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Supplemental Data

DNA Demethylation in Zebrafish Involves the Coupling of a Deaminase, a Glycosylase, and Gadd45

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Supplemental Experimental Procedures

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as described earlier (Rai et al., 2006) with digoxigenin (Roche) labeled probes against *neurogenin-1* and s*ox-2*.

Co-immunoprecipitation and Western blotting

Human colorectal cancer RKO cells were transfected at ~90-95% confluency with the plasmids as designated and whole cell extracts were prepared within 24 hours post transfection using IPH buffer (50mM Tris, pH 8.0, 150mM NaCl, 5mM EDTA, 0.5% NP-40, Protease inhibitors, phosphatase inhibitors). Cells were harvested using PBS, pelleted, resuspended in IPH buffer containing protease inhibitors and phosphatase inhibitors, sonicated and spun to pellet the debris. Supernatant was and pre-cleared using washed sheep-anti rabbit dynabeads (Invitrogen). Proteins were then incubated with the designated antibody overnight at 4^{o}C. Immunocomplexes were then pelleted using the rabbit dynabeads, boiled in LDS samples buffer (Invitrogen) and then separated on a 4-12% denaturing polyacrylamide gel. Proteins were then transferred on a PVDF membrane which was then immunoblotted in the designated antibodies.

Methylated DNA Immunoprecipitation (Me-DIP)

Genomic DNA was prepared using Puregene DNA isolation kit (Gentra), sonicated to 300bp-1000bp length and purified using PCR purification kit (Qiagen). Four micrograms of this DNA was incubated with 10ug of 5-Methylcytosine antibody (Eurogentech) in IP

buffer (20 mM Tris (pH7.5), 140 mM NaCl, 0.05% Triton X-100) for 4hrs at 4^{o}C. DNA-antibody complexes were then pulled down using BSA and poly dAdT saturated sheep-anti-mouse dynabeads (Invitrogen), beads washed three times in IP buffer and then eluted by proteinase K digestion for 3 hrs at 50^{o}C and subsequent purification using PCR purification kit (Qiagen). Eluted DNA were then subjected to PCR for target identification.

Morpholino, plasmid and mRNA injections

Morpholinos were obtained from Gene-tools LLC Ltd. Morpholinos were designed against AID (5'-GTTTTGTTTTCGCTTACCTGTCCAG-3'), Apobec2a (5'-GCTGCTGCCCTTTCTATCGGCCATC-3'), Apobec2b (5'-CTTGCTGTCCTTTTTGTCTGCCATG-3'), Gadd45α (5'-ATATAAAAACATACCTTTCCGTTGC-3'), Gadd45αlike (5'-CACCGAGTCCATCCTGGAAAACCAC-3'), Gadd45β (5'-AAAGGAACTTACTTTGTATCAGTAA-3'), Gadd45γ (5'-TCCGGCAGTCTGCATTCTGGAGAAA-3'), zMbd4 (5'-AGAGAGAAACACACCTGCTCGCTGC-3'), AID Scr Mo (5'-CTGACTTCCCGTTGTTATCTGTGTT-3'), Mbd4 Scr Mo (5'-CACACACGGTGGCAAATACGCGTCA-3'), Apobec2a mismatch Mo (5'-GCTCCTCCCCTTTGTATCCGCGATC-3'), Apobec2b mismatch Mo (5'-CTTGgTGTCgTTTTaGTgTGCgATG-3') and Control (5'-CCTCTTACCTCAG TACAATTTATA-3'). For plasmid injections and transfections full length cDNAs were cloned as follows: zAID (Ensembl gene ID: ENSDARG00000015734) in pcDNA-nV5-DEST (Invitrogen), Apobec2a (GenBank Accession No: FJ469677; Ensembl gene ID: ENSDARG00000018881; GenBank accession no: NM_001013314) in pDEST-40

(Invitrogen), Apobec2b (GenBank Accession No: FJ469678; Ensembl gene ID: ENSDARG00000034604; GenBank Accession no: XM_690335) in pCMV-3XHA (modified to add two extra HA tags in pCMV-HA from Stratagene), Gadd45α (Ensembl gene ID: ENSDARG00000034604) in pDEST-26 (Invitrogen) or pCMV-3XHA and MBD4 (GenBank Accession no: NM_003925) in pCMV-3XMyc (modified to add two extra Myc tags in pCMV-Myc from Stratagene) and pCMV-3XHA. For mRNA injections cDNAs for zAID, zApobec2a, zApobec2b, zGadd45α (Ensembl gene ID: ENSDARG00000069991), zGaddd45αlike (Ensemble gene ID: ENSDARG00000043581) and zGadd45β (GenBank accession number: AY714220) were cloned in pCRII-TOPO vector (Invitrogen). Our partially sequenced zebrafish Mbd4 clones are an assembly of three ESTs present in GenBank database: CA473601, EG576141, AL921290, and Mbd4 morpholino is located in the first exon-intron boundary of the first EST. AID, Apobec2a/2b, MBD4 and Gadd45a mRNAs need to be made fresh in order to see efficient demethylation.

Figure S1. Methylated plasmid induces demethylation in zebrafish

(A) pCMV-Luc was *in vitro*-methylated using HpaII methylase which converts CCGG sites to $C^{me}CGG$ sites. Full methylation was verified by HpaII resistance and MspI susceptibility. Unmethylated (U) and methylated (Me) plasmids were subjected to southern blotting using a 736 bp probe against the luciferase gene cDNA coded on the plasmid (same as M-DNA; see Figure 1). (B) Fertilized zebrafish embryos were injected with 150 pg of *in vitro*-methylated pCMV-Luc (prepared as in panel A), and genomic DNA was isolated at indicated time points. Genome-wide methylation status was verified

by the same procedure, with ethidium bromide staining revealing cleavage, and on the plasmid itself by Southern blotting using the same probe as in panel A. Note that both genome-wide and plasmid demethylation (steady state measurement) is first detected at ~8 hpf and peaks at 13 hpf, whereas remethylation occurs by 28 hpf.

Figure S2. Expression patterns of AID, zMbd4 and TDG during zebrafish development

Relative expression of AID (A), zMbd4 (B) and TDG (C) were determined by semi-quantitative RT-PCR in cDNAs made from embryos the stages of development shown. Values indicated are normalized to 28S levels. Error bars: +/– one standard deviation.

Figure S3. Efficiency of morpholino knockdown of deaminase proteins and MBD4

(A-B) Morpholino knockdown efficiency of Apobec2a (4pg) (A) and Apobec2b (2pg) (B) was assessed at protein level by western blotting using whole cell extracts derived from wild type or morpholino-injected embryos. Anti-Apobec2a and anti-Apobec2b were specific rabbit antibodies generated from recombinant zebrafish proteins. Anti-Vinculin and anti-panH3 antibodies were used as loading controls. In panel B arrow indicates the band for Apobec2b whereas asterisk indicates a non-specific band. (C-D) Morpholino knockdown of AID and MBD4 was assessed at 80% epiboly in cDNA made from total RNA obtained from wild type embryos or embryos injected with AID Mo (4 pg) or MBD4 Mo (4pg). Spliced and unspliced DNA length is shown. 28S was used as a loading control.

Figure S4. AID morphants, zMbd4 morphants, and Gadd45α morphants show neurogenesis defects

(A) Gross morphological defects are shown by a bright field image of AID, Gadd45α and zMbd4 morphants at 24 hpf. These defects can be reversed to a large extent by injection of mRNAs encoding the indicated proteins that are refractory to the morpholino. AID and Gadd45α mRNA were of zebrafish origin, whereas hMbd4 mRNA was of human origin. AID Scr morpholino and MBD4 Scr morpholino injected embryos showed wild type morphology (not shown). (B-C) Whole mount *in situ* hybridizations were performed for *neurogenin-1* (B; a proneural marker) and *sox-2* (C; a gene expressed in neural progenitors) on AID, Gadd45α and zMbd4 morpholino (4pg each) injected embryos or wild type embryos at 80% epiboly (a stage when wild type and morphant embryos look relatively similar in gross morphology). Note the complete absence of *neurogenin-1* expression and drastic reduction in *sox-2* expression in these embryos. These defects can be compensated by injection of corresponding mRNAs (refractory to the morpholino) as indicated in panel A. zAID and zGadd45a mRNA were injected at 25pgs each whereas hMBD4 mRNA was injected at 50pgs for rescue of respective morphants. AID Scr morpholino and MBD4 Scr morpholino injected embryos showed wild type expression for *ngn-1* and *sox-2* (not shown).

Figure S5. Protein expression levels of catalytic mutant derivatives

Expression levels of wild type and catalytically-inactive derivatives of AID (A), Apobec2a (B), Apobec2b (C) and Mbd4 (D) were compared by transfection in RKO cells and detection in total cellular extracts by antibodies against the epitope tags as indicated.

Figure S6. Morpholino knockdown efficacy of Gadd45 family members

Knockdown efficiencies of morpholinos against Gadd45α (4pg) (A), Gadd45αlike (4pg) (B), Gadd45β (2pg) (C), and Gadd45γ (2pg) (D) was assessed at 80% epiboly in cDNA made from total RNA obtained from wild type embryos or ones injected with respective morpholinos. Spliced and unspliced DNA length is shown. 28S (A), Gadd45γ (B-C) and Gadd45β (D) were used as a loading controls. Panels A, B, and D contain lanes cut and pasted next to each other from the same gel.

Figure S7. Physical interactions between AID and Mbd4 and enhancement by Gadd45α

Western blot showing the co-immunoprecipitation of V5-AID and Myc-hMbd4 (A-B), V5-Apobec2a and Myc-hMbd4 (C-D) and HA-Apobec2b and Myc-MBD4 (E-F) in the absence (A, C, E) or presence (B,D,F) of Gadd45α when overexpressed in RKO cells. (G-J) Interaction between Myc-hMbd4 and HA-tagged Gadd45α (G), V5-AID and HA-Gadd45α (H), V5-Apobec2a and HA-Gadd45α (I) and HA-Apobec2b and His-Gadd45α (J) were detected in RKO cell extracts overexpressing the two proteins, suggesting that Gadd45α can individually interact with deaminases or hMbd4. Immune complexes were pulled down using the IP antibody as shown, and interacting proteins were detected in western blot format (WB) antibody as shown.

Figure S8. Identification of gene targets of AID and MBD4 by methylated DNA immunoprecipitation (Me-DIP)

(A-K) Genomic DNA prepared from wild type embryos or those injected (at 80% epiboly) with AID scr mo (sequence scrambled control morpholino; 2pg), AID morpholino (2pg), MBD4 scr mo (sequence scrambled control morpholino; 2pg) or MBD4 morpholino (2pg) were subjected to immunoprecipitation using an antibody directed against 5-methylcytosine and subsequently PCR analysis was performed for multiple genes including *neurod2* (A, ~200bp upstream of TSS), *sox1a* (B, ~450bp downstream of TSS), *hoxb2a* (C, ~3700bp upstream of TSS), *atoh1a* (D, ~350 bp upstream of TSS), pyruvate carboxylase (E, ~4800 bp upstream of TSS), nucleoside phosphorylase (F, ~300 bp upstream of TSS), *noggin2* (G, ~500bp downstream of TSS), *foxd3* (H, ~10 bp upstream of TSS), *sox2* (I, ~3350 bp upstream of TSS), *lin-28* (J, ~400 bp upstream of TSS) and *carbonic anhydrase 7* (K, ~50 bp upstream of TSS). Y-axis shows enrichment at these loci relative to a control *neurod2* locus (R2, figure 6). Of the loci tested *neurod2*, *sox1a*, *hoxb2a*, *atoh1a*, *pyruvate carboxylase*, *nucleoside phosphorylase*, and *noggin2* promoters/genes showed selective enrichment, whereas *foxd3*, *sox2*, *lin-28* and *carbonic anhydrase 7* did not. Several of these loci were verified by bisulphite sequencing (Figure S9). Primer information for the Me-DIP PCR is provided in Supplementary Table II. Graph shows one representative biological experiment (three biological repeats), with the average of two technical replicates shown. Error bars +/- SD.

Figure S9. Target validation of AID and MBD4 by bisulphite sequencing

(A-H) Genomic DNA prepared from wild type embryos or those injected (at 80% epiboly) with AID Scr Mo (sequence scrambled control morpholino; 2pg) or AID

Morpholino (2pg) were subjected to bisulphite conversion and subsequent PCR amplification, cloning and sequencing. Each line represents one clone and each circle represents one CpG dinucleotide. Open circles represent unmethylated cytosine whereas closed circles represent methylated cytosines in the context of CpG dinucleotides. Corresponding to the Me-DIP data (Figure S8), *sox1a, hoxb2a, atoh1a,* and *pyruvate carboxylase* showed hypermethylation in AID morphants compared to wild type or control morpholino injected embryos, whereas *foxd3, sox2, lin-28* and *carbonic anhydrase 7* remained unmethylated. Primer information for the PCR is provided in Supplementary Table II.

Supplementary Table I: Statistics of Morpholino phenotypes.

	Neurogenin-1	*Sox-2*
Control Mo	100% n=42	97% n=39
AID Mo	12% n=50	13% n=38
AID Mo + zAID RNA	86% n=64	83% n=124
Gadd45a Mo	11% n=52	33% n=48
Gadd45a Mo + zGadd45a RNA	77% n=61	78% n=50
Mbd4 Mo	14% n=43	15% n=53
Mbd4 Mo + hMbd4 RNA	91% n=56	87% n=64

Percentage shown depicts embryos showing positive staining for the marker indicated. All embryos from at least three different injections were counted and the number is represented by n. zAID and zGadd45a are of zebrafish origin whereas hMbd4 is of human origin.

Supplementary Table II: Primer Sequence Information.

	Forward (5' to 3')	*Reverse (5' to 3')*
RT-PCR		
AID	agtgtgctcatgacccaga	cacaacgcacccaagtga
Apobec2a	atggccgatagaaagggc	cctgtgatggtctcgaatg
Apobec2b	atggcagacaaaaaggacag	cggtctcctacaatgatctc
MBD4	aagagacgctcttccatga	ttatgatcgtctggagtgac
TDG	atggatgaaaggctgtatggatc	tcctctggatgtacaggcat
Gadd45a	atgacttttgaagaaccgtgtgg	gatctggagggccacat
Gadd45alike	tccgttctggattttacatgc	acccgattttgggtttcagt
Gadd45b	tctcacagtcggcgtttatg	cggctctcctcacagtaggt
Gadd45g	caacgacatcaacatcgttcg	tcagcgttcaggcagagtaa
M-DNA	atggaagacgccaaaaacataa	cgtgatggaatggaacaaca
Bisulfite		
Line-1	tttaattagatggtagtttttatatt	aacaaaccaacctaaaatataaa
	ggttggagatggtttatttt	ttcctaaaccacaaatataaacat
M-DNA	atggaagatgttaaaaatataaa	taataaaataaaacaacacttaaaat
	gatgttaaaaatataaagaaaggt (nested)	aaacaacacttaaaatcacaatat (nested)
KenoDr1	gtttgtattgaattattggt	aaaatcattttccttaaaaatcaa
	gagaagggataaatggattat (nested)	aaatcaactctaatccctcta (nested)
Neurod2	gtttttaaataggtataggt	caaacaaaattacataccta
	aggtataggttaggttatgt (nested)	catacctactcttataccaaa (nested)
Sox1a	ttttgtttaaaaaggataagtat	aatacatactaatcatctct
	gttagaggttggagagttt (nested)	taatcatctctctcaaatctc (nested)
Hoxb2a	ggataagtttttttatggttagt	aaacttcctataaaaaaaaaccc
	tggttagttttattagtggga (nested)	ccctaataataaaccaatacct (nested)
Atoh1a	aaagattgtttggggtaat	aattattatacccaaactcta
	gtaattaaaaggtttttgtgtgt (nested)	aactctacctttttttccataata (nested)
Pyruvate Carboxylase	ttggataaagtaaggaaagg	tcttaccaaatcaatattatcct
	aaggaaagggttggttttgt (nested)	caatattatcctctctttccttc (nested)
Foxd3	gattttattttgggttgttttagt	ttttctcctcctcaaaaaac
	tttgggttgttttagtagtaaag (nested)	ctcctcctcaaaaaactac (nested)

Sox2	ggaggataagaattataatt	tattcaacttcctataaaaaaa
	aagagtggttgtggatttt (nested)	acctattactctacaaatatcta (nested)
Lin-28	gtttttgatggatagaaataa	gatggatagaaataatttgaattat
	atataatccaaacaaaaccaa (nested)	ctcttcatataataatcaaacata (nested)
Carbonic anhydrase	atgagtagatttaatgatgt	caaaataccacatacaaaaa
	ggattttattaggtgaaaaaaat (nested)	cacatacaaaaattcaccaa (nested)
ChIP		
pCMV-Luc (Fig. 5)		
Positive (w/CCGG)	agatcgtggattacgtcgc	cttggcctttatgaggatctc
Negative (w/o CCGG)	cattgacgtcaatgggagtttg	ttagccagagagctctgc
Neurod2		
Positive (P1; Fig. 6 in promoter)	atacatttgtggctgctgtgt	acatcctgacgatatatggaga
Negative (P2; Fig. 6 in intron)	ggttgaaaatggacattctgc	gctaaacagtgctgaattagg
MeDIP		
Neurod2	atacatttgtggctgctgtgt	acatcctgacgatatatggaga
Sox1a	gcatgatgatggaaacggac	ttccactcagcaccgagt
Hoxb2a	ggaagaccttcaatcagcgt	caatgacaagaacacagcgtc
Atoh1a	gtgtgaagacggctgaatac	tcctcacgccatgttttgga
Pyruvate carboxylase	tgaatcgagtggctcgaag	catccgctctcaaggtttac
Nucleoside phosphorylase	ctcacatgcctttgtgtacc	attcctgaagcgtcgctct
Noggin2	tgtgaccaagaccttcctg	cgtgggtaaacagtgcaatc
Foxd3	aagccttcggactggagaa	gaccacgtcgatatccacat
Sox-2	tttgcacctgtacctccgaa	gaaatccacagccactcttg
Lin-28	gacaacctaccatattagcttgc	tcaagcatgtgcttcaacgg
Carbonic anhydrase 7	cagtggatttcaccaggtga	aggtgaaggaagagcgatga
Negative Control 1 (neurod2 negative region)	ggttgaaaatggacattctgc	gctaaacagtgctgaattagg
Negative Control 2 (1Kb region in Chr 1 with 3 CpGs)	aacagagtggcaggcatcattta	acagtcatatttcttggaagacccc
Negative Control 3 (1Kb region in Chr 14 with no CpGs)	ctgaactttcatcaaattggtttcc	gtgatggcaaacttaaagtccttca
C to T detection (CCGG position)		
Forward Strand		

32	ccaaaaacataaagaaaggccca	atatccttgcctgatacctg
272	aaactctcttcaattctttatgcca	atatccttgcctgatacctg
522	acatctcatctacctccca	atatccttgcctgatacctg
Reverse Strand		
272	atggaagacgccaaaaacataa	aaataacgcgcccaacacca
522	atggaagacgccaaaaacataa	tggcacaaaatcgtattcattaaaacca
698	atggaagacgccaaaaacataa	caacacttaaaatcgcagtatcca

Supplemental Figures

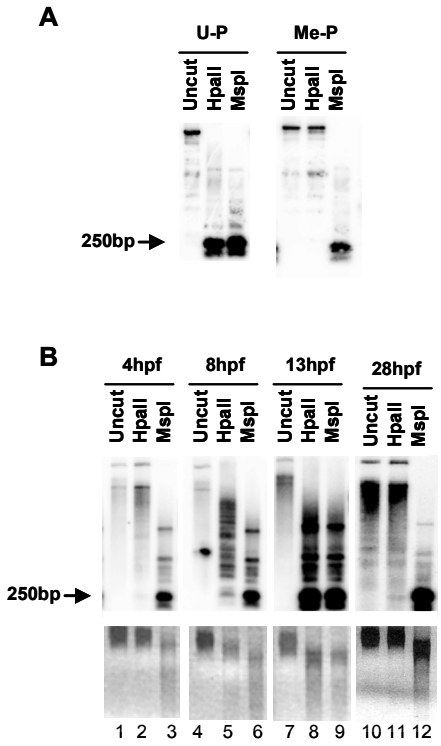


Figure S1. Rai et al.

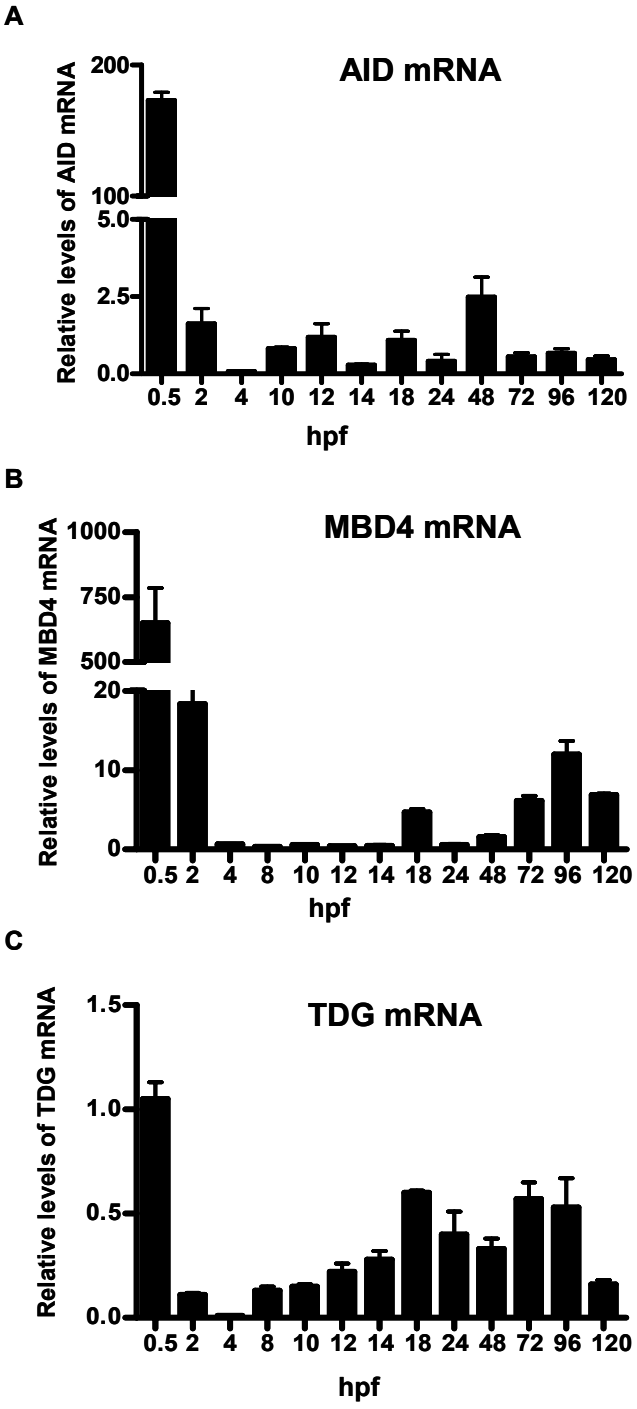


Figure S2. Rai et al.

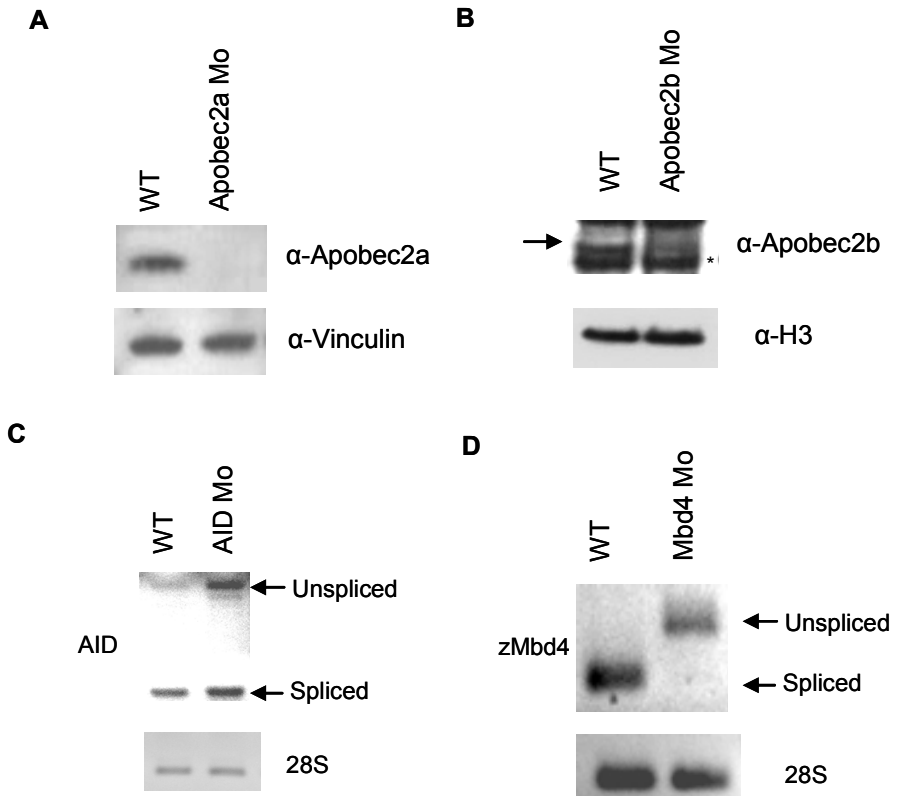


Figure S3. Rai et al.

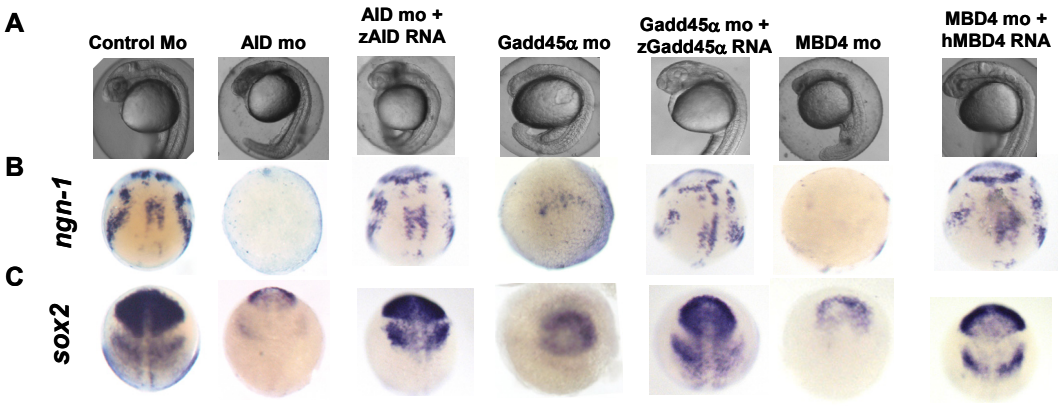
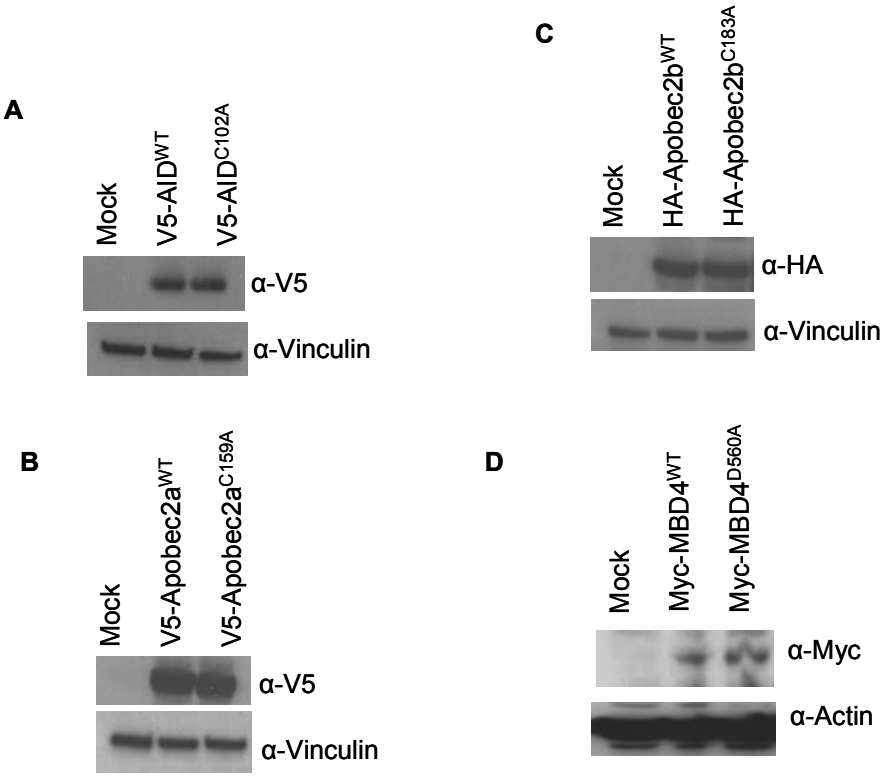


Figure S4. Rai et al.



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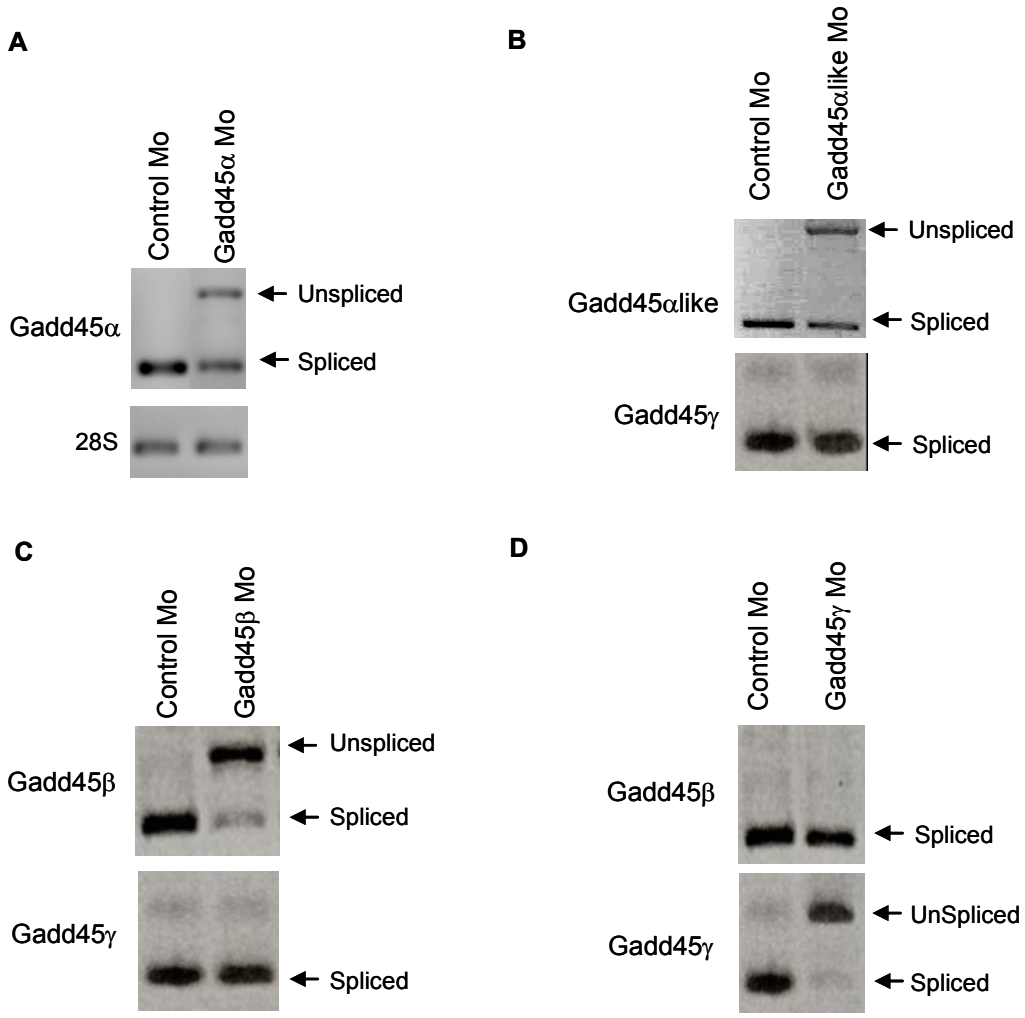

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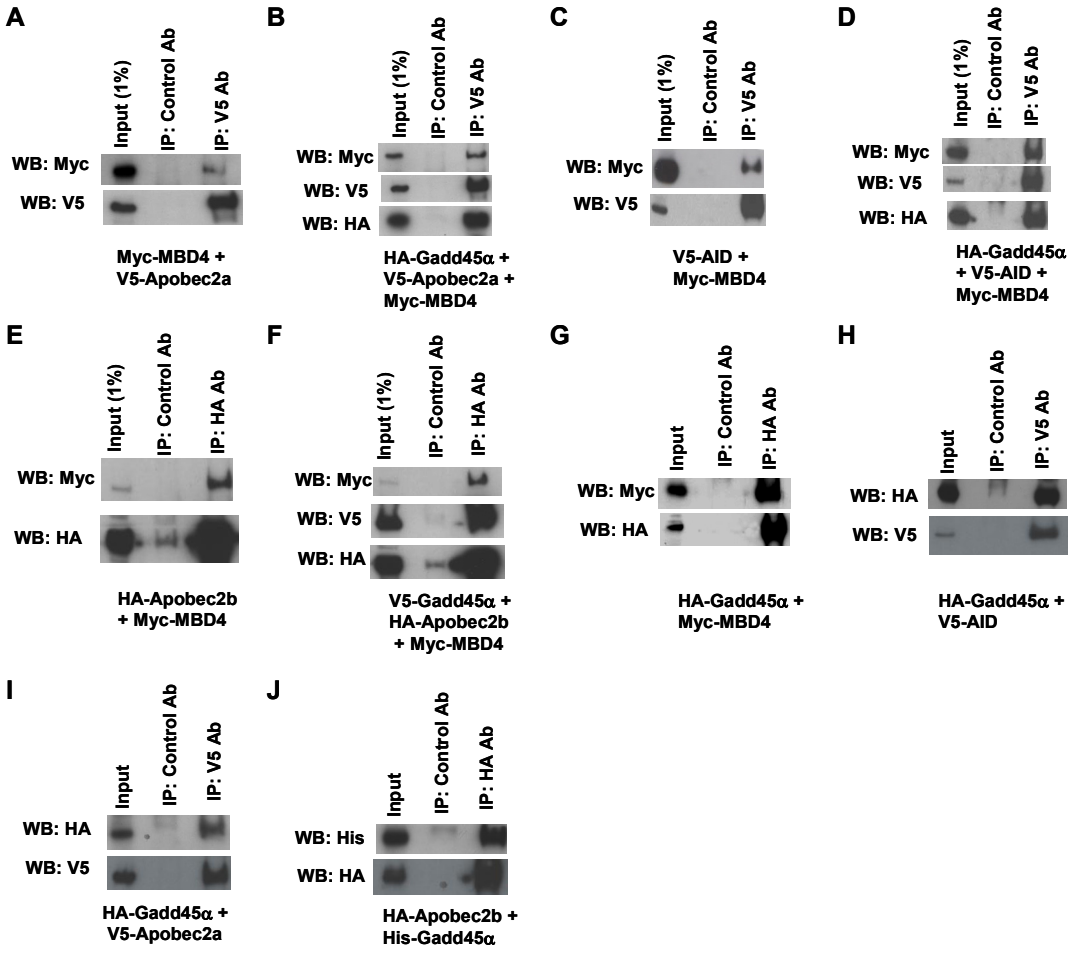

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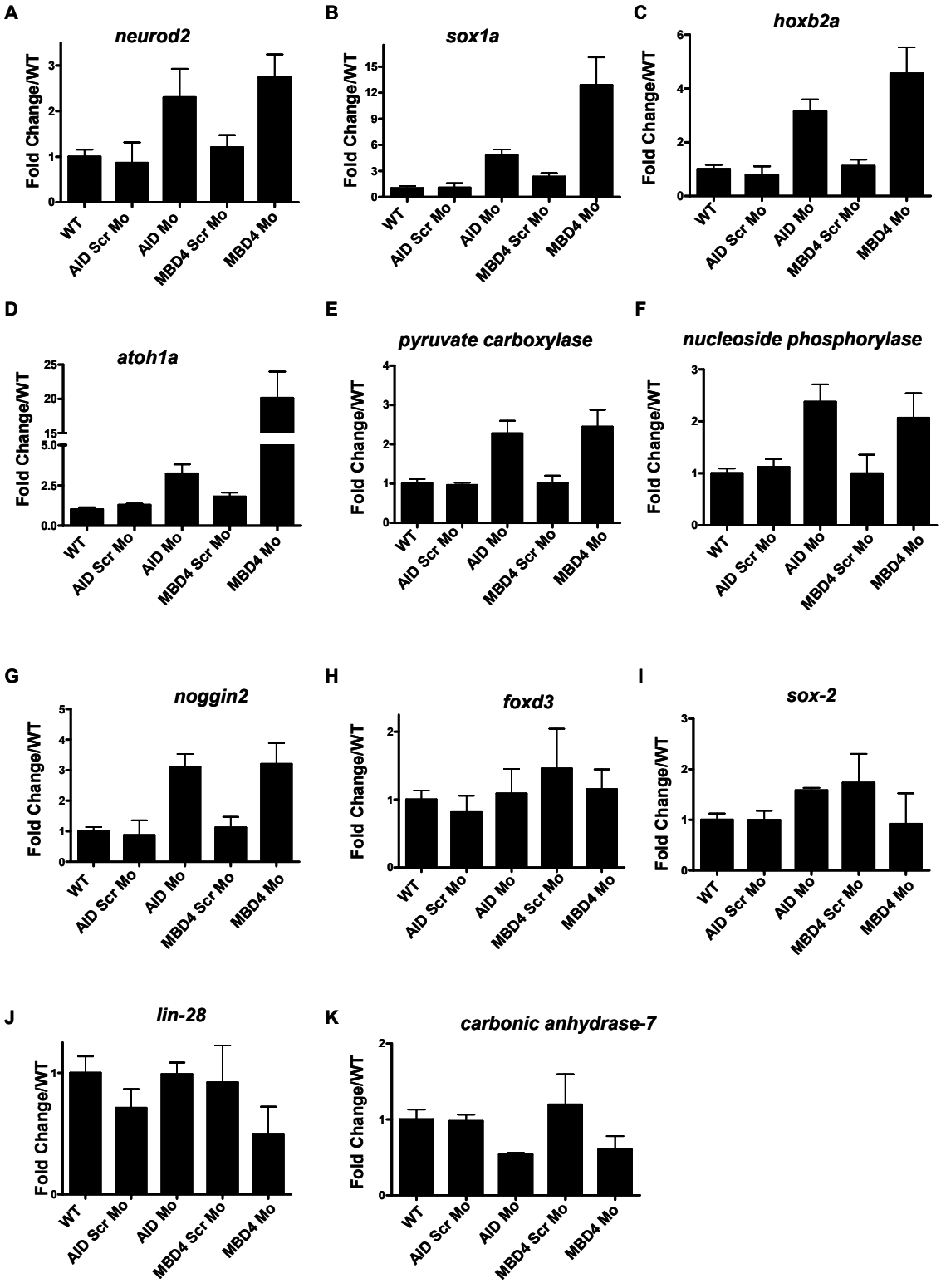

Figure S8. Rai et al.

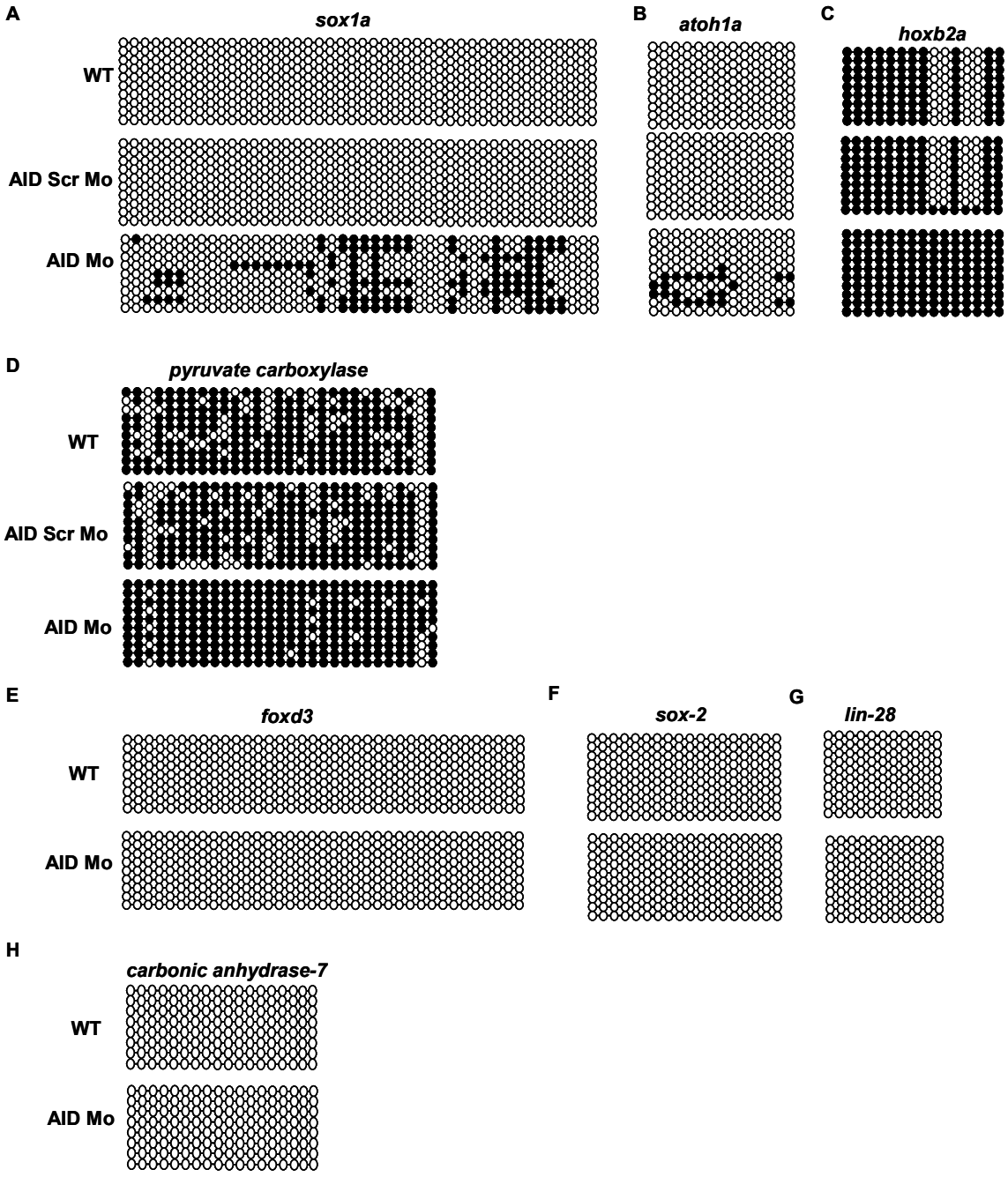

Figure S9. Rai et al.