scoliosis phenotypes. To identify gene expression profiles directly associated with spinal curve progression, we repeated RNA-seq experiments comparing ptk7 and ptk7+ Tg(foxj1a::ptk7) mutant animals, which are genetically and phenotypically equivalent beyond ptk7 transgene expression and spine development (fig. S4A) (12). RNA was collected from trunk and tail tissues at time points correlating with scoliosis onset (~0.7 cm in length; fig. S4B), as well as mild and severe curve progression (~0.8 cm in length; fig. S4, C and D). Notably, differential gene expression and qRT-PCR analyses confirmed a significant and dynamic scoliosis-associated immune response in ptk7 mutants, including activation of acute-phase inflammation markers at the onset of spinal curvature, complement system activation at severe curve progression, and the up-regulation of NOD (nucleotide-binding oligomerization domain)–like receptors and other immune response genes across developmental time points (Fig. 2, D and E; fig. S4, E to G; and data file S1).

To determine how inflammation and immune responses correlate with spinal curvature, we directly monitored macrophage activity in juvenile ptk7 mutant versus ptk7+/+ sibling fish using the mpeg1:eGFP reporter transgene (20). Notably, eGFP+ macrophages accumulated locally at the site of spinal curve formation in ptk7 mutants (Fig. 3, A and B), consistent with an early role for inflammation in the pathogenesis of IS. Transverse sections through the trunks of these animals revealed increased accumulation of eGFP+ macrophages specifically...
within the spinal cord of ptk7 mutant fish (Fig. 3, C to F, and fig. S5). These results, in conjunction with RNA-seq data, suggest that ptk7 mutants experience a neuroinflammatory response at the onset of spinal curve formation.

**Focal neuroinflammatory signals are sufficient to induce IS-like spinal curvature**

If neuroinflammation drives scoliosis in ptk7 mutant fish, then induction of immune responses within the spinal cord of wild-type animals should be sufficient to cause spinal curvature. To test this, we generated CNS expression clones of (i) the proinflammatory cytokine interferon-γ 1-2 (ifng1-2) (21) and (ii) mCherry-tagged immunoresponsive gene 1-like (irg1l), which regulates macrophage and neutrophil recruitment to wounded epithelia (22) and is strongly up-regulated in scoliotic ptk7 mutants (Fig. 2, D and E). We constructed Tol2-based transposable elements composed of a ubiquitous βactin2 promoter driving expression of a floxed eGFP reporter element (loxP-eGFP-loxP or LGSL) followed by ifng1-2 or mCherry-irg1l cassettes. When these transposons are injected into one-cell–staged embryos, widespread and mosaic eGFP expression can be detected (Fig. 4, A, C, and D, and fig. S6). However, within the spinal cord of Tg(fox1a::iCre) embryos, lineage-specific Cre recombinase activity results in recombination of reporter elements, focal expression of proinflammatory molecules, and local recruitment of eGFP+ macrophage (Fig. 4C and fig. S6, B and C”).

To determine whether CNS-specific expression of proinflammatory cues can induce scoliosis, injected embryos were raised and screened for reporter expression and spinal curve formation (Fig. 4).
Injected Cre-negative animals demonstrated mosaic expression of eGFP across all tissues and failed to develop scoliosis (Fig. 4, E and G; n = 137). In contrast, Tg(foxj1α::iCre) animals injected with Tg(β:LSL mCherry-irg1l) developed spinal curvatures at 3 weeks post fertilization (Fig. 4F; n = 7 of 38), suggesting that recombination of the eGFP cassette and resulting mCherry-irg1l expression in foxj1α+ lineages is sufficient to induce spine curvature. Notably, small clones of mCherry-irg1l+ cells could be identified in the CNS at the apex of developing axial curvatures (Fig. 4D). Similarly, Tg(foxj1α::iCre) animals injected with Tg(β:LSL ifng1-2) also developed scoliosis (Fig. 4H; n = 12 of 44). The association between proinflammatory signals and spinal curvature is specific to the CNS, as mosaic endothelial-specific expression of mCherry-irg1l (fig. S6, D to G; n = 53 of 53) or ifng1-2 failed to cause scoliosis. Together, our data suggest that focal, neuroinflammatory signals are sufficient to drive idiopathic-like spinal curvatures.

Simple immunomodulating therapies suppress scoliosis onset and curve severity

To determine whether suppression of inflammatory responses can block scoliosis in our IS model, ptk7 mutant zebrafish were treated with the nonsteroidal anti-inflammatory drug (NSAID) acetylsalicylic acid (aspirin). Aspirin was administered to juvenile fish at 10 days of age (~4 mm standard length), before the onset of spinal curvatures, through simple dispensation into tank water at a final concentration of 100 mg/liter. Control and experimental cohorts were reared in tanks through simple dispensation into tank water at a final concentration of 100 mg/liter. Notably, by 40 dpf, only 17% (n = 8 of 47) of NAC-treated ptk7 mutants had developed spinal curvatures compared to 81% (n = 77 of 95) of the control population (79% reduction in incidence; Fig. 6A). Moreover, removal of NAC from the experimental cohort resulted in rapid onset of scoliosis in 49% of animals (Fig. 6A; n = 19 of 39). These data indicate a significant and specific role for NAC treatment in suppressing spinal curve formation.

The low penetrance of scoliosis in the NAC-treated cohort precluded meaningful statistical analysis of spinal curve severity. We therefore devised a treatment regimen whereby fish were not administered NAC until after spinal curve onset (Fig. 6B). ptk7 mutants were censused daily and, upon onset of scoliosis, removed from the population and split into experimental and control groups. Experimental cohorts were administered NAC (200 mg/liter), as described above. All fish were then collected at 20 days after curve onset to assess curve severity (fig. S7). Treatment with NAC after scoliosis onset significantly reduced the severity of spinal curve progression (Fig. 6C).

Fig. 5. Aspirin treatment significantly affects spinal curve formation. (A) Kaplan-Meier estimator analysis demonstrating significant differences in the incidence and onset of scoliosis in control (n = 87) versus aspirin-treated (n = 111) ptk7 mutant fish (N = 5). (B) Box and whisker plot demonstrating a significant difference in the age of scoliosis onset between control (n = 90) and aspirin-treated (n = 88) ptk7 mutants (P = 1.8 × 10−12, two-tailed t test). *** P < 0.001. (C) Scoliotic fish were fixed at 30 days after phenotype onset to assess curve severity. Quantification of combined Cobb angles in untreated (n = 14) versus aspirin-treated (n = 12) ptk7 scoliotic fish revealed no significant difference in curve severity (P = 0.1779, two-tailed t test).
Together, our results implicate neuroinflammatory signals as the pathobiological mechanism driving idiopathic-like spinal curve formation in zebrafish models of IS. In ptk7 mutant animals, we postulate that neuroinflammation is caused by focal neurodegeneration or demyelination events, which occur in the spinal cord downstream of observed CSF flow and/or homeostasis defects. How proinflammatory signals within the CNS ultimately influence spine development remains to be determined. Irregularities in the distribution of cytokines/signals within the CSF, or perhaps asymmetric hydrodynamic forces operating within the central canal of the spinal cord, must be relayed to overlying musculature and axial skeleton to induce curvatures. Notably, an evolutionarily conserved population of neurons called “CSF-contacting neurons” (CSF-cNs) line the central canal of the spinal cord, project a sensory cilia into the CSF, and can detect changes in pH as well as physical curvature of the body axis (25–29). Although the function of CSF-cNs is not well understood, they have been demonstrated to modulate spinal circuits that underlie locomotion and posture, and zebrafish pdk2l1 mutations, which compromise mechanosensory functions of CSF-cNs, have recently been associated with exaggerated spine curvatures (26, 30). Because CSF-cNs are uniquely situated to both detect CSF abnormalities within the central canal and influence overlying muscular contraction, we have hypothesized that abnormal CSF-cN activity (i.e., in response to physical changes to CSF flow or chemical composition, downstream of proinflammatory signals) may contribute to spinal curve progression (8).

Although a definitive link between neuroinflammation and human IS requires further study, inflammatory origins of scoliosis are not inconsistent with human clinical and genetic studies. Hyper-IgE (immunoglobulin E) syndrome, characterized by frequent bacterial infection and inflammation, has been associated with developmental deformities, conservation of the mechanism could have profound impacts on the future prevention and treatment of human scoliosis. The therapeutic potential of NAC in treating IS remains to be determined. Irregularities in the distribution of cytokines/signals within the CNS ultimately influence spine development remain uncertain (31). For example, IS-associated variants linked to GPR126 have been functionally interrogated largely in the context of musculoskeletal origins of scoliosis (4, 33). However, because myelination requires GPR126 (34), regional demyelinating events downstream of GPR126 dysfunction could activate microglial inflammatory signals that initiate IS-like spinal curvatures (32). To date, the biological consequences of most IS-associated variants identified in genetic and genome-wide association studies remain uncertain (1). For example, IS-associated variants linked to GPR126 have been functionally interrogated largely in the context of musculoskeletal origins of scoliosis (4, 33). However, because myelination requires GPR126 (34), regional demyelinating events downstream of GPR126 dysfunction could activate microglial inflammatory signals that initiate IS-like spinal curvatures. Novel neuroinflammatory origins of IS identified in this study thus warrant a reexamination and interpretation of human IS clinical and genetic data.

Strikingly, we have demonstrated that treatment with common NSAIDs can have a significant and positive impact on the incidence and severity of scoliosis in our ptk7 mutant models. Notably, prophylactic treatment with NAC reduced the incidence of scoliosis in experimental cohorts by 79%. Furthermore, administration of NAC after scoliosis onset significantly reduced the severity of spinal curve progression. NAC is considered a well-tolerated and safe medication, with emerging clinical use for treating neurological and neurodevelopmental disorders in both children and adults [reviewed in (23, 35)]. The therapeutic potential of NAC in treating IS remains to be determined. However, given that simple immunomodulating therapies prove effective in managing zebrafish idiopathic-like spinal deformities, conservation of the mechanism could have profound impacts on the future prevention and treatment of human scoliosis.  

**DISCUSSION**

Together, our results implicate neuroinflammatory signals as the pathobiological mechanism driving idiopathic-like spinal curve formation in zebrafish models of IS. In ptk7 mutant animals, we postulate that neuroinflammation is caused by focal neurodegeneration or demyelination events, which occur in the spinal cord downstream of observed CSF flow and/or homeostasis defects. How proinflammatory signals within the CNS ultimately influence spine development remains to be determined. Irregularities in the distribution of cytokines/signals within the CSF, or perhaps asymmetric hydrodynamic forces operating within the central canal of the spinal cord, must be relayed to overlying musculature and axial skeleton to induce curvatures. Notably, an evolutionarily conserved population of neurons called “CSF-contacting neurons” (CSF-cNs) line the central canal of the spinal cord, project a sensory cilia into the CSF, and can detect changes in pH as well as physical curvature of the body axis (25–29). Although the function of CSF-cNs is not well understood, they have been demonstrated to modulate spinal circuits that underlie locomotion and posture, and zebrafish pdk2l1 mutations, which compromise mechanosensory functions of CSF-cNs, have recently been associated with exaggerated spine curvatures (26, 30). Because CSF-cNs are uniquely situated to both detect CSF abnormalities within the central canal and influence overlying muscular contraction, we have hypothesized that abnormal CSF-cN activity (i.e., in response to physical changes to CSF flow or chemical composition, downstream of proinflammatory signals) may contribute to spinal curve progression (8).

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**MATERIALS AND METHODS**

**Animal care**

Zebrafish husbandry and experimental protocols were approved by the Hospital for Sick Children’s Animal Care Committee, and all
protocols were performed in accordance with Canadian Council on Animal Care guidelines. \textit{ptk7} (9), \textit{Tg(foxj1a::ptk7} (12), \textit{Tg(ubi::Switch)C1701} (16), \textit{Tg(hsp68-Cre)} (17), \textit{Tg(mpeg1:eGFP)} (20), and \textit{Tg(kdr::Cre)} (36) mutant and transgenic fish used in these studies have already been published.

**Molecular cloning**

A full-length open reading frame (ORF) of \textit{irg1l} was amplified from complementary DNA template prepared from 3-dpf zebrafish embryo RNA using SuperScript IV following the manufacturer’s instructions using primers \textit{GGGGAACAGCTTTGTCTGAAAGTGGGGCCACCATGGTTCCAGGTACAGAGATC} (forward) and \textit{GGGGCACTTTGTATAAATAAGTGGCTATTGGCATTTGGGCAAGCAG} (reverse). An ORF was amplified from \textit{pTol2-ifng1-2} and \textit{bryo RNA} using \textit{SuperScript IV} following the manufacturer’s instructions to prepare the complementary DNA template prepared from 3-dpf zebrafish embryos. \textit{Irg1l} was amplified from a full-length open reading frame (ORF) of \textit{Molecular cloning} was subcloned into \textit{pCS2+} vectors and used as templates for in vitro transcription. Two untagged \textit{mCherry-irg1l} and \textit{gene induction (\textit{ptk7})). Briefly, untagged \textit{mCherry-irg1l} and \textit{gene induction} were performed by RNA overexpression, followed by qRT-PCR assays of the up-regulated and immune-related genes (see table S1 for primer sequences).

**Quantitative reverse transcription polymerase chain reaction**

qRT-PCR for RNA-seq verification was performed using 10 ng of total RNA in a one-step qRT-PCR \textit{(Luna Universal One-Step RT-qPCR Kit, New England Biolabs (NEB)) and performed on a Roche LightCycler 96 system. Primers were designed for verifying some of the up-regulated and immune-related genes (see table S1 for primers used). All graphs are representative of two independent experiments with three technical replicates.

**Transgenesis**

The Cre-excisable destination vector \textit{pDEST Tol2LGSR} was generated from \textit{pDEST Tol2pA2}. A fragment encoding for the zebrafish \textit{\gamma-crystallin promoter}, \textit{eGFP}, \textit{loxp} site, and overlapping sequence was purchased and synthesized from Blue Heron Gene Synthesis, digested with \textit{Bgl II} and \textit{Nco I} (NEB), and ligated into the corresponding sites in \textit{pDEST Tol2pA2} to generate an intermediate vector. A second fragment encoding for a \textit{loxP} site, \textit{mCherry}, and overlapping sequence was purchased and synthesized similarly, digested with \textit{Nsi I} and \textit{Sal I} (NEB), and ligated into the intermediate vector to generate the final Gateway-compatible \textit{pDEST Tol2LGSR}. This vector permits Cre-dependent removal of transgenes with a visual readout of lens color change. Upon the introduction of Cre recombinase, transgene cassettes that reside between the vector \textit{loxP} sites are excised with the GFP, resulting in \textit{mCherry} being driven by the \textit{\gamma-crystallin} promoter.

Final transgenes were assembled using standard \textit{Tol2} kit Gateway-compatible vectors via LR reactions (37). To generate \textit{Tg(foxj1a::icre)} zebrafish, \textit{p5E-fosxj1aP} (12), \textit{pME-icre} (a gift from K. Kwan), and \textit{p3E-polyA} were recombined into \textit{pDEST Tol2 CG2 transgenesis vector}. To generate \textit{Tg(floxed-fosxj1a::ptk7)} zebrafish, \textit{p5E-fosxj1aP} (12), \textit{pME-pk7(9)}, and \textit{p3E-polyA} were recombined into \textit{pDEST Tol2LGSR transgenesis vector}. Embryos were injected at the one-cell stage with 25 pg of assembled transgene and 25 pg of Tol2 mRNA. Embryos were scored at 48 hpf for reporter expression (GFP+ hearts or eyes) and were subsequently grown to adulthood. Individuals were bred to TU wild-type zebrafish to generate stable F1 lines. Subsequent F1 lines harboring \textit{Tg(floxed-fosxj1a::ptk7)} were bred and maintained in \textit{ptk7} (floxed) mutants. Two independent \textit{Tg(foxj1a::icre)} lines that had equivalent expression were generated.

**Imaging**

Imaging of double transgenic \textit{Tg(ubi::Switch);Tg(foxj1a::icre)} animals was performed on an Axio Zoom.V16 and an LSM 710 confocal microscope (Zeiss). Z-stacks were collected and processed in Zen (Zeiss).

**Global and local heat shocks**

Global heat shocking was performed by rapidly immersing 2.5-dpf embryos into 38°C egg water for 30 min, followed by gradual recovery to 28.5°C. Embryos were screened for successful heat shocking by recombination of the eGFP to \textit{mCherry} lens reporter and grown to adulthood. Local heat shocking was performed by using a modified soldering iron (SP12 Mini Soldering Iron, Weller) connected to a DC regulated power supply (Digital Triple Output DC Power Supply, Extech). Soldering iron tips were calibrated for temperature using a soldering tip thermometer (Digital Solder Tip Celsius Thermometer, Hakko FG100), and voltage output was set to 22.5 V corresponding to 44°C (as warmer temperatures were required for heat shock induction because of tip cooling after immersion into egg water). Before heat shock, embryos were aligned in an agarose plate with wells containing egg water, with tricaine anaesthetic either head-up for head heat shocks or tail-up for trunk heat shocks. Local heat shocking was performed by resting the soldering iron tip on the embryos for 3 min per individual embryo, followed by removal from the agarose plate and recovery at 28.5°C. Embryos were screened for successful heat shock induction by recombination of the eGFP to \textit{mCherry ubi::Switch reporter} and grown to adulthood. See fig. S2 for experimental setup.

**Transposon-mediated clonal expression**

Cre-dependent transposons used in mosaic clonal expression were assembled using standard \textit{Tol2} kit Gateway-compatible vectors via LR reactions. To generate \textit{Tg(\betaactin2::LGSL mCherry-irg1l)}, \textit{p5E-\betaactin2} (37), \textit{pME-loxp-eGFP-pA-loxp}, and \textit{p3E-mCherry-irg1l} were recombined into \textit{pDEST Tol2pA2} transgenesis vector. To generate

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bryos were further screened at 72 hpf for mCherry+ cells within actin2 actively). In experiments using Tg(foxj1a::mCherry-irg1l) or Tg(foxj1a::mCherry-irg1l) injection vector. Embryos produced from a Tg(iCre) outcross were and p3E-ifng1-2::actin2, pME-loxP-eGFP-pA-loxP, LGSL ifng1-2 Tg(foxiCre) injected at the one-cell stage with 12 pg of either Tg(foxj1a::ptk7 + Tg(foxj1a::ptk7) were selected and processed for RNA-seq differential gene expression analysis. RNA-seq yielded approximately 60 to 70 million reads per sample.

**RNA-seq differential gene expression analysis**

Aligned reads were quantified using SeqMonk. Differential gene expression analysis was performed with the Bioconductor package DESeq2. GO term enrichment analysis was acquired using the PANTHER classification system and PANTHER Tools gene list analysis. The heatmap was created using R. Lists of genes annotated with “GO:0006953 Acute-phase response,” “GO:0006956 Complement activation,” and “GO:0006954 Inflammatory response” were downloaded from the Gene Ontology Consortium (38), and genes within these lists, which were significantly (P < 0.05) up-regulated at onset of scoliosis, at mild curvature, or at severe curvature, were chosen to create the heatmap. Volcano plots were created using R, and lists of genes for associated GO terms were downloaded from the Gene Ontology Consortium.

**Drug and chemical treatments**

Fish for chemical/drug treatments were housed off-system in 1 liter of system water in 6-liter tanks with up to 40 fish per tank, and water was changed once per day. Acetylsalicylic acid (100 mg; CAS 50-78-2, Sigma-Aldrich) was administered once per day to experimental fish during water changes. NAC (CAS 616-91-1, Sigma-Aldrich) was prepared in a stock solution of 10 g/liter in system water and brought up to a pH of 7 with NaOH and then stored at 4°C. NAC was administered once per day at a final concentration of 200 mg/liter with water changes. Fish were fed according to the regular feeding schedule throughout the experiment. During water changes, fish were monitored for onset of scoliosis, and overall length was measured. For analysis of curve severity, fish were separated on the basis of age of curve onset and, at 20 or 30 days after curve onset, fixed in 4% paraformaldehyde in 1× phosphate-buffered saline (PBS) for alizarin red staining.

Alizarin red staining and Cobb angle measurements

Alizarin red staining was performed as previously described (10). Cobb angles were measured using SCODIAC software. Cobb angle measurements from lateral and dorsal images were summed together to achieve an overall accumulative Cobb angle measurement for each scoliotic fish (fig. S7).

**Transverse cross sections and quantification of macrophage localization**

Tg(mpeg1:eGFP) fish were euthanized and fixed in 4% paraformaldehyde overnight at 4°C. Fish were then stained with alizarin red at room temperature overnight and washed once in 1× PBS. Stained samples were embedded in 4% agarose, and agarose blocks were stored overnight in 1× PBS at 4°C. Sections (150 μm) were obtained using a Leica VT1200S vibrating blade microtome and mounted in Fluoroshield with DAPI (4′,6-diamidino-2-phenylindole; F6057, Sigma-Aldrich). Z-stacks of approximately 50 μm, 1.375-μm step size, were captured for each section using a Nikon A1 confocal microscope with 20× objective. Macrophage numbers were first counted in maximum intensity z-stack projection images of individual sections. Macrophage numbers were verified by reexamining the entire z-series to ensure that outstretched processes were properly assigned to individual macrophage cell bodies.
Supplementary materials

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/12/eaa1781/DC1

Fig. S1. Cre/lox lineage tracing of mutant fish expressing the Tg(foxj1a::ptk7/+ cells are broadly present throughout the central and peripheral nervous system.

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