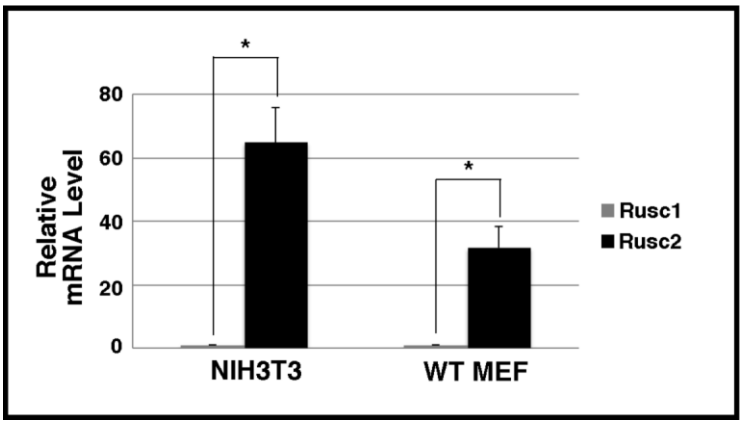
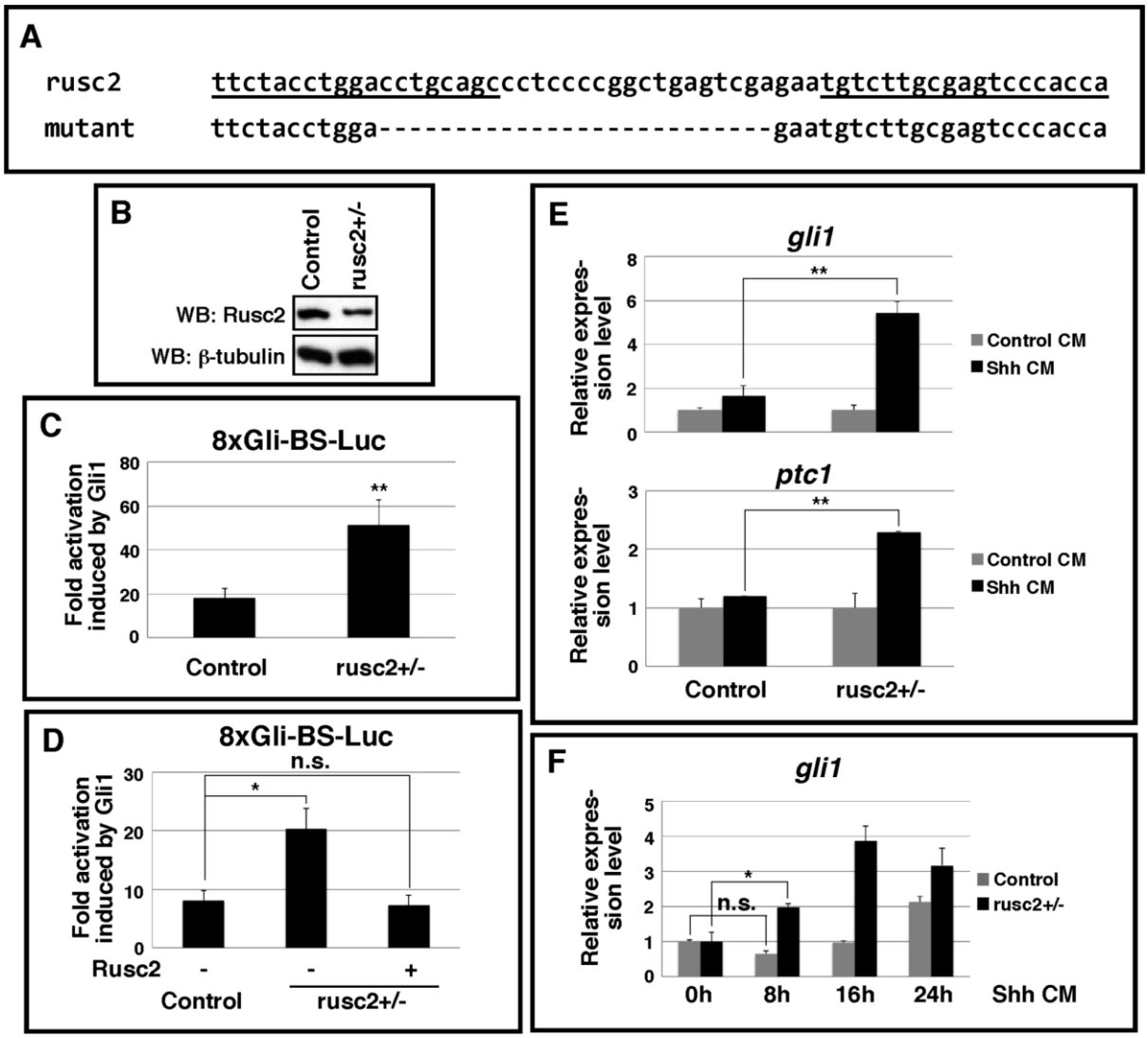
Supplemental Figures



Supplemental Figure 1.

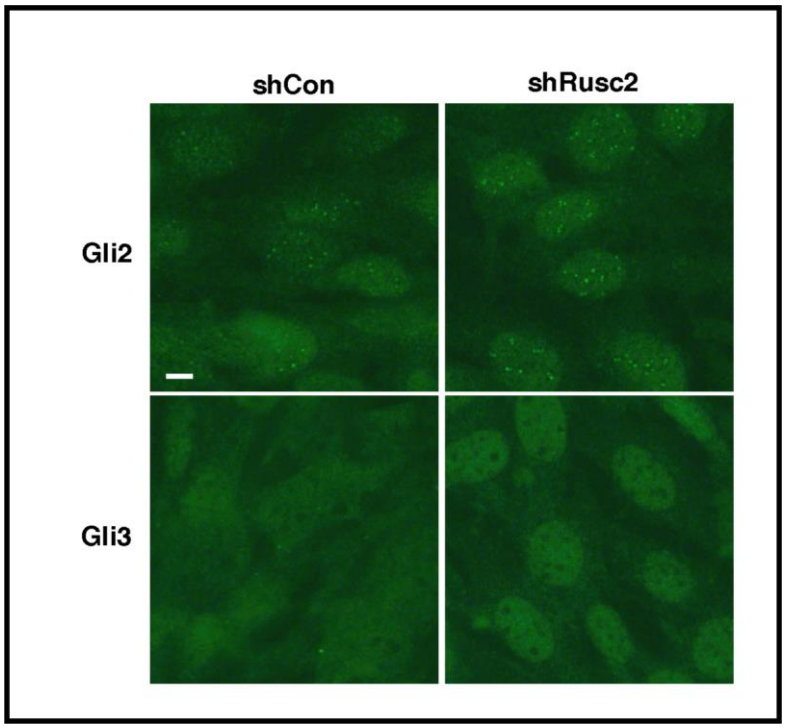
Expression of *rusc1* and *rusc2* in MEFs and NIH3T3 cells. Real-time RT-PCR showing that *rusc2*, but not *rusc1*, is abundantly expressed in NIH3T3 and MEFs. In the real-time PCR experiment, pCS2-Rusc1 and pCS2-Rusc2 plasmids (0.4 pg) were used as the control for normalization. Data are shown as mean±SD. *$p<0.05$.



Supplemental Figure 2.

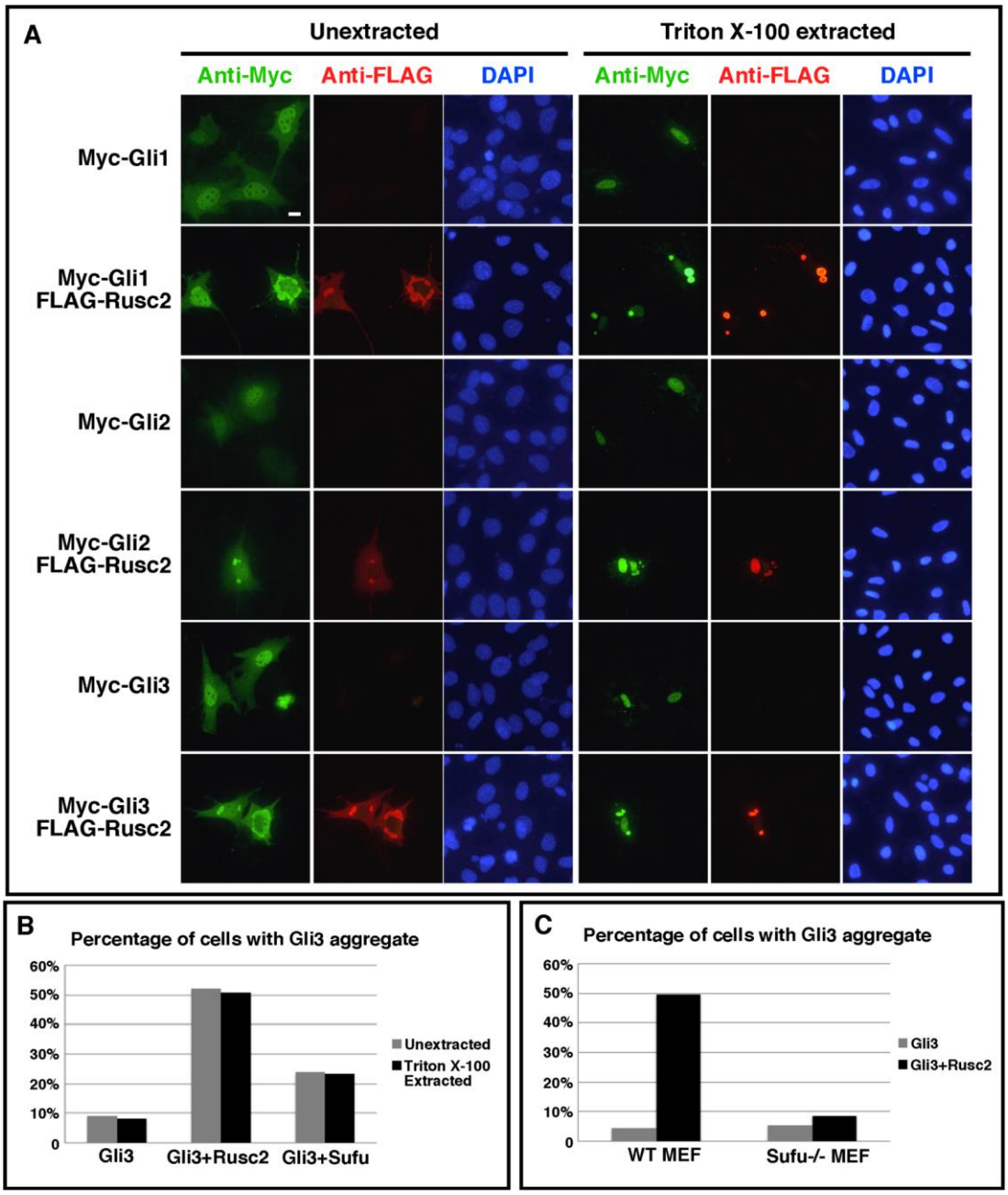
Enhanced Hh response in rusc2 heterozygous mutant MEFs. A. Schematic diagram showing the sequences of the wild type and mutant rusc2 alleles. Sequences targeted by the left and right TALEN arms are underlined. To establish rusc2 mutant cell lines, wild type MEFs were transfected with TALEN and selected by puromycin. Several cell lines were established from TALEN-transfected single cells. The targeted loci of these cells were sequenced. A cell line carrying a mutated rusc2 allele was identified. When testing the Hh response of this rusc2 heterozygous mutant MEF cell line, we used an un-mutated MEF cell line established through the same procedure as the control. **B.** Western blot to show that the expression of Rusc2 is reduced in rusc2 heterozygous mutant MEFs. **C.** Dual-luciferase assay showing that transfection of Gli1 (100 ng) into control MEFs activated the 8xGli-BS-Luciferase reporter by 19 folds. In the rusc2 heterozygous mutant MEFs, the

same amount of Gli1 activated the 8xGli-BS-Luciferase reporter by 51 folds. Data is shown as mean±SD. **p<0.01. **D.** Overexpression of FLAG-hRusc2 rescued the response of the rusc2 heterozygous mutant MEFs to Gli1 in an 8xGli-BS-Luciferase reporter assay. Data is shown as mean±SD. *p<0.05, n.s., non-significant. **E**. Real-time RT-PCR results showing that a low dose of Shh-conditioned medium, which was insufficient for activating *gli1* and *ptc1* expression in control MEFs, markedly increased the expression of *gli1* and *ptc1* in rusc2 heterozygous mutant MEFs. Data is shown as mean±SD. **p<0.01. **F**. Real-time RT-PCR results showing the expression of *gli1* in control and rusc2 heterozygous mutant MEFs at various time points after Shh conditioned medium treatment. Compared to control MEFs, rusc2 heterozygous mutant MEFs showed accelerated *gli1* activation kinetics in response to Shh conditioned medium treatment. Data is shown as mean±SD. *p<0.05, n.s., non-significant.



Supplemental Figure 3.

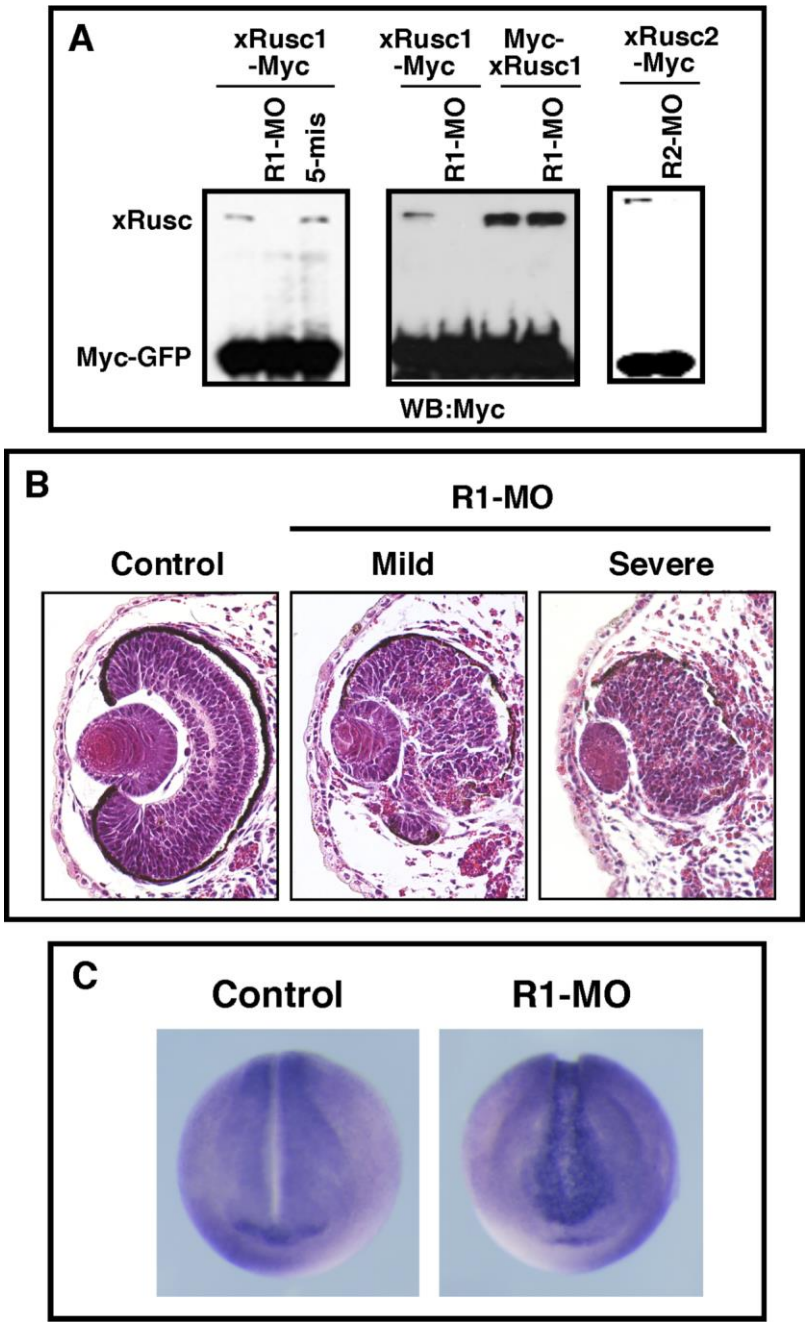
Knockdown of rusc2 does not significantly alter the subcellular localization of endogenous Gli2 and Gli3. Immunofluorescence showing subcellular localization of endogenous Gli2 (upper panels) and Gli3 (lower panels) in control shRNA (left) and Rusc2 shRNA infected cells. Scale bars: 10μm.



Supplemental Figure 4.

Overexpression of rusc2 induces cytoplasmic Gli1, Gli2, and Gli3 protein aggregates, which are resistant to Triton X-100 extraction. A. Immunofluorescence showing that overexpression of Rusc2 altered the subcellular distribution of myc-Gli1, myc-Gli2, and myc-Gli3 in NIH3T3 cells. When expressed alone, Gli proteins were enriched in the nucleus. Overexpression of hRusc2 decreased the amount of Gli proteins in the nucleus and induced cytoplasmic Gli protein aggregates. In the Triton extraction experiment, cells were pre-extracted with 0.5% Triton X-100 in a cytoskeleton stabilizing buffer for 3 minutes at 4°C

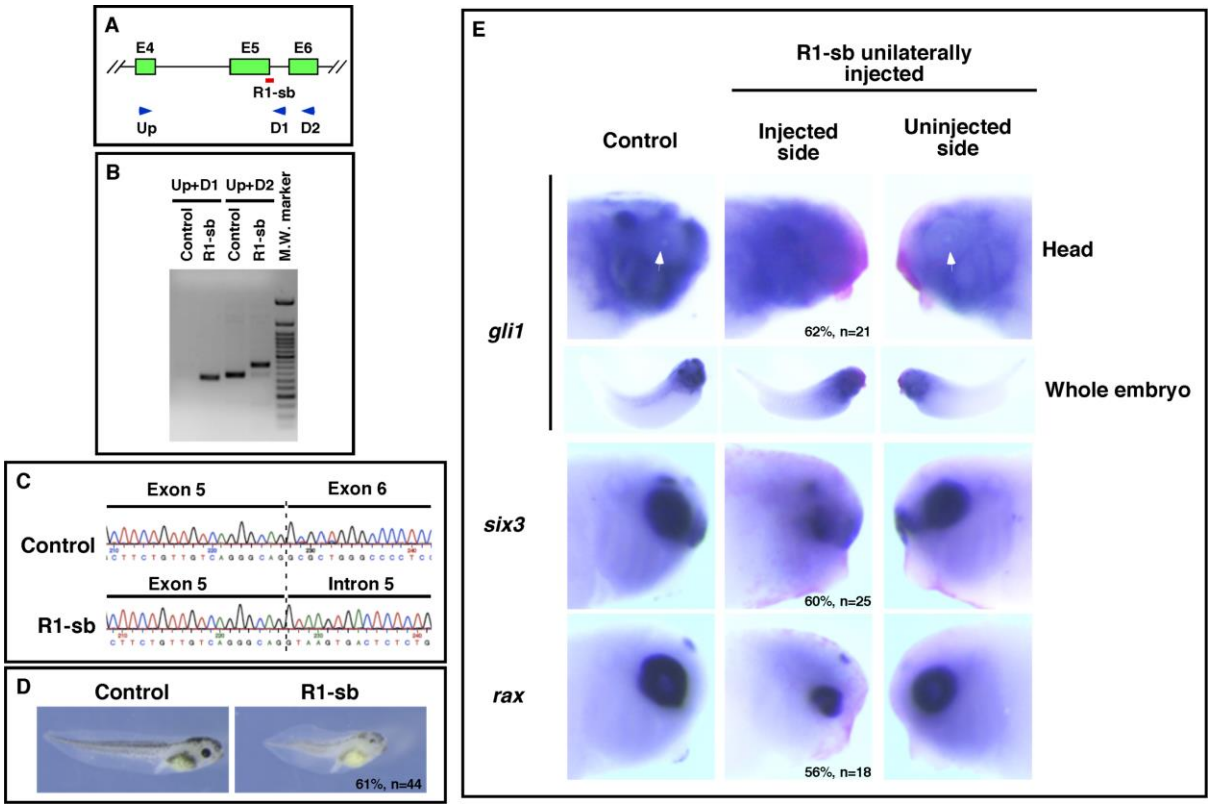
prior to fixation. This treatment condition was sufficient for removing all cytosolic GFP in myc-GFP transfected cells (not shown). However, Gli protein aggregates remained in the cytoplasm after cells were extracted with Triton. Scale bars: 10µm. **B** and **C** are quantification of the results shown in Fig. 5C and Fig. 5D, respectively. Bar graphs indicate the percentage of cells showing cytoplasmic Gli3 protein aggregates. In these experiments, we counted 200 Gli3-transfected cells from each sample.



Supplemental Figure 5.

Effects of xRusc morpholinos. A. Western blot showing that injection of Rusc1 morpholino (R1-MO, 20 ng) and Rusc2 morpholinos (R2-MO, 20 ng) into *Xenopus* embryos blocked translation of C-terminal myc-tagged xRusc1 (left panel) and xRusc2 (right panel), respectively. R1-MO blocked translation of a C-terminal myc-tagged xRusc1, but not a N-terminal myc-tagged xRusc1 (middle panel). In these experiments, morpholinos were injected at the 1-cell stage. At the 2-cell stage, a mixture of Rusc (1 ng) and myc-GFP RNA (50 pg)

were injected into embryos. **B**. Histological analysis of eyes from a control embryo (left) and R1-MO injected embryos with mildly (middle) and severely (right) affected eyes. **C**. In situ hybridization showing the expression of *gli1* in a control embryo (left) and an embryo bi-laterally injected with 40 ng of R1-MO (right). Embryos were analyzed at stage 18.



Supplemental Figure 6.

Knocking down xRusc1 by injection of R1-sb enhances Hh signaling and impairs eye development. A. Schematic diagram showing the design of R1-sb, which blocks *rusc1* splicing. Arrowheads indicate primers used in RT-PCR to validate the effect of R1-sb on splicing of *rusc1*. **B.** RT-PCR result showing the effect of R1-sb on *rusc1* splicing. Fertilized eggs were injected with R1-sb (80 ng) and harvested at stage 33 for RT-PCR. **C.** Sequences of the PCR products (primers Up + D1) amplified from control and R1-sb injected embryos, showing insertion of intron 5 into *rusc1* mRNA in R1-sb injected embryos. **D.** Whole embryo morphology of a control tadpole and a tadpole that was injected with 20 ng of R1-sb bilaterally at the 4-cell stage. Both dorsal blastomeres were injected. **E**. In situ hybridization showing the expression of *gli1*, *six3*, and *rax* in control and R1-sb injected embryos. A mixture of R1-sb (20ng) and RNA encoding n-ß-gal (500 pg) was injected into one of the dorsal blastomeres at the 4-cell stage. Embryos were harvested at stage 33. Both un-injected and injected sides of injected embryos are shown. In stage 33 control embryos, *gli1* is not expressed in the eye, forming a prominent "*gli1*-free" domain in the head (pointed by arrows). In R1-sb injected embryos, the *gli1*-free domain disappears. Cells in the head region express *gli1* nearly uniformly.