Published in final edited form as:

Comp Biochem Physiol B Biochem Mol Biol. 2013 September ; 166(1): . doi:10.1016/j.cbpb.2013.07.007.

Global and gene specific DNA methylation changes during zebrafish development

Xiefan Fang¹, Jone Corrales¹, Cammi Thornton¹, Brian E. Scheffler², and Kristine L. Willett^{1,*}

¹Department of Pharmacology and Environmental Toxicology Research Program, School of Pharmacy, University of Mississippi, University, MS 38677, USA

²USDA-ARS Genomics and Bioinformatics Research Unit, Stoneville, MS 38776, USA

Abstract

DNA methylation is dynamic through the life of an organism. Previous studies have primarily focused on DNA methylation changes during very early embryogenesis. In this study, global and gene specific DNA methylation in zebrafish (*Danio rerio*) embryos, larvae and adult liver were compared. The percent methylation of cytosines was low in 2 to 4.3 hours post fertilization (hpf) zebrafish embryos and was consistently higher in zebrafish older than 6 hpf. Furthermore, quantitative real-time PCR (qPCR) results showed relatively high DNA methyltransferase 1 (*dnmt1*) and low glycine *N*-methyltransferase (*gnmt*) mRNA expression in early embryogenesis. By studying methylation patterns and gene expression of five developmentally important genes, namely *vasa*, Ras-association domain family member 1 (*rassf1*), telomerase reverse transcriptase (*tert*), *c-jun* and *c-myca*, we found that the timing of changes in DNA methylation patterns was gene specific, and changes in gene expression were not necessarily correlated with the DNA methylation patterns.

Keywords

DNA methylation; zebrafish; development; *vasa*; *rassf1*; *tert*; *c-jun*; *c-myca*

1. Introduction

DNA methylation is the best studied epigenetic mechanism, and the only one that can covalently modify DNA. DNA methylation is mostly found at cytosine residues that are followed by guanines (CpG sites) (Gruenbaum et al., 1981), and approximately 2–5% of all cytosines are methylated in mammalian genomes (Fuke et al., 2004). CpG islands (CGI) are regions with densely clustered ‘CG’ dinucleotides that are typically unmethylated in active genes. DNA methylation patterns are established during early development by *de novo* DNA methyltransferases (*dnmts*). The already established methylation pattern can be copied from the parental strand into the daughter strand after each cell division by a semiconservative maintenance *dnmt* (Razin and Riggs, 1980). Therefore, DNA methylation patterns are stably

© 2013 Elsevier Inc. All rights reserved.

*Corresponding author: Kristine L. Willett, Box 1848, 303 Faser Hall, Department of Pharmacology, University of Mississippi, University, MS, 38677, USA, kwillett@olemiss.edu, Tel: (662) 915-6691; Fax: (662) 915-5148.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

memorized and have long-term impact on an organism's phenotype without any changes in the genotype.

However, DNA methylation patterns are also dynamic during development. In mammals, DNA methylation patterns experience dramatic changes in the inner cell mass (lineage-specific) during early development and in the early primordial germ cells (PGCs, germ-line specific) after sex determination (Anway and Skinner, 2006). After fertilization, both paternal and maternal genomes undergo a rapid global loss of methylation and hit the lowest point by the blastocyst stage (Mayer et al., 2000; Sanz et al., 2010). At the morula and blastocyst stages, extensive acquisition of *de novo* DNA methylation occurs and eventually about 70% or more of CpGs are rapidly remethylated (Shi and Wu, 2009). A second round of genome-wide methylation reprogramming occurs in PGCs after sex determination. The methylation level remains low in the PGCs until *de novo* methylation occurs during germ cell maturation and gametogenesis (Rousseaux et al., 2005; Lees-Murdock and Walsh, 2008). Currently, the significance of genome-wide methylation reprogramming is not well understood but disruptions in this highly coordinated process may contribute to developmental defects or embryo death (Haaf, 2006). DNA methylation patterns are believed to be established in early embryogenesis, but modest gains and losses of CpG methylation were observed in the transition of embryonic stem cells to differentiated cells (Meissner et al., 2008; Mohn et al., 2008). Although most epigenetic marks are maintained after birth, DNA methylation patterns are still changeable by external environment interventions, and the epigenome is the most plastic during early postnatal development (Fraga et al., 2005; Bjornsson et al., 2008).

At the other end of the life spectrum, DNA methylation changes are part of normal aging and tend to associate with age-onset pathologic phenotypes (Gravina and Vijg, 2010). The expression and activity of *dnmt1* decline and errors of methylation maintenance increase in aging individuals (Lopatina et al., 2002). As a result, global hypomethylation is detected in most vertebrate tissues with aging (Wilson et al., 1987; Barbot et al., 2002; Bollati et al., 2009). The relaxation of epigenetic control of genes contributes to abnormal gene expression (Bennett-Baker et al., 2003), functional decline in organs, and progression of age-related diseases (Wang et al., 2008).

Zebrafish are an important model organism for developmental biology and related scientific fields because its genome has approximately 70% homology to human genes (Howe et al., 2013). Mechanisms of DNA methylation in fish are generally conserved with that of mammals (Walter et al., 2002; Varriale and Bernardi, 2006; Aniagu et al., 2008; Zemach et al., 2010). For example, most of the effector proteins in the methylation machinery have been identified in zebrafish (Goll and Halpern, 2011). In the past decade, a few studies have investigated the DNA methylation changes during early embryogenesis in zebrafish. After initial debates, it is now clear that global DNA demethylation and reestablishment are conserved in fish (McGowan and Martin, 2002; Mhanni and McGowan, 2004; MacKay et al., 2007). However, there are no studies so far to measure the DNA methylation changes in later developmental stages and aging adult zebrafish. Therefore, in this study, we measured the global and gene specific DNA methylation in embryos, larvae and adult livers (1.5-year old).

For specific gene studies, five important developmental genes, i.e. *vasa*, Ras association domain family member 1 (*rassf1*), telomerase reverse transcriptase (*tert*), *c-jun* and *c-myc*, were selected for sodium bisulfite sequencing and gene expression analysis. Expression of *vasa* is essential for germline development and is used as a marker gene to distinguish the germ cell (including PGCs) population (Fujiwara et al. 1994; Castrillon et al., 2000). The tumor suppressor gene *rassf1* was selected because its promoter methylation has strong

correlation with gene expression in mammals, and it is one of the most frequently silenced genes by promoter hypermethylation in many cancer types (Richter et al., 2009). *Tert* is a catalytic subunit of telomerase, and its promoter activity is partially regulated by DNA methylation and histone modulation (Kyo et al., 2008). Both *c-jun* and *c-myc* are proto-oncogenes essential for embryogenesis because genetic deletion of either of them can lead to embryonic lethality (Eferl et al., 1999; Soucek and Evan, 2010). The target regions of the five genes represent sequences with different CpG richness, and they are located on five different chromosomes. Importantly, the CGI distribution, splicing and protein homology of the five genes are well conserved between zebrafish and mammals (Supplemental Table 1 and Supplemental Figure 1).

In this study, a statistically significant increase in global methylation was found by 6 hours post fertilization (hpf) in zebrafish. Yet, a unique timing of methylation pattern establishment in the five specific genes, *vasa*, *rassf1*, *tert*, *c-jun* and *c-myc*, was observed. Finally, there was an inverse relationship between DNA methylation and gene expression in *vasa*. Our results indicate that the process of DNA remethylation is dynamic, complicated, and time-specific for individual genes. Future studies are needed to understand the biological significance of global DNA methylation during early development.

2. Material and methods

2.1. Zebrafish care

AB line wild-type zebrafish (*Danio rerio*) were purchased from Zebrafish International Resource Center (ZFIN, Eugene, OR, USA) and raised under the approved IACUC protocol. Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL, USA) with zebrafish water (pH 7.0–7.5, 60 parts per million (ppm), Instant Ocean, Cincinnati, OH) at 28 °C (range 24–30 °C), 14:10 light-dark cycle. Adult fish were fed twice daily with tropical flake fish food and live brine shrimp. Larvae were fed with Artemia-0 powdered food (20–80 micron size, Bio-Marine, Hawthorne, CA, USA) and/or live brine shrimp depending on their age.

2.2 Genomic DNA isolation

To determine global DNA methylation status through sequential time points, genomic DNA from zebrafish embryos (2, 3.3, 4.3, 6, 11.7, 24, and 36 hpf; n=3 pools, 30 or 50 embryos/pool), hatching early-larvae (48, 60, and 72 hpf; n=3 pools, 10 early-larvae/pool), larvae (96 hpf; n=3 pools, 10 larvae/pool), and adult female and male livers (n=3, no pooling) was extracted with DNAzol (Molecular Research Center, Cincinnati, OH, USA) according to manufacturer's protocol except the following modifications: homogenized embryos were digested overnight with proteinase K (1.8 mg/mL) to eliminate the high content of yolk protein; PolyAcryl carrier (Molecular Research Center) was used to isolate small quantities of DNA such as in embryos; and DNA was further purified with the DNA Clean & Concentrator kit (ZYMO Research, Irvine, CA, USA). For bisulfite sequencing, genomic DNA from zebrafish embryos (n=3 pools, 30–50 embryos/pool), larvae (n=3 pools, 10 larvae/pool) and livers (n=3, no pooling) was isolated with DNeasy Blood & Tissue Easy Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. For both methods, DNA was treated with RNAase to remove RNA contaminants. DNA concentrations were quantitated with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.3 Global DNA methylation measurement

Global DNA methylation was measured with the MethylFlash™ Methylated DNA Kit (Epigentek Group, Farmingdale, NY, USA, Cat. No. P-1034) by following the manufacturer's protocol. The OD intensity of the colorimetric reaction was measured by a

Biotech ELx800 plate reader (Winooski, VT, USA). The OD intensity of each sample was normalized to 100 ng of DNA. The cytosine methylation percentage was calculated by using the formula: cytosine methylation % = [(OD sample – OD negative control/100ng)/(OD positive control – negative control) x 2/5ng] X 100; 100 ng is the amount of DNA per sample, 2 is a factor to normalize 5-methylcytosine in the positive control to 100%, and 5 ng is the amount of positive control, which contains 50% unmethylated and 50% methylated cytosine. Three biological replicates were measured in duplicate.

2.4 Exon, intron identification and gene homology

The information of exon, intron, and splice variants of the specific genes were found on Ensembl (<http://useast.ensembl.org/index.html>). Gene homologs or protein pairwise alignments were done on NCBI HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>) or SDSC Biology WorkBench.

2.5 CpG island identification and classification

CpG islands were found by using the Methyl Primer Express v1.0 (Applied Biosystems, Foster City, CA, USA). The criteria to determine CpG islands were set as follows: DNA region length longer than 300 bp but shorter than 2000 bp; greater than 50% C+G content; and observed CpG/expected CpG of 0.65 (Gardiner-Garden and Frommer, 1987). Promoters or regions were further classified into three categories to distinguish strong CpG islands, weak CpG islands and sequences with low abundance of CpG sites (Takai and Jones, 2002). High CpG promoters/regions (HCP) contain a 500 bp sequence with GC content above 55%, CpG ratio above 0.75, and a CpG observed to expected ratio greater than 0.6. Intermediate CpG promoters/regions (ICP) contain a region below 500 bp and have moderate CpG richness (GC content less than 55%) with CpG ratio between 0.48 and 0.75 and have a CpG observed to expected ratio between 0.4 and 0.6. Low CpG promoters/regions (LCP) do not have a region of 500 bp with a CpG ratio above 0.48 or CpG observed to expected ratio above 0.4 (Weber et al., 2007).

2.6 Sodium bisulfite sequencing

Bisulfite sequencing was performed as described in (Fang et al., 2013). Genomic DNA was treated with sodium metabisulfite and cleaned with EZ Bisulfite DNA Clean-up Kit (ZYMO Research). Previously validated bisulfite specific primers were used for PCR amplification (Fang et al., 2013). PCR products were ligated into pGEM® T Easy Vector System (Promega, Madison, WI, USA) and cloned into DH5 *E. coli* competent cells (Invitrogen, Grand Island, NY, USA). Each treatment or time-point had three biological samples and at least eight white colonies from each sample were selected for sequencing. Plasmid DNA was isolated in 96-well format using a modified alkaline-lysis extraction procedure with Qiagen reagents and sequenced in the ABI 3730xl sequencer using BigDye v3.1 Terminator/Buffer Ready Rxn Cycle Sequencing kit (Applied Biosystems). Sequence data were analyzed with DNASTAR (SeqMan, Madison, WI, USA). CpG methylation percentage was calculated as (total number of methylated CpG)/(number of CpG sites in each gene X number of colonies sequenced).

2.7 Quantitative reverse transcription real time PCR (qPCR)

RNA from embryos (20–50 embryos/pool), larvae (10 larvae/pool) or livers (no pooling) was isolated with RNeasy (Molecular Research Center) and purified with RNeasy Mini Kit (Qiagen) by following the manufacturer's protocols. Total RNA was reverse transcribed to cDNA by using TaqMan® Reverse Transcription Reagents (Applied Biosystems). Relative abundance of target genes to 18S rRNA transcripts in the cDNA libraries was determined by

qPCR with SYBR®Green and validated qPCR primers in a GeneAmp 7500 Sequence Detection System (Applied Biosystems) (Fang et al., 2013).

2.8 Statistical analysis

Results were analyzed by GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and presented as mean $\pm$ standard error of the mean (SEM). Statistical differences between treatment groups were determined using one-way ANOVA followed by Neuman-Keuls post hoc test ($p < 0.05$). Data of the qPCR were calculated with the 2^{-CT} method. Statistical differences between treatments or time-points were determined on the linearized 2^{-CT} values. The correlation coefficient between *vasa* promoter methylation and gene expression was calculated using parametric correlation analysis (Pearson).

3. Results

3.1 Constitutive global DNA methylation changes during development

The average percentage of methylated cytosines was 0.61% in 2–4.3 hpf zebrafish embryos (Figure 1). The methylation increased significantly to an average of 2.06% in 6 to 96 hpf whole zebrafish, and adult female and male livers. Additionally, the percent of methylated cytosines between 4.3 hpf embryos and adult male liver was not significantly different.

3.2 Constitutive zebrafish *dnmt1* and glycine N-methyltransferase (*gnmt*) mRNA expression during development

Transcripts of *dnmt1* mRNA were maternally transferred into the eggs with high abundance in unfertilized zebrafish eggs (Figure 2A). Transcription of *dnmt1* was initially high and then dropped significantly at 4 hpf and remained at a low level until a transient increase at 60 hpf (pec-fin stage).

The expression of *gnmt* was low throughout early embryo development (Figure 2B). Between 12 and 24 hpf, *gnmt* mRNA expression rose significantly by 5-fold. Peak *gnmt* expression was at 60 hpf, but like *dnmt1*, expression also decreased again at 96 hpf.

Comparing the relative expression between *dnmt1* and *gnmt*, *dnmt1* was maternally deposited about 7-fold more in unfertilized eggs compared to *gnmt* (Figure 2C). Starting at 24 hpf, *gnmt* exceeded *dnmt1* expression by 1.6-fold. At 96 hpf, *gnmt* expression was about 5.6-fold higher than *dnmt1*.

3.3 De novo DNA methylation in the *vasa* promoter during zebrafish development

The potential methylation target region of the *vasa* promoter is illustrated in Figure 3 and further explained in Table 1. Only ~6% of the 5 CpG sites in the *vasa* promoter were methylated in zebrafish embryos aged from 3.3 to 8 hpf (Figure 4A and Supplemental Figure 2). By 12 hpf, the CpG methylation percentage increased significantly to 37.5%. Methylation continued to increase with development to 68.3% at 48 hpf and 79.8% at 96 hpf. The promoter was also highly methylated in the 1.5-year old female livers (76.8%).

3.4 Constitutive *vasa* mRNA expression during zebrafish development

Transcripts of *vasa* mRNA were maternally deposited into the oocyte with high abundance in unfertilized eggs (Figure 4B). After fertilization, *vasa* expression significantly increased at 3.3 hpf, indicating embryonic transcription of *vasa* mRNA. The mRNA began to drop slightly at 4 hpf, and the highest relative decrease of *vasa* mRNA expression occurred at 12 hpf. The decrease in expression continued, and the lowest expression was detected at 96 hpf which corresponded to only 0.2% of the *vasa* transcription in the 3.3 hpf embryos.

Consistent with theory, the trends of *vasa* DNA methylation and mRNA expression during embryogenesis were opposite from each other (Figure 4C). As the DNA methylation increased, *vasa* expression decreased significantly. The Pearson correlation coefficient of *vasa* promoter methylation with gene expression was -0.885 ($P < 0.00001$).

3.5 Constitutive DNA methylation and gene expression of *rassf1*, *tert*, *c-jun*, and *c-myca* during development

The target regions of these four genes are illustrated and described in Figure 3 and Table 1. The 26 CpG sites located in the first promoter (CGI-1) of *rassf1* were hypomethylated at 3.3 hpf in mid-blastula embryos, 96 hpf larvae, and 1.5-year old female zebrafish livers (Figure 5A and Supplemental Figure 3A) and not significantly different. In contrast, the CpG sites in the second promoter (CGI-2) of *rassf1* were highly methylated (Figure 5B and Supplemental Figure 3B). The percentage of CpG methylation reached 98.0% at 3.3 hpf and remained highly methylated at 5 hpf (96.5%), 6 hpf (98.5%), and 96 hpf (92.5%). In adult livers, the methylation percentage dropped significantly to 73.3%. The *rassf1-001* mRNA increased significantly at 5 hpf, peaked at 96 hpf, and then decreased significantly in adult livers (Figure 6A). Similarly, total *rassf1* mRNA expression was low at 3.3 hpf, then increased dramatically at 5 hpf, but the total expression dropped significantly later (Figure 6B). The percentage of *rassf1-001* represented in the total *rassf1* expression at different time-points is shown in Figure 6C.

For *tert* promoter, all the CpG sites except CpG #1 were highly methylated in all the time-points tested (Supplemental Figure 4). Neither the methylation pattern nor the percentage was different between the three time-points (Figure 7A). However, *tert* gene expression was significantly different (Figure 7B). Expression of *tert* at 96 hpf was 3.5-fold higher than that at 3.3 hpf. *Tert* expression in adult livers was extremely low, and was only 5.3% and 1.5% of the expression in 3.3 hpf embryos and 96 hpf larvae, respectively.

The 36 CpG sites located in the CGI in the *c-jun* first exon were hypomethylated (Figure 7C and Supplemental Figure 5). Neither the methylation pattern nor the percentage was different between the three time-points. However, like *tert*, gene expression was significantly different (Figure 7D). Expression of *c-jun* at 96 hpf was 379-fold higher compared to the expression at 3.3 hpf. In adult livers, expression was lower than 96 hpf larvae, but still 39-fold higher than the expression in 3.3 hpf embryos.

The 26 CpG sites located in the CGI in the *c-myca* second exon were also hypomethylated (Figure 7E and Supplemental Figure 6). The overall methylation percentage was statistically higher in the 3.3 hpf embryos than the percentage in 96 hpf larvae and adult livers. As a possible result of this hypomethylation, *c-myca* expression at 96 hpf was higher than that at 3.3 hpf (Figure 7F).

3.6 Efficiency of sodium bisulfite treatment

The rate of conversion of non-CpG cytosines was examined to evaluate the efficiency of bisulfite conversion. Three PCR products (eight colonies chosen from each product) derived from *vasa* (207 bp), *rassf1* CGI-1 (314 bp), *rassf1* CGI-2 (438 bp), *tert* (233 bp), *c-jun* (425 bp) and *c-myca* (444 bp) were sequenced. The amplified regions have 43, 94, 51, 101 and 91 non-CpG cytosines, respectively. The efficiencies of bisulfite conversion were all above 99.8% (Supplemental Table 2). This demonstrates that the sodium bisulfite conversion of cytosines was very efficient and did not generate artifacts due to incomplete conversion.

3.7 CG loss in genomic DNA

When alignments were done between converted Genbank sequences (non-CpG C was converted to T) and our sequence data in DNASTar, dinucleotide polymorphisms were observed in *rassf1*, *tert* and *c-myca* due to CG losses in our fish. In *rassf1* CGI-2, 36.2% of the CpG #16 was mutated to TT (Table 2). In *tert*, CpG #7 was completely changed to TA. In *c-myca*, 81.0% of CpG #26 was changed to TT.

4. Discussion

In this study, global and gene specific DNA methylation was measured at different developmental time-points in zebrafish. Our data showed that overall about 0.53%, 0.54%, 0.75% of the cytosines were methylated in the genome of 2, 3.3, and 4.3 hpf embryos, respectively (Figure 1), which increased approximately four times at 6 hpf and later life stages. These results are consistent with Mhanni and McGowan (2004) and Wu and coworkers (2011) who showed that cytosine methylation increased from 2 and 3.3 hpf to 6 hpf. Liver tissue from 1.5 year old zebrafish was used to represent differentiated adult conditions. It should be noted that both percent methylation and gene expression would be expected to be variable between adult tissues, however, adult liver global cytosine methylation was not statistically different than embryo and larval results after 6 hpf.

We measured the expression of two DNA methylation related enzymes during embryogenesis aiming to associate their expression profiles with DNA methylation changes. Due to the genome duplication events, there are eight known *dnmt* orthologs in zebrafish (Shimoda et al., 2005; reviewed in Goll and Halpern, 2011). We have focused on *dnmt1* in this study. Of the *dnmt* orthologs, there is only a single copy of *dnmt1* that is highly similar to human *dnmt1*. This is also the most studied *dnmt* ortholog in zebrafish, whereas less is known about the rest of *dnmts* though their differential activity may be involved in tissue-specific differentiation (Wu et al., 2011). On the other hand, zebrafish has only one copy of *gnmt*. Gnmt is the enzyme responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH) and sarcosine, thus it preserves the ratio of SAM/SAH, a sensitive indicator of cellular methylation capacity. Constitutively, we found that both *dnmt1* and *gnmt* were maternally transferred mRNAs and expressed throughout development (Figure 2). However, their expression profiles were distinct. The average expression of *dnmt1* was high from 8-cell (1.25 hpf) to mid-blastula (3.3 hpf) stage and was approximately 5-fold higher than the average expression from sphere (4 hpf) to early larvae (96 hpf) stage. In contrast, *gnmt* expression was low in the beginning and increased significantly from 24 hpf. Expression of *dnmt1* was higher than *gnmt* until 24 hpf when *gnmt* expression began to exceed *dnmt1*. Although *dnmt1* is dominantly a maintenance methyltransferase, recent studies reported that *dnmt1* also plays an active role in *de novo* methylation (Fatemi et al., 2002; Athanasiadou et al., 2010). Low expression of *gnmt* during early embryogenesis could be expected to help preserve a high SAM/SAH ratio, whereas *dnmt1* will consume SAM as a methyl donor to remethylate DNA. Therefore, the relatively low expression of *gnmt* together with high expression of *dnmt1* may contribute to DNA remethylation in early zebrafish embryos.

In addition to measuring the global DNA methylation changes, we have also studied the DNA methylation patterns of individual genes during zebrafish development. Five important developmental genes, i.e. *vasa*, *rassf1*, *tert*, *c-jun* and *c-myca*, were selected for sodium bisulfite sequencing analysis, which is able to generate single nucleotide resolution of individual CpG methylation status.

The *vasa* promoter is a low CpG promoter (LCP) (Table 3) and would be predicted to be highly methylated, which was supported by our results. The *de novo* DNA methylation in

the *vasa* promoter did not occur in the blastula stage (3.3 hpf) when the majority of the methylation patterns are typically established. In fact, the *vasa* promoter did not begin to be methylated until 11 hpf and reached its plateau methylation level by 48 hpf. The DNA methylation pattern was thereafter not different than the livers of adult zebrafish. Relative hypomethylation of *vasa* at 3.5 hpf (mid-late blastula) compared to differentiated ZF-4 zebrafish fibroblast cells was also found by Lindeman and coworkers (2010). Consistent with the increasing promoter methylation during development, *vasa* mRNA expression decreased continuously over the same period. The most dramatic decline of *vasa* mRNA occurred at 12 hpf, the same time-point when the promoter methylation percentage first became significantly higher than the previous stages. The opposite trends of DNA methylation and gene expression suggest that *vasa* promoter methylation status was associated with zygotic *vasa* expression. This is also consistent to our previous finding that benzo[a]pyrene –treated zebrafish larvae (96 hpf) had decreased promoter methylation in *vasa* and increased *vasa* gene expression (Fang et al., 2013). A consideration worth noting is that in these studies the genomic DNA was extracted from whole embryos whereas the *vasa* mRNA is assumed to be predominately synthesized by the PGCs that represent only a small fraction compared to somatic tissues. Therefore, in future studies it will be informative to extract genomic DNA from isolated PGCs to further substantiate the relationship between *vasa* methylation and gene expression.

The single copy of *rassf1* gene can generate multiple transcript variants by using two different CGI promoters, and this is well conserved among human, rat, mouse and zebrafish (Supplemental Figure 1). Zebrafish *rassf1* has five transcripts and possibly three CpG rich promoters crossing each TSS (Supplemental Figure 7). Due to the high identity between the five transcripts, it was a challenge to design specific qPCR primers to target each one of the *rassf1* variants. It was only possible to measure the transcription of *rassf1-001* by designing primers located within exon 1 (Fang et al. 2013). We also measured the total expression of all the *rassf1* variants simultaneously using the primer pairs located at exon 5 and 6. Our results showed that *rassf1* CGI-1 was almost 100% unmethylated, which is similar to the normal methylation pattern in the first promoter of human *rassf1* (Peters et al., 2007). In contrast, *rassf1* CGI-2 was heavily methylated (>92%). In contrast to the pattern noted for *vasa*, the methylation pattern for *rassf1* CGI-2 was established during early embryogenesis, and the methylation percentage decreased in aging fish livers. However, there was no apparent correlation between changes of promoter methylation patterns and *rassf1* mRNA expression.

The proximal promoter of *tert* was highly methylated (except in CpG #1), and its consistent methylation pattern was also established by 3.3 hpf. Although the methylation patterns were unchanged, *tert* mRNA expression was significantly different at the three time-points tested. The expression of *tert* was the highest in 96 hpf larvae when extensive cellular proliferation required *tert* to add telomere ends to the newly-replicated DNA. On the contrary, *tert* expression was the lowest in adult livers presumably because the aging animals were losing telomerase activity (Anchelin et al., 2011). The results from *rassf1* and *tert* contradicted the general notion that promoter DNA methylation and gene expression are inversely correlated (Kass et al., 1997). Others too have found that some promoter methylation patterns do not correlate well with gene expression (Weber et al., 2007). Therefore, for *rassf1* and *tert*, DNA methylation may not be the dominate factor regulating their gene expression during development. Accordingly, histone modifications and miRNAs play important roles in regulating transcription of *rassf1* and *tert* (Li et al., 2012; Daniel et al., 2012).

The majority of previous studies have focused on promoter DNA methylation, however, recent studies found the importance of gene body methylation. In plants, gene body DNA methylation was positively correlated with gene transcription (Zhang et al., 1999; Zilberman

et al., 2007). However, conflicting results were found in vertebrates. Some studies reported that actively transcribed genes have extensive DNA methylation within their gene bodies, supporting the positive correlation between methylation and transcription (Lister et al., 2009; Rauch et al., 2009). Yet, results from *in vitro* studies showed that methylation of the gene body was sufficient to repress gene expression in the absence of promoter methylation (Kesht et al., 1985; Graessmann et al., 1994). Another study found that methylation of alternative promoters discovered within gene bodies was associated with gene silencing (Maunakea et al., 2010). Therefore, gene body methylation is important for transcriptional regulation. Here we measured the gene body methylation in two genes, namely, *c-jun* and *c-myca*.

We found that the CpG sites were nearly 100% unmethylated in the coding region of *c-jun*, and the methylation patterns were maintained from 3.3 hpf to 1.5 year-old zebrafish liver. However, the *c-jun* mRNA expression was significantly different in distinct life stages. This indicates that the low methylation in the *c-jun* gene body is permissive to gene expression, but the actual magnitude is likely determined by other factors such as miRNAs and histone modification (Yamaguchi et al., 2005; Kappelmann et al., 2012).

Due to genome duplication, zebrafish have two copies of *c-myc*. We studied *c-myca* because it is phylogenetically closer to mammalian *c-myc* (Supplemental Table 1). The target region was in the second exon, which is within the coding region. The results showed that the methylation percentage of *c-myca* in 3.3 hpf embryos was significantly higher than in 96 hpf larvae and adult livers. When demethylation occurred, gene expression was significantly higher at 96 hpf compared to 3.3 hpf, supporting the theory that gene body methylation also has a reverse correlation with gene expression for *c-myca*.

From our results, the relationship between CpG density and methylation outcome agrees with the earlier study done in human promoters (Weber et al., 2007). Weber and coworkers found that LCP were almost exclusively methylated, ICP were less likely to be methylated (21%), and the majority of HCPs (97%) had very low methylation. Our results showed a similar trend. For example, the LCP in *vasa* was highly methylated in larvae and liver; some ICPs, like *rassf1* CGI-1 and *c-myca* gene body, were hypomethylated and some ICPs, like *rassf1* CGI-2 and *tert*, were hypermethylated; the HCP in *c-jun* gene body was hypomethylated. Work in zebrafish found that at (3.3 hpf) and after (5.3 hpf) the midblastula transition there was an increase in methylated HCPs relative to LCPs (Andersen et al., 2012). By comparing the constitutive DNA methylation and mRNA expression in the five genes, we noticed that promoter hypermethylation in *vasa* and *tert* were associated with low mRNA expression (Figure 8). In contrast, hypomethylation in *rassf1* CGI-1, intragenic *c-jun* and *c-myca* were linked to high mRNA expression. The high content of unmethylated cytosines within a gene body may increase the copy number of mRNA being transcribed and improve the mRNA stability (Duan and Antezana, 2003; Bauer et al., 2010).

In this study, we found that evolutionary CpG loss was apparent in the zebrafish genome. Similar to the finding in mammals, CpG mutations preferentially target the methylated cytosines in zebrafish. For example, CpG losses were identified in both the heavily methylated *rassf1* CGI-2 and *tert* promoters. Even the CpG loss in *c-myca* was located at the end of the target region which had a higher methylation percentage. More instances of CpG loss will likely be discovered as genome-wide methylation studies become more widely used.

5. Conclusion

While global DNA methylation begins to increase after 3.3 hpf in zebrafish embryos and reached a plateau between 6 and 96 hpf, the timing of establishment of promoter

methylation patterns was gene specific. Of the five genes studied, we found that *de novo* methylation of *rassf1*, *tert*, *c-jun*, and *c-myca* occurred by 3.3 hpf during early embryogenesis. However, in the *vasa* promoter, the methylation pattern was established at later stages of development. In addition, changes in gene expression during embryo-larval development did not correlate with DNA methylation patterns in each gene except for *vasa* and *c-myca*. Therefore, as the role of DNA methylation and gene expression in development is more extensively studied, it will be important to investigate the changes at gene and CpG island specific resolution as well as consider other mechanisms of epigenetic modulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Fanny Liu from USDA-ARS Genomics and Bioinformatics Research Unit for her work on plasmid isolation and sequencing. We also want to thank all the undergraduate fish feeders: Courtney Johnson, Kate Goode, Hallie Freyaldenhoven, Susan Jenkins, Richard Kyle, Matthew Thornton, Cynthia Tran, Wesley Walker and Zhizhong Shan.

This project was supported in parts by Grant Numbers R01ES012710, R03ES018962 and R21ES019940 from the National Institute of Environmental Health Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences, the National Institutes of Health, or the Agricultural Research Service. This work was also partly supported by South Central Technology Transfer Award from Society of Toxicology, and Graduate Student Council Research Grant from the University of Mississippi, 6402-21310-003-00 and project of the Agricultural Research Service.

Abbreviations

CpG	Cytosine phosphate guanine
CGI	CpG island
<i>dnmt</i>	DNA methyltransferase
hpf	Hours post fertilization
HCP	High CpG promoter
ICP	Intermediate CpG promoter
PGCs	Primordial germ cells
<i>gnmt</i>	Glycine N-methyltransferase
LCP	Low CpG promoter
<i>rassf1</i>	Ras association domain family member 1
<i>tert</i>	Telomerase reverse transcriptase
TSS	Transcriptional start site

References

- Andersen IS, Reiner AH, Aanes H, Alestrom P, Collas P. Developmental features of DNA methylation during activation of the embryonic zebrafish genome. *Genome Biol.* 2012; 13(7):R65. [PubMed: 22830626]
- Anchelin M, Murcia L, caraz-Perez F, Garcia-Navarro EM, Cayuela ML. Behaviour of telomere and telomerase during aging and regeneration in zebrafish. *PLoS One.* 2011; 6:e16955. [PubMed: 21347393]

- Aniagu SO, Williams TD, Allen Y, Katsiadaki I, Chipman JK. Global genomic methylation levels in the liver and gonads of the three-spine stickleback (*Gasterosteus aculeatus*) after exposure to hexabromocyclododecane and 17-beta oestradiol. *Environ Int.* 2008; 34:310–317. [PubMed: 17482264]
- Anway MD, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology.* 2006; 147:S43–S49. [PubMed: 16690803]
- Athanasiadou R, de SD, Myant K, Merusi C, Stancheva I, Bird A. Targeting of de novo DNA methylation throughout the Oct-4 gene regulatory region in differentiating embryonic stem cells. *PLoS One.* 2010; 5:e9937. [PubMed: 20376339]
- Barbot W, Dupressoir A, Lazar V, Heidmann T. Epigenetic regulation of an IAP retrotransposon in the aging mouse: progressive demethylation and de-silencing of the element by its repetitive induction. *Nucleic Acids Res.* 2002; 30:2365–2373. [PubMed: 12034823]
- Bauer AP, Leikam D, Krinner S, Notka F, Ludwig C, Langst G, Wagner R. The impact of intragenic CpG content on gene expression. *Nucleic Acids Res.* 2010; 38:3891–3908. [PubMed: 20203083]
- Bennett-Baker PE, Wilkowski J, Burke DT. Age-associated activation of epigenetically repressed genes in the mouse. *Genetics.* 2003; 165:2055–2062. [PubMed: 14704185]
- Bjornsson HT, Sigurdsson MI, Fallin MD, Irizarry RA, Aspelund T, Cui H, Yu W, Rongione MA, Ekstrom TJ, Harris TB, Launer LJ, Eiriksdottir G, Leppert MF, Sapienza C, Gudnason V, Feinberg AP. Intra-individual change over time in DNA methylation with familial clustering. *JAMA.* 2008; 299:2877–2883. [PubMed: 18577732]
- Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, Baccarelli A. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech Ageing Dev.* 2009; 130:234–239. [PubMed: 19150625]
- Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci U S A.* 2000; 97:9585–9590. [PubMed: 10920202]
- Daniel M, Peek GW, Tollefsbol TO. Regulation of the human catalytic subunit of telomerase (hTERT). *Gene.* 2012; 498:135–146. [PubMed: 22381618]
- Duan J, Antezana MA. Mammalian mutation pressure, synonymous codon choice, and mRNA degradation. *J Mol Evol.* 2003; 57:694–701. [PubMed: 14745538]
- Eferl R, Sibilina M, Hilberg F, Fuchsbichler A, Kufferath I, Guertl B, Zenz R, Wagner EF, Zatloukal K. Functions of c-Jun in liver and heart development. *J Cell Biol.* 1999; 145:1049–1061. [PubMed: 10352021]
- Fang X, Thornton C, Scheffler BE, Willett KL. Benzo[a]pyrene decreases global and gene specific DNA methylation during zebrafish development. *Environ Toxicol Pharmacol.* 2013; 36:40–50. [PubMed: 23542452]
- Fatemi M, Hermann A, Gowher H, Jeltsch A. Dnmt3a and Dnmt1 functionally cooperate during de novo methylation of DNA. *Eur J Biochem.* 2002; 269:4981–4984. [PubMed: 12383256]
- Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA.* 2005; 102:10604–10609. [PubMed: 16009939]
- Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, Noce T. Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila vasa* and its specific expression in germ cell lineage. *Proc Natl Acad Sci USA.* 1994; 91:12258–12262. [PubMed: 7991615]
- Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, Miyazaki T, Ogura C, Okazaki Y, Jinno Y. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Ann Hum Genet.* 2004; 68:196–204. [PubMed: 15180700]
- Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. *J Mol Biol.* 1987; 196:261–282. [PubMed: 3656447]
- Goll MG, Halpern ME. DNA methylation in zebrafish. *Prog Mol Biol Transl Sci.* 2011; 101:193–218. [PubMed: 21507352]

- Graessmann A, Sandberg G, Guhl E, Graessmann M. Methylation of single sites within the herpes simplex virus tk coding region and the simian virus 40 T-antigen intron causes gene inactivation. *Mol Cell Biol.* 1994; 14:2004–2010. [PubMed: 7509450]
- Gravina S, Vijg J. Epigenetic factors in aging and longevity. *Pflugers Arch.* 2010; 459:247–258. [PubMed: 19768466]
- Gruenbaum Y, Stein R, Cedar H, Razin A. Methylation of CpG sequences in eukaryotic DNA. *FEBS Lett.* 1981; 124:67–71. [PubMed: 7215556]
- Haaf T. Methylation dynamics in the early mammalian embryo: implications of genome reprogramming defects for development. *Curr Top Microbiol Immunol.* 2006; 310:13–22. [PubMed: 16909904]
- Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assuncao JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Meida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliott D, Threadgold G, Harden G, Ware D, Mortimer B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthavadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Uzun Z, Eser C, Geiger H, Geisler M, Karotki L, Kirm A, Konantz J, Konantz M, Oberlander M, Rudolph-Geiger S, Teucke M, Osogawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LL, Postlethwait JH, Nusslein-Volhard C, Hubbard TJ, Roest CH, Rogers J, Stemple DL. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* 2013; 496:498–503. [PubMed: 23594743]
- Kappelmann M, Kuphal S, Meister G, Vardimon L, Bosserhoff AK. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression. *Oncogene.* 2012
- Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? *Trends Genet.* 1997; 13:444–449. [PubMed: 9385841]
- Keshet I, Yisraeli J, Cedar H. Effect of regional DNA methylation on gene expression. *Proc Natl Acad Sci USA.* 1985; 82:2560–2564. [PubMed: 3857599]
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995; 203:253–310. [PubMed: 8589427]
- Kyo S, Takakura M, Fujiwara T, Inoue M. Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. *Cancer Sci.* 2008; 99:1528–1538. [PubMed: 18754863]
- Lees-Murdock DJ, Walsh CP. DNA methylation reprogramming in the germ line. *Epigenetics.* 2008; 3:5–13. [PubMed: 18259118]
- Li Q, Zhu F, Chen P. miR-7 and miR-218 epigenetically control tumor suppressor genes RASSF1A and Claudin-6 by targeting HoxB3 in breast cancer. *Biochem Biophys Res Commun.* 2012; 424:28–33. [PubMed: 22705304]
- Lindeman LC, Winata CL, Aanes H, Mathavan S, Alestrom P, Collas P. Chromatin states of developmentally-regulated genes revealed by DNA and histone methylation patterns in zebrafish embryos. *Int J Dev Biol.* 2010; 54:803–813. [PubMed: 20336603]
- Lister R, Pelizzola M, Downen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Ntosiwicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker

- JR. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009; 462:315–322. [PubMed: 19829295]
- Lopatina N, Haskell JF, Andrews LG, Poole JC, Saldanha S, Tollefsbol T. Differential maintenance and de novo methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts. *J Cell Biochem*. 2002; 84:324–334. [PubMed: 11787061]
- MacKay AB, Mhanni AA, McGowan RA, Krone PH. Immunological detection of changes in genomic DNA methylation during early zebrafish development. *Genome*. 2007; 50:778–785. [PubMed: 17893737]
- Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T, Costello JF. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature*. 2010; 466:253–257. [PubMed: 20613842]
- Mayer W, Fundele R, Haaf T. Spatial separation of parental genomes during mouse interspecific (*Mus musculus* x *M. spretus*) spermiogenesis. *Chromosome Res*. 2000; 8:555–558. [PubMed: 11032324]
- McGowan RA, Martin CC. DNA methylation and genome imprinting in the zebrafish, *Danio rerio*: some evolutionary ramifications. *Biochem Cell Biol*. 2002; 75:499–506. [PubMed: 9551175]
- Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*. 2008; 454:766–770. [PubMed: 18600261]
- Mhanni AA, McGowan RA. Global changes in genomic methylation levels during early development of the zebrafish embryo. *Dev Genes Evol*. 2004; 214:412–417. [PubMed: 15309635]
- Mohn F, Weber M, Rebhan M, Roloff TC, Richter J, Stadler MB, Bibel M, Schubeler D. Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. *Mol Cell*. 2008; 30:755–766. [PubMed: 18514006]
- Peters I, Rehmet K, Wilke N, Kuczyk MA, Hennenlotter J, Eilers T, Machtens S, Jonas U, Serth J. RASSF1A promoter methylation and expression analysis in normal and neoplastic kidney indicates a role in early tumorigenesis. *Mol Cancer*. 2007; 6:49. [PubMed: 17634119]
- Rauch TA, Wu X, Zhong X, Riggs AD, Pfeifer GP. A human B cell methylome at 100-base pair resolution. *Proc Natl Acad Sci USA*. 2009; 106:671–678. [PubMed: 19139413]
- Razin A, Riggs AD. DNA methylation and gene function. *Science*. 1980; 210:604–610. [PubMed: 6254144]
- Richter AM, Pfeifer GP, Dammann RH. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta*. 2009; 1796:114–128. [PubMed: 19344752]
- Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. *Gene*. 2005; 345:139–153. [PubMed: 15716030]
- Sanz LA, Kota SK, Feil R. Genome-wide DNA demethylation in mammals. *Genome Biol*. 2010; 11:110. [PubMed: 20236475]
- Shi L, Wu J. Epigenetic regulation in mammalian preimplantation embryo development. *Reprod Biol Endocrinol*. 2009; 7:59. [PubMed: 19500360]
- Shimoda N, Yamakoshi K, Miyake A, Takeda H. Identification of a gene required for de novo DNA methylation of the zebrafish no tail gene. *Dev Dyn*. 2005; 233:1509–1516. [PubMed: 15937923]
- Soucek L, Evan GI. The ups and downs of Myc biology. *Curr Opin Genet Dev*. 2010; 20:91–95. [PubMed: 19962879]
- Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA*. 2002; 99:3740–3745. [PubMed: 11891299]
- Varriale A, Bernardi G. DNA methylation and body temperature in fishes. *Gene*. 2006; 385:111–121. [PubMed: 17067764]
- Walter RB, Li HY, Intano GW, Kazianis S, Walter CA. Absence of global genomic cytosine methylation pattern erasure during medaka (*Oryzias latipes*) early embryo development. *Comp Biochem Physiol B Biochem Mol Biol*. 2002; 133:597–607. [PubMed: 12470822]
- Wang SC, Oelze B, Schumacher A. Age-specific epigenetic drift in late-onset Alzheimer's disease. *PLoS One*. 2008; 3:e2698. [PubMed: 18628954]

- Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, Schubeler D. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet.* 2007; 39:457–466. [PubMed: 17334365]
- Wilson VL, Smith RA, Ma S, Cutler RG. Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem.* 1987; 262:9948–9951. [PubMed: 3611071]
- Wu SF, Zhang H, Hammoud SS, Potok M, Nix DA, Jones DA, Cairns BR. DNA methylation profiling in zebrafish. *Methods Cell Biol.* 2011; 104:327–339. [PubMed: 21924171]
- Yamaguchi K, Lantowski A, Dannenberg AJ, Subbaramaiah K. Histone deacetylase inhibitors suppress the induction of c-Jun and its target genes including COX-2. *J Biol Chem.* 2005; 280:32569–32577. [PubMed: 15994313]
- Zemach A, McDaniel IE, Silva P, Zilberman D. Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science.* 2010; 328:916–919. [PubMed: 20395474]
- Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes Dev.* 1999; 13:1924–1935. [PubMed: 10444591]
- Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S. Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nat Genet.* 2007; 39:61–69. [PubMed: 17128275]

Figure 1. Constitutive global DNA methylation changes during zebrafish development. Each bar $\pm$ SEM represents $n = 3$ (Bars with different letters are significantly different, $p < 0.05$).

Figure 2.

Constitutive *dnmt1* (A) and *gnm1* (B) expression during zebrafish embryogenesis measured by qPCR. Expression of each gene was normalized to internal control 18S rRNA and relative to the expression level in unfertilized eggs. Developmental stages corresponding to time-points are indicated on top of the bars (Kimmel et al., 1995). Panel C shows the ratio of *dnmt1/gnm1* mRNA expression. The dotted line in C indicates ratio=1 (equal expression of the two genes). Bars with different letters are significantly different ($p < 0.05$, $n = 3$ pools, 20–30 embryos/pool).

Figure 3.
Schematic figure of the sodium bisulfite sequencing regions for the five target genes.

Figure 4. Constitutive zebrafish *vasa* promoter methylation percentage (A) and mRNA expression (B). Expression of *vasa* mRNA was measured by qPCR, normalized to 18S rRNA and relative to the expression level at 96 hpf (n = 3 pools). Bars with different letters are significantly different. Panel C is the maximal response curves for *vasa de novo* methylation and mRNA expression during zebrafish embryogenesis. The Pearson correlation coefficient of *vasa* promoter methylation with gene expression was -0.885 ($p < 0.00001$).

Figure 5. Constitutive CpG methylation percentage (A) in zebrafish *rassf1* CGI-1 (first promoter; n=3 pools). Panel B is constitutive CpG methylation percentage in zebrafish *rassf1* CGI-2 (second promoter; n=3 pools; * p<0.05).

Figure 6.

Constitutive mRNA expression of *rassf1-001* (A) and total *rassf1* (B) in zebrafish. The percentage of *rassf1-001* in total *rassf1* is shown in (C). Expression of each gene was normalized to 18S rRNA and relative to 3.3 hpf expression level. Bars with different letters are statistically significantly different (n= 3 pools; p<0.05).

Figure 7. Constitutive zebrafish methylation percentage of *tert* (A), *c-jun* (C), *c-myca* (E) and mRNA expression of *tert* (B), *c-jun* (D), *c-myca* (F). Gene expression was measured by qPCR, normalized to 18S rRNA and relative to 3.3 hpf expression level. Bars with different letters are statistically significantly different (n= 3 pools; p<0.05).

Figure 8.

Comparison of DNA methylation (A, C, E) and gene expression (B, D, F) at 3.3 hpf, 96 hpf and adult livers. For DNA methylation graphs, bars represent CpG methylation percentage. For mRNA expression graphs, bars represent expression of genes which were normalized to internal control 18S rRNA and relative to *tert* expression at the same time-point. Bars with different letters are statistically significantly different (n= 3 pools; P<0.05).

Table 1

Candidate genes in the bisulfite sequencing study.

Gene	Chromosome	Region of interest	Within CpG island?	CpG sites	Gene category
<i>rassf1</i>	22	-179 to +135 cross the first promoter	Yes	26	Tumor suppressor gene
<i>rassf1</i>	22	-123 to +315 cross the second promoter	Yes	17	Tumor suppressor gene
<i>vasa</i>	10	-369 to -163 in 5' promoter	No	5	PGC marker
<i>tert</i>	19	-1136 to -904 in 5' promoter	Yes	13	Potential proto-oncogene
<i>c-jun</i>	20	+970 to +1394 in exon 1	Yes	36	Proto-oncogene
<i>c-myc</i>	24	+517 to +960 in exon 2	Yes	26	Proto-oncogene

Table 2

CG loss in our zebrafish.

Gene	SNPs	Prevalence	Locations
<i>rassf1</i> CGI 2	CG/TT	63.8/36.2%	+2060, +2061
<i>tert</i>	CG/TA	0/100%	−1017, −1016
<i>c-myc</i>	CG/TT	19.0/81.0%	+931, +932

Locations numbered forward or back from transcription start site (+1).

Table 3

Analysis of target CpG islands investigated in this study.

Gene	Length (bp)	C+G%	CpG%	CpG ratio ^a	Type of CGI ^b
<i>rassf1</i> CGI 1	552	48.37	7.44	1.28	ICP
<i>rassf1</i> CGI 2	438	50.91	3.89	0.60	ICP
<i>tert</i>	970	46.49	3.51	0.65	ICP
<i>c-jun</i>	1610	55.47	7.02	0.92	HCP
<i>c-myc</i>	920	51.63	4.68	0.70	ICP
<i>vasa</i>	212	32.08	2.36	0.92	LCP

^aThe CpG ratio was calculated as the (number of CpGs X number of base pairs)/(number of Cs X number of Gs).

^bAs assigned based on Weber et al., 2007.