Supplementary Information for

Dnmt3 and G9a cooperate for tissue-specific development in zebrafish

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Supplementary Methods

Genomic DNA isolation and global DNA methylation assay (LC-MS)

Embryos were collected at 72hpf and genomic DNA was isolated using the Puregene DNA isolation kit (Gentra systems). The global DNA methylation assay was performed using liquid chromatography-mass spectrometry as described previously (Song et al., 2005).

Bisulfite sequencing

Total genomic DNA was isolated from wild type or *dnmt3* morpholino injected embryos at 24hpf using puregene DNA isolation kit (Qiagen). Two micrograms of genomic DNA was brought to a volume of 50μL with water, 3.5μL of 3M NaOH was added and the mixture was incubated at 70°C for 10 min. 520μL of freshly prepared 3M sodium bisulfite, pH 5.0 (Sigma) and 30μL of freshly prepared 10mM hydroquinone (Sigma) were then added, mineral oil was overlaid, and the mixture incubated at 50°C for 16 hrs. The reaction mixture was then desalted using a DNeasy spin column (Qiagen). The DNA was desulphonated by adding 0.1 volumes of 3M NaOH and incubating at 37°C for 10 min. The DNA was then ethanol precipitated by adding 0.1 volumes of 4M NaOAc, 20μg of glycogen, 2 volumes of ethanol, and incubated at -20°C overnight. The DNA pellet was washed with 75% ethanol and resuspended in a volume of 30 ul 10mM Tris pH 8.5. PCR primers for *no tail* are described in (14) and are as follows: GTTGTTAAAGTAATAGTA TTTAATGGGATT (forward) and CACTTAA TATAATATCAAATCTCAACTTAC (reverse).

Statistical Analyses

In Supplementary Table I and II, n represents addition of the number of embryos used in two or three different experiments. P-values were calculated using student's t-test. P-value for morphants

is compared with control injected embryos, while p-values for the rescue experiments are compared with the morphants.

Supplementary Figure Legends

Supplementary Figure S1: Expression pattern of *dnmt3* in developing zebrafish embryos. Whole mount *in situ* analysis of *dnmt3* expression in different stages of zebrafish development. Shown here are 8-cell, bud, 10-somites, 22-somites, 24hpf, 48hpf and 72hpf stages. For first four stages only lateral views are shown whereas for last three time points dorsal and lateral images are shown to depict tissue-specific expression. Black arrows show expression in brain, red arrows denote expression in the eye and blue arrows point to the expression in the gut.

Supplementary Figure S2: *G9a* and *dnmt3* morphants have wild type 5-methyl-deoxycytosine and histone H3K9 di- or tri-methyl levels. (A) LC-MS assay was performed on genomic DNA isolated from *dnmt3* morphants and wild type embryos at 24hpf and 72hpf. Note that no change in global 5-methyl-deoxycytosine levels was detected in *dnmt3* morphants compared to wild type embryos at the time points tested. (B) Schematic presentation of bisulfite sequencing data for *ntl* CpG island in wild type and *dnmt3* morphants at 24hpf. Black and white circles represent methylated and unmethylated cytosines in CpG sequences, respectively. (C) Expression of *ntl* (ventral view) in control and *dnmt3* morphant embryos at 24hpf as detected by whole mount *in situ* hybridization. (D) Protein was isolated from wild type and *dnmt3* morphants at 80hpf and H3K9me2 and H3K9me3 levels were measured by antibodies specific to these marks. Decreasing amounts (as shown in microgram quantities) of the proteins were loaded for proteins from *dnmt3* morphants or wild type embryos. (E) LC-MS assay was performed on genomic DNA isolated from *g9a* morphants and wild type embryos at 24hpf and 72hpf. Note that no change in global 5-methyl-deoxycytosine levels was detected in *g9a* morphants compared to wild type embryos at the time points tested. (F) Protein was isolated from wild type and *g9a*

morphants at 80hpf and H3K9me2 and H3K9me3 levels were measured by antibodies specific to these marks. Twenty five micrograms of total protein isolated from *g9a* morphants or wild type embryos was loaded in each lane. No detectable change was observed in global levels of H3K9me2 or H3K9me3 in *dnmt3* or *g9a* morphants when compared to wild type embryos. Pan-H3 C-terminal antibody was used for total H3 content.

Supplementary Figure S3: *Dnmt3* morphants are not defective in the patterning of brain. Whole mount *in situ* of *krox20* (at 3 somites), *dlx2*, *pax6.2,* and *pax2.1* (all three at 24hpf) expression in Dnmt3 and control morphants. *Krox20* (rhombomeres 3 and 5), *dlx2* (diencephalons and telencephalon, blue arrow), *pax6.2* (dorsal diencephalons, red arrow), and *pax2.1* [midbrain-hindbrain boundary (pink arrow) and hindbrain neurons (green arrow)] are expressed normally in *dnmt3* morphants.

Supplementary Figure S4: Examples of positive and negative staining. Examples of embryos counted as positive or negative is shown for *ascl1a*, *ascl1b*, *crx* and *neurod*.

Supplementary Figure S5: *Dnmt3* morphants are defective in brain neurogenesis. Whole mount *in situ* analysis of, *ascl1a* and *ascl1b* expression in a second *dnmt3* morpholino (translation blocker, 8ng) or control morpholino injected embryos at 30hpf.

Supplementary Figure S6: *Dnmt3* morphants show abnormal brain structure. (A) Bright light image of *dnmt3* and control morphants at 24hpf shows that brain structure was not formed correctly. Note that Control morphants are transparent and internal structures can be seen whereas *dnmt3* morphants are filled with black spots which depict cells undergoing apoptosis or necrosis. Embryos co-injected with *dnmt3* morpholino and *p53* morpholino together show reduced cell death. (B) *p53* morpholino knockdown. PCR was performed on cDNA made from embryos

injected with control morpholino or *p53* morpholino and *dnmt3* morpholino at 24hpf. Unspliced and spliced DNA length for p53 is shown by arrows.

Supplementary Figure S7: Expression pattern of *g9a* in developing zebrafish embryos. Whole mount *in situ* analysis of *g9a* expression in different stages of zebrafish development. Shown here are 1-cell, bud, 10-somites, 24hpf, 48hpf and 72hpf stages. For first three stages only lateral views are shown whereas for last three time points dorsal and lateral images are shown to depict tissue-specific expression. Black arrows show expression in brain, red arrows denote expression in the eye and blue arrows point to the expression in the gut.

Supplementary Figure S8: *G9a* morphants are defective in brain neurogenesis. Whole mount *in situ* analysis of, *ascl1a* and *ascl1b* expression in a second *g9a* morpholino (splice blocker, 8ng) or control morpholino injected embryos at 30hpf.

Supplementary Table SI: Statistical Summary of the morphant phenotypes

	ascl1a	*ascl1b*	*ngn-1*	*pcna*	*pax6.2*^{T}	*crx*	*neurod*	*atoh7*	*irbp*	*fabp2*	*trypsin*	*insulin*	*fabp10*
Control Mo	98% n=57	97% n=65	100% n=39	100% n=33	100% n=41	100% n=47	100% n=50	94% n=31	95% n=38	100% n=72	100% n=54	97% n=36	100% n=35
***dnmt3* Mo1**	10% n=90	13% n=108	86% n=42	25% n=48	15% n=60	16% n=75	19% n=84	70% n=60	30% n=81	81% n=89	8% n=78	95% n=40	91% n=35
***dnmt3* Mo2**	17% n=30	18% n=28	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
***dnmt3* Mo1 + p53Mo**	14% n=122	15% n=131	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
***dnmt3* Mo1 + Dnmt3^{wt}**	73% n=75	77% n=125	n/a	81% n=31	86% n=35	90% n=89	88% n=90	n/a	89% n=56	n/a	90% n=39	n/a	n/a
***dnmt3* Mo1 + Dnmt3^{C819S}**	20% n=66	25% n=71	n/a	18% n=28	21% n=27	18% n=85	22% n=71	n/a	29% n=41	n/a	18% n=28	n/a	n/a
***dnmt3* Mo1 + DNMT3B^{WT}**	82% n=49	79% n=81	n/a	90% n=40	89% n=37	91% n=97	85% n=88	n/a	83% n=36	n/a	89% n=45	n/a	n/a
***dnmt3* Mo1 + DNMT3B^{C651S}**	20% n=45	19% n=70	n/a	14% n=35	17% n=41	17% n=54	22% n=58	n/a	20% n=25	n/a	10% n=30	n/a	n/a
***dnmt3* Mo1 + DNMT1^{WT}**	15% n=82	19% n=85	n/a	n/d	n/d	18% n=71	21% n=65	n/a	n/d	n/a	n/d	n/a	n/a
Dnmt3 Mo1 + G9a^{WT}	59% n=110	66% n=152	n/a	n/d	77% n=65	68% n=79	73% n=85	n/d	n/d	n/a	89% n=45	n/a	n/a
***dnmt3* Mo1 + G9a^{C1133S}**	25% n=72	21% n=89	n/a	n/d	31% n=71	34% n=65	35% n=62	n/d	n/d	n/a	23% n=34	n/a	n/a
***dnmt3* Mo1 + G9A^{WT}**	68% n=104	72% n=136	n/a	n/d	74% n=54	81% n=80	77% n=91	n/d	n/d	n/a	n/d	n/a	n/a
***dnmt3* Mo1 + G9A^{C1114A}**	23% n=91	24% n=107	n/a	n/d	18% n=49	28% n=61	25% n=61	n/d	n/d	n/a	n/d	n/a	n/a
***dnmt3* Mo + Suv39h1**	14% n=49	13% n=79	n/a	n/d	20% n=50	11% n=67	16% n=71	n/d	n/d	n/a	n/d	n/a	n/a
***dnmt3* Mo1 + Lef-1 Mo1**	65% n=43	72% n=40	n/a	n/d	n/d	n/d	n/d	n/d	n/d	n/a	n/d	n/a	n/a
***dnmt3* Mo1 + Lef-1 Mo2**	55% n=42	63% n=38	n/a	n/d	n/d	n/d	n/d	n/d	n/d	n/a	n/d	n/a	n/a
***dnmt1* Mo**	95% n=65	89% n=73	97% n=37	85% n=35	84% n=38	86% n=52	86% n=58	86% n=28	6% n=48	3% n=96	18% n=62	100% n=57	89% n=19
***dnmt1* Mo + Dnmt3^{WT}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	10% n=60	10% n=76	n/d	n/a	n/a
***dnmt1* Mo + G9a^{WT}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	77% n=66	76% n=87	23% n=43	n/a	n/a
***dnmt1* Mo + G9a^{C1133A}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	15% n=41	26% n=58	n/d	n/a	n/a

***dnmt1* Mo + G9A^{WT}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	84% n=71	79% n=119	n/d	n/a	n/a
***dnmt1* Mo + G9A^{C1114A}**	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	29% n=56	23% n=78	n/d	n/a	n/a
***suv39h1* Mo**	88% n=51	86% n=52	95% n=41	98% n=44	93% n=43	98% n=56	95% n=61	97% n=32	5% n=38	25% n=57	22% n=37	100% n=37	76% n=21
***g9a*Mo1**	37% n=111	33% n=120	88% n=57	17% n=29	17% n=30	25% n=53	21% n=57	97% n=31	9% n=33	8% n=98	9% n=53	n/d	n/d
***g9a* Mo2**	30% n=34	32% n=38	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
***g9a* Mo1 + G9a^{WT}**	73% n=71	73% n=82	n/a	78% n=32	85% n=41	90% n=61	89% n=67	n/a	87% n=46	89% n=56	93% n=41	n/a	n/a
***g9a* Mo1 + G9a^{C1133A}**	36% n=56	31% n=58	n/a	14% n=35	23% n=43	21% n=47	22% n=40	n/a	21% n=38	24% n=38	24% n=29	n/a	n/a
***g9a* Mo1 + G9A^{WT}**	78% n=58	77% n=65	n/a	90% n=40	89% n=45	92% n=76	90% n=72	n/a	91% n=55	88% n=42	82% n=33	n/a	n/a
***g9a* Mo + G9A^{C1114A}**	39% n=49	36% n=42	n/a	19% n=37	21% n=38	22% n=51	20% n=50	n/a	20% n=35	23% n=22	26% n=31	n/a	n/a
***g9a* Mo1 + *lef1* Mo1**	66% n=38	66% n=35	n/a	n/d	n/d	n/d	n/d	n/a	n/d	n/d	n/d	n/a	n/a
***g9a* Mo1 + *lef1* Mo2**	72% n=28	80% n=25	n/a	n/d	n/d	n/d	n/d	n/a	n/d	n/d	n/d	n/a	n/a

Values shown indicate the percentage of n embryos staining positively for the indicated marker.
[$]n is the total number of embryos used in two or three different replicates of an experiment.
[#]p values were calculated based on student's t-test comparing the test group to control. In rescue experiments p values are calculated in comparison to knock down embryos. Confidence level was set to 99% (i.e. $p<0.01$).
n/a indicates not applicable.
n/d indicates not detected.
As per nomenclature guidelines zebrafish genes are annotated in lowercase font while human genes are annotated in uppercase font.
Please note that some of the data has been published earlier (Rai et al., 2006) and is repeated here for comparison purposes.

Supplementary Table SII: Statistics of *dnmt3* morphants phenotypes.

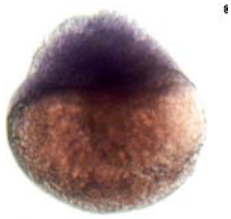
Marker	Control Mo	*dnmt3* Mo	*dnmt1* Mo
krox20	100% n=25	96% n=28	n/d
dlx2	100% n=15	94% n=18	n/d
pax6.2 (24hpf)	100% n=18	90% n=20	n/d
pax2.1	100% n=15	89% n=17	n/d
dhand	100% n=16	16% n=25	93% n=30
Alcian Blue (jaw)	100% n=16	100% n=17	16% n=32
gata6	100% n=21	84% n=25	95% n=22

Values shown indicate the percentage of n embryos staining positively for the indicated marker.
[$]n is the total number of embryos used in two or three different replicates of an experiment.
[#]p values were calculated based on student's t-test comparing the test group to control. In rescue experiments p values are calculated in comparison to knock down embryos. Confidence level was set to 99% (i.e. $p<0.01$).
n/a indicates not applicable.
n/d indicates not detected.
As per nomenclature guidelines zebrafish genes are annotated in lowercase font while human genes are annotated in uppercase font.

References

Rai, K., Nadauld, L. D., Chidester, S., Manos, E. J., James, S. R., Karpf, A. R., Cairns, B. R. and Jones, D. A. (2006). Zebra fish Dnmt1 and Suv39h1 regulate organ-specific terminal differentiation during development. *Mol Cell Biol* **26**, 7077-85.

Song, L., James, S. R., Kazim, L. and Karpf, A. R. (2005). Specific method for the determination of genomic DNA methylation by liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Chem* **77**, 504-10.



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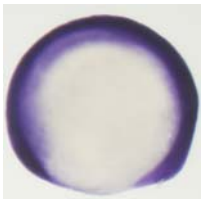

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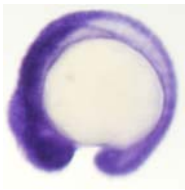

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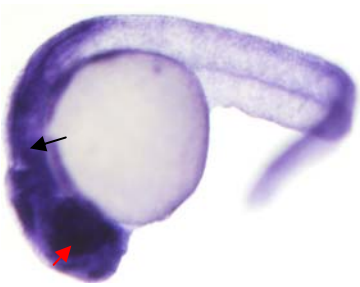
22 Somites



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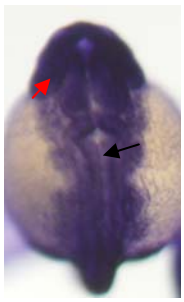

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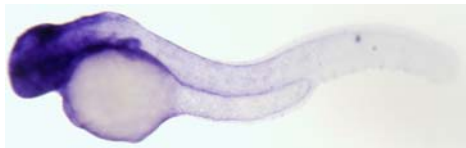

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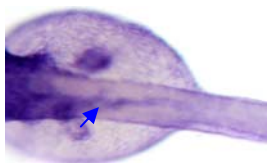

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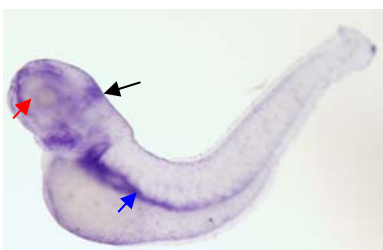

Lateral

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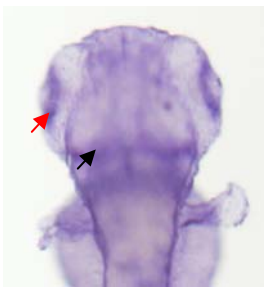

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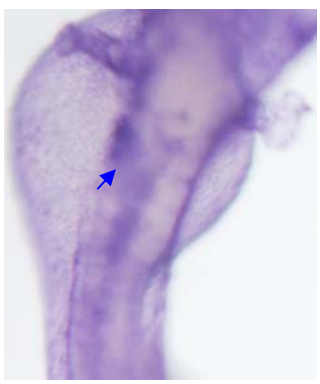
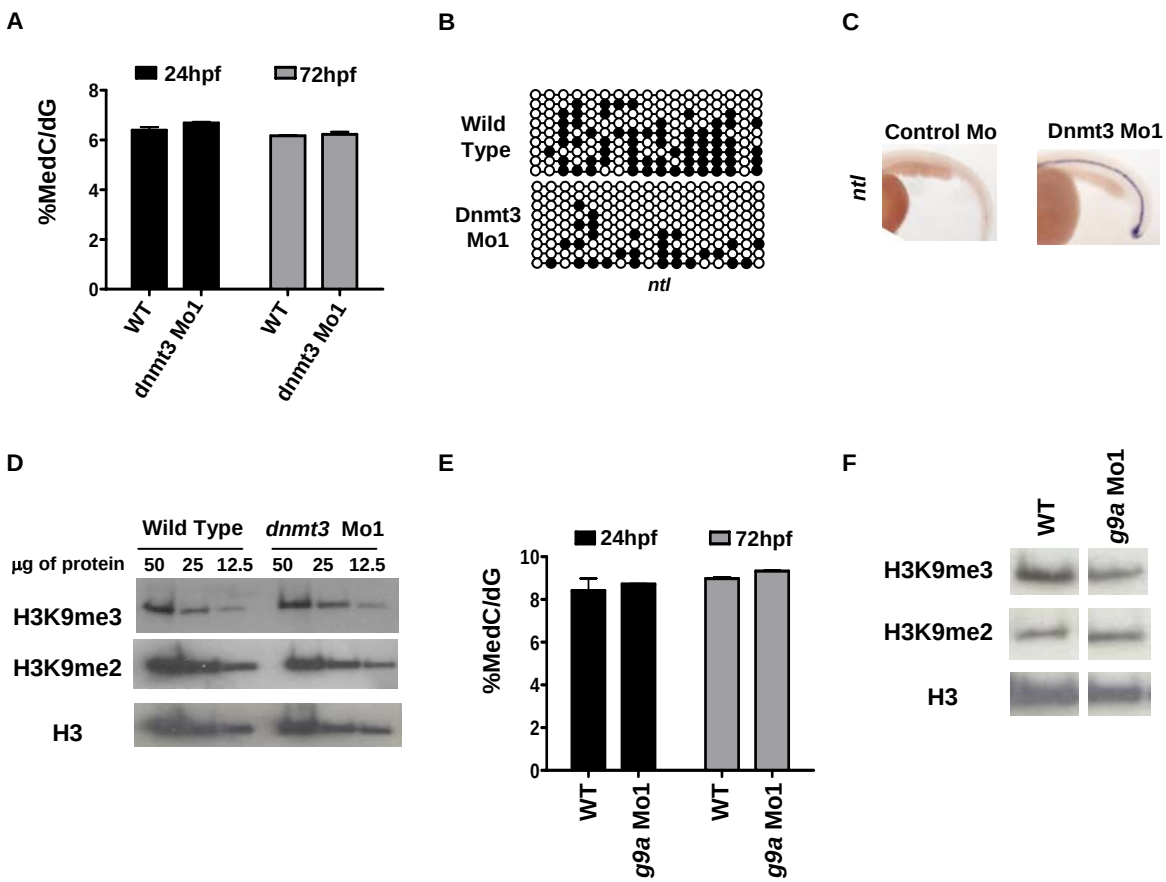


72 hpf

dnmt3

Rai et al. Supplementary Figure S1



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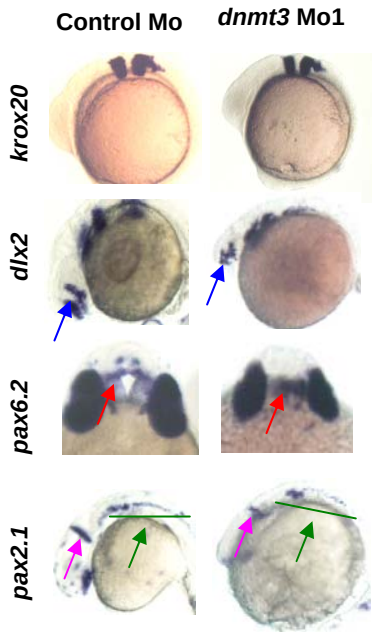

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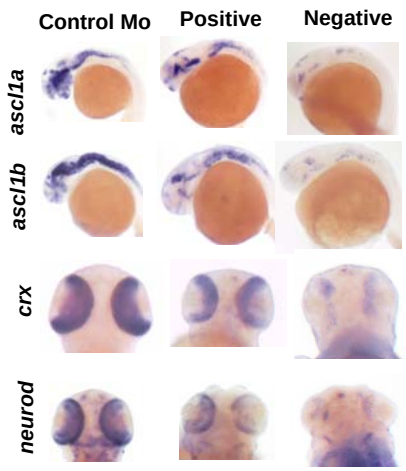

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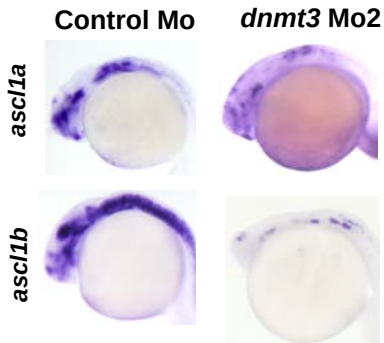

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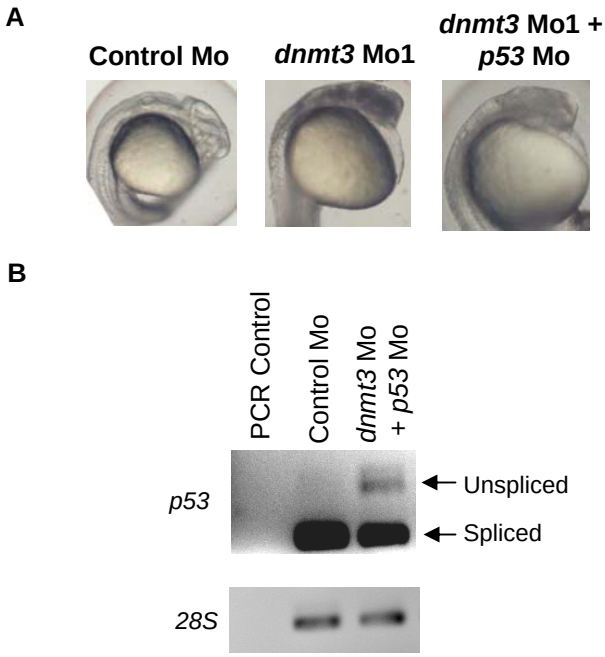

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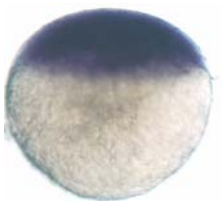

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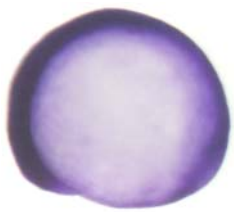

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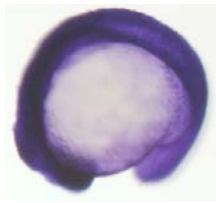
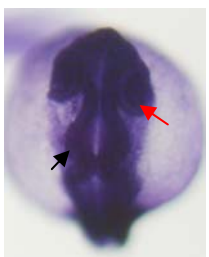


10-somites

Lateral

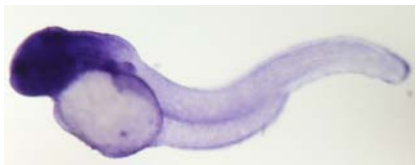
Dorsal



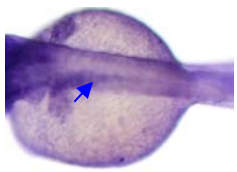
24 hpf

24 hpf

Lateral

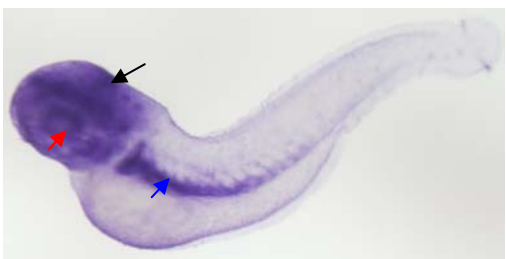


Dorsal



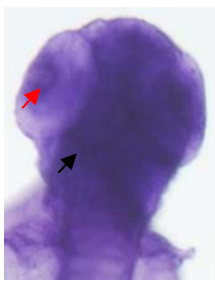
48 hpf

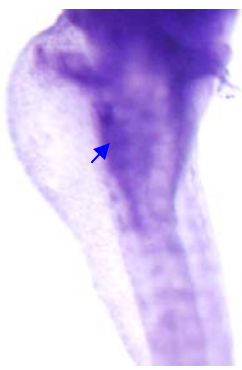
Lateral



72 hpf

Dorsal





72 hpf

g9a

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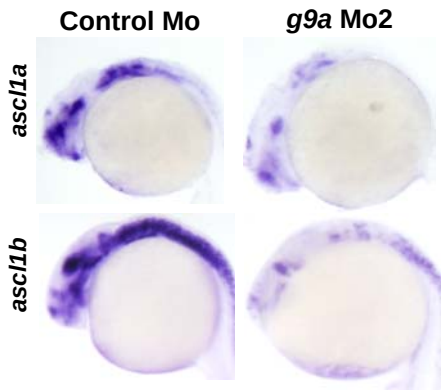

Rai et al. Supplementary Figure S8