Shoc2 is a pivotal regulator of neural crest specification, migration, and differentiation

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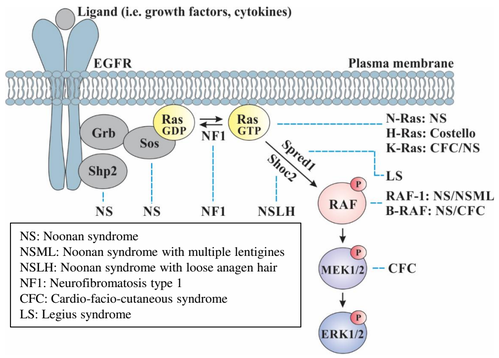
Abstract/Introduction

RASopathies collectively account for one of the most common genetic disorders. Patients exhibit diverse developmental defects including cartilage and bone dimorphisms, growth delays, irregular skin pigmentation, and defective enteric nervous systems. Most RASopathy patients' affected tissues arise from a transient, stem cell-like progenitor cell population, neural crest (NC). Shoc2 is a non-enzymatic scaffold protein essential for development in which gene mutations have been identified in RASopathy (Noonan syndrome with loose anagen hair) patients. However, the molecular details delineating Shoc2-mediated signals in NC are undetermined. This study focuses on elucidating the molecular mechanism by which Shoc2-mediated signals regulate the development of NC.

We investigated how the loss of Shoc2 affects the expression of NC regulatory network genes at different stages of NC development. In our experiments, we depleted Shoc2 using a morpholino (MO) and analyzed gene expression by whole *in situ* hybridization (WISH) and RT-qPCR analysis. We found that the expression of early neural plate border (NPB) formation regulators *prdm1a*, *pax7*, and *foxD3* were affected by the loss of Shoc2. Embryos depleted of Shoc2 also had nonuniform spatial organization of expreseed NC markers *foxD3* and *sox10* and deficient cell migratory gene *dlx2a* during the subsequent NC cell specification and migration processes. Additionally, *krox20* expression was deficient during NC cell terminal differentiation into mature Schwann cells. This suggests that NC cells are unable to overcome the early NC defects from Shoc2 deficient misregulated signaling. Finally, we determined that Shoc2-mediated signaling is essential for the regulation of cartilage proteoglycan genes' (*acana* and *acanb*) and collagen expression.

For the first time, this study has defined the foundational mechanism of Shoc2-deficient RASopathy phenotypes in zebrafish embryos that recapitulate patient Noonan syndrome with loose anagen hair. We conclude that Shoc2 is required for the NPB formation and NC cell specification, migration, and terminal differentiation. Finally, the changes of *acana, acanb,* and *col2a1* expression indicate that Shoc2-transmitted signals are essential for the regulation of the tightly controlled structural proteins that are required for craniofacial anatomy. Ultimately, depleting Shoc2 leads to abnormal NC development and deficient NC-derived tissues.

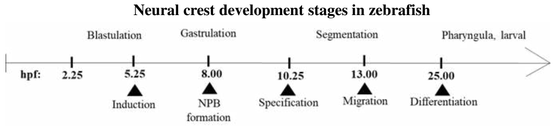
Shoc2 mutations in Noonan-like RASopathy patients



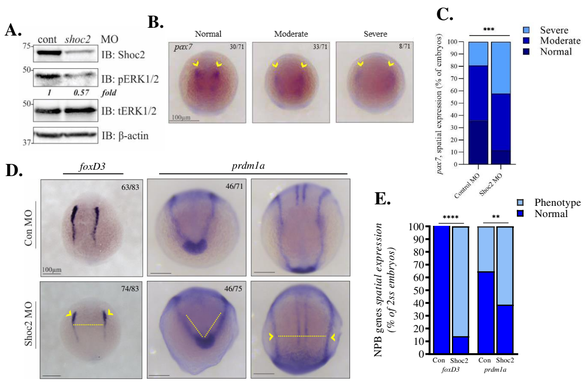
Zebrafish as a vertebrate development model to study Shoc2

- Shoc2 is well conserved in zebrafish (*D. rerio*)
- Robust reproduction and rapid development
- External development with optical clarity during embryogenesis
- Easy genetic manipulation by microinjection

Neural crest development stages in zebrafish



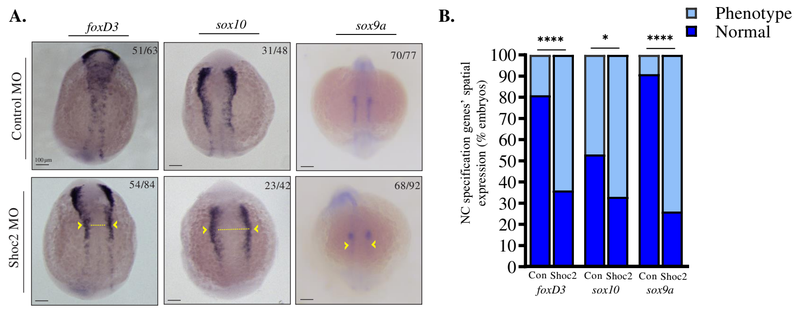
1. Molecular defects in NPB formation in shoc2 morphants



shoc2 or control (con) MO were injected into 1-2 cell stage embryos. **(A.)** Injected embryos were harvested for immunoblotting at 72hpf. **(B. & D.)** The expression of NPB regulators *pax7*, *foxD3*, and *prdm1a* were analyzed by whole *in situ* hybridization (WISH) at the 2 somite stage. Abnormal spatial distribution of gene expression was detected. (**C & E.)** Embryos were qualitatively grouped (from A. and D respectively) and statistical significance (p≤ 0.05) was determined by chi-square analysis. Yellow arrow heads and lines indicate specific points of differences in expression patterns.

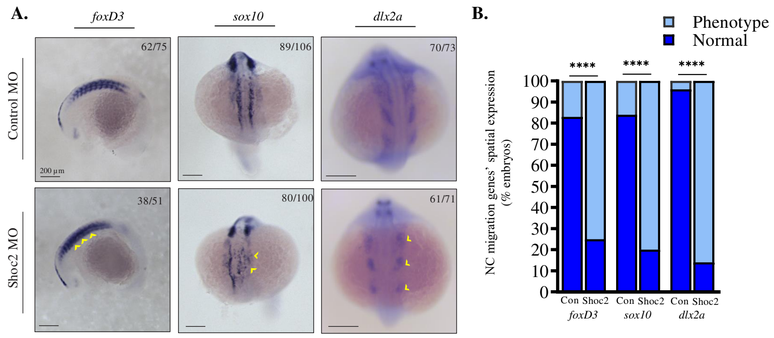
Methods / Results

2. Gene expression abnormalities of *foxD3, sox10,* and *sox9a* in *shoc2* morphants



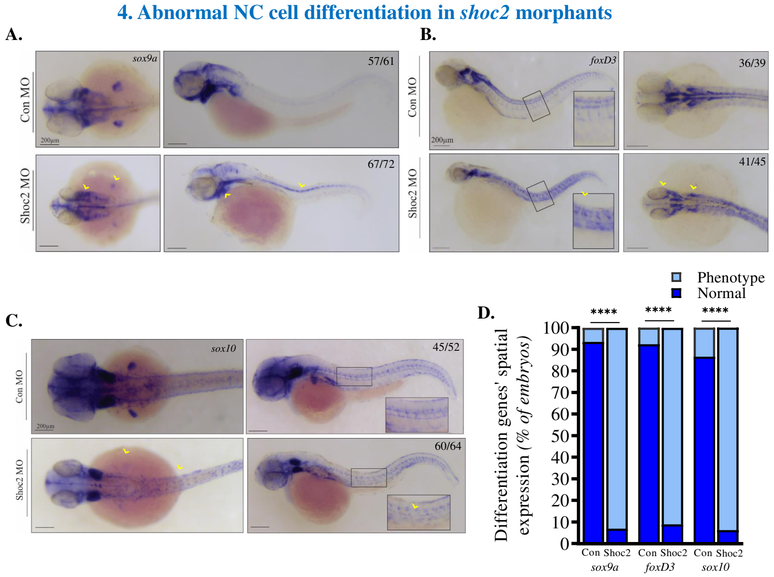
(A.) *shoc2* or control MO were injected into 1-2 cell stage embryos. The expression of NC specification regulators *foxD3, sox10,* and *sox9a* (pre-chondrogenic marker) were analyzed by WISH. Abnormal expression was detected in 5 somite embryos (*foxD3* and *sox10*) and 18 somites (*sox9a.)* **B.)** Embryos were qualitatively grouped and statistical significance (p< 0.05) was determined by chi-square analysis. Yellow arrow heads and lines indicate specific points of differences in expression patterns.

3. Gene expression abnormalities in early migrating NC cells in *shoc2* morphants



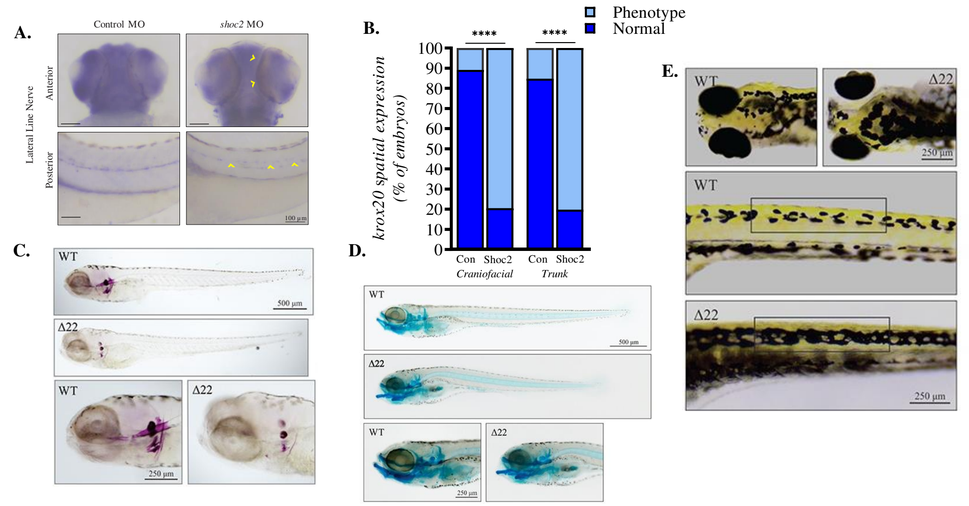
(A.) *shoc2* or control MO were injected into 1-2 cell stage embryos. The expression of NC migratory markers *foxD3*, *sox10,* and *dlx2a* (migratory maker of osteochondral progenitor cells) were analyzed by WISH at 18 somites. Delayed expression was detected. **(B.)** Embryos were qualitatively grouped and statistical significance (p< 0.05) was determined by chi-square analysis. Yellow arrow heads and lines indicate specific points of differences in expression patterns.

4. Abnormal NC cell differentiation in *shoc2* morphants



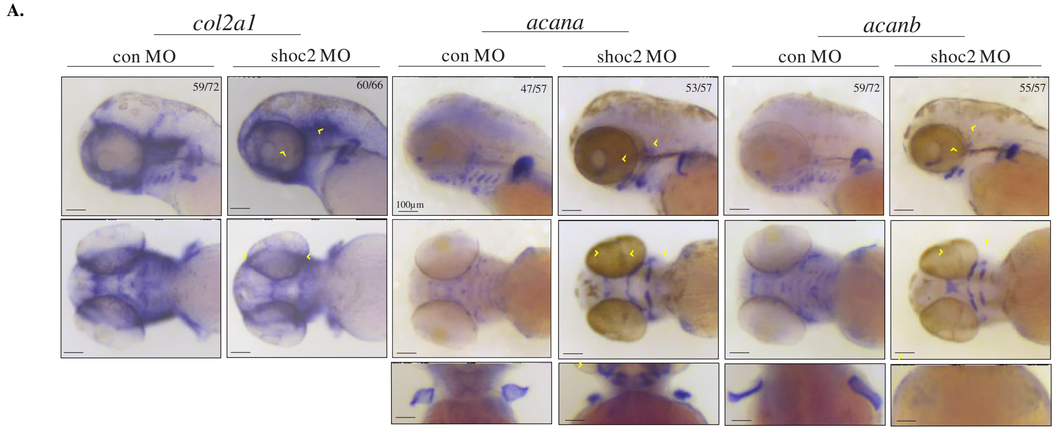
shoc2 or control (con) MO were injected into 1-2 cell stage embryos. The expression of NC cell differentiation markers **(A.)** *sox9a*, **(B.)** *foxD3*, and **(C.)** *sox10* were analyzed by WISH at 2dpf. **(D.)** Embryos were qualitatively grouped and statistical significance (p< 0.05) was determined by chi-square analysis. Yellow arrow heads and lines indicate specific points of differences in expression patterns. *sox9a* is essential for NC progenitor specification and cartilage morphogenesis. *foxD3* is needed for the NC derivatives including cartilage, glia, enteric neurons, and iridophores. *sox10* is present in neurons, melanocytes, and glia.

6. Defects in peripheral neurons, chromatophores and craniofacial cartilages and bone in *shoc2* morphants



(A.) *shoc2* or control MO were injected into 1-2 cell stage embryo. The expression marker of mature Schwann cells, *krox20* required for myelination, *was* analyzed by WISH at 3dpf. *Krox20* expression pattern was abnormal and downregulated. Yellow arrow heads and lines indicate specific points of differences in expression patterns. **(B.)** Embryos were qualitatively grouped and statistical significance (p< 0.05) was determined by chi-square analysis. **(C.)** Alizarin staining identified significantly deformed/delayed cranial bone elements in Shoc2 null embryos (Δ22) at 6dpf. **(D.)** Alcian blue staining of head cartilage identified significant differences in craniofacial development of Shoc2 null larvae (Δ22) at 6dpf. **(E.)** 6 dpf larvae showing melanophores in WT and Shoc2 null embryos (Δ22). Unlike WT larvae, *shoc2* mutants presented with closed gaps in melanocyte pattern of head and lateral stipe (see brackets).

7. Alterations in expression of collagen and proteoglycans in *shoc2* morphants and mutants



(A.) *shoc2* or control MO were injected into 1-2 cell stage embryos. The expression of extracellular matrix genes *col2a1, acana,* and *acanb* were analyzed by WISH at 3dpf. ECM gene expression in craniofacial structures has abnormal patterning and appears decreased as compared to the con MO embryos. **(B.)** Embryos from A. were qualitatively grouped and statistical significance (p≤ 0.05) was determined by chi-square analysis. Yellow arrow heads indicate specific points of differences in expression patterns. **(C.)** RT-qPCR was done on cDNA from Shoc2 null (stop codon mutant) embryos raised to 5 and 6dpf. Expression was normalized to *gapdh*. ECM expression in Shoc2 null embryos is heavily upregulated by 6dpf. However, at 5dpf ECM expression is decreased or not different from wildtype. Error bars represent means with SD. p ≤0.05 (Student's t-test).

Acknowledgements

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Conclusions / Future Directions

Our data suggest that Shoc2 is critical for early embryonic NC development from NPB formation through final NC cell differentiation. Loss of *shoc2* resulted in misregulated NPB formation, and NC cell specification, migration, and terminal differentiation. Furthermore, Shoc2 is critical for ECM expression. Together, this is the first collection of data demonstrating the significance of Shoc2 at all stages of NC development. This work will be continued to further understand the specific molecular mechanism by which Shoc2 regulates ECM expression and NC development.