Rassf7a promotes spinal cord regeneration and controls spindle orientation in neural progenitor cells

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Supplementary Materials

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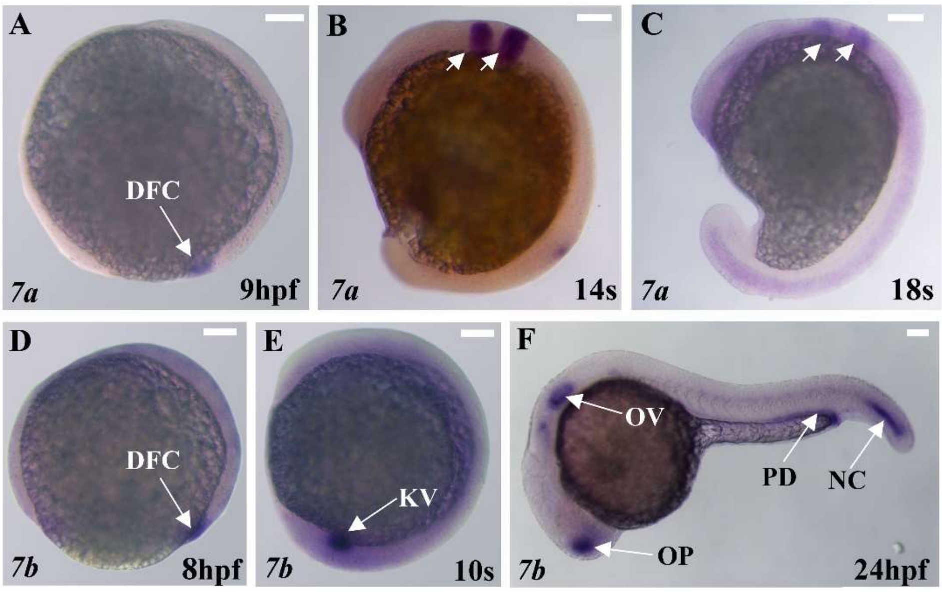
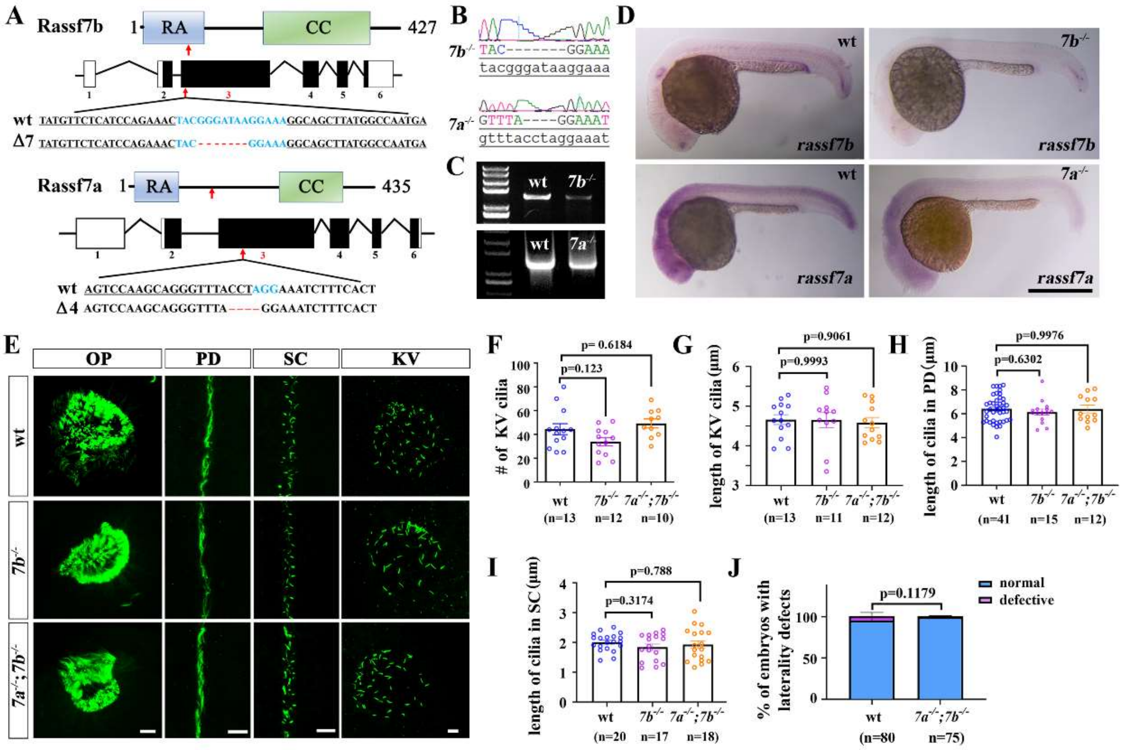
Appendix Figures



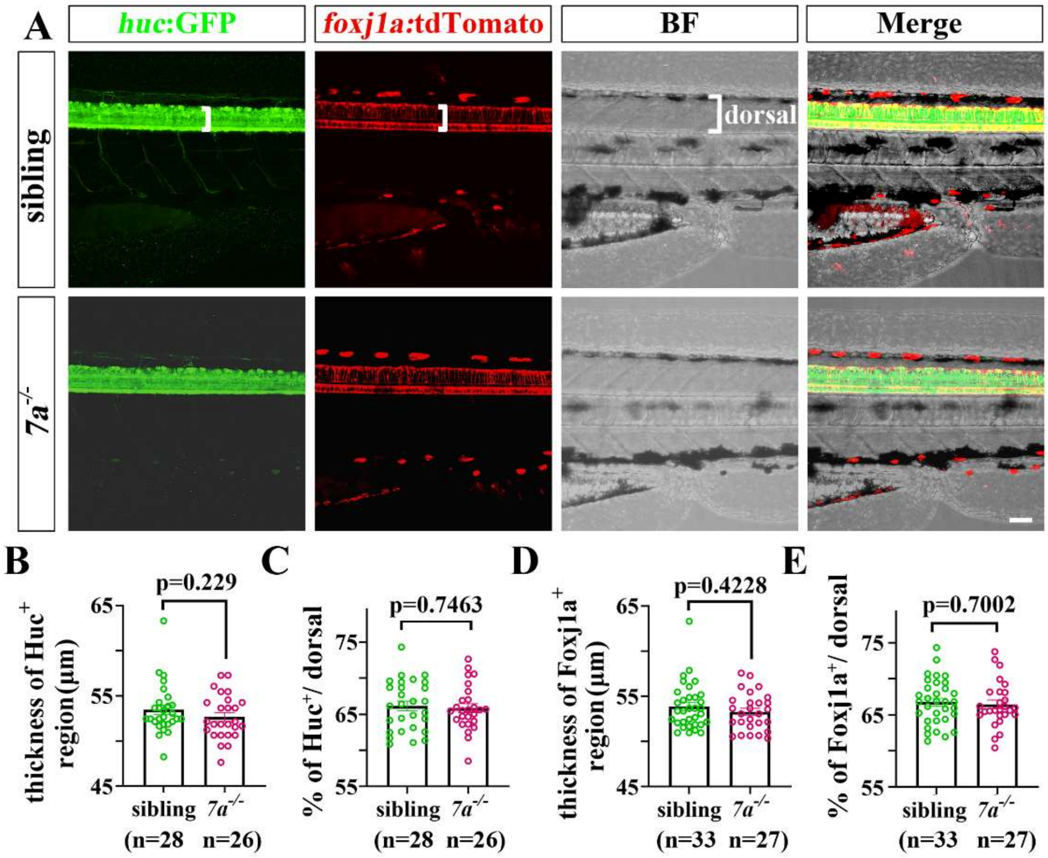
Appendix Figure S1. Expression pattern of *rassf7a* and *rassf7b*

(A-C) Whole mount *in situ* hybridization results showing expression of *rassf7a* at different stages as indicated. (B) Double staining results showing the colocalization of *rassf7a* (purple) and *krox20* (red) in rhombomeres 3 and 5 at a 14-somite stage (14 s) embryo. (D-F) Whole mount *in situ* hybridization results showing expression of *rassf7b* at different stages as indicated. Arrowheads in (B, C) represent rhombomeres 3 and 5. DFC, dorsal forerunner cells; KV, kupffer's vesicle; NC, notochord; OP, olfactory pit; OV, otic vesicle; PD, pronephric duct. Scale bars: 100 µm.



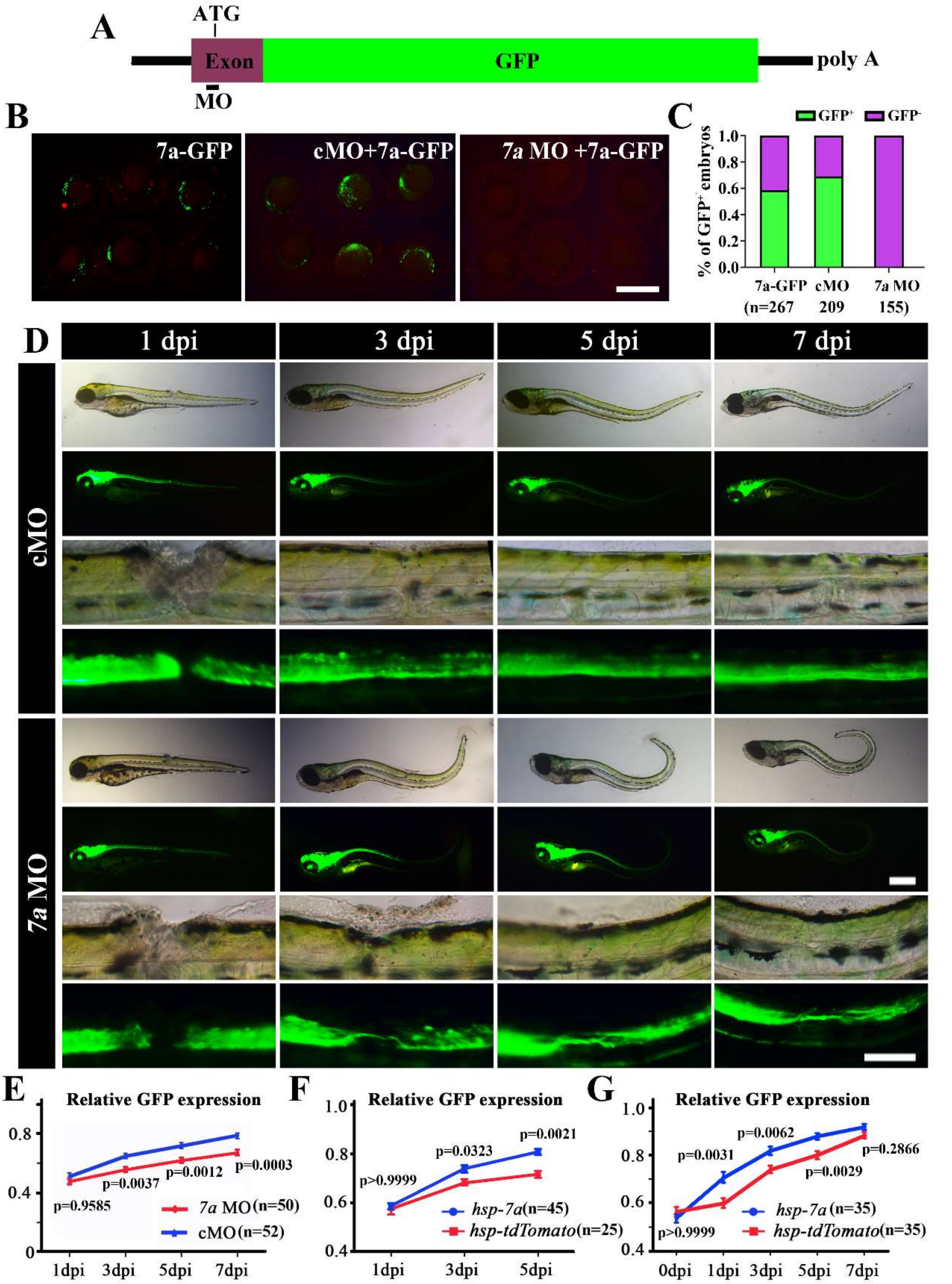
Appendix Figure S2. Generation of *rassf7a* and *rassf7b* mutants

(A) Diagram showing the protein domains, genomic structures and sequences of wild-type and corresponding *rassf7b* (top) and *rassf7a* (bottom) mutants. Red arrows indicate mutation sites. Dark boxes indicate open reading frames. Underlined sequences indicate TALEN binding sites (top, *rassf7b*) or Cas9 binding sites (bottom, *rassf7a*). RA, Ras association domain; CC, coiled-coil domain. (B) Sanger sequencing results confirming the deletion of target region in *rassf7a* and *rassf7b* mutant transcripts. (C) PCR analysis showing the amplification of mutant transcripts from 24 hpf wild-type and mutant larvae as indicated. (D) Whole mount *in situ* hybridization results showing the expression of *rassf7a* and *rassf7b* in wild-type and mutant larvae as indicated. (E) Confocal images showing cilia in different tissues of wild-type and mutant larvae as indicated. Cilia were visualized with anti-acetylated tubulin antibody. (F) Dot plots showing the number of cilia in Kupffer's vesicle in wild-type and mutant embryos as indicated. (G-I) Dot plots showing length of cilia in different tissues as indicated. (J) Bar graph showing the percentages of embryos with laterality defects characterized by abnormal *lefty-2* expression in wild-type or *rassf7a;rassf7b* double mutants. *P* values for One-way ANOVA with Dunnett's test or Dunnett's T3 test (F-I) and Fisher's Exact test (J) are indicated. Data are shown as mean ± S.E.M. For detailed statistics, see Supplementary Table 2 (Table S2). Each experiment was performed independently 4 times. Each data point represents an individual fish. Scale bars: 500 µm in (D),10 µm in (E).



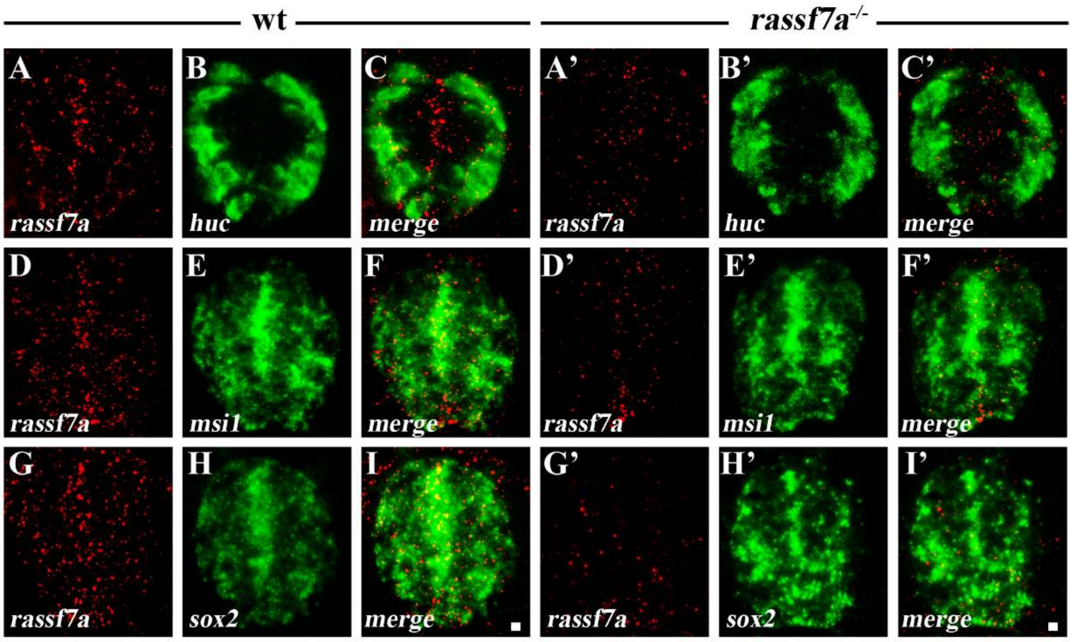
Appendix Figure S3. Spinal cords developed normally in *rassf7a* mutants

(A) Representative images showing bright field and fluorescence signals of *rassf7a* mutants or siblings carrying Tg(*huc:GFP*) and Tg(*foxj1a:HA-tdTomato*) at 3 dpf. White brackets in fluorescence and bright field images indicate measurement regions of spinal cord and dorsal thickness respectively. (B, D) Dot plots showing the thickness of fluorescent regions as indicated in (A). (C, E) Dot graphs showing percentages of fluorescence thickness in *rassf7a* mutants or siblings as indicated. *P* values for unpaired Mann-Whitney test (B, D) and unpaired Student's t-test (C-E) are indicated. Data are shown as mean ± S.E.M. Each data point represents an individual fish. Scale bars: 50 μm in (A).



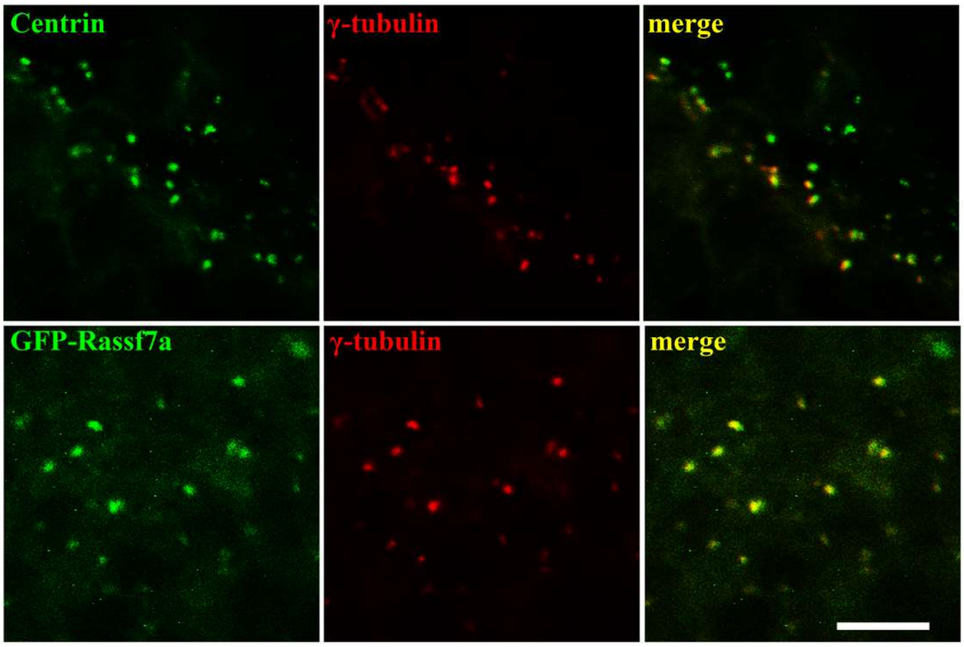
Appendix Figure S4. Recovery defects of neural cells in *rassf7a* morphants after injury

(A) Diagram showing the construct used for detecting the efficiency of *rassf7a* morpholino, which binds to the same exon containing translational start site. (B) Images of 2-somite stage embryos injected with GFP reporter construct and control (cMO) or *rassf7a* morpholino (*7a* MO) as indicated. (C) Bar graph showing the percentages of embryos with GFP fluorescent signals in different groups as indicated. (D) Representative images showing bright field and fluorescence images of control or *rassf7a* MO injected Tg(*huc*:*GFP*) transgenic larvae at different time points after SCI. The magnified views of the lesion sites were shown at the bottom. (E) Relative GFP expression level at the lesion sites in control or *rassf7a* morphants at different time points after injury. (F-G) Relative GFP expression level of the Tg(*huc*:*GFP*) transgene at the lesion sites of mutant (F) or wild-type larvae (G) overexpressed with *rassf7a* or *tdTomato* genes under heat shock promoters. *P* values for Two-way ANOVA with Bonferroni's multiple comparisons test (E-G) are indicated. Data are shown as mean ± S.E.M. Each experiment was performed independently 3 times. Each data point represents an average of each group as indicated. Scale bars: 200 μm in (B), 500 μm in (D).



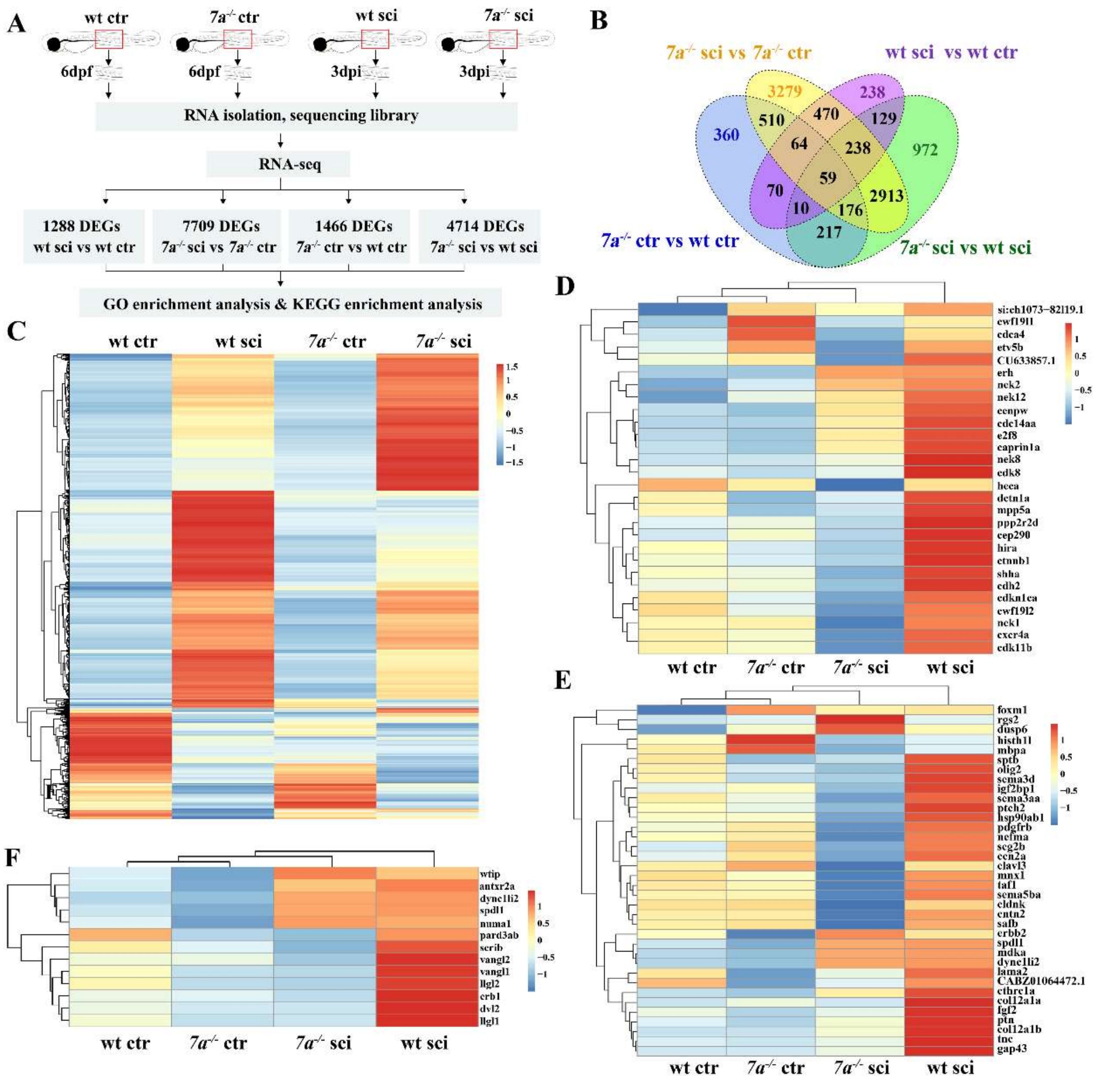
Appendix Figure S5. Expression of *rassf7a* in the spinal cord at 24 hpf

(A-I’) Double fluorescent *in situ* hybridization results showing the expression of *rassf7a* (red) and different neural markers (green) on the cross sections through the spinal cord of 24 hpf wild-type (A-I) and mutant (A’-I’) zebrafish larvae. Scale bars: 10 µm.



Appendix Figure S6. Rassf7a localizes to the centrosomes in zebrafish

Confocal images showing the colocalization analysis between Rassf7a and centrosomal markers. Zebrafish embryos were injected with either Centrin-GFP or GFP-Rassf7a constructs and stained with anti-γ-tubulin antibodies (red) to label the centrosomes at 24 hpf. Scale bar: 10 µm.



Appendix Figure S7. Transcriptome analysis of differentially expressed genes during spinal cord regeneration

(A) Strategy used for total RNA isolation and transcriptome analysis. (B) Venn diagrams showing the number of DEGs in control and injured groups as indicated. (C) Heat map analysis of transcriptomic data from wild-type and *rassf7a* mutant embryos at 3 days after spina cord injury. (D) Expression heat map of genes involved in mitotic process in wild-type and *rassf7a* mutant embryos. (E) Expression heat map of genes involved in axon elongation in wild-type and *rassf7a* mutant embryos. (F) Expression heat map of genes involved in cell polarity in wild-type and *rassf7a* mutants.

Appendix Tables

Appendix Table S1

Primer sequences used for mutant detection

Name	Forward sequence	Reverse sequence
rassf7a	5’-CTGGTCGTTATGTTCTCATTC-3’	5’-GACTGTACACCTCCTCTTTAG- 3’
rassf7b	5’-TCATGTAGAGAGACCCCTTACTG- 3’	5’-TGCCCTTGTTGTCGAAGAATC- 3’

Primers for *rassf7a* BP reaction

Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTccaccATGAGAATCCAGACTTTATTA

Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTaCCGCCAAGACGTTTCCCTGGA

Primers for *rassf7a* MO efficacy verification

rassf7a Exon 2:
Forward: 5’-ATCGATTCGAATTCGCCACGAATCCAGACTTTATTATGTATTTCTGATCTG-3’

Reverse: 5’- GCCCTTGCTCACCATAATGGCTTGGGCAAGTGCAATGACA-3’

pCS2:
Forward: 5’-CCATTATGGTGAGCAAGGGC-3’
Reverse: 5’-CGTGGCGAATTCGAATCG-3’

Primers for probe synthesis

Name	Forward sequence	Reverse sequence
rassf7a	5’-GTAGTAGGCAAAACAAGAAG-3’	5’-TCAGATTCATTACCGCCAAG-3’
rassf7b	5’-TCATGTAGAGAGACCCCTTACTG-3’	5’-GCTAATGAAATATATACGCAGG-3’
huc	5’-GAAGACCTGCAAATCGAAGGAC-3’	5’-GAATGTACAGGGAGCTCAGTAG-3’
sox2	5’-GGTGGGGTAGACTTTCAAGAA-3’	5’-CCAGCAGTGTAGTAAAAAGAG-3’
msi1	5’-CAAATGGAATCGGAAGGCAG-3’	5’-CTGTACCTGTCACTTGTTTC-3’

Primers for RT-PCR and qRT-PCR

Name	Forward sequence	Reverse sequence
rassf7a	5’- GAGAAGACTTTGGGGAGGGCTG-3’	5’- GGAATTGTTTGGCAGTGGGACG-3’
rassf7b	5’- CGTTTGAAGAGGTGGATAAGGC-3’	5’- GACCTCTGGATGAAGACTGGAC-3’

Appendix Table S2

Detailed statistics

Figure	**Sample size**	**Statistical test**	**Post-hoc Test**	**Comparison**	***P* value**
Fig 1I	wt: n=48, $7a^{-/-}$: n=47	two-tailed unpaired Mann-Whitney test			*P* <0.0001
Fig 1J	wt: n=48, $7a^{-/-}$: n=47	two-tailed unpaired Mann-Whitney test			*P* <0.0001
Fig 1K	wt: n=48, $7a^{-/-}$: n=47	Two-way ANVOA	Bonferroni's multiple comparisons test	active: wt vs $7a^{-/-}$	*P* <0.0001
				static: wt vs $7a^{--/}$	*P* <0.0001
Fig 2B	wt: n=32, $7a^{-/-}$: n=38	Two-way ANOVA	Bonferroni's multiple comparisons test	0 dpi wt vs $7a^{-/-}$	*P* >0.9999
				1 dpi: wt vs $7a^{-/-}$	*P* >0.9999
				3 dpi: wt vs $7a^{-/-}$	*P* =0.1675
				5 dpi: wt vs $7a^{-/-}$	*P* =0.0766
				7 dpi: wt vs $7a^{-/-}$	*P* >0.9999
Fig 2C	wt: n=26, $7a^{-/-}$: n=24	Two-way ANOVA	Bonferroni's multiple comparisons test	0 dpi: wt vs $7a^{-/-}$	*P* =0.8965
				1 dpi: wt vs $7a^{-/-}$	*P* =0.0115
				3 dpi: wt vs $7a^{-/-}$	*P* <0.0001
				5 dpi: wt vs $7a^{-/-}$	*P* <0.0001
				7 dpi: wt vs $7a^{-/-}$	*P* <0.0001
Fig 3B	*huc*:GFP: wt: n=31, $7a^{-/-}$: n=25	two-tailed unpaired Student's t-test		*huc*:GFP/wt vs *huc*:GFP/$7a^{-/-}$	*P* <0.0001
	gfap:GFP: wt: n=49, $7a^{-/-}$: n=37	two-tailed unpaired Mann-Whitney test		*gfap*:GFP/wt vs *gfap*:GFP/$7a^{-/-}$	*P* =0.9326
Fig 3C	*huc*:GFP: wt: n=15, $7a^{-/-}$: n=16	two-tailed unpaired Student's t-test		*huc*:GFP/wt vs *huc*:GFP/$7a^{-/-}$	*P* =0.0015
	gfap:GFP: wt: n=24, $7a^{-/-}$: n=21	two-tailed unpaired Student's t-test		*gfap*:GFP/wt vs *gfap*:GFP/$7a^{-/-}$	*P* =0.8505
Fig 3D	*huc*:GFP: wt: n=13, $7a^{-/-}$: n=9	two-tailed unpaired Student's t-test		*huc*:GFP/wt vs *huc*:GFP/$7a^{-/-}$	*P* =0.9993
	gfap:GFP: wt: n=32, $7a^{-/-}$: n=36	two-tailed unpaired Mann-Whitney test		*gfap*:GFP/wt vs *gfap*:GFP/$7a^{-/-}$	*P* =0.4144
Fig 3E	wt: n=13; $7a^{-/-}$: n=9	Two-way ANOVA	Bonferroni's multiple	1dpi: wt vs $7a^{-/-}$	*P* <0.0001

			comparisons test	3dpi: wt vs $7a^{-/-}$	*P* =0.0048
				5dpi: wt vs $7a^{-/-}$	*P* >0.9999
Fig 3F	wt: n=24; $7a^{-/-}$: n=21	Two-way ANOVA	Bonferroni's multiple comparisons test	1dpi: wt vs $7a^{-/-}$	*P* >0.9999
				3dpi: wt vs $7a^{-/-}$	*P* >0.9999
				5dpi: wt vs $7a^{-/-}$	*P* >0.9999
Fig 3H	wt: n=26; $7a^{-/-}$: n=16	two-tailed unpaired Student's t-test			*P* =0.0005
Fig 3I	wt: n=27; $7a^{-/-}$: n=13	two-tailed unpaired Student's t-test			*P*<0.0001
Fig 4P	$Sox2^+$: n=3 experiments Huc^+: n=3 experiments	two-tailed unpaired Student's t-test with Welch's correction			*P*<0.0001
Fig 4Q	ctr: n=3 experiments injured: n=3 experiments	two-tailed unpaired Student's t-test with Welch's correction			*P* =0.8590
Fig 4R	ctr: n=3 experiments injured: n=3 experiments	two-tailed unpaired Student's t-test with Welch's correction			*P* =0.0028
Fig 5C	wt: n=18 cells; $7a^{-/-}$: n=17 cells	two-tailed unpaired Student's t-test			*P* =0.0232
Fig 5F	wt: n=21 cells, N=14 fish $7a^{-/-}$: n=21 cells, N=23 fish	two-tailed unpaired Mann-Whitney test			*P* =0.0002
Fig 6C	wt: n=38 cells, N=14 fish $7a^{-/-}$: n=49 cells, N=23 fish	two-sided Fisher's Exact test			*P* =0.0021
Fig 6D	ctr MO: n=18 cells *7a* MO: n=30 cells	two-sided Fisher's Exact test			*P* =0.006
Fig 7D	siControl: n=3 experiments siRASSF7: n=3 experiments	two-tailed unpaired Student's t-test			*P* =0.0034
Fig 7G	siControl: n=4 experiments siRASSF7: n=4 experiments	two-tailed unpaired Student's t-test			*P* =0.0013
Fig 7H	siControl: n=4 experiments, siRASSF7: n=4 experiments	two-tailed unpaired Student's t-test			*P* =0.0006

Fig 7J	siControl: n=1737 siRASSF7: n=988	two-tailed unpaired Mann-Whitney test			*P* <0.0001
Fig 7K	siControl: n=6 experiments siRASSF7: n=6 experiments	two-sided Fisher's Exact test			*P* <0.0001
Fig S2F	wt: n=13; *7b*$^{-/-}$: n=12 *7a*$^{-/-}$;*7b*$^{-/-}$: n=10	One-way ANOVA	Dunnett's test	wt vs *7b*$^{-/-}$	*P* =0.123
			Dunnett's test	wt vs *7a*$^{-/-}$;*7b*$^{-/-}$	*P* =0.6184
Fig S2G	wt: n=13; *7b*$^{-/-}$: n=11; *7a*$^{-/-}$;*7b*$^{-/-}$: n=12	One-way ANOVA	Dunnett's test	wt vs *7b*$^{-/-}$	*P* =0.9993
			Dunnett's test	wt vs *7a*$^{-/-}$;*7b*$^{-/-}$	*P* =0.9061
Fig S2H	wt: n=41; *7b*$^{-/-}$: n=15; *7a*$^{-/-}$;*7b*$^{-/-}$: n=12	One-way ANOVA	Dunnett's test	wt vs *7b*$^{-/-}$	*P* =0.6302
			Dunnett's test	wt vs *7a*$^{-/-}$;*7b*$^{-/-}$	*P* =0.9976
Fig S2I	wt: n=20; *7b*$^{-/-}$: n=17; *7a*$^{-/-}$;*7b*$^{-/-}$: n=18	One-way ANOVA	Dunnett's T3 test	wt vs *7b*$^{-/-}$	*P* =0.3174
			Dunnett's T3 test	wt vs *7a*$^{-/-}$;*7b*$^{-/-}$	*P* =0.788
Fig S2J	wt: n=80; *7a*$^{-/-}$;*7b*$^{-/-}$: n=75	two-sided Fisher's Exact test			*P* =0.1179
Fig S3B	sibling: n=28; *7a*$^{-/-}$: n=26	two-tailed unpaired Mann-Whitney test			*P* =0.229
Fig S3C	sibling: n=28; *7a*$^{-/-}$: n=26	two-tailed unpaired Student's t-test			*P* =0.7463
Fig S3D	sibling: n=33; *7a*$^{-/-}$: n=27	two-tailed unpaired Mann-Whitney test			*P* =0.4228
Fig S3E	sibling: n=33; *7a*$^{-/-}$: n=27	two-tailed unpaired Student's t-test			*P* =0.7002
Fig S4E	ctr MO: n=52 *7a* MO: n=50	Two-way ANOVA	Bonferroni's multiple comparisons test	1 dpi: cMO vs *7a* MO	*P* =0.9585
				3 dpi: cMO vs *7a* MO	*P* =0.0037
				5 dpi: cMO vs *7a* MO	*P* =0.0012
				7 dpi: cMO vs *7a* MO	*P* =0.0003
Fig S4F	*hsp-7a*: n=45 *hsp-tdTomato*: n=25	Two-way ANOVA	Bonferroni's multiple comparisons test	1 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* >0.9999
				3 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.0323
				5 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.0021
Fig S4G	*hsp-7a*: n=35	Two-way ANOVA	Bonferroni's multiple	0 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* >0.9999

	hsp-tdTomato: n=35		comparisons test	1 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.0031
				3 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.0062
				5 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.0029
				7 dpi: *hsp-7a* vs *hsp-tdTomato*	*P* =0.2866
Fig EV1E	straight: wt: n=23, $7a^{-/-}$: n=21	two-tailed unpaired Student's t-test		straight: wt vs $7a^{-/-}$	*P* =0.0006
	curved: wt: n=23, $7a^{-/-}$: n=23	two-tailed unpaired Student's t-test		curved: wt vs $7a^{-/-}$	*P* =0.0487
Fig EV1F	straight: wt: n=23, $7a^{-/-}$: n=21	two-tailed unpaired Student's t-test		straight: wt vs $7a^{-/-}$	*P* =0.0005
	curved: wt: n=23, $7a^{-/-}$: n=23	two-tailed unpaired Student's t-test		curved: wt vs $7a^{-/-}$	*P* =0.0487
Fig EV1G	straight wt: n=23, $7a^{-/-}$: n=21	Two-way ANVOA	Bonferroni's multiple comparisons test	active: wt vs $7a^{-/-}$	*P* =0.0029
			Bonferroni's multiple comparisons test	static wt vs $7a^{-/-}$	*P* =0.0029
	curved wt: n=23, $7a^{-/-}$: n=23	Two-way ANVOA	Bonferroni's multiple comparisons test	active wt vs $7a^{-/-}$	*P* =0.0222
			Bonferroni's multiple comparisons test	static wt vs $7a^{-/-}$	*P* =0.0224
Fig EV1I	straight wt: n=58, $7a^{-/-}$: n=38 curved wt: n=32, $7a^{-/-}$: n=61	two-tailed unpaired Mann-Whitney test		wt straight vs wt curved	*P* =0.0773
		two-tailed unpaired Mann-Whitney test		$7a^{-/-}$ straight vs $7a^{-/-}$ curved	*P* =0.0022
		two-tailed unpaired Mann-Whitney test		wt straight vs $7a^{-/-}$ straight	*P* <0.0001
		two-tailed unpaired Student's t-test		wt curved vs $7a^{-/-}$ curved	*P* <0.0001
Fig EV1J	straight wt: n=49, $7a^{-/-}$: n=31 curved wt: n=31, $7a^{-/-}$: n=41	two-tailed unpaired Student's t-test		wt straight vs wt curved	*P* =0.1086
		two-tailed unpaired Student's t-test		$7a^{-/-}$ straight vs $7a^{-/-}$curved	*P* =0.1459
		two-tailed unpaired Student's t-test		wt straight vs $7a^{-/-}$ straight	*P* <0.0001
		two-tailed unpaired Student's t-test		wt curved vs $7a^{-/-}$ curved	*P* <0.0001
Fig EV2C	axons: wt: n=48,$7a^{-/-}$: n=43	two-sided Fisher's Exact test			*P* =0.6444

	glia: wt: n=48, *7a*$^{-/-}$: n=43	two-sided Fisher's Exact test		*P* =0.8285
Fig EV2D	wt: n=35; *7a*$^{-/-}$: n=27	two-tailed unpaired Mann-Whitney test		*P* =0.4089
Fig EV2F	straight wt: n=12, *7a*$^{-/-}$: n=31 curved wt: n=27, *7a*$^{-/-}$: n=31	two-tailed unpaired Student's t-test	wt straight vs wt curved	*P* =0.0696
		two-tailed unpaired Student's t-test	*7a*$^{-/-}$ straight vs *7a*$^{-/-}$ curved	*P* =0.0886
		two-tailed unpaired Student's t-test	wt straight vs *7a*$^{-/-}$ straight	*P* =0.0233
		two-tailed unpaired Student's t-test	wt curved vs *7a*$^{-/-}$ curved	*P* =0.0299
Fig EV5B	ctr MO: n=16 cells *7a* MO^{-}: n=24 cells	two-tailed unpaired Mann-Whitney test		*P* =0.0017
Fig EV5E	ctr MO: n=15 cells *7a* MO: n=23 cells	two-tailed unpaired Student's t-test		*P* =0.0056