DNA methylation dynamics underlie metamorphic gene regulation programs in *Xenopus* tadpole brain

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**A R T I C L E  I N F O**

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**A B S T R A C T**

Methylation of cytosine residues in DNA influences chromatin structure and gene transcription, and its regulation is crucial for brain development. There is mounting evidence that DNA methylation can be modulated by hormone signaling. We analyzed genome-wide changes in DNA methylation and their relationship to gene regulation in the brain of *Xenopus* tadpoles during metamorphosis, a thyroid hormone-dependent developmental process. We studied the region of the tadpole brain containing neurosecretory neurons that control pituitary hormone secretion, a region that is highly responsive to thyroid hormone action. Using Methylated DNA Capture sequencing (MethylCap-seq) we discovered a diverse landscape of DNA methylation across the tadpole neural cell genome, and pairwise stage comparisons identified several thousand differentially methylated regions (DMRs). During the pre-to pro-metamorphic period, the number of DMRs was lowest (1,163), with demethylation predominating. From pre-metamorphosis to metamorphic climax DMRs nearly doubled (2,204), with methylation predominating. The largest changes in DNA methylation were seen from metamorphic climax to the completion of metamorphosis (2960 DMRs), with 80% of the DMRs representing demethylation. Using RNA sequencing, we found negative correlations between differentially expressed genes and DMRs localized to gene bodies and regions upstream of transcription start sites. DNA demethylation at metamorphosis revealed by MethylCap-seq was corroborated by increased immunoreactivity for the DNA demethylation intermediates 5-hydroxymethylcytosine and 5-carboxymethylcytosine, and the methylcytosine dioxygenase ten eleven translocation 3 that catalyzes DNA demethylation. Our findings show that the genome of tadpole neural cells undergoes significant changes in DNA methylation during metamorphosis, and these changes likely influence chromatin architecture, and gene regulation programs occurring during this developmental period.

1. Introduction

Methylation of DNA is an important epigenetic modification that involves the addition of a methyl group to the carbon-5 position of cytosine (5-methylcytosine; 5-mC). In vertebrates, 5-mC is found predominantly in the context of cytosine-guanine (CpG) dinucleotides, located in intergenic regions, and within genes and transposable elements (Feng et al., 2010b; Suzuki and Bird, 2008; Zemach et al., 2010). Global changes in DNA methylation have been shown to be important for establishing tissue-specific gene expression patterns during embryogenesis in mammals (Goll and Bestor, 2005) and in *Xenopus* (Bogdanovic et al., 2011). Regions with large CpG content often found within gene promoters are known as ‘CpG islands’, and methylation of CpG islands often leads to gene repression, while demethylation leads to gene activation (Guo et al., 2011; Lister et al., 2013; Song et al., 2011a; Szulwach et al., 2011).
Methylation of DNA is catalyzed by DNA methyltransferases (DNMTs), of which there are three that have enzymatic activity in mammals. The de novo DNMTs, DNMT3a and DNMT3b, establish the pattern of DNA methylation during embryogenesis, which is then preserved through subsequent rounds of cell division by DNMT1 (Rottach et al., 2009; Watanabe et al., 2002). DNA methylation imposes long-term, stable transcriptional silencing through physical blockade of transcription factor binding, and recruitment of methyl-CpG binding proteins, which in turn recruit corepressors or histone deacetylases to modify local chromatin into a transcriptionally silent state (Rottach et al., 2009). DNA methylation of gene regulatory regions typically leads to gene repression, and DNA demethylation gene activation (Maeder et al., 2013; Shi et al., 2014; Suzuki and Bird, 2008; Xu et al., 2012). DNA demethylation can be active or passive; active DNA demethylation is a DNA replication-independent enzymatic removal of 5-mC, while passive DNA demethylation occurs when the newly synthesized DNA does not inherit the pattern of DNA methylation during cell division, which may occur through exclusion of DNMT1 from the cell nucleus (Wu and Zhang, 2014).

Active DNA demethylation involves a complex set of enzymatic steps that depends in part on the ten-eleven translocation (TET) family of dioxygenases (Lu et al., 2015). These enzymes catalyze active DNA demethylation by oxidizing 5-mC to the intermediates 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (He et al., 2011a; Ito et al., 2010, 2011; Lu et al., 2015; Maiti and Drohat, 2011). The isocitrate dehydrogenase family of enzymes (IDH1/2/3) provide the substrate α-ketoglutarate for TET-mediated oxidation of 5-mC, and are necessary for TET-dependent 5-hmC production (Figuerola et al., 2011; Lian et al., 2012). The 5-hmC is removed by two different pathways. It can be further oxidized by TET enzymes to 5-fC and to 5-caC (He et al., 2011b; Ito et al., 2011), and then removed through the base-excision repair pathway catalyzed by thymine DNA glycosylase (TDG) (He et al., 2011b; Maiti and Drohat, 2011), or by nucleotide excision repair pathways catalyzed by growth arrest and DNA damage (GADD45α/β/γ) enzymes (Schafer, 2013). Alternatively, 5-hmC may be deaminated to unmethylated cytosine by the combined actions of activity-induced cytidine deaminase (AID) and the apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) (Wu and Zhang, 2014).

The methylation state of DNA plays a critical role in animal development, particularly the nervous system. The DNMTs are essential for normal development; mice carrying mutations for these genes are embryonic lethal or die shortly after birth (Li et al., 1992; Okano et al., 1999). Rett syndrome in humans is caused by mutations in the X-linked gene that codes for methyl-CpG binding protein 2 (MeCP2), which binds to methylated cytosines through its methyl-CpG binding domain (Amir et al., 1999). Neural cell-specific deletion of DNMT genes in mice causes abnormal neurological development accompanied by defective cell differentiation, neurodegeneration and deficits in learning and memory (Fan et al., 2005; Feng et al., 2010a; Hutnick et al., 2009; Nguyen et al., 2007). In mouse and human there are dramatic changes in DNA methylation in the brain during the neonatal/early postnatal period. A recent, comprehensive genome-wide mapping of 5-mC revealed a large increase in cytosine-adenine (CpA) methylation in developing mouse and human brain, coincident with the period of synaptogenesis and synaptic pruning (Lister et al., 2013).

While DNA methylation is recognized as an essential epigenetic modification that regulates gene expression, how methylation is regulated is poorly understood. We previously showed that thyroid hormone (T3) can directly induce transcription of the dnmt3a gene in Xenopus and mouse neural cells (Kyono et al., 2016a, 2016b). Thyroid hormone is essential for neurological development in mammals, and deficiency of T3 during the neonatal/early postnatal period in humans leads to a condition of severe mental and growth retardation known as cretinism (Bernal, 2009; Porterfield and Hendrich, 1993). Amphibian tadpole metamorphosis is entirely dependent on T3, and it is an important developmental model system for investigating T3 actions (Buchholz, 2017). Developmental processes that occur in the tadpole brain during metamorphosis mirror those in mammals, with T3 regulating cell proliferation, migration, myelination, synaptogenesis, and cell death (Denver, 1998; Denver et al., 2009). The hormone binds to nuclear hormone receptors (thyroid hormone receptors - TRs) that modify chromatin structure and regulate gene transcription (Cheng et al., 2010). The gene regulation programs during amphibian metamorphosis underlie tissue morphogenesis (Shi, 2000), and correlate with histone modifications that are thought to be due primarily to T3 action (Shi, 2009). There are dynamic changes in DNA methylation during early Xenopus development (Bogdanovic et al., 2011, 2012), but to our knowledge, changes in DNA methylation during metamorphosis have not been investigated in any tissue. Such changes, if they occur, are likely to be important for gene regulation during metamorphosis, and for establishing stable, cell type-specific gene expression patterns in the juvenile adult.

In the current study, we used genome-wide Methylated DNA Capture sequencing (MethylCap-seq), and several biochemical and histochemical approaches, to investigate developmental changes in DNA methylation in the brain of Xenopus tadpoles during metamorphosis. We also conducted RNA sequencing (RNA-seq) to look for associated changes in gene transcription during metamorphosis. We focused on the brain region containing the preoptic area, thalamus and hypothalamus, which contains neurosecretory neurons that control hormone production during metamorphosis, and is highly responsive to T3 action (Denver, 1998; Denver et al., 2009). Using MethylCap-seq, we identified several thousand differentially methylated regions (DMRs). We also found negative correlations between changes in DNA methylation and gene expression. The largest changes in DNA methylation occurred from metamorphic climax to the completion of metamorphosis, with DNA demethylation predominating. During this interval we found increases in mRNAs for genes that encode enzymes involved with DNA demethylation, and also immunoreactivity for the DNA demethylation intermediates 5-hydroxymethylcytosine (5-hmC) and 5-carboxymethylcytosine (5-caC), and the methylcytosine dioxygenase ten eleven translocation 3 (TET3).

2. Materials and Methods

2.1. Animal care and use

We obtained X. tropicalis tadpoles by in-house breeding or from Xenopus One (Dexter, MI), reared them in dechlorinated tap water (water temperature 25 °C, pH 7) and maintained them on a 13L:11D photoperiod. We fed tadpoles ad libitum with pulverized frog brittle (NASCO, Fort Atkinson, WI) or Sera Micron plankton food. Tadpoles were staged using the developmental staging system of Nieuwkoop and Faber (Nieuwkoop and Faber, 1994) (NF). We euthanized tadpoles before tissue collection by rapid decapitation. The animals were not anesthetized before euthanasia because anesthetics can cause changes in gene expression, and perhaps also DNA methylation. This method, and all procedures involving animals were conducted under an approved animal use protocol (PRO00006809) in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Michigan.

2.2. Methylated DNA capture for deep sequencing and targeted analysis

To investigate genome-wide changes in DNA methylation in tadpole brain during metamorphosis, we conducted Methylated DNA Capture assay (MethylCap) using the MethylCap kit (Diagenode, Denville, NJ) following the manufacturer’s instructions. We microdissected the region of the tadpole brain containing the preoptic area/thalamus/ hypothalamus (Supplemental Fig. 1A) at four stages of metamorphosis: NF stages 50 (pre-metamorphosis), 56 (pro-metamorphosis), 62 (metamorphic climax) and 66 (metamorphosis complete, juvenile adult). We included three biological replicates for each developmental stage, and for each
biological replicate we pooled tissue from 15 animals for NF stage 50, 5 for stage 56, and 6 for stages 62 and 66.

We isolated genomic DNA using the DNeasy Blood and Tissue kit (Qiagen), and sonicated the DNA to an average size of 400 bp using a Covaris S2 sonicator (Covaris, Woburn, MA). To monitor DNA capture efficiency during the MethylCap procedure, we ‘spiked’ the MethylCap reaction with a cocktail of four lambda phage DNAs containing varying numbers of GC dinucleotides, and either methylated or not (described below), as described by Taiwo et al. (2012) with minor modifications. Using PCR, we generated two unique DNA fragments that differed in CpG density using lambda phage DNA as template (one fragment with low, and one with high CpG density; see Supplemental Table 4 for oligonucleotide primer sequences). We conducted in vitro methylation of the two DNA fragments by incubating them with 10 U of M. Smal CpG methyltransferase (New England Biolabs [NEB], Ipswich, MA) and S-adenosylmethionine (SAM; 640 μM) for 8 h (we replenished the SAM after 4 h). We purified the DNA fragments by phenol/chloroform/isooamyl alcohol precipitation (Invitrogen Life Technologies, Carlsbad, CA), resuspended them in restriction enzyme buffer EB from Qiagen (Valencia, CA), and verified in vitro methylation by digestion of the fragments with 10 U of HinfI1 (NEB), a methylation-sensitive restriction enzyme; methylated DNA fragments were resistant to digestion, while unmethylated DNA fragments were not (data not shown). The two un-methylated, low and high CpG density DNA fragments served as negative controls. We combined the four DNA fragments into a cocktail (two methylated and two un-methylated) at a concentration of 0.2 pM for each DNA fragment, and added this to Buffer B of the MethylCap kit (Diagenode) to a final concentration of 0.0067 pM before proceeding with the capture reaction.

Using 1 μg of sonicated genomic DNA for each sample (each with the cocktail of lambda phage DNA described above added), we captured methylated DNA with magnetic beads, then eluted the captured DNA sequentially with three concentrations of NaCl; we retained DNA from the high salt elution buffer (900 mM NaCl) for deep sequencing. This salt concentration selects for DNA sequences with high CpG density, and therefore we may not have isolated regions of the genome with lower CpG density. However, Bogdanovic and colleagues (Bogdanovic et al., 2011) found no differences in overall distribution of DNA methylation in Xenopus embryos among different salt concentrations of elution buffer. We purified the eluates and the input DNA using the QIAquick PCR Purification kit (Qiagen). Real-time qPCR analysis targeting the three spiked-in DNA fragments showed that the capture efficiency was similar among samples (Supplemental Fig. 1B).

For MethylCap-seq, we sequenced captured DNA samples from tadpole brain at the four stages of metamorphosis, and we also selected three input DNA samples for sequencing, one from each of three NF stages, 50, 56 and 62. The sequencing libraries were constructed on the Apollo instrument (an automated system) using IntegenX reagents (now Wafergen, Fremont, CA) at the University of Michigan DNA sequencing core. We sequenced the libraries on Illumina HiSeq 2000 using High Output mode (50 bp, single-end).

2.3. Bioinformatics analysis of MethylCap-seq data

We conducted quality processing on raw reads (fastq) files from each sample to remove duplicate reads, poor quality bases, and adapter sequences using dedupe.sh and bbduk.sh from the BBTools suite (v37.90). Quality assessments were done on raw and processed data using FastQC (v0.11.7) and MultiQC (v1.5). We aligned the quality-controlled processed reads to the X. tropicalis genome (v4.1) using hisat2 (v2.1.0) and the –dta option with all other defaults. Uniquely mapped reads were selected for further analysis. We sorted aligned reads and indexed them using the samtools sort and samtools index commands, respectively (samtools v1.8). Read depth-normalized bigwig files were created from sorted alignment files for ease of viewing in genome browsers using the bamCoverage command from deepTools (v3.0.2).

We called methylation peaks using PePr (v1.1.18) (Zhang et al., 2014) with default parameters except for –shiftdown and –windowsize, which were each set to 200. For each stage, peaks were called with both the –broad and –sharp flags. We then combined broad and sharp peaks (at each stage) using the bedops –not-element-of 1 command (v2.4.34) to add all sharp peaks that were not partially contained within broad peaks and combine them with the broad peaks.

We called differentially methylated regions (DMRs) using PePr (v1.1.18) as above with the exception that we used the –normalization flag, which was set inter-group, and –diff flag. As with peak calling, DMRs were called with the –broad and –sharp flags set, and combined in the same manner. We filtered the DMRs to accept only those with a p-value ≤ 0.0005.

For each stage, we calculated the % GC content of each peak using the bedtools nuc command from the bedtools suite (v2.27.1). From these values, we calculated the mean % GC content of peaks at each stage. For CpG and Cpa enrichment calculations, two different enrichment scores were calculated: CpG|A frequency enrichment and CpG|A observed/expected ratio enrichment. We used the bedtools nuc command to calculate the number of occurrences of each nucleotide (A,T,C,G,N), and the number of occurrences of CpG and CpA, within each peak. From these values, we calculated CpG|A frequency for each stage by normalizing the number of bases in a CpG|A context within peaks to the total length of the peaks using a formula recreated based on that developed in the MEDIPS package (Lienhard et al., 2014). See formula 1a and 1b below.

CpG frequency = (sum(number of CpG) / (sum(peak lengths)) * 100 (1a)

CpA frequency = (sum(number of CpA) / (sum(peak lengths)) * 100 (1b)

We calculated a CpG|A observed/expected value for each stage by multiplying the number of CpG|A occurrences in peaks by the total length of the peaks, and dividing that by the product of the total number of C and the total number of (G+A) in the peaks. See formula 2a and 2b below.

CpG observed/expected value = (sum(number of CpG) / (sum(peak lengths)) / (sum(C) * sum(G)) (2a)

CpA observed/expected value = (sum(number of CpA) / (sum(peak lengths)) / (sum(C) * sum(A)) (2b)

These same CpG|A frequency and CpG|A observed/expected ratios were calculated for the genomic region not containing peaks. We then calculated enrichment scores by dividing the CpG|A frequency of peaks by the CpG|A frequency of the genomic region not containing peaks using a formula recreated based on that developed in the MEDIPS package (Lienhard et al., 2014). This was done for each stage. See formulas 3 and 4 below.

CpG frequency Enrichment Score = CpG frequencypeaks / CpG frequencygenome (3a)

CpA frequency Enrichment Score = CpA frequencypeaks / CpA frequencygenome (3b)

CpG observed/expected value Enrichment Score = CpG observed/expected valuepeaks / CpG observed/expected valuegenome (4a)

CpA observed/expected value Enrichment Score = CpA observed/expected valuepeaks / CpA observed/expected valuegenome (4b)

To investigate peak and DMR distributions relative to genomic features, we used the bedmap command from the bedops suite (v2.4.34) with the –prec 3 and –fraction–either 0.50 flag values set to identify peaks or DMRs as overlapping a genomic feature (intron, exon, tss, promoter, TSS, gene body, etc.) if more than 50% of the peak overlapped the genomic feature, or vice versa. Genomic features correspond to the MNHN gene model annotation published by Buisine and colleagues (Buisine et al., 2015).

With the exception of a few steps, we implemented most of the
workflow in Snakemake v4.7.0.

2.4. RNA-sequencing library preparation

To investigate the relationship between changes in gene expression and DNA methylation, we conducted RNA sequencing (RNA-seq) on RNA isolated from X. tropicalis tadpole brain during metamorphosis. We micro-dissected the preoptic area/thalamus/hypothalamus of the tadpole brain (Supplemental Fig. 1A) at the same four stages of metamorphosis as for MethylCap-seq. We included three biological replicates for each stage of metamorphosis. After extraction of total RNA using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), we further purified the total RNA using the RNeasy Mini kit (Qiagen), then submitted samples to the University of Michigan DNA sequencing core for sequencing library preparation and deep sequencing. All samples had RNA Integrity Numbers (RIN) of 8 or greater as analyzed using the TapeStation (Agilent). Each library was prepared from 130 ng of RNA using the Illumina TruSeq mRNA Sample Prep v2 kit (Illumina Inc., San Diego, CA), where total RNA was poly-A selected, fragmented and reverse transcribed with random primers. The 3’ ends of the cDNA libraries were adenylated, ligated with adapters that contain hexanucleotide barcodes, and PCR-amplified to construct the final libraries. The quality of the final libraries was assessed using the TapeStation (Agilent) and quantified by qPCR using a commercial kit (Kapa Biosystems, Wilmington MA). All libraries were pooled and sequenced to produce single-end 50 bp reads in two lanes on the Illumina HiSeq 2000 platform with High Output mode using version 3 reagents.

2.5. Bioinformatics analysis of RNA-seq data

We conducted quality processing on raw reads (fastq) files from each sample to remove poor quality bases and adapter sequences using cutadapt (v1.11). We did quality assessments on all raw and processed data using FastQC (v0.11.7) and MultiQC (v0.8). The quality control processed reads were aligned to the Xenopus tropicalis genome (v4.1) and quantified using bowtie2/RSEM (v2.1.0) with default parameters. We conducted gene-level quantification using a GTF file containing each of 22,820 X. tropicalis genes of a custom annotation (the MNHN gene model) built by Nicolas Buisine and Laurent Sachs based on high-throughput RNA paired-end tag sequencing that identified the 5’ and 3’ ends of transcripts expressed in different X. tropicalis tissues, including the brain (Buisine et al., 2015). A gene-level abundance matrix was generated using the abundance_estimates_to_matrix.pl script from the Trinity suite (v2.2.0).

We sorted and indexed aligned reads using the samtools sort and samtools index commands from samtools (v1.8), respectively. Read depth-normalized bigwig files were created from sorted alignment files for ease of viewing in genome browsers using the bamCoverage command from deepTools (v3.0.2). We conducted differential expression analysis using the R package DESeq2 (v1.22.0) to identify differentially expressed genes with a false discovery rate ≤ 0.01.

With the exception of a few steps, we implemented most of the workflow in Snakemake v4.7.0.

2.6. Bioinformatics analysis of relationship between DMRs and differentially expressed genes (DEGs)

To investigate possible relationships between DMRs and DEGs, we used the bedmap command from the bedops suite (v2.4.34) with –echo and –echo-map flags set to intersect DMRs within 50 kb (–range 50000) of DEGs or non-DEGs. Similarly, we used bedmap to identify DMRs within the gene-body of DEGs or non-DEGs. Using these values, we ran a Fisher’s Exact Test to test 1) whether there is a difference in the proportion of DMRs within 50 kb (associated with) of DEGs and non-DEGs and 2) whether there is a difference in the proportion of DMRs within the gene-body of DEGs and non-DEGs.

We also analyzed possible correlations between DMRs and DEGs considering their direction of change (i.e., we tested the hypothesis that DNA demethylation is correlated with gene activation, and vice versa). For each comparison, we used the bedmap command from the bedops suite (v2.4.34) with –echo and –echo-map flags set to intersect DMRs with the following regions from DEGs for the same comparison: 5 kb upstream of the DEG, the DEG gene-body, and 5 kb upstream + the DEG gene-body. Pearson correlation coefficients and p-values were then calculated for each comparison between the DMR height (log2 A/B) and the DEG fold-change (log2 A/B) value for the intersecting region. We plotted the values and fit a regression line to the data. We also calculated Pearson correlation coefficients and p-values for each comparison between the DMR direction (1 for positive DMR height [log2 A/B value], −1 for negative DMR height [log2 A/B value]) and the intersecting DEG region fold-change direction (1 for positive DEG log2 fold-change A/B, −1 for negative log2 fold-change A/B). We plotted the values and fit a regression line to the data.

2.7. Targeted analysis of DNA methylation using bisulfite sequencing

We bisulfite-converted 400 ng of genomic DNA extracted from X. tropicalis tadpole brain using the BisulFlash DNA modification kit (Epigentek, Farmingdale, NY) (three biological replicates per developmental stage). We used Methprimer software (Li and Dahiya, 2002) to design oligonucleotide primers to specifically amplify DNA that had been converted by bisulfite treatment (oligonucleotide primer sequences are given in Supplemental Table 4), and conducted PCR using GoTaq DNA polymerase (Promega) with the following PCR conditions: (94°C for 2 min; 49 cycles of 94°C for 30 s, 55 or 60°C 30 s, 72°C 45 s). We purified the PCR products using the Qiagen PCR purification kit (Qiagen) and subcloned them into the pGEM-Teasy vector (Promega) following the manufacturer’s instructions. After transformation of E. coli with the resulting plasmids, we conducted colony PCR on bacterial colonies using SP6 and T7 oligonucleotide primers, purified the PCR products and conducted Sanger sequencing using SP6 or T7 primers. We sequenced 5–7 colonies for each biological replicate.

2.8. Targeted analysis of DNA methylation by 5-mC-sensitive restriction digest

We used 5-mC-sensitive restriction digest followed by qPCR (5-mC chop-qPCR) to analyze changes in DNA methylation in tadpole brain at discrete genomic regions. We incubated 1 μg of genomic DNA in a 50 μl reaction with 10 units of HinfI or HpyCH4IV (NEB) at 37°C overnight, purified the DNA using phenol/chloroform/isoamyl alcohol (Invitrogen, Life Technologies, Carlsbad, CA) and resuspended in 40 μl of buffer AE (Qiagen, 10mM Tris, 0.5mM EDTA, pH 9.0). To measure the extent of restriction digest, we conducted genomic DNA qPCR using primers flanking the restriction sites (see Supplemental Table 4 for oligonucleotide primer sequences), which was normalized to the quantity of the DNA amplicon at an adjacent genomic region that did not contain restriction sites.

2.9. Reverse transcriptase real-time quantitative PCR (RTqPCR)

We used RTqPCR to validate gene expression changes identified by RNA-seq, and also for targeted analysis of developmental changes in genes that code for enzymes involved with DNA demethylation. We isolated RNA from the preoptic area/thalamus/hypothalamus of pools of 2–5 brains for each biological replicate; the number pooled depended on the stage/size of the animal, and was held constant within a developmental stage. We treated 1 μg total RNA with 20 units of DNase I (Promega) to remove genomic DNA, then synthesized cDNA using the High Capacity cDNA Synthesis Kit (Applied Biosystems Inc. [ABI], Foster City, CA). We conducted real-time qPCR using Absolute Blue qPCR SYBR Low ROX Mix (ABgene Thermo Scientific, Surrey, UK) and the Fast 7500
Brains out of the skull and further
We transferred the brains to 30% sucrose in 0.6X PBS, incubated at 4°C overnight followed by saturation in a 2:1 solution of sucrose:Tissue-Tek Cryo-OCT compound (Fisher Scientific) overnight at 4°C. We embedded the brains in a mold in OCT compound, snap froze and stored them at −80°C until cryosectioning. We made 16 μm transverse cryosections on Superfrost slides (ThermoFisher Scientific) and stored the slides at −80°C until processing for IHC.

For IHC, we air-dried the slides for 30 min, rehydrated them in 0.6X PBS and subjected them to antigen retrieval by immersing slides in 0.01 M sodium citrate (pH 6) at 95°C for 10 min. We let the slides cool to room-temperature, then blocked with Superblock (Pierce Chemical Co.) plus 5% normal goat serum and 0.3% Tween-20 in 0.6X PBS for 1 h at room temperature. After blocking, we incubated slides overnight at 4°C with one of the following: rabbit polyclonal antiserum to 5-hmC (1:500; Diagenode C15410205-20), rabbit polyclonal antiserum to 5-c5C (1:500; Diagenode C15410204-20) or protein A-purified IgG from a rabbit (200 μg/ml; Diagenode C15410204-20) or protein A-purified IgG from a rabbit

2.10. Immunohistochemistry (IHC)

We obtained brains for IHC by dissecting the heads of X. tropicalis tadpoles at six different NF stages of metamorphosis (50, 54, 56, 58, 62 and 66). We fixed tadpole heads in 4% paraformaldehyde made in 0.6X phosphate buffered saline (PBS) overnight at 4°C, then dissected the brains out of the skull and further fixed them in 4% PFA for 2–3 h at 4°C. We transferred the brains to 30% sucrose in 0.6X PBS, incubated at 4°C overnight followed by saturation in a 2:1 solution of sucrose:Tissue-Tek Cryo-OCT compound (Fisher Scientific) overnight at 4°C. We embedded the brains in a mold in OCT compound, snap froze and stored them at −80°C until cryosectioning. We made 16 μm transverse cryosections on Superfrost slides (ThermoFisher Scientific) and stored the slides at −80°C until processing for IHC.

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Antibodies
Rabbit polyclonal antiserum to 5-hmC

Critical Commercial Assays
MethylCap kit
Diagenode, 300ppm
C02020010 (AF-100-0048)

TruSeq mRNA Sample Prep v2 kit
Illumina Inc., San Diego, CA
RS-122-2001

DNA modification kit
Epigentek, Farmingdale, NY
P-1026-050

Deposited Data
Raw and analyzed MethylCap-seq data
This paper
GSE139267

Raw and analyzed RNA-seq data
This paper
GSE140120

Experimental Models: Cell Lines
Experimental Models: Organisms/Strains
Xenopus tropicalis, Nigerian strain
Xenopus One (Dexter, MI)

Xenopus laevis
Xenopus One (Dexter, MI)

Oligonucleotides
Primers for RTqPCR – see Supplemental Table 4
This paper
N/A

Primers for synthesizing spike-in controls using lambda DNA as template – see Supplemental Table 4
This paper
N/A

Primers for monitoring spike-in controls after methylated DNA capture using RTqPCR – see Supplemental Table 4
This paper
N/A

Primers for methylation-sensitive restriction digest/qPCR assays – see Supplemental Table 4
This paper
N/A

Primers for MethylCap-qPCR assay – see Supplemental Table 4
This paper
N/A

Primers for bisulfite sequencing – see Supplemental Table 4
This paper
N/A

Primers for subcloning xTET3 cDNA – see Supplemental Table 4
This paper
N/A

Recombinant DNA

Software and Algorithms
BBTools suite (v37.90)
https://sourceforge.net/projects/bbmap/

SAMtools v1.8
Li et al., 2009
https://sourceforge.net/projects/samtools/

PePr (v1.1.18)
Zhang et al. (2014)

bowtie2/RSEM (v2.1.0)
Langmead and Salzberg, 2012
https://github.com/bowtie-bio/bowtie2

Methprimer software
Li and Dahiya (2002)
https://www.urogene.org/methprimer/
3. Results

3.1. MethylCap-seq revealed a dense and diverse DNA methylation landscape across the tadpole neural cell genome

We analyzed the region of the tadpole brain containing the preoptic area, thalamus and hypothalamus (Supplemental Fig. 1A). The MethylCap assay enriches methylated cytosines using the recombinant methyl-CpG-binding domain of human MeCP2. Capture efficiency did not differ between developmental stages (Supplemental Fig. 1B). Genome-wide profiling of DNA methylation by MethylCap-seq during metamorphosis revealed a dense and diverse DNA methylation landscape across the tadpole neural cell genome. High quality reads were mapped on the reference genome with stringent parameters to minimize noise (see Materials and Methods). Independent profiles derived from three biological replicates per stage were highly correlated ($r = 0.9781$, Spearman correlation coefficient). The MethylCap-seq data reported in this study have been deposited in the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI), accession number GSE139267.

The GC content of peaks was not different among developmental stages and averaged 44.52% (Supplemental Table 1). Within the peak sequences, CpG dinucleotides were present at a higher frequency relative to the genome at each developmental stage, while Cpa dinucleotides were not (Fig. 1A and B). The CpG and Cpa enrichment statistics are given in Supplemental Table 2. These data support that the MethylCap-seq technique achieved enrichment of methylated regions of the genome containing high CG content. The mean number of peaks declined with development, although this was not statistically significant (Nieuwool-Faber [NF] 50–111,789; NF 56–111,962; NF 62–110,835; NF 66–106,016; Fig. 1B).

The mean number of peaks was equally distributed among genes and intergenic regions, and this distribution did not change with development (Fig. 1B). Within genes (defined as the beginning of the first exon through the end of the last exon), the majority of peaks were found within introns. Approximately 20% of the peaks were in proximal promoter regions (defined as ≤ 1 kb upstream of the transcription start site – TSS), 7% in more distal promoter regions (defined as between 1 and 5 kb upstream of the TSS), and about 20% were in CpG islands (regions identified by the University of California, Santa Cruz Genome Browser; Fig. 1C; Supplemental Fig. 2). The length and height of the peaks did not differ between developmental stages (Supplemental Fig. 3). We found four general patterns of DNA methylation across the tadpole neural cell genome: within gene bodies localized to exons (Supplemental Fig. 4A), within gene bodies localized to introns (Supplemental Fig. 4B), in intergenic regions (see rightmost peaks, Supplemental Fig. 4A&B) and little or no methylation (Supplemental Fig. 4C).

3.2. Changes in DNA methylation in tadpole brain during metamorphosis analyzed by MethylCap-seq

Our analysis revealed >10,000 distinct genomic regions that displayed statistically significant ($p < 0.0005$) variation in the level of DNA methylation. The number of differentially methylated regions (DMRs) increased with the progression of metamorphosis, with the largest number of DMRs (2,960) appearing between metamorphic climax (NF stage 62) and the completion of metamorphosis (NF stage 66; Fig. 2A). At the beginning of metamorphosis (NF stage 50–56) DNA demethylation predominated; of 1163 DMRs, 738 became demethylated and 425 became methylated (Fig. 2B). Between pro-metamorphosis (NF stage 56) and metamorphic climax (NF stage 62), this pattern shifted, where DNA methylation predominated; of 2904 DMRs, 694 became demethylated and 2210 methylated. The DNA methylation pattern changed during the completion of metamorphosis (NF stage 62–66), where DNA methylation predominated; of 2960 DMRs, 2425 became demethylated and 535 methylated. If we compare pre-metamorphosis (NF stage 50) with metamorphic climax (NF stage 62), we find that DNA methylation predominated (923 demethylated vs. 1281 methylated). If we compare pre-metamorphosis (NF stage 50) with the completion of metamorphosis (NF stage 66), we find that DNA methylation predominated (1633 demethylated vs. 697 methylated). Several examples of changes in peak heights for MethylCap-seq and RNA-seq reads mapped to the reference genome are shown in Supplemental Figs. 5 and 6.

The shift toward demethylation at the completion of metamorphosis was also seen in our bulk genome biochemical analyses, where we saw a decrease in 5-mC content between pre-metamorphosis (NF stage 50–52) and immediately after metamorphic climax (NF stage 63–64) measured by 5-mC enzyme immunoassay (ELA), luminometric assay (LUMA) and cytosine extension assay (Supplemental Fig. 7).

We also saw a decrease in the total height, but not the length of the DMRs during metamorphosis (shown in the small bar graphs at the top of the graph panels in Supplemental Fig. 8), supporting greater DNA demethylation from pro-metamorphosis to the completion of metamorphosis. Similar to the total methylation peaks, we found that the DMRs were distributed roughly equally between genes and intergenic regions, and there were no differences in the relative distribution between these regions when comparing stages of metamorphosis (Fig. 2A; Supplemental Fig. 9). There was no change in the distance from TSSs of intergenic DMRs in any of the comparisons (Supplemental Fig. 10).

3.3. Gene expression changes in the tadpole preoptic area/thalamus/hypothalamus during metamorphosis analyzed by RNA sequencing

To investigate relationships between DNA methylation and gene expression we conducted RNA-seq on X. tropicalis tadpole brain (preoptic area/thalamus/hypothalamus) at four stages of metamorphosis (NF stages 50, 56, 62 and 66). For RNA-seq we generated an average of 27.4 million reads per sample, which we then mapped to the X. tropicalis genome. Using DESeq software (Anders and Huber, 2010) we found that 5220 of 22,820 annotated genes (23%) showed changes in expression in tadpole brain during metamorphosis. Pairwise comparisons by developmental stage of the number of genes up or downregulated are shown in Fig. 3A, and all data are presented in Supplemental Table 3. This analysis showed that, of all DEGs, approximately half were upregulated, and half were downregulated. We conducted RTqPCR for 14 DEGs (6 up-, 6 down- and 2 non-regulated) chosen based on their magnitude change (low, medium and high). All DEGs tested by RTqPCR showed the expected developmental changes as found by RNA-seq (Supplemental Fig. 11; gene annotation and primer sequences in Supplemental Table 4). The RNA-seq data reported in this study have been deposited in GEO at NCBI, accession number GSE140120. A full analysis of gene expression changes, gene ontology and pathways will be published elsewhere.

3.4. DNA methylation and gene transcription

We analyzed the relationship between DMRs and DEGs by calculating the Odds Ratios and 95% confidence intervals for DMRs’ association with DEGs versus non-DEGs, at each of the pairwise stage comparisons (Supplemental Fig. 12). We looked at the region 50 kb upstream of the TSS, or within the gene body. An Odds Ratio of 1.5 means that a DMR is 1.5 times more likely to be associated with a DEG than a non-DEG. For most stage comparisons, DMRs were enriched within 50 kb upstream of the TSS of DEGs vs non-DEGs, and this association was even greater if the DMR was
located within the gene-body (Supplemental Fig. 12).

To test the hypothesis that increased DNA methylation is correlated with downregulation of gene expression, and vice versa, we examined correlations between the direction and magnitude of change in DNA methylation and gene expression. For this analysis we binned data based on DMR location, looking at DMRs within DEG gene bodies, or DMRs at 0–5 kb upstream of DEG TSSs. We found statistically significant negative correlations between DMRs and DEGs within gene bodies when

Fig. 1. Genome-wide patterns of DNA methylation in tadpole brain during metamorphosis analyzed by MethylCap-seq. We conducted MethylCap-seq on DNA isolated from X. tropicalis tadpole brain (preoptic area/thalamus/hypothalamus) at four stages of metamorphosis (n = 3 per stage). A. CpG dinucleotides (left panel) are enriched at MethylCap-seq peaks at each stage of development, while CpA dinucleotides (right panel) are not. Shown are plots of the peak CpG (left panel) and CpA (right panel) frequencies for each stage of metamorphosis (NF - Nieuwkoop-Faber). The CpG and CpA frequency values were binned (50 bins) prior to plotting. B. The mean number of total MethylCap-seq peaks across the genome, and peaks found within genes (presented by gene region or feature; 50% or more of the peak had to occur within the gene region or feature to be counted) at four stages of metamorphosis. C. The number of MethylCap-seq peaks and their distribution between different regions of the genome did not differ between developmental stages. Gene refers to the gene-body, which we defined as the region from the first exon through the last exon. The promoter region was defined as 5 kb upstream from the end of the first exon (i.e., it included the first exon). Intergenic refers to regions not within any gene-body, and non-intergenic is regions within a gene body. CpG islands are regions identified by the UCSC genome browser.
comparing pre-metamorphosis (NF stage 50) and metamorphic climax (NF stage 62; \( p = 4.4 \times 10^{-7} \)), and pre-metamorphosis and the completion of metamorphosis (NF stage 66; \( p = 0.00057 \)) (Fig. 3B). There were no statistically significant correlations among DMRs and DEGs in the 0–5 kb upstream region in these comparisons. When we looked at the period from pro-metamorphosis (NF stage 56) to metamorphic climax (NF stage 62) we found statistically significant negative correlations between DMRs and DEGs, for DMRs within gene bodies (\( p = 5.4 \times 10^{-13} \)) and also within the 0–5 kb upstream regions (\( p = 0.00045 \); Fig. 3C). Examples of screenshots of MethlCap-seq and RNA-seq reads mapped to the reference genome for genomic regions showing a negative correlation between DNA methylation and gene transcription are given in Fig. 3D and Supplemental Fig. 5 (Supplemental Fig. 6 shows an example of a positive correlation, and two examples of no correlation between DNA methylation and gene transcription).

3.5. Analysis of changes in DNA methylation using targeted, biochemical assays

We looked at changes in DNA methylation using several biochemical assays. We chose two genes that are known T3 response genes, DNA methyltransferase 3a (\( \text{dnmt3a} \)) and Krüppel-like factor 9 (\( \text{klf9} \)). The frog \( \text{dnmt3a} \) gene has two T3 response elements (TREs), one located \( 7.1 \) kb from the TSS, designated TRE-A, and one located \( +5.1 \) kb from the TSS, designated TRE-B (22) (Fig. 4A). The TRE in the frog \( \text{klf9} \) gene is located \( 5.9 \) kb upstream of the TSS within a 180 bp, ultraconserved enhancer module called the \( \text{klf9} \) synergy module (KSM) (Bagamasbad et al., 2015).

Our MethlCap-seq analysis showed that the \( \text{dnmt3a} \) gene was heavily methylated in pre-metamorphic tadpole brain (NF stage 50), but exhibited a progressive decline in methylation during metamorphosis (Fig. 4B). The largest demethylation occurred at regions flanking the CpG island (“CpG island shores”) located near \( \text{dnmt3a} \) TRE-A; methylation at
Fig. 3. Gene expression changes in X. tropicalis tadpole brain during metamorphosis analyzed by RNA-seq and their relationship to changes in DNA methylation analyzed by MethylCap-seq. We conducted RNA-seq on total RNA isolated from X. tropicalis tadpole brain (preoptic area/thalamus/hypothalamus) at four stages of metamorphosis (n = 3 per stage). We used DESeq software to identify genes whose mRNA levels changed during metamorphosis (differentially expressed genes - DEGs). A. Shown are pie charts with pairwise comparisons among the four stages of metamorphosis of DEGs. The numbers in the red areas of the pie charts represent genes upregulated in Stage B, while the numbers in the blue areas of the pie charts represent genes downregulated in Stage B. Gene regulations changes increased as metamorphosis proceeded, with roughly half the genes upregulated and half downregulated. B. Correlation between overlapping DMRs and DEGs by direction and magnitude of change within gene bodies comparing pre-metamorphosis (NF 50) with metamorphic climax (NF 62) and pre-metamorphosis and the completion of metamorphosis (NF 66). Shown are scatter plots, Pearson correlation coefficients, and p-values for each comparison, with DEG fold-change on the y-axis and differentially methylated region (DMR) height on the x-axis (Log2A/B, where A is NF 62 or 66 and B is NF 50). The values were plotted and a regression line was fit (black line). A 95% Confidence Interval (light-gray region) is also shown around the regression line. There were no statistically significant correlations for these comparisons in the region 5 kb upstream of the transcription start site (TSS; data not shown). C. Correlation between overlapping DMRs and DEGs by direction and magnitude of change within gene bodies and at 5 kb upstream of TSSs comparing pro-metamorphosis (NF 56) with metamorphic climax (NF 62). D. Three examples of a negative correlation between DNA methylation and gene transcription in tadpole brain during metamorphosis (top and middle panels: DNA demethylation; bottom panel: DNA methylation). Shown are screenshots of MethylCap-seq (blue) and RNA-seq (red) reads at discrete genomic regions at four stages of metamorphosis. Reads were mapped to the reference genome using the UCSD Genome Browser. NF stage – Nieuwkoop-Faber developmental stage. Numbers in brackets represent the peak height range. Gene IDs and symbols: (top) XLOC_002166; unknown, (middle) XLOC_030199.1; avpr1a, (bottom) new_exon_scaffold_851_1255.1; unknown.
the *dnmt3a* TRE-B also decreased with metamorphosis, but the change was less than at TRE-A. We confirmed demethylation occurring proximal to the TRE-A using targeted bisulfite sequencing (Fig. 4C). Of the five CpG dinucleotides located in the upstream (‘west’) shore, the first three CpGs (CpG-1, CpG-2, and CpG-3) became demethylated between pre-metamorphosis (NF stage 50) and metamorphic climax (NF stage 62). In pre-metamorphic tadpoles 93.3% of CpG1, 100% of CpG2, and 93.3% of CpG3 were methylated; whereas, at metamorphic climax 33.3% of CpG1, 66.7% of CpG2 and 77.8% of CpG3 were methylated. The other two CpGs in this region (CpG4 and CpG5) did not show changes in methylation between the two stages.

We also confirmed demethylation at *dnmt3a* using targeted MethylCap-qPCR analysis (the locations of oligonucleotide primers used for this and the assays described below are shown in Supplemental Fig. 13). Regions proximal to the two *dnmt3a* TREs became demethylated between pro-metamorphosis and metamorphic climax (NF stages 56 and 62) (Fig. 4D). The reduction in the MethylCap-qPCR signal was greater at TRE-A than at TRE-B, which is consistent with the MethylCap-seq data. We also confirmed DNA demethylation during metamorphosis at regions proximal to the two *dnmt3a* TREs using 5-mC-sensitive restriction digest followed by qPCR (5-mC chop-qPCR; Fig. 4E). DNA methylation at both TRE-A and TRE-B showed progressive declines from NF stage 54 through
the completion of metamorphosis (NF stage 66), and as with the MethylCap assays, the decrease in methylation was greater at TRE-A. The DNA demethylation at this locus correlated with a developmental increase in *dnmt3a* mRNA analyzed by RTqPCR (Fig. 4F).

Unlike *dnmt3a*, the entire *klf9* gene, including the KSM, exhibited very low MethylCap-seq signal that was not different from background (input) and did not change during metamorphosis (Supplemental Fig. 14A). Targeted MethylCap-qPCR analysis also found no developmental changes at the *klf9* locus (Supplemental Fig. 14B). Using 5-mC chop-qPCR, we found a small, but statistically significant decrease in DNA methylation at the KSM during metamorphosis (Supplemental Fig. 14C). The *klf9* mRNA level increased strongly (~20 fold) during metamorphosis (Supplemental Fig. 14D). Analysis of the homologous regions (*dnmt3a* and the KSM) in the genome of the related species *X. laevis* using targeted MethylCap-qPCR showed similar changes to those observed in *X. tropicalis* (Supplemental Fig. 15).

### 3.6. The mRNAs for genes that encode enzymes that catalyze active DNA demethylation increase in tadpole brain during metamorphosis

We used RTqPCR to analyze the mRNA levels for genes that encode enzymes that catalyze DNA demethylation (*tet2, tet3, idh1/2/3, gadd45α/β/γ, tdg, idax, aid* and *apobec2*) at five stages of metamorphosis (NF stages 50, 54, 56, 62 and 66). We found that the mRNA levels for all genes, except *aid* and *apobec2* which did not change, were lowest at NF stage 50, then showed progressive increases during metamorphosis, and were maximal at metamorphic climax (NF stage 62; ~2–3 fold increases relative to NF stage 50) when circulating T3 concentration is maximal (Leloup, 1977) (Fig. 5). Of the 10 genes that increased during metamorphosis, mRNAs for 8 genes remained at similar elevated levels in the post-metamorphic frog (NF stage 66), with *gadd45γ* and *idh2* showing statistically significant declines (Fig. 5).

### 3.7. Immunoreactivity for 5-hmC, 5-caC and TET3 increased in tadpole brain during spontaneous metamorphosis

We used immunohistochemistry (IHC) to analyze changes in immunoreactivity (ir) of the DNA demethylation intermediates 5-hmC and 5-caC, and the methylcytosine dioxygenase TET3 in tadpole brain during metamorphosis. We generated rabbit antisera to *Xenopus* TET3 (see Supplemental Methods) and we verified the titer and specificity by EIA, dot blot, immunocytochemistry, IHC and ChIP assays conducted on chromatin isolated from *X. laevis* embryos (Supplemental Fig. 16).

We first analyzed the distribution in tadpole brain of 5-hmC-ir, 5-caC-ir and TET3-ir by conducting IHC on transverse sections from NF stage 62 tadpoles (Supplemental Fig. 17). We chose this stage to map these antigens in the brain because it is when the mRNA levels for genes that encode enzymes involved with DNA demethylation are highest (Fig. 5). We saw no 5-hmC-ir or 5-caC-ir in the most rostral regions of the telencephalon (data not shown). There was pronounced 5-hmC-ir and 5-caC-ir in several brain regions including the region of the pallium, amygdala and bed nucleus of the stria terminalis, anterior preoptic area (location of neurosecretory neuron cell bodies; Supplemental Fig. 18&19, region E), the suprachiasmatic nucleus (Supplemental Fig. 18&19, region H) thalamic nuclei and the ventral hypothalamic nucleus (Supplemental Fig. 18&19, regions I and K).

![Fig. 5. The mRNAs for genes that code for enzymes involved with active DNA demethylation increase during metamorphosis in tadpole brain.](image-url)
The TET3-ir showed a similar distribution pattern in the tadpole brain as 5-hmC-ir and 5-caC-ir (Supplemental Fig. 20). It was undetectable in the most rostral regions of the telencephalon (brain regions A, B & C in Supplemental Fig. 17; data not shown) and was first detected in the most rostral pallium. We saw TET3-ir throughout the pallium, the anterior preoptic area (Supplemental Fig. 20, region E) and the succeeding brain regions (Supplemental Fig. 20, regions H, I, K, M), with pronounced signal in the thalamic nuclei (anterior, ventromedial, posterior, lateral), the ventral hypothalamic nucleus and the tegmentum. The TET3-ir was low or absent in the hindbrain and spinal cord (data not shown).

We chose the tadpole brain region containing the thalamic nuclei and ventral hypothalamic nucleus (Supplementary Fig. 17, region K) for IHC analysis during spontaneous metamorphosis, as this region showed high 5-hmC-ir, 5-caC-ir and TET3-ir in metamorphic climax stage animals (Supplemental Figs. 18, 19 & 20). This region contains neurosecretory neuron cell bodies and axons that project to the median eminence to control pituitary hormone secretion, and it is highly responsive to T3 action (Denver, 1998).

The 5-hmC-ir in tadpole brain was low or nondetectable during pro-metamorphosis (NF stages 54 and 56; Fig. 6), then increased at NF stage 58 and reached a maximum at metamorphic climax (NF stage 62). The intensity of 5-hmC-ir was highest in the thalamic nuclei, with lower signal in the ventral hypothalamus. We detected 5-caC-ir at earlier developmental stages than 5-hmC-ir (Fig. 6; or TET3-ir, see below). At NF stage 50, we saw low but distinct 5-caC-ir that was localized to the thalamic nuclei. The signal increased during metamorphosis and reached a maximum at metamorphic climax. Throughout metamorphosis the 5-caC-ir increased in the thalamic nuclei and appeared in the ventral hypothalamus.

Similar to 5-hmC-ir, there was faint TET3-ir in early pro-metamorphic tadpole brain (NF stages 54 and 56), which then increased at NF stage 58 and reached a maximum at NF stage 62 (Fig. 6). These changes in TET3-ir parallel changes in tet3 mRNA (Fig. 5). The TET3 signal was highest in the thalamic nuclei, with lower signal in the ventral hypothalamus. Due

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**Fig. 6.** Immunoreactivity for 5-hmC, 5-caC and TET3 increases in tadpole brain during spontaneous metamorphosis. We conducted immunohistochemistry on the region of the tadpole brain containing the thalamic nuclei and ventral hypothalamus (section K in Supplementary Fig. 17; abbreviations are defined in Supplementary Table 5) at different stages of metamorphosis. Shown are representative brain sections stained with DAPI and with antibodies to the three different antigens, captured at 4X magnification. Scale bar = 0.5 mm. Graphs to the right of the brain images provide quantification of the immunoreactivity. Bars represent the means ± SEM (n = 4/developmental stage). Data were analyzed by one-way ANOVA (p < 0.001). Letters indicate results of the post-hoc test (Fishers’s LSD); means with the same letter are not significantly different (p < 0.05).
to technical problems we only had $n = 2$ for NF stage 66; the 5-hmC-ir and 5-caC-ir were similar to NF stage 62 in these samples, while the TET3-ir was lower.

4. Discussion

The regulation of cytosine methylation in DNA is crucial for normal neural development and function (Bogdanovic et al., 2011; Feng et al., 2005; Guo et al., 2011; Lister et al., 2013; Shin et al., 2014; Song et al., 2011b; Szulwach et al., 2011). Here we present the first genome-wide analysis of changes in DNA methylation, paired with analysis of changes in gene expression in the brain of a nonmammalian vertebrate during postembryonic development. This developmental period in *Xenopus*, metamorphosis, is characterized by profound changes in gene regulation (Brown et al., 1995; Shi, 2000; Wen et al., 2019), further evidenced here by our RNA-seq analysis, and it shares many similarities with the late fetal/neonatal developmental period of humans (Sachs and Buchholz, 2017; Tata, 2006). We focused on the part of the tadpole brain that houses the neurosecretory neurons that control pituitary hormone secretion, a brain region that is highly dependent on T3 for its development and maturation (Denver, 1998; Etkin, 1965, 1968; Kikuyama et al., 1979). Using MethylCap-seq, we discovered changes in DNA methylation at discrete regions of the tadpole neural cell genome during metamorphosis, with the largest changes occurring during the period from metamorphic climax to the completion of metamorphosis, when DNA demethylation accounted for over 80% of the methylation changes. The changes in DNA methylation were negatively correlated with changes in gene expression, with the strongest relationship seen for methylation changes occurring within gene bodies of DEGs. In agreement with our genome-wide analyses, biochemical and immunohistochemical analyses showed a decrease in 5-mC, but increases in the DNA demethylation intermediates 5-hmC and 5-caC, and also TET3 during metamorphosis. Taken together, these findings provide additional evidence for predominant DNA demethylation during metamorphosis. The DNA demethylation that we observed is likely dependent, at least in part, on increased expression of genes that encode enzymes that catalyze DNA demethylation.

4.1. MethylCap-seq revealed a dense and diverse DNA methylation landscape across the genome of tadpole neural cells

We found that CpG, but not CpA dinucleotides, were enriched within the DNA sequences covered by the MethylCap-seq peaks. This is in contrast to mouse and human, where investigators found pronounced CpA methylation in the frontal cortex that increased during the early postnatal period (Lister et al., 2009); the significance of non-CpG methylation in mammalian neurons and embryonic stem cells is not understood (Shin et al., 2014). In vertebrate genomes, CpG dinucleotides are often present in clusters (termed ‘CpG islands’), and many CpG islands overlap with promoters of constitutively active (i.e., housekeeping) genes; these regions are generally free of DNA methylation, which is thought to maintain the promoters active (Deaton and Bird, 2011). In *Xenopus* tadpole brain during metamorphosis we found that gene promoters and CpG islands had low methylation compared with other regions of the genome, which is consistent with earlier findings in *Xenopus*, zebrafish and mammals (Bogdanovic et al., 2011, 2016). The MethylCap-seq peaks in tadpole brain were approximately equally distributed between genes and intergenic regions.

4.2. MethylCap-seq showed changes in DNA methylation in tadpole brain during metamorphosis, with predominant DNA demethylation from climax to completion

Our MethylCap-seq analysis discovered significant changes in DNA methylation at thousands of loci in tadpole brain during metamorphosis. During the pre- to pro-metamorphic transition there was more demethylation, which may represent active DNA demethylation supported by TETs and other enzymes in the demethylation pathway; these genes are expressed during this developmental period, albeit at relatively low levels. On the other hand, these changes could be due to passive DNA demethylation, since neural cell proliferation peaks during this period (Denver et al., 2009). From pro-metamorphosis to metamorphic climax methylation predominated, which may be driven by the activation of *dnmt3a* transcription during pro-metamorphosis and metamorphic climax, when circulating [T3] is maximal; *Xenopus dnmt3a* is directly regulated by T3 via two TREs (Kyono et al., 2016a).

From metamorphic climax to the completion of metamorphosis we saw predominant DNA demethylation, which is supported by MethylCap-seq analysis, biochemical analyses of 5-mC (directly by ELA, and indirectly by LUMA and cytosine extension assay), and increased levels of the DNA demethylation intermediates 5-hmC and 5-caC analyzed by IHC. These changes during late metamorphosis are likely dependent on the increased expression of enzymes that catalyze DNA demethylation, evidenced by increases in mRNAs for these genes, and also increased TET3 protein.

We also found, using targeted analyses, that genomic regions in proximity to known TREs of T3-regulated genes (kfl9 and *dnmt3a*) (Bagmasbad et al., 2015; Kyono et al., 2016a) became demethylated during metamorphosis. Thyroid hormone response elements are critical regulatory regions mediating T3 action. The most common TRE found in vertebrate genomes consists of two contiguous 6-bp half sites (AGGTCA) separated by a 4-bp spacer (DR-4). DNA demethylation near TREs, as occurred at the *dnmt3* and *kfl9* TREs in this study, could modulate chromatin accessibility, and perhaps the function of TRs (it may not influence TR binding to DNA, since DR-4 TREs do not contain CG dinucleotides.) On the other hand, DNA demethylation at these sites may be induced by liganded TR action in chromatin (S. Raj, Y. Kyono and R.J. Denver, unpublished data). Studies in HEK293T cells support that TRα can directly interact with TET3, and that this may influence TR function in chromatin (Guan et al., 2017). Our preliminary findings support that T3 can promote DNA demethylation at discrete loci in *Xenopus* tadpole brain (S. Raj, Y. Kyono and R.J. Denver, unpublished data; but see Kasai et al. (2015)).

4.3. Roles for DNA methylation in animal development

Our findings support that changes in DNA methylation are linked to the gene regulation programs during tadpole metamorphosis, which underlie the dramatic morphogenetic changes that occur during this developmental period. Critical roles for DNA methylation in development have been demonstrated in several vertebrate species. For example, DNA methylation plays a pivotal role in the long-term transcriptional silencing seen with genomic imprinting, X chromosome inactivation, and the silencing of repetitive elements (Bird, 2002; Weber and Schueller, 2007). The depletion of enzymes that catalyze DNA methylation or DNA demethylation leads to a diversity of developmental disorders, including severe neurological defects (Edwards et al., 2017; Fedotova and Illarionokhina, 2018; Kumar et al., 2018; Ross and Bogdanovic, 2019; Wu and Zhang, 2017; Wu et al., 2018). Many studies support that DNA methylation is a central regulator of cell identity (Bogdanovic and Lister, 2017; Wu et al., 2018; Zeng and Chen, 2019). This is hypothesized to be directly related to the modulation of the chromatin environment, and consequently gene transcription (Bogdanovic and Lister, 2017). Bogdanovic and colleagues (Bogdanovic et al., 2016) found that transcriptional activation of genes involved with body plan formation in zebrafish, X. tropicalis and the mouse depends on active DNA demethylation of functionally conserved enhancer elements.

4.4. Role for DNA methylation in the regulation of gene transcription

Our findings show that during the metamorphic transition, decreased DNA methylation correlated with upregulation of gene expression;
whereas, increased DNA methylation at a locus correlated with down-regulation of gene expression. We found negative correlations within gene bodies and the 0–5 kb upstream region (proximal and distal promoter), and this relationship was stronger for gene bodies. Our findings provide compelling support, using a vertebrate developmental model system, for the hypothesis that DNA demethylation promotes gene activation in vivo, while DNA methylation is associated with gene repression.

Mounting evidence supports that changes in DNA methylation at gene promoters and enhancers can influence transcription (Bogdanovic et al., 2016; Hackett et al., 2013; Wu and Zhang, 2017; Wu et al., 2018). DNA methylation at gene regulatory regions follow this general rule, as there is an inverse correlation between expression of nearby genes and DNA methylation at cell-type specific enhancers, promoters and within gene bodies (Guo et al., 2011; Hon et al., 2013; Lister et al., 2013). The DNA demethylation intermediate 5-hmC is enriched at enhancers, promoters and bodies of actively transcribed genes, which is consistent with DNA demethylation being associated with transcriptional activation (Nestor et al., 2016; Taylor et al., 2016; Tsagaratou et al., 2014). There is evidence from zebra fish and mouse that TET proteins, which catalyze conversion of 5-mC to 5-hmC and other DNA demethylation intermediates, play pivotal roles in enhancer activation through modulation of chromatin accessibility (Bogdanovic et al., 2016; Li et al., 2016). In vitro methylation of target DNA sequences represses transcription in Xenopus embryos (but not in oocytes) (Bogdanovic et al., 2011). Whole-genome bisulfite sequencing found that the genome of Xenopus embryos undergoes DNA demethylation during development, and that these differentially methylated regions were enriched in epigenomic marks indicative of enhancers (H3K4me1, p300) (Bogdanovic et al., 2016).

There are two mechanisms that have been proposed by which DNA methylation at gene regulatory regions can influence transcription. First, nuclear proteins that bind 5-mC (methyl-CpG binding proteins: MBDs) (Hendrich and Bird, 1998) are known to interact with protein complexes that contain the corepressor Sin3A, which in turn recruits histone deacetylases to generate a repressive chromatin state (Nan et al., 1998). Second, transcription factor binding to DNA may be impaired by cytosine methylation when the recognition motif contains CpG dinucleotides (Bogdanovic and Lister, 2017).

Genes that are activated during tadpole metamorphosis display different kinetic profiles, that may be related in part to DNA methylation at the locus. That is, there may be a correlation between the level of DNA methylation and how early the gene is activated during metamorphosis. This may be illustrated by the low methylation level at klf9, which shows very early developmental activation and rapid kinetics after T3 treatment (Bagamasbad et al., 2008, 2015; Furlow and Kanamori, 2002; Hu et al., 2016). This is in contrast to dmn3a, which is heavily methylated, and exhibits late activation during metamorphosis and slow kinetics after T3 treatment (Kyono et al., 2016a). This hypothesis requires further investigation.

Our RNA-seq experiment documents the dramatic changes in gene expression that underlie development of the Xenopus tadpole preoptic area/thalamus/hypothalamus. Between pre-metamorphosis and metamorphic climax more than 5000 genes change their expression level, and this number is nearly equally divided between up and downregulated genes. This is the first analysis of its kind in any tadpole tissue (other studies have looked only at gene regulation responses to exogenous T3 in pre-metamorphic tadpoles); a full analysis of the RNA-seq data will be published elsewhere.

4.5. Molecular mechanisms for DNA demethylation in tadpole brain during metamorphosis

We found that mRNA levels for genes that encode enzymes involved in the active DNA demethylation pathway increased in tadpole brain during metamorphosis, with highest levels during metamorphic climax, when circulating T3 is highest (Leloup, 1977). We also show that for one of the genes, tet3, the increase in its mRNA level coincided with increased TET3 protein in tadpole brain. TET3 catalyzes the oxidation of 5-mC to the active DNA demethylation intermediates 5-hmC and 5-caC (Ross and Bogdanovic, 2019; Wu and Zhang, 2017). Consistent with the increase in tet3 mRNA and TET3 protein during metamorphosis, and the role of TET3 in direct catalysis of DNA demethylation, we found increases in the DNA demethylation intermediates 5-hmC and 5-caC in tadpole brain during metamorphosis.

To our knowledge, this is the first demonstration, in a vertebrate developmental model system, of a developmental increase in the expression of enzymes involved with DNA demethylation, that is coincident with an increase in DNA demethylation intermediates, and consequently DNA demethylation. It is noteworthy that immunoreactivity for TET3, 5-hmC and 5-caC was minimal in rostral regions of the telencephalon, the hindbrain and spinal cord in tadpole brain at metamorphic climax, but was high in the part of the brain that houses the thalamic nuclei, the ventral hypothalamus and the tegmentum, regions of the tadpole brain that are known to be highly responsive to T3 (Denver, 1998; Etkin, 1968; Etkin et al., 1965). Diotel and colleagues (Diotel et al., 2017) used IHC to detect 5-hmC in neurons of pre-metamorphic X. laevis tadpoles (NF stage 50) and newly metamorphosed frogs (NF stage 66) in the same brain regions as the current study; this marker of DNA demethylation was excluded from proliferating cells. The 5-hmC level in mouse brain was highest in neurons, and it strongly increased postnatally when neurons were maturing and forming synapses (Cadena-del-Castillo et al., 2014; Lister et al., 2009; Song et al., 2011b; Szulwach et al., 2011). Furthermore, 5-hmC is enriched within gene bodies, and exhibited a positive correlation with gene transcription (Hahn et al., 2013, 2014; Song et al., 2011b; Szulwach et al., 2011). The increase that we saw in TET3 in tadpole brain supports that this enzyme is responsible, at least in part, for the increase in 5-hmC and 5-caC, and DNA demethylation that we saw after metamorphic climax.

In mammals there are three TET genes, and all are expressed in the brain, primarily in neurons, with TET3 being the most abundant (Kaas et al., 2013; Li et al., 2014; Mi et al., 2015; Szwagierczak et al., 2010); these enzymes support the high levels of 5-hmC found in neuronal cells (Szulwach et al., 2011; Szwagierczak et al., 2010). In Xenopus, only tet2 and tet3 genes have been identified. The TET proteins play critical roles in modulating DNA methylation, and have profound influence on animal development (Kaas et al., 2013; Li et al., 2014; Mi et al., 2015; Tan and Shi, 2012). In mouse embryonic stem cells in which Tet 1, 2 & 3 were knocked out, and in tet1, 2 & 3 triple morphant zebrafish embryos, the levels of 5-mC at promoters, enhancers and Dnasel-hypersensitive sites were significantly higher compared to wild type embryos (Bogdanovic et al., 2016; Dawlaty et al., 2014; Lu et al., 2014). Inhibition of TET enzymes in the brain leads to dysregulation of methylation at, and transcription of genes involved with synaptogenesis and synaptic function (Campbell and Wood, 2019). TET3 has been shown to play a critical role in the maintenance of neural progenitors and the terminal differentiation of neurons (Antunes et al., 2019; Li et al., 2015; Li et al., 2014). This enzyme has also been shown to function as a synaptic activity sensor, and is required for homeostatic synaptic plasticity (Campbell and Wood, 2019; Yu et al., 2015). In Xenopus, Xu and colleagues (Xu et al., 2012) demonstrated an essential role for TET3 in eye and neural development.

5. Conclusions

The genome of neural cells located in the region of the preoptic area/thalamus/hypothalamus of X. tropicalis tadpole brain undergoes changes in DNA methylation during metamorphosis that are negatively correlated with changes in gene expression. DNA methylation predominated during the pro-metamorphic period, possibly mediated by increased expression of dmn3a, which is activated by T3 during these stages of metamorphosis. DNA demethylation predominated from metamorphic climax to the completion of metamorphosis, and this change may depend, in part, on the upregulation of genes that encode enzymes involved with
active DNA demethylation. The activation of these genes, for example tett3, is likely responsible for the increased levels of TET3 protein, and the generation of the DNA demethylation intermediates 5-hmC and 5-caC. Taken together, our data support that changes in DNA methylation are likely crucial for the coordination of gene regulation programs that underlie tissue morphogenesis.

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Declaration of competing interest
None.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2020.03.013.

References


Wen, L., He, C., Sifuentes, C.J., Denver, R.J., 2019. Thyroid hormone receptor alpha is required for thyroid hormone-dependent neural cell proliferation during tadpole metamorphosis. Front. Endocrinol. 10.


