Appendix A

Supplemental Tables and Figures for Chapter 2

Supplemental Table 2.1. Primer sequences (SYBR Green assay) used for real-time, quantitative PCR analysis of gene expression (RT-qPCR) and chromatin immunoprecipitation (ChIP) assays.

For RT-qPCR

Xenopus laevis

Dnmt3a mRNA
Forward: 5' GGT CTA TGA AGT GAG GCA GAA GTG 3'
Reverse: 5' TTC AAA CTG CCG CAA GAA ATA C 3'

Alpha-actinin mRNA
Forward: 5' GGA CAA TTA TCC TGC GTT TTG C 3'
Reverse: 5' CCT TCT TTG GCA GAT GTT TCT TC 3'

Mouse

Dnmt3a mRNA
Forward: 5' CCT GTG GTG CAC TGA AAT GG 3'
Reverse: 5' TCG CCA AGC GGC TCA T 3'

GAPDH mRNA
Forward: 5' TGT GTC CGT CGT GGA TCT GA 3'
Reverse: 5' CTT CAC CAC CTT CTT GAT GTC ATC 3'

For ChIP assay

Xenopus laevis

Dnmt3a Region A
Forward: 5' CCC CTC TCT CTG CCC AAC A 3'
Reverse: 5' GTG TCC TTA TTA ACC CCG TTA TCA CT 3'

Dnmt3a Region B
Forward: 5' TGA CTC TGC GCT GCA GTG A 3'

Reverse: 5’ AGG CTG CGT CCC CCT TAC 3’

Dnmt3a Region C
Forward: 5’ CTC CGT TTC CCC TGC AAA T 3’
Reverse: 5’ ACA TAC ACA TTA ATC ACT CCA TTT CCC 3’

Mouse

Dnmt3a +30.1 kb TSS
Forward: 5’ CAG AGG AGG GAA CAC TGT ATA A 3’
Reverse: 5’ CAA ACA CAA GCC CAG ATG TC 3’

Dnmt3a +39.0 kb TSS (negative control region)
Forward: 5’ AGT GAG GCA GGA CTT TCT AGG TAT G 3’
Reverse: 5’ ACC TCT GCT TTG CTC CTA CTC AA 3’

Dnmt3a +49.0 kb TSS
Forward: 5’ CCA ACT TCC TCC AGG GTT ATT T 3’
Reverse: 5’ GCC CTT GCT GGG TTA TTC T 3’

Supplemental Table 2.2. Genomic coordinates of TR binding sites associated with the *dnmt3a* loci identified by high-throughput sequencing analyses

TR ChIA-PET conducted on premetamorphic *Xenopus tropicalis* tail fin (Sachs and colleagues, unpublished data) (coordinates are based on XenTro2 build)

Region A: Scaffold_971: 107,805-108,924

Region B: Scaffold_971: 120,574-120,823

Region C: Scaffold_971: 124,856-125,536

TR ChIP-seq conducted on mouse liver chromatin (Ramadoss et al., 2014) (Mm9 build)

Peak 1: chr12: 3,806,012-3,806,644

Peak 2: chr12: 3,807,928-3,809,079

Peak 3: chr12: 3,812,247-3,813,164

Peak 4 (+30.1 kb TSS): chr12: 3,837,246-?

Peak 5: chr12: 3,841,058-3,841,690

Peak 6: chr12: 3,859,292-3,860,559

Peak 7: chr12: 3,866,399-3,867,232

Peak 8: chr12: 3,868,339-3,869,565

Peak 9: chr12: 3,889,702-3,890,300

Peak 10: chr12: 3,894,480-3,895,087

Peak 11: chr12: 3,911,787-3,912,497

Peak 12: chr12: 3,919,268-3,919,956

TR ChIP-seq conducted on mouse cerebellar cell line C17.2 (Chatonett et al., 2013) (Mm9 build)

Peak 13 (+49.0 kb TSS): chr12: 3,855,855-3,856,520

Supplemental Table 2.3. Oligonucleotides used for plasmid constructs used in transfection-reporter assay and in *in situ* hybridization histochemistry (ISHH); Oligonucleotides used for electrophoretic mobility shift assay (EMSA)

PCR primers used to amplify *X. laevis dnmt3a* cDNA for subcloning into pGEM-Teasy cloning vector

Forward: 5' TTT CCC ATC ATG CCC AGA GAA CGA 3'
Reverse: 5' GCC GTC GTC ATC GTA TTG GT 3'

PCR primers used to amplify genomic fragments for subcloning into pGL4.23/pCpGL luciferase vectors (restriction sites are underlined)

Xenopus tropicalis
Dnmt3a region A (pGL4.23)
Forward: 5' TAT GGT ACC GGG CCC TAC AGA GCA TAT AGT 3'
Reverse: 5' CAA TGA GCT CCA TTT CTG TGA GAC TGC CTA TCC 3'

Dnmt3a region B (pGL4.23)
Forward: 5' AAG GTA CCA CAG TCA GTT CTG CTC ATG TTG GG 3'
Reverse: 5' TTC TCG AGC TGC ACA TAC CCT TCC CTG TGT AT 3'

Mouse
Dnmt3a +30.1 kb TSS (pCpGL)
Forward: 5' CCC AGA TCT TGG GAC AAA CTC CTC TCA TC 3'
Reverse: 5' GCC CAA GCT TGC TCT AGC CTT TGT CTA ACC 3'

Dnmt3a +49.0 kb TSS (pGL4.23)
Forward: 5' AGC GGT ACC GGG ATT TAT ACC CAC CAA CT 3'
Reverse: 5' TGA CGA GCT CCA GCT GTC TTC TGG AAG GCC A 3'

PCR primers used for site-directed mutagenesis of pGL4.23/pCpGL luciferase vectors (lowercase letters indicate mutated nucleotides)

Xenopus tropicalis
Dnmt3a T_3RE-A1
Forward: 5' CAG AGC GGG GGc TAG CTG AGa ACT GGC CTG CA 3'
Reverse: 5' TGC AGG CCA GTt CTC AGC TAg CCC CCG CTC TG 3'

Dnmt3a T_3RE-B2
Forward: 5' CCC CAG TGG GTT AtC TGG TTT GgC TCT GCG CTG CA 3'
Reverse: 5' TGC AGC GCA GAG cCA AAC CAG aTA ACC CAC TGG GG 3'

Mouse

Dnmt3a +30.1 kb T_3RE 2

Forward: 5’ CTG CCT CTT CAG GtA GAT GTG ACT aCA GGA CAT CTG GG 3’
Reverse: 5’ CCC AGA TGT CCT GtA GTC ACA TCT aCC TGA AGA GGC AG 3’

Dnmt3a +30.1 kb T_3RE 3
Forward: 5’ GCC TCT TCA GGG AGA TaT GAC TTC AGa ACA TCT GGG CTT G 3’
Reverse: 5’ CAA GCC CAG ATG TtC TGA AGT CAt ATC TCC CTG AAG AGG C 3’

Dnmt3a +30.1 kb T_3RE 4
Forward: 5’ GGA GAT GTG ACT TCA GaA CAT CTG GaC TTG TGT TTG CTC AGC 3’
Reverse: 5’ GCT GAG CAA ACA CAA GtC CAG ATG TtC TGA AGT CAC ATC TCC 3’

Dnmt3a +30.1 kb T_3RE 6
Forward: 5’ GCC TGT GGA AGc CAG CTG AGa TCA CGG GCA GG 3’
Reverse: 5’ CCT GCC CGT GAt CTC AGC TGg CTT CCA CAG GC 3’

Dnmt3a +49.0 kb T_3RE wild-type
Forward: 5’ GGG TGC ATT CTG AGG cCA CTC TTG aTA AGA ATA ACC CAG C 3’
Reverse: 5’ GCT GGG TTA TTC TTA tCA AGA GTG gCC TCA GAA TGC ACC C 3’

Oligonucleotides used for EMSA (lowercase letters indicate non-native nucleotides added to the 5’ends for [^{32}P] labeling by Kelnow fill-in)

Xenopus tropicalis

Dnmt3a T_3RE-A1 wild-type
Forward: 5’ gat cAC AGA GCG GGG GTT AGC TGA GGA CTG GCC TGC AC 3’
Reverse: 5’ gat cGT GCA GGC CAG TCC TCA GCT AAC CCC CGC TCT GT 3’

Dnmt3a T_3RE-A1 mutant (mutated sequence is underlined)
Forward: 5’ gat cAC AGA GCG GGG GCT AGC TGA GAA CTG GCC TGC AC 3’
Reverse: 5’ gat cGT GCA GGC CAG TTC TCA GCT AGC CCC CGC TCT GT 3’

Dnmt3a T_3RE-B2 wild-type
Forward: 5’ gat cTG CAG CGC AGA GTC AAA CCA GGT AAC CCA CTG GG 3’
Reverse: 5’ gat cCC CAG TGG GTT ACC TGG TTT GAC TCT GCG CTG CA 3’

Dnmt3a T_3RE-B2 mutant (mutated sequence is underlined)
Forward: 5’ gat cTG CAG CGC AGA GCC AAA CCA GAT AAC CCA CTG GG 3’
Reverse: 5’ gat cCC CAG TGG GTT ATC TGG TTT GGC TCT GCG CTG CA 3’

Mouse

Dnmt3a +30.1 kb T_3RE 1
Forward: 5’ gat cCG CTT TGA AGC AGA GGA GGG AAC ACT GTA T 3’
Reverse: 5’ gat cAT ACA GTG TTC CCT CCT CTG CTT CAA AGC G 3’

Dnmt3a +30.1 kb T_3RE 2-4
Forward: 5' gat cCT TCA GGG AGA TGT GAC TTC AGG ACA TCT GGG CTT GTG TTT 3'
Reverse: 5' gat cAA ACA CAA GCC CAG ATG TCC TGA AGT CAC ATC TCC CTG AAG 3'

Dnmt3a +30.1 kb T_3RE 5
Forward: 5' gat cGT AAC TCT AGC CTT GCC TGT CTT ACA GGG C 3'
Reverse: 5' gat cGC CCT GTA AGA CAG GCA AGG CTA GAG TTA C 3'

Dnmt3a +30.1 kb T_3RE 6
Forward: 5' gat cCC TGT GGA AGT CAG CTG AGG TCA CGG GCA G 3'
Reverse: 5' gat cCT GCC CGT GAC CTC AGC TGA CTT CCA CAG G 3'

Dnmt3a +30.1 kb T_3RE 7
Forward: 5' gat cAG ATT CTG GGA CTG ATA GAG TGA GCG TAT T 3'
Reverse: 5' gat cAA TAC GCT CAC TCT ATC AGT CCC AGA ATC T 3'

Dnmt3a +49.0 kb T_3RE wild-type
Forward: 5' gat cGT GCA TTC TGA GGT CAC TCT TGC TAA GAA TAA CCC A 3'
Reverse: 5' gat cTG GGT TAT TCT TAG CAA GAG TGA CCT CAG AAT GCA C 3'

Dnmt3a +49.0 kb T_3RE mutant (mutated sequence is underlined)
Forward: 5' gat cGT GCA TTC TGA GGC CAC TCT TGA TAA GAA TAA CCC A 3'
Reverse: 5' gat cTG GGT TAT TCT TAT CAA GAG TGG CCT CAG AAT GCA C 3'

PCR primers used to generate genomic fragments for competitive EMSA

Xenopus tropicalis

Region A with T_3RE-A1 (wild-type and mutant)
Forward: 5' GCC CTA CAG AGT AAT GTT GCT TAT 3'
Reverse: 5' CCT CAC TCT GCC GTC ATC TA 3'

Region A with T_3RE-A2
Forward: 5' ACT TCC CAG TTT GCC CTC T 3'
Reverse: 5' AAA GCT GCT TCC CAC AAT CC 3'

Region A with T_3RE-A3
Forward: 5' TAT CGG GCA CAC TGG TAC A 3'
Reverse: 5' CCC ACA ACC ACA CTT TCC 3'

Region B with T_3RE-B1
Forward: 5' AAG GTA CCA CAG TCA GTT CTG CTC ATG TTG GG 3'
Reverse: 5' CCC TCC CAT CGC TGC ACT T 3'

Region B with T_3RE-B2 (wild-type and mutant)
Forward: 5' AAG TGC AGC GAT GGG AGG G 3'

Reverse: 5’ AAT CAT TCT TGG CTG CGC CC 3’

Region B with T_3RE-B3
Forward: 5’ GGG CGC AGC CAA GAA TGA TT 3’
Reverse: 5’ CCT GCA CAT ACC CTT CCC TGT GTA T 3'

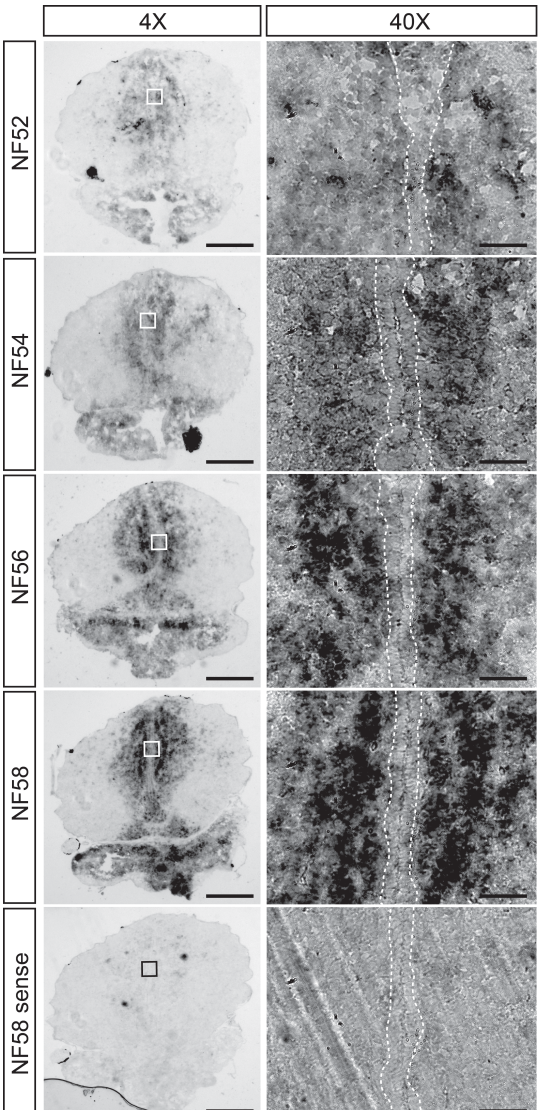
Region B with no T_3RE
Forward: 5’ ATA CAC AGG GAA GGG TAT GTG CAG G 3’
Reverse: 5’ TTA CCA AAC TGC AAC TCC CAG CAG 3’

Mouse

+30.1 kb TSS region with T_3RE (wild-type, and mutant 2, 3, 4 and 6)
Forward: 5’ CAG AGG AGG GAA CAC TGT ATA A 3’
Reverse: 5’ CCC TCA TAA TTG GAC CAG AC 3’

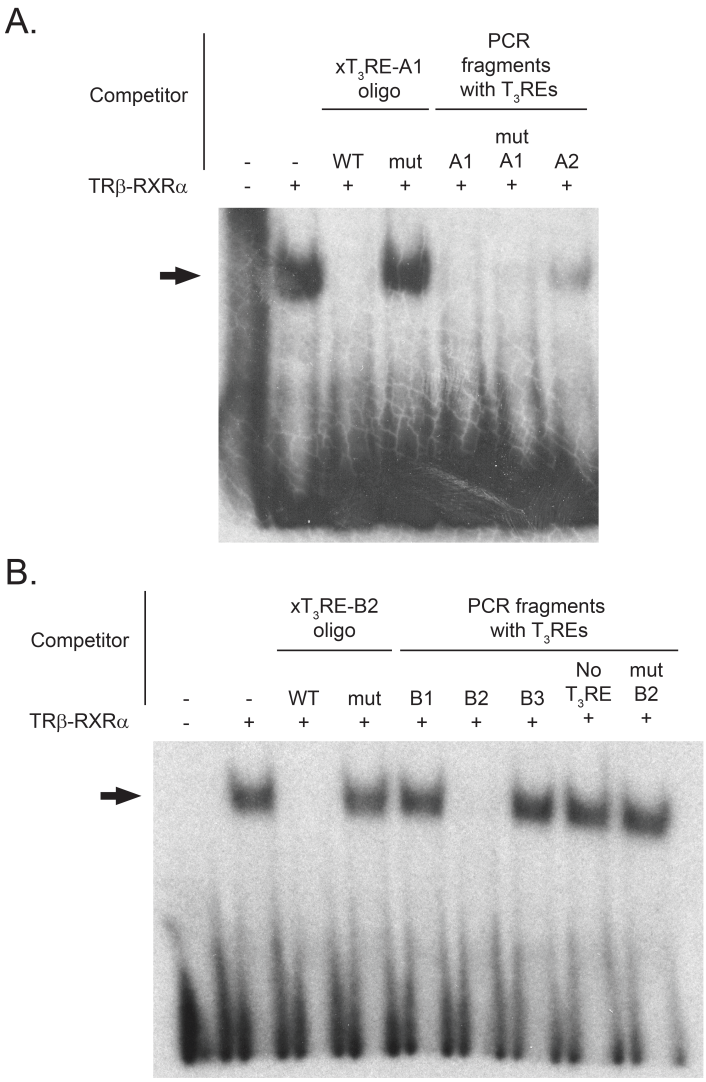
+49.0 kb TSS region with T_3RE (wild-type and mutant)
Forward: 5’ AGC GGT ACC GGG ATT TAT ACC CAC CAA CT 3’
Reverse: 5’ GGC TGC TGT GGA GTA AGT A 3’

+49.0 kb TSS with no T_3RE
Forward: 5’ TAC TTA CTC CAC AGC AGC C 3’
Reverse: 5’ CCA AAT GGC TCT TAG ACT GG 3’

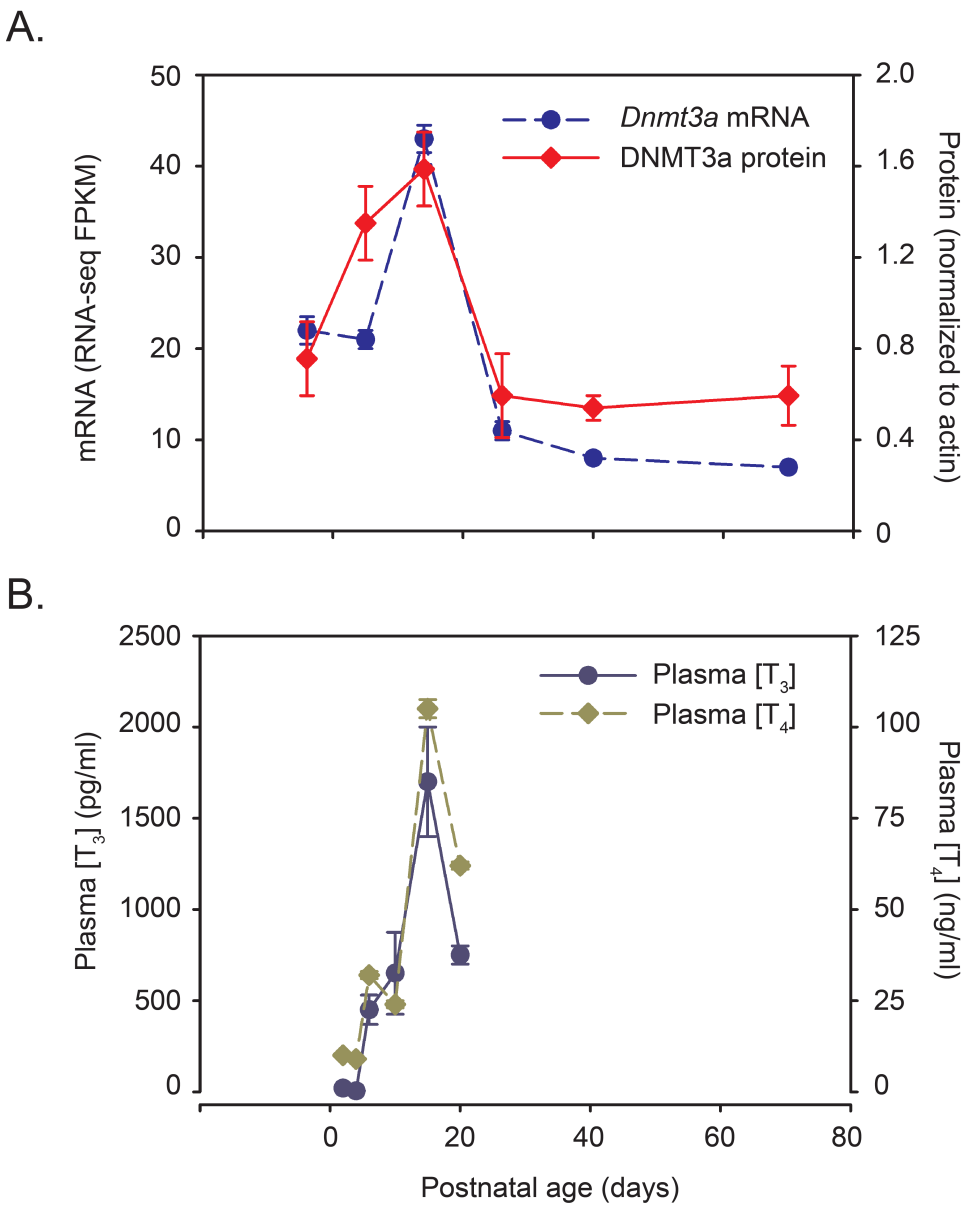


Supplemental Fig. 2.1: *Dnmt3a* mRNA exhibits developmental increases in *Xenopus* tadpole brain. I conducted ISHH for *dnmt3a* mRNA on *X. laevis* tadpole brain between premetamorphosis and late prometamorphosis (NF stages 52 and 58) during metamorphosis. Shown are representative micrographs of transverse sections at the region of the ventral hypothalamus and thalamic nuclei (region 1 in Fig. 1B). Right panels (40X) show magnified view of regions indicated by boxes in left panels. Areas within dotted lines indicate the ventricular and subventricular zones (VZ/SVZ). Note that *dnmt3a* mRNA is excluded from the VZ and SVZ. Scale bar = 200 μm (4X), 20 μm (40X).

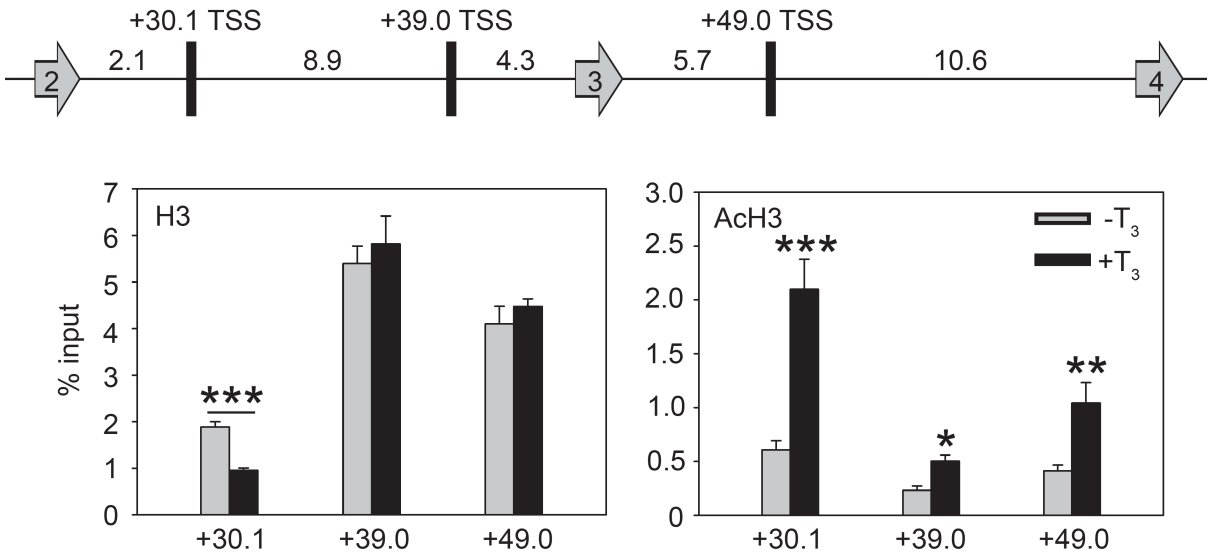
Supplemental Fig. 2.2. Identification of conserved T_3REs associated with the frog *dnmt3a* gene. By analyzing data from a ChIA-PET experiment conducted on tadpole tail fin chromatin with TR as the target protein (L. Sachs and N. Buisine, unpublished data) I identified three putative TR binding sites at the *X. tropicalis dnmt3a* locus (regions A, B and C). Shown are sequence alignment of homologous, conserved genomic loci found in the *X. laevis* genome (Build 5.0) for regions A and B. Alignment for region C is not shown because I did not find TR association at this locus in tadpole brain (see Results and Fig. 2C) and did not further investigate this locus. The *Xenopus laevis* genome is pseudotetraploid (*X. tropicalis* is diploid), and therefore, there are two homologous regions for each region. Boxes indicate the locations of predicted T_3RE half sites based on *X. tropicalis* sequences). The T_3REs are numbered in 5' to 3' direction according to the *X. tropicalis* reference genome (XenTro2 Build). In region A I found another putative T_3RE (xT_3RE-A3), but it is not shown in the alignment because it is located outside the conserved region.


A.
Competitor
xT3RE-A1 oligo
PCR fragments with T3REs
mut
- - WT mut A1 A1 A2
TRβ-RXRα - + + + + + +
B.
Competitor
xT3RE-B2 oligo
PCR fragments with T3REs
No T3RE
mut B2
- - WT mut B1 B2 B3
TRβ-RXRα - + + + + + + + +

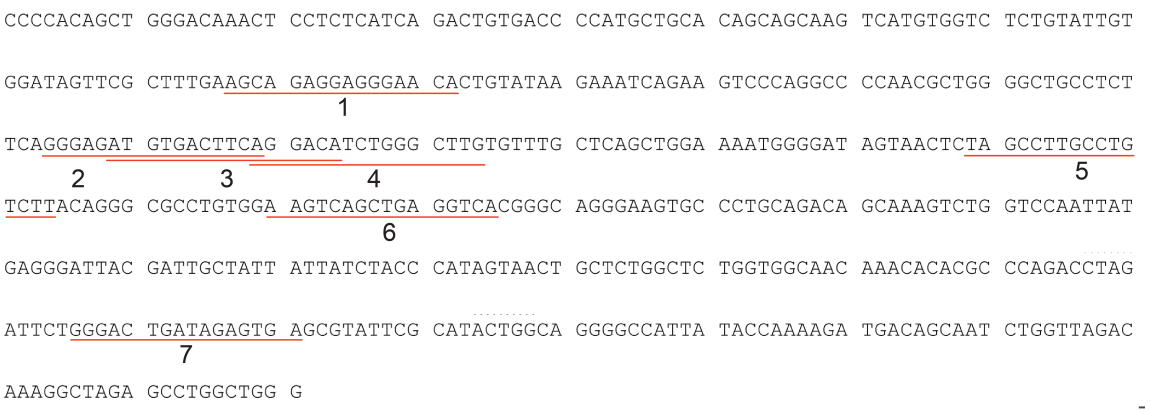
Supplemental Fig. 2.3: Radioinert duplex olingonucleotides or DNA fragments containing the frog *dnmt3a* T_3REs displace TRβ-RXRα from the radiolabled probe. I conducted competitive EMSA using a [^{32}P]-labeled oligonucleotide probe corresponding to the xT_3RE-B2 sequences. Before gel eletrophoresis the probe was incubated with *in vitro* synthesized *Xenopus* TRβ plus RXRα with or without indicated radioinert oligonucleotides or PCR-generated genomic fragments that encompass the predicted T_3REs (see Supplemental Fig. 2 for location of the predicated T_3REs; probe sequences and primer sequences that were used to generate PCR fragments are given in Supplemental Table 2.2). Mutant oligonucleotides or genomic fragments contained single base pair mutations that were introduced into each of the two half sites of the indicated T_3REs; these mutations abolished T_3-dependent trasactivity of regions A and B of the *X. tropicalis dnmt3a* locus (see Results and Fig. 3B). The arrows indicate the location of supershifted bands due to TRβ-RXRα binding to the probes. (**A.**) A radioinert oligonucleotide (1 μM) corresponding to the native xT_3RE -A1 sequence completely displaced TRβ-RXRα binding to the probe, while that for the mutant xT_3RE-A1 did not. Radioinert PCR-generated genomic fragments (19 nM) that encompass the native and mutant xT_3RE-A1 sequences both completely displaced TRβ-RXRα binding to the probe, while that for xT_3RE-A2 sequence had a reduced activity (**B.**) A radioinert oligonucleotide (1 uM) corresponding to the native xT_3RE-B2 sequence completely displaced TRβ-RXRα binding to the probe, while that for the mutant xT_3RE-B2 sequence did not. Radioinert PCR-generated genomic fragments (22 nM) that encompass the native xT_3RE -B2 sequence completely displaced TRβ-RXRα binding to the probe, while those for xT_3RE -B1, xT_3RE -B3 and the mutant xT_3RE -B2 sequences had no activity. A PCR fragment that did not contain a predicted T_3RE was used as a negative control.



Supplemental Fig. 2.4: Developmental increases in *Dnmt3a* mRNA and protein correlate with increase in circulating plasma [T_3] and [T_4] in neonatal mouse. (**A.**) *Dnmt3a* mRNA and protein levels peak at PND14 in neonatal mouse frontal cortex. The mRNA level was measured using RNA-seq (FPKM, fragments per kilobase of exon per million fragments mapped) and protein level was measured by Western blot (normalized to actin protein) (**B.**) Circulating plasma [T_3] and [T_4] levels also reach their highest levels at PND14 in mouse as measured by radioimmunoassay. Points represent the mean ± SEM (n ≥ 4) (Both figures were adapted and modified with permission from A: Lister et al., (2013); and B: Hadj-Sahraoui et al. (2000)).

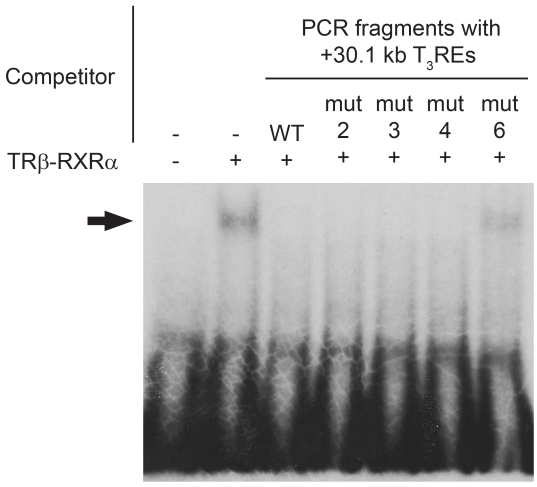


Supplemental Fig. 2.5: T_3 treatment reduces histone H3 (H3) and increases acetylated H3 (AcH3) at the +30.1 kb from the TSS of the mouse *Dnmt3a* gene in Neuro2a[TRβ1] cells. The schematic diagram (top) shows putative TR binding sites (rectangles) identified by TR ChIP-seq experiments conducted on mouse liver (REF) and C17.2 mouse cerebellar cell line (REF) that are located between exons 2 and 4 (arrows) of the mouse *Dnmt3a* gene (distance between features are in kb). I conducted targeted ChIP assays for histone 3 (H3) and acetylated H3 (AcH3) at +30.1, +39.0 and +49.0 kb from the TSS of the mouse *Dnmt3a* gene using chromatin isolated from Neuro2a[TRβ1] cells treated with vehicle (0.1% DMSO) or T_3 (30 nM) for 24 hrs before harvest. Purified IgG antibodies (negative control) yielded < 0.03% of input DNA (data not shown). Bars represent the mean ± SEM ChIP signal expressed as a percentage of input DNA quantity. Asterisks above the means indicate statistically significant differences in ChIP signals between treatments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's independent sample *t*-test).

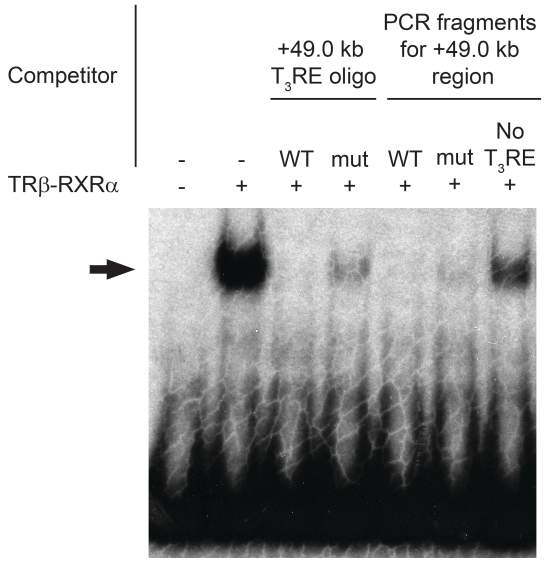


Supplemental Fig. 2.6: Identification of putative T_3REs in +30.1 kb TSS region of the mouse *Dnmt3a* gene. Shown is the DNA sequence of the 500 bp region centered at +30.1 kb from the TSS of the mouse *Dnmt3a* gene and location of the seven predicted T_3REs (underlined) found within this region. Oligonucleotide sequences for competitive EMSA in Fig. 6A were designed to encompass these predicted T_3REs (Supplemental Table 2.2).

A.

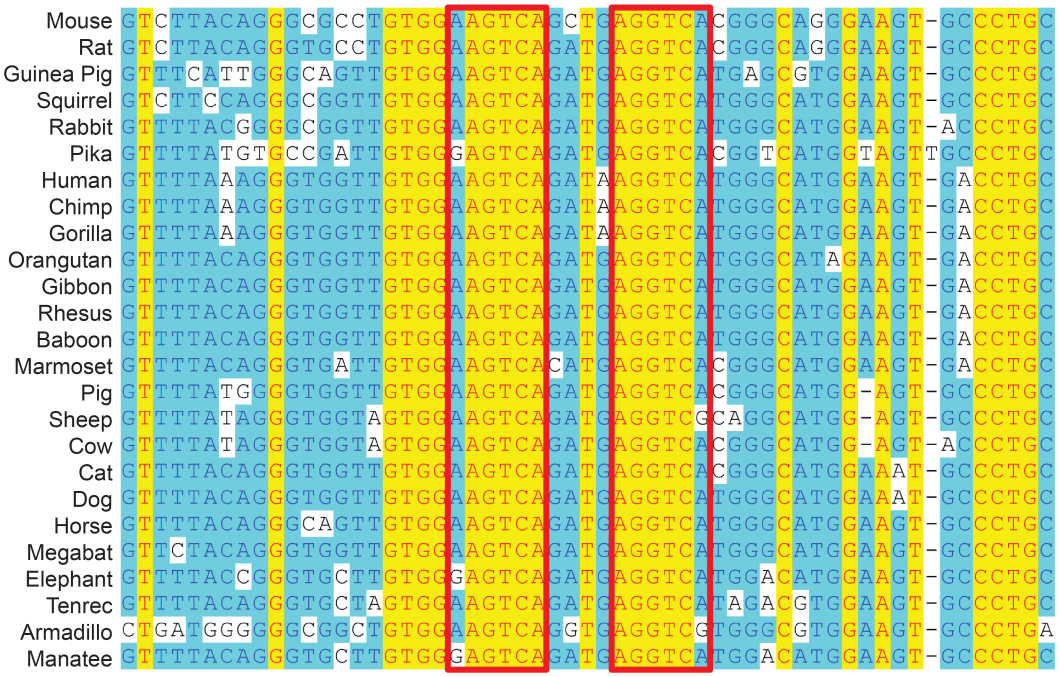

Competitor
PCR fragments with +30.1 kb T3REs
mut mut mut mut
- - WT 2 3 4 6
TRβ-RXRα
- + + + + + +

B.

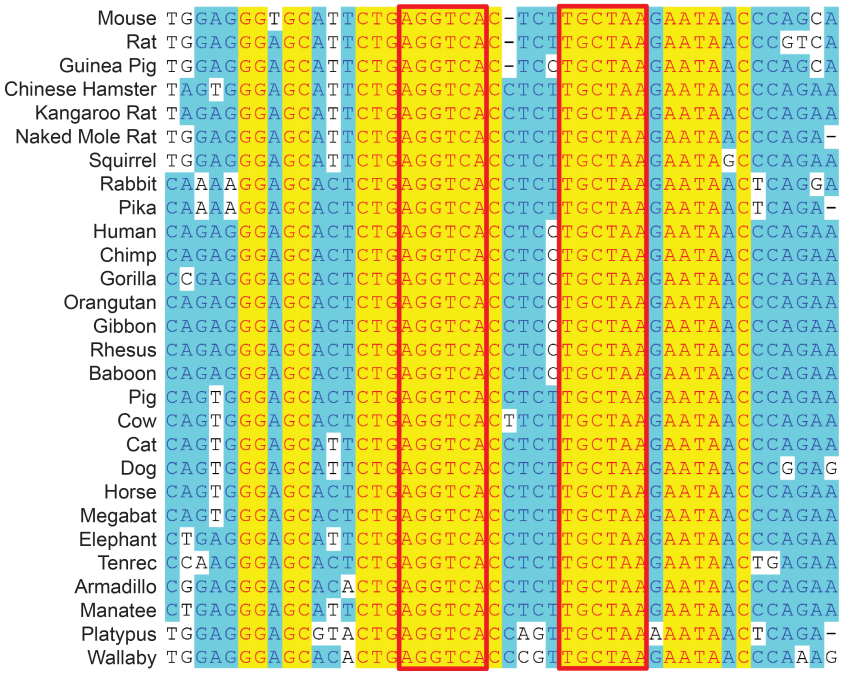

Competitor
+49.0 kb T3RE oligo
PCR fragments for +49.0 kb region
No
- - WT mut WT mut T3RE
TRβ-RXRα
- + + + + + +

Supplemental Fig. 2.7: Radioinert oligonucleotides or DNA fragments that encompass the predicted mouse *Dnmt3a* T_3REs displaces TRβ-RXRα heterodimers from the radiolabeled probe (**A.**) **I** conducted competitive EMSA using a [^{32}P]-labeled oligonucleotide probe corresponding to the *X. tropicalis dnmt3a* xT_3RE -B2 sequence (probe) and radioinert PCR-generated genomic fragments (277 bp) that encompass the sequences of putative T_3RE s 2, 3, 4 and 6 found within a 500 bp genomic region centered at +30.1 kb from the TSS of the mouse *Dnmt3a* gene (see Supplemental Fig. 6 for location of these predicted T_3REs; primer sequences that were used to generate PCR fragments are given in Supplemental Table 2.2). Mutant PCR fragments contained single base pair mutations that were introduced into each of the two half sites of the indicated T_3REs. Mutations of T_3REs 2, 3 and 4 reduced T_3-dependent trasactivity of the +30.1 kb region, while mutation of T_3RE 6 almost completely abolished it (see Results and Fig. 6B). Before gel electrophoresis the probe was incubated with *in vitro* synthesized *Xenopus* TRβ plus RXRα with or without the indicated radioinert PCR fragments (21.5 nM). The arrow indicates the location of supershifted bands due to TRβ-RXRα binding to the probe. Radioinert PCR fragments that contained native T_3RE sequences and those for the mutant T_3RE 2, 3, and 4 completely displaced TRβ-RXRα binding to the probe, while that for mutant T_3RE 6 had a reduced activity. (**B.**) I conducted competitive EMSA using the probe and radioinert oligonucleotides corresponding to the mouse +49.0 kb T_3RE sequence (1 μM) or PCR-generated genomic fragments (180 bp) that encompass correspond the mouse +49.0 kb T_3RE. The arrow indicates the location of supershifted bands due to TRβ-RXRα binding to the probe. Mutant PCR fragments contained single base pair mutations that were introduced into each of the two half sites of the +49.0 kb T_3RE, which reduced T_3-dependent transactivity of the +49.0 kb region (see Results and Fig. 6D). Radioinert oligonucleotide with the native sequence completely displaced TRβ-RXRα binding to the probe, while that for mutant sequence had a reduced activity. Radioinert PCR fragment (34 nM) that contained the native +49.0 kb T_3RE sequence completely displaced TRβ-RXRα binding to the probe, while that for the mutant sequence reduced TRβ-RXRα binding to the probe. A PCR fragment that did not contain a predicted T_3RE was used as a negative control, which did not have activity.

A.



B.



Supplemental Fig. 2.8: Sequence conservation of mouse *Dnmt3a* T_3REs among mammals. Shown are DNA sequence alignment of T_3REs found at the +30.1 kb (**A.**) and +49.0 kb (**B.**) from the TSS of the mouse *Dnmt3a* gene. Boxes indicate the location of predicated T_3RE half sites based on the mouse sequences. Note that the +49.0 kb T_3RE is DR+4 for mouse, rat and Chinese hamster, while it is DR+5 for other species.