

# Decrease in cytosine methylation at CpG island shores and increase in DNA fragmentation during zebrafish aging

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**Abstract** Age-related changes in DNA methylation have been demonstrated in mammals, but it remains unclear as to the generality of this phenomenon in vertebrates, which is a criterion for the fundamental cause of senescence. Here we showed that the zebrafish genome gradually and clearly lost methylcytosine in somatic cells, but not in male germ cells during aging, and that

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age-dependent hypomethylation preferentially occurred at a particular domain called the CpG island shore, which is associated with vertebrates' genes and has been shown to be hypomethylated in humans with age. We also found that two CpG island shores hypomethylated in zebrafish oocytes were de novo methylated in fertilized eggs, which suggests that the zebrafish epigenome is reset upon fertilization, enabling new generations to restart with a heavily methylated genome. Furthermore, we observed an increase in cleavage of the zebrafish genome to an oligonucleosome length in somatic cells from the age of 12 months, which is suggestive of an elevated rate of apoptosis in the senescent stage.

**Keywords** Aging · Zebrafish · DNA methylation · CpG island shore

## Introduction

The majority of cytosines in CpG dinucleotides in vertebrate genomes are methylated at the 5 position by cytosine methyltransferases, except for CpGs in the CpG-rich region called the “CpG island,” which often encompasses the promoters and transcription start sites of many vertebrate genes (Illingworth and Bird 2009). Most promoter region CpG islands are in an unmethylated state irrespective of transcriptional activity, while a small fraction of these is densely methylated and associated with gene silencing (Shen et al. 2007). The artificial methylation of CpG island promoters was shown to repress transcription in living cells (Stein et al. 1982),

and the demethylation of endogenous methylated CpG islands using DNA methyltransferase inhibitors restored the expression of the gene (Hansen and Gartler 1990). Thus, the aberrant methylation or demethylation of CpG islands can be a cause of gene misregulation and has been attributed to human pathogenic conditions represented by cancers (Jones and Baylin 2007). Similarly, changes in DNA methylation have been considered to be a cause of senescence since the Vanyushin group first found an age-related decrease in methylcytosine levels in the somatic tissues of the salmon (Berdishev et al. 1967). This phenomenon was later shown to be true for cellular senescence and mammalian aging (Wilson and Jones 1983). Subsequently, with the advent of bisulfite sequencing, age-related methylation changes have been located at distinctive positions in mammalian genomes: certain CpG islands were more likely to be methylated with age (Christensen et al. 2009; Hernandez et al. 2011), whereas age-related hypomethylation was observed at the CpG island “shore” region of a comparatively low CpG density that is present near traditional CpG islands, typically within 2 kb (Alisch et al. 2012; Pirazzini et al. 2012; Heyn et al. 2012). CpG island shores were first recognized as regions showing human tissue- and cancer-specific methylation (Irizarry et al. 2009). Besides CpG island shores, several reports have described age-related hypomethylation at repetitive sequences in human blood samples (Bollati et al. 2009; Jintaridh and Mutirangura 2010; Gentilini et al. 2012). Furthermore, a comprehensive study that performed whole genome bisulfite sequencing of a centenarian and newborn showed that cytosines unmethylated in the centenarian, but not in the newborn, covered all genomic compartments, such as exonic, intronic, and intergenic regions (Heyn et al. 2012). Thus, loci showing age-related methylation changes appear to be scattered in mammalian genomes, and it is still elusive if any specific hyper- or hypomethylated domains exist relevant to senescence. In addition, it remains unclear if age-related methylation changes are widely seen in vertebrates, which will be a requirement for the fundamental cause of senescence. To address these issues, we examined the zebrafish, *Danio rerio*, in this study based on the indication that if a certain type(s) of methylation change exists that is primarily involved in senescence, it should be seen in the lower vertebrate, and such a change(s) may be more easily found by comparing distantly related species rather than closely related ones. Other merits of utilizing fish included the availability

of basic information regarding zebrafish senescence (Kishi et al. 2003; Tsai et al. 2007) and possibilities for future genetic and pharmacological manipulations. We did not detect global hypomethylation in the zebrafish genome during development, which had been previously shown (Macleod et al. 1999); however, we detected a clear loss of methylation at the CpG island shores of some genes after the developmental stage in the tissues of aging zebrafish. Thus, methylation changes in this domain may be directly involved in vertebrate senescence. Furthermore, we observed genome DNA fragmentation in zebrafish of an advanced age, a likely result of apoptosis, which is another phenomenon also observed in aged mammals (Tagliabue et al. 1996; Itzhaki et al. 2003).

## Experimental procedures

### Zebrafish maintenance

Zebrafish of the wild-type AB/Tü were used throughout this study. Fish at the same stage were derived from a single clutch of eggs. Fish were bred naturally and early stage fry were fed with paramecia, and then with living brine shrimp three times per day. Water temperature was maintained at 28.5 °C with a 14-h light/10-h dark cycle in a central fish facility. At around 10 weeks of age, male and female fish were separately housed at a density of ~20 fish per 3-l glass tank. They were crossed twice per month on average from 3 to 4 months old until reproductive activity declined (12~15 months old).

### Preparation of genomic DNA

Zebrafish embryos and adult tissues were digested with Proteinase K and SDS to extract genomic DNA. Zebrafish oocytes were obtained by gently pushing the female's belly with our fingers. Follicle cells were carefully removed using forceps under a dissecting microscope. Chorions were removed by Pronase E before the isolation of genomic DNA from fertilized eggs and cleavage stage (128-cell) embryos (Westerfield 2000).

### Methylation analysis

After digestion with methylation-sensitive enzymes, 100 ng of DNA fragments were separated on 1.2 % agarose gels with one or two DNA size markers (New

England Biolabs, Ipswich, MA, USA), stained with ethidium bromide (0.5 µg/ml), and then destained in water. All enzymes used were purchased from New England Biolabs. For bisulfite sequencing, 200 ng of genomic DNA was processed using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) to convert unmethylated cytosines into uracils. We used the MethPrimer program to choose sequences of primer pairs for bisulfite sequencing (Li and Dahiya 2002). Primer sequences and PCR conditions are shown in the Supplemental Table. PCR fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and were used for the transformation of *Escherichia coli*. Plasmids isolated from 24 transformants were sequenced with the DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Piscataway, NJ, USA) on an ABI 310 genetic analyzer or ABI3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). QUMA was used for the analysis of sequence data and drawing figures (Kumaki et al. 2008).

#### Statistical analysis

All analyses were performed using Prism 5 software (GraphPad Software, San Diego, CA, USA). The methylation level of each amplicon was the ratio of methylated cytosines to the total number of CpG dinucleotides present in 20 or more clones. The Kruskal–Wallis test was used to make nonparametric comparisons of the median methylation levels of a region to be investigated between more than two groups. A two-tailed *t* test was employed for comparisons between two groups.

#### Southwestern hybridization for the detection of DNA fragmentation

One hundred nanograms of genomic DNA was labeled with 50 pmol of digoxigenin (DIG)-dUTP (Roche Diagnostics, Mannheim, Germany) by 0.5 U of Klenow fragment (3'→5' exo<sup>-</sup>) (New England Biolabs) in a volume of 4 µl (10 mM Tris–HCl, 5 mM MgCl<sub>2</sub>, 7.5 mM DTT, pH 7.5 at 25 °C) at 37 °C for 15 min. After the addition of 1 µl of 5× sample loading buffer (25 % glycerol, 5 mM EDTA, pH 8.0, 0.5 % SDS), the reaction mixture was directly loaded onto a 2 % agarose gel and run at 50 V for 60 min. The gel was stained with EtBr for 10 min, and then rinsed in water. DNA was transferred and fixed onto Biodyne B (Pall, Port Washington, NY, USA) by Stratalinker UV Crosslinker (Agilent Technologies,

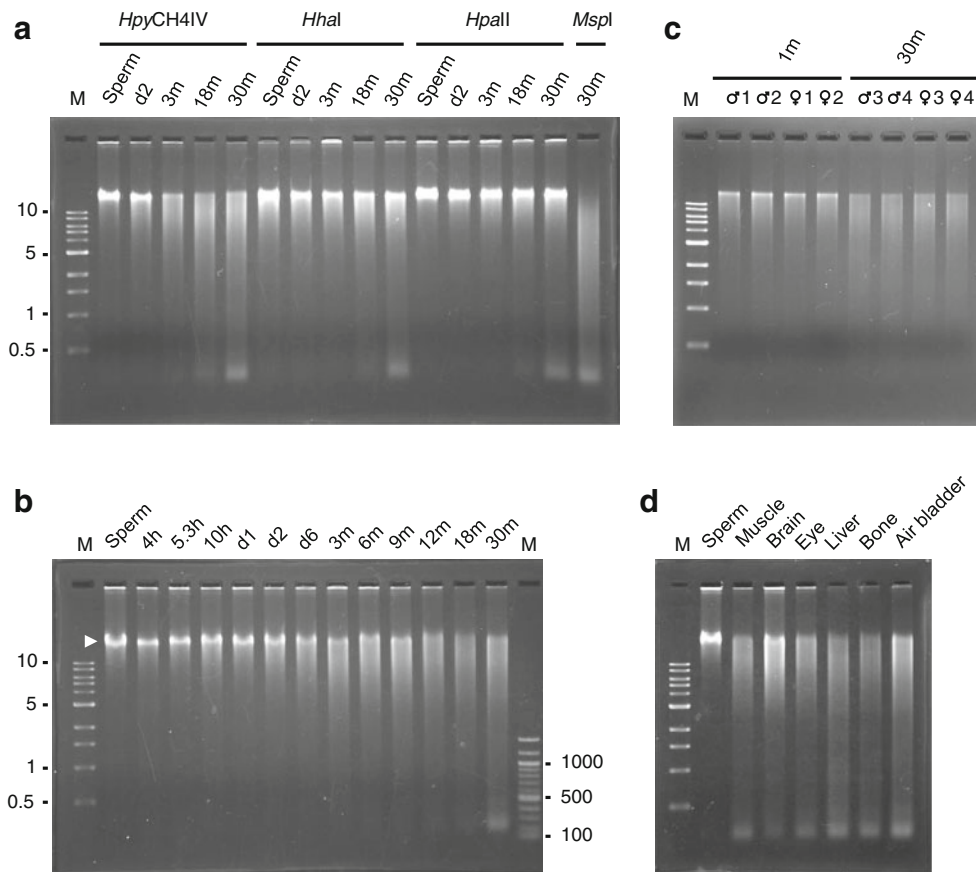
Santa Clara, CA, USA). The membrane was rinsed with 2× SSC, and DIG-labeled DNA fragments were detected by an anti-DIG antibody conjugated with alkaline phosphatase (Roche Diagnostics) following the manufacturer's instructions.

## Results

### Changes in global methylation levels in the zebrafish genome with age

To examine if global methylation levels in the zebrafish genome changed with aging, we extracted genomic DNA from the sperm, 2-day post fertilization (d2) embryos, and muscle of 3-, 18-, and 30-month-old fish. We regarded 30-month-old zebrafish as aged fish since they live for approximately 3 years on average in conventional aquarium systems including ours (Tsai et al. 2007). Muscle was chosen as a representative of somatic cells since it is a predominant structure of the fish species. Sperm was extracted from the same 30-month-old fish from which muscle was isolated. DNA was digested with each of three methylation-sensitive restriction enzymes, *Hpy*CH4IV, *Hha*I, and *Hpa*II, neither of which was able to cut at each recognition site if there was a methylated cytosine inside. Digested DNA was then run on an agarose gel and stained with ethidium bromide (Fig. 1a). One of the enzymes, *Hpy*CH4IV, whose recognition site is ACGT, digested the genomes of adult fish (3 months or older) more frequently than those of sperm and d2 embryos, as judged by DNA smearing patterns on gels. Furthermore, the enzyme cleaved the genomes of 18- and 30-month-old fish slightly more frequently than the genomes of 3-month-old fish. These results indicated that age-dependent genomic hypomethylation occurred in zebrafish somatic cells, but not in male germ cells.

*Hha*I, whose recognition site is GCGC, also cleaved the genomic DNA of 18- and 30-month-old fish slightly more than that of 3-month-old fish. However, this enzyme did not reveal an appreciable difference in digestion patterns between embryos and 3-month-old fish. The other enzyme, *Hpa*II, whose recognition site is CCGG, detected little increase in DNA cleavage with age. This was consistent with the result of a previous study in which *Hpa*II was used to investigate differences in global DNA methylation between zebrafish embryos and adults (Macleod et al. 1999). These results showed



**Fig. 1** Age-dependent DNA hypomethylation in the zebrafish genome. **a** The genomic DNA of zebrafish sperm, 2-day post fertilization embryos (d2), and muscle of 3-, 12-, and 30-month-old fish (3 m, 12 m, and 30 m, respectively) were digested with each of the three methylation-sensitive restriction enzymes indicated and were run on an agarose gel. *HpyCH4IV* digestion clearly showed age-dependent hypomethylation. *MspI*, an isoschizomer of *HpaII*, is a methylation-insensitive enzyme. **b** Genomic DNAs of sperm, embryos (h; hours post fertilization), and adult muscle of the indicated ages were digested with *HpyCH4IV*. Hypomethylation was recognizable as early as d2

and became evident at d6 (larval stage). A high molecular weight DNA band was seen until 9 months (lanes 2~11, white arrowhead). No discernible difference in the smearing pattern was observed after 12 months (lanes 12~14). **c** Liver DNA from four individuals of 1- and 30-month-old zebrafish were digested and electrophoresed as in **b**. **d** Genomic DNA isolated from various tissues of an aged zebrafish (30-month-old) was digested with *HpyCH4IV* and run on an agarose gel. The molecular marker of DNA loaded on the first lane of each gel was a 1-kb ladder. The 100-bp DNA ladder was also loaded on the last lane in **b**

that age-related demethylation events were governed by the immediate nucleotide composition, and it should be noted that age-demethylated CpG dinucleotides in humans were preferably preceded and followed by A and T, respectively (Alisch et al. 2012), which constitutes the tetranucleotide digested by *HpyCH4IV*.

To trace age-dependent hypomethylation in detail, we prepared DNA from the embryos of several developmental stages and muscle of aging adult fish at various stages and digested them with *HpyCH4IV* (Fig. 1b). We found that the genome was heavily

methylated in early zebrafish embryos, from the sphere stage (4 hpf—hours post fertilization) to d1. Hypomethylation of the genome could be recognized as early as d2, the end of the developmental stage, and became evident by d6, the early larval stage. Hypomethylation of the genome then appeared to proceed gradually until 12 months, the stage when high molecular weight fragments became obscure, after which no further digestion was discernible by smearing patterns on agarose gels. As shown in Fig. 1c, the liver DNAs of 30-month-old fish were cleaved more

than those of 1-month-old fish, indicating that age-related hypomethylation was not restricted to muscle. We next compared the methylation levels of different tissues in a single 30-month-old zebrafish by digestion with *Hpy*CH4IV (Fig. 1d). DNA from somatic tissues was cleaved as much as muscle DNA, except for brain DNA, which was cleaved less than the others. In contrast to somatic DNA, the sperm genome appeared to be resistant to digestion.

#### Changes in regional methylation levels of the zebrafish genome with age

We first suspected that the regions of genomic hypomethylation that we detected may be located at repetitive sequences since age-dependent hypomethylation at repeats have been found in human blood samples (Bollati et al. 2009; Jintaridith and Mutirangura 2010; Gentilini et al. 2012). To examine this possibility, we performed bisulfite sequence analysis against four short interspersed nuclear elements (SINEs) in zebrafish (Supplementary Fig. S1): three individual DANA elements (Izsvák et al. 1996; Shimoda et al. 1996) and a conserved portion of SINE3-1a elements (Kapitonov and Jurka 2003). To compare interindividual differences in methylation levels and to test the significance of methylation changes between groups of different stages, we purified genomic DNA from four individual embryos (sex was unknown) and four adult fish (two males and two females) and analyzed at least 20 clones per region from each of four embryos or adult fish, which came to at least 80 clones per region in total. However, all the repeats examined were heavily methylated both in embryos and in aged fish (Supplementary Fig. S1).

We then turned our attention to unique regions of the genome and noticed that one of our previous studies, which was undertaken for another purpose, had recorded lower methylation levels at a region spanning exons 7 and 8 (exon 7~8) in the eukaryotic translation elongation factor 1  $\alpha$ 1, like 1 (commonly referred to as *EF1 $\alpha$* ) gene in adult zebrafish (67 %) than those at the same region in embryos (97 %) (Yamakoshi and Shimoda 2003). This prompted us to analyze the methylation levels of the *EF1 $\alpha$*  locus in embryos and adult fish of different ages by bisulfite sequencing (Fig. 2 and Supplementary Fig. S2a, b). Results showed that apparent age-related hypomethylation occurred in a region that flanks a CpG island located ~1 kb downstream of the *EF1 $\alpha$*  gene (Fig. 2). Such a region was defined as the “CpG island

shore” (Irizarry et al. 2009). No clear decline in methylation with age was observed in regions outside the CpG island shore region (Supplementary Fig. S2a, b). A slight decrease in methylation was already observed in the region of the d6 larvae relative to d2 embryos (Fig. 2). This was in accordance with the *Hpy*CH4IV digestion results of the genome that showed decreases in global methylation levels between d2 and d6 (Fig. 1b). In contrast to the 3' CpG island shore, the shore region of the promoter-associated 5' CpG island showed no age-related methylation changes (Supplementary Fig. S2a).

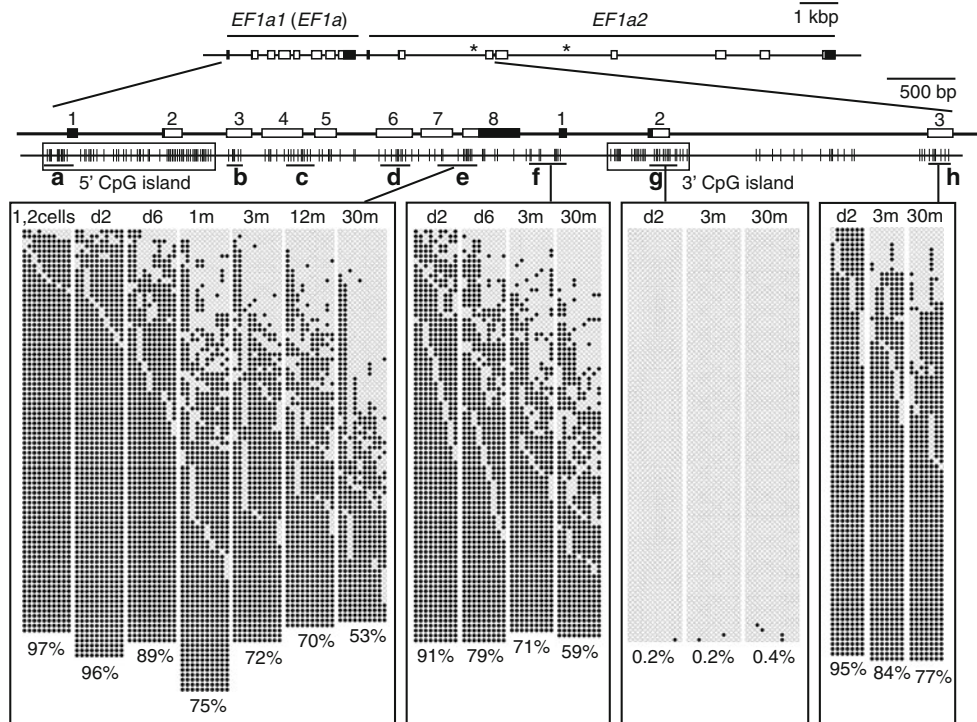
#### Presence of a tandemly duplicated *EF1 $\alpha$* homologue

It is possible that the 3' CpG island was a CpG island of another gene localized closely to *EF1 $\alpha$* . Indeed, the GenBank database annotated an *EF1 $\alpha$*  homologue immediately downstream of the *EF1 $\alpha$*  gene, eukaryotic translation elongation factor 1  $\alpha$ 1, like 2, which we referred to as *EF1 $\alpha$ 2*, and it was clear that the 3' CpG island of the locus was an authentic CpG island of *EF1 $\alpha$ 2* (Fig. 2 and Supplementary Fig. S2a, b). Hereafter, we referred to upstream *EF1 $\alpha$*  as *EF1 $\alpha$ 1*.

Two isoforms of *EF1 $\alpha$*  have been identified in mammals and are referred to as eEF1A-1 (eukaryotic elongation factor) and eEF1A-2. eEF1A-1 is expressed ubiquitously as is zebrafish *EF1 $\alpha$ 1*, whereas eEF1A-2 is specifically expressed in the muscle and heart and weakly in the brain (Khalyfa et al. 2001). Since zebrafish *EF1 $\alpha$ 2* is highly expressed in muscle but not in the liver (data not shown), *EF1 $\alpha$ 2* appears to be the orthologue of mammalian eEF1A-2.

#### Age-dependent hypomethylation incident to CpG island shores

We then examined if age-related hypomethylation occurred at other CpG island shores in muscle. For this analysis, four single-copy zebrafish genes were randomly chosen: *fli1a* (friend leukemia integration), *fgf3* (fibroblast growth factor),  *$\beta$ -actin2*, and *rpl13a* (ribosomal protein L13a). *fli1a* and *fgf3* are tissue-specific genes that are exclusively expressed in the vasculature and subsets of neurons in the brain in adult zebrafish, respectively (Lawson and Weinstein 2002; Topp et al. 2008). A CpG island overlapped with exon 1 of *fli1a*, free of methylation at both embryonic and adult stages (Fig. 3). In contrast, a region in intron 1 of *fli1a*, which corresponds to the CpG island shore region



**Fig. 2** Age-dependent hypomethylation at a CpG island shore region in the *EF1α* locus. The methylation levels of various regions in the zebrafish *EF1α* locus in fertilized eggs (1, 2 cells), embryos (d2), young larvae (d6), and muscle of immature (1-month-old) and adult fish (3-, 12-, and 30-month-old) were analyzed by bisulfite sequencing. Schematics of *EF1α1* (*EF1α*) and *EF1α2* are shown at the top. Asterisks represent positions where unread sequences in the Sanger database were determined in this study. Filled and open rectangles indicate untranslated and translated regions, respectively, and numbers on exons are also shown. Vertical lines indicate the positions of CpG dinucleotides. Black horizontal bars with small letters indicate the regions analyzed. Filled and open circles show methylated and

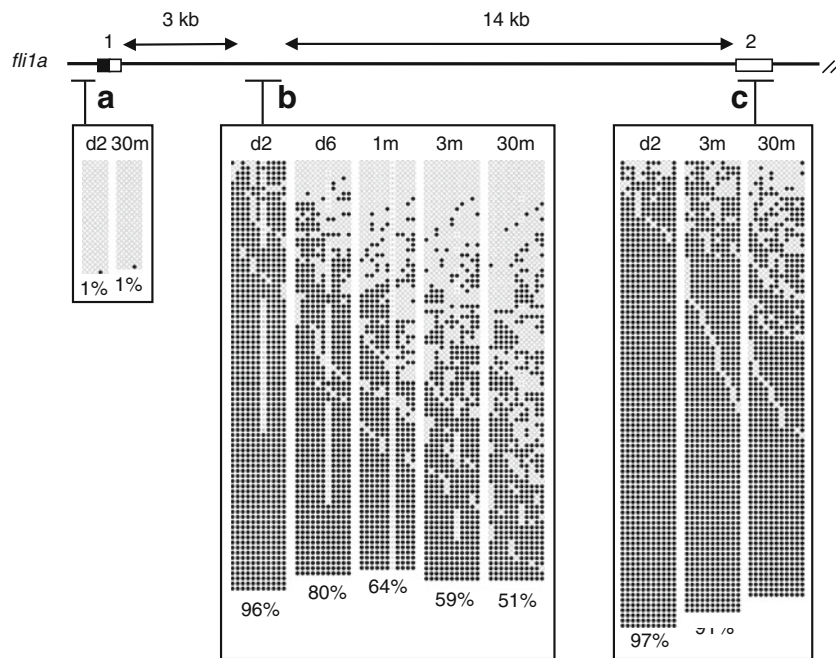
unmethylated cytosines, respectively. Crosses indicate the positions where CpG was absent due to DNA polymorphism. A total of four single embryos, four larvae, and four adult fish were analyzed to obtain methylation profiles for each region, and at least 20 clones were sequenced per fish for each region. In addition to a conventional CpG island that covered exons 1 and 2 of the *EF1α1* gene (5' CpG island, Fig. S2A), there was another CpG island downstream of the gene (3' CpG island). Age-related hypomethylation was observed at a limited region juxtaposed to the 3' CpG island, a region called the "CpG island shore." The percentages of mean methylation levels were shown at the bottom

and was heavily methylated at the embryonic stage, showed a marked reduction in methylation in d6 larvae, which gradually became pronounced with aging (Fig. 3). Differences in methylation levels in this region were significant ( $p < 0.05$ ) between groups consisting of four 1-month-old and four 30-month-old fish (Fig. 4). Exon 2 of *flil1a* was heavily methylated irrespective of age.

The second gene, *fgf3*, had a typical CpG island at the 5' region that covers exons 1 and 2 (Supplementary Fig. S3; Yamakoshi and Shimoda 2003). Although exon 3 of *fgf3* was located within 500 bp of exon 2, it was heavily methylated in embryos. We found that an upstream part of exon 3, which corresponded to the

CpG island shore, showed approximately a 20 % reduction in methylation in muscle cells by 3 months. This reduction was less than that observed in the CpG island shores of *EF1α2* and *flil1a*, and no further hypomethylation was observed in the muscle of 30-month-old fish. No significant hypomethylation was detected in the middle part of exon 3.

The third gene, *β-actin2*, which encodes a cytoskeletal structural protein, had a cluster of CpG dinucleotides at the 5' region that constituted a CpG island (Supplementary Fig. S4). We found that exon 3, where CpG dinucleotides were sparsely distributed, was heavily methylated in d2 embryos, but showed approximately a 50 % reduction in methylation in muscle cells by



**Fig. 3** Age-dependent hypomethylation at a CpG island shore region in the *flil1a* locus. The methylation levels of three regions in the zebrafish *flil1a* locus in embryos (d2), young larvae (d6), and muscle of immature (1-month-old) and adult fish (3- and 30-month-old) were analyzed and presented as in Fig. 2. Regions a, b, and c correspond to the CpG island, CpG island

shore, and non-CpG island, respectively. Only a single embryo and an adult fish were analyzed for region a. A total of four single embryos and four fish were analyzed to obtain methylation profiles for regions b and c. At least 20 clones were sequenced per sample for each region

3 months. At this CpG island shore region, the methylation level was further reduced by 30 months. Exon 5 also showed age-dependent hypomethylation, albeit to a lesser extent.

The fourth gene, *rpl13a*, which codes a subunit of ribosomal 60S protein, had a CpG island at the 5' region covering exons 1 and 2 (Supplementary Fig. S5). In *rpl13a*, CpG dinucleotides were sparsely distributed outside the CpG island, and we did not detect age-related methylation changes at exon 6 of the *rpl13a* gene, which corresponded to the CpG island shore region. All together, age-associated hypomethylation was observed in muscle at four out of six shores.

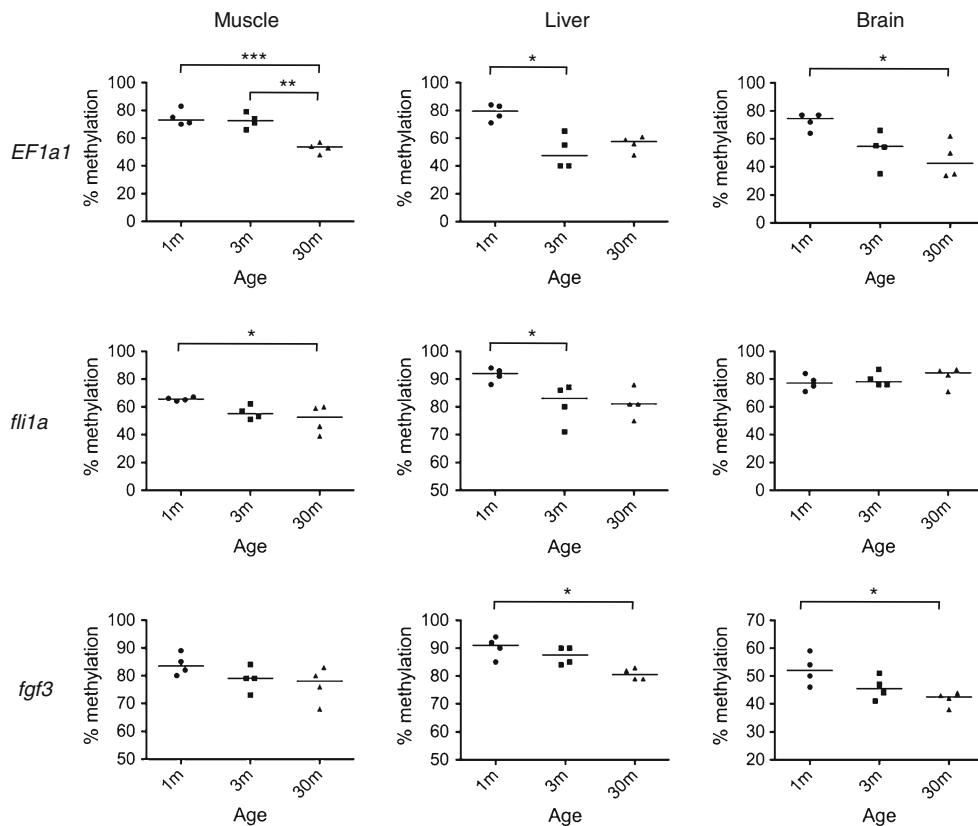
#### Hypomethylation at CpG island shores in nonmuscle cells

We next investigated methylation levels at the CpG island shores of *EF1 $\alpha$ 2* and *flil1a* in other tissues of a 30-month-old male zebrafish (Supplementary Fig. S6) and found that CpG island shores were more or less hypomethylated in any type of somatic cells, but not in

germ cells. This result appeared to reflect the global methylation patterns shown in Fig. 1d. To see if hypomethylation in these tissues was age related, we quantified methylation levels at the CpG island shores of *EF1 $\alpha$ 2*, *flil1a*, and *fgf3* in the livers and brains of 1-, 3-, and 30-month-old fish using the bisulfite sequencing method (Fig. 4). As in muscle, methylation levels at the CpG island shores of these tissues decreased with age, except for the CpG island shore of *flil1a* in the brain, and differences in methylation levels between these groups at different ages were significant at most regions examined.

#### De novo methylation of CpG island shores upon fertilization

We then compared the methylation levels of sperm and oocytes (Fig. 5). Both *EF1 $\alpha$ 2* and *flil1a* shores were densely methylated in the germ cells of young and aged male fish, which indicated that male germ cells were exempt from age-related hypomethylation in adult zebrafish. In contrast, the same regions were



**Fig. 4** CpG island shore regions showed age-related hypomethylation not only in muscle, but also in the liver and brain. The methylation levels of CpG island shore regions in *EF1 $\alpha$ 1* (region e in Fig. 2), *fli1a* (region b in Fig. S3), and *fgf3*

(region b in Fig. S4) in the zebrafish muscle, liver, and brain were examined by bisulfite sequencing. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . Four fish per stage were analyzed. Horizontal bars show medians

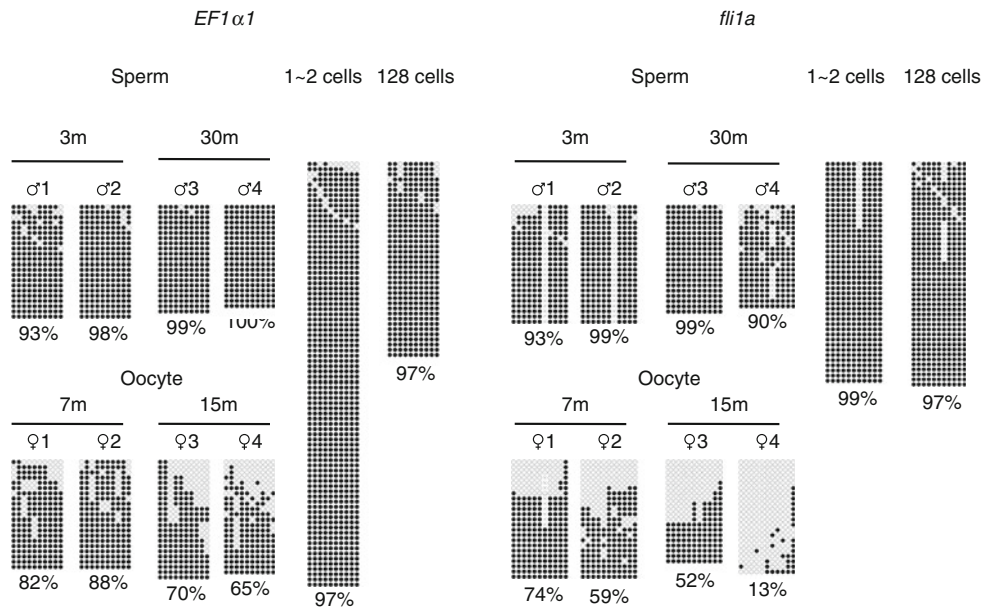
moderately methylated in oocytes. Since repetitive sequences, SINE3-1a and four long interspersed nuclear elements (Sugano et al. 2006; Rai et al. 2008), in oocytes were heavily methylated as in sperm (Supplementary Fig. S7), it is possible that the oocyte genome manifested age-related methylation changes at CpG island shores similar to somatic cell genomes. It should be noted that *EF1 $\alpha$ 2* and *fli1a* CpG island shores were also fully methylated in fertilized eggs and cleavage stage embryos (Fig. 5). These results imply that the maternal genome is de novo methylated in fertilized eggs and that new generations restart with a heavily methylated genome.

#### Fragmentation of the zebrafish genome with age

Although we demonstrated age-related increases in DNA digestion by methylation-sensitive enzymes (Fig. 1), there was a possibility that DNA samples from adult

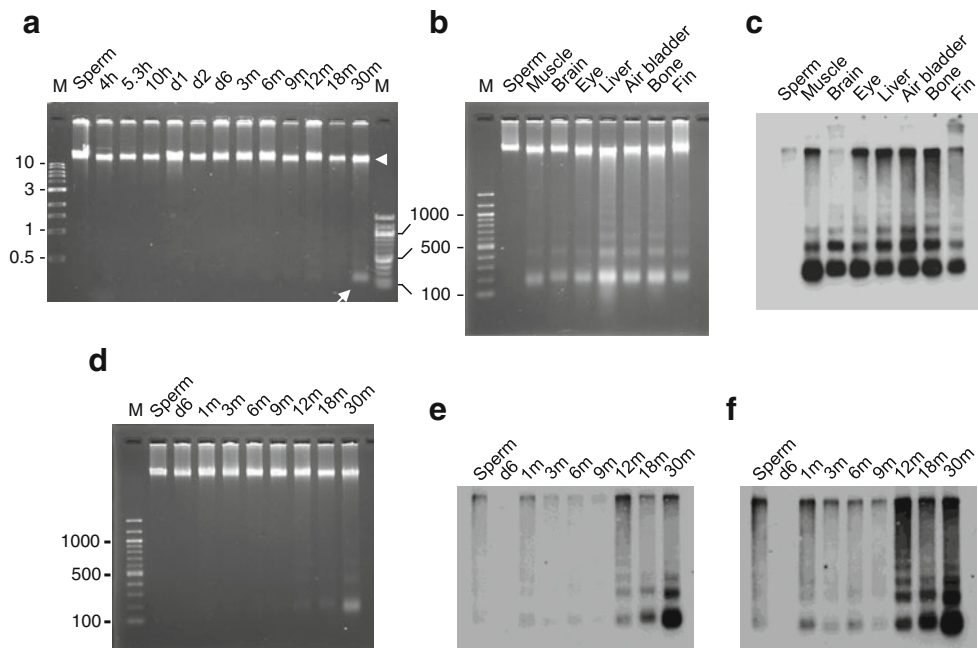
muscle were degraded. To confirm the intactness of DNA samples, we ran the same genomic DNA as the ones used in Fig. 1, but in an undigested state, on a 1 % agarose gel. As shown in Fig. 6a, a high molecular DNA band was observed in each lane with a slight smearing pattern, indicating the relative intactness of the samples. However, we noticed that a small distinct band of approximately 200 bp existed in the lane to which DNA from a 30-month-old fish was applied (Fig. 6a). This indicated that this small DNA was already present in the genomic DNA before digestion. To characterize the small DNA band in detail, we ran undigested genomic DNA from various tissues of the same zebrafish on a 2 % agarose gel (Fig. 6b). The results showed that DNA from all somatic cells, but not from sperm, contained approximately 200 bp DNA, and larger DNA fragments spacing at ~200 bp were also detected in some tissues. This periodic DNA banding was reminiscent of the characteristic DNA ladder observed in apoptosis (Wyllie 1980), in





**Fig. 5** CpG island shores moderately methylated in oocytes were heavily methylated in fertilized eggs. The methylation levels of CpG island shores in *EF1α1* (region e in Fig. 2) and

*fli1a* (region b in Fig. 3) were determined in germ cells extracted from adult fish of the indicated ages and in early embryos



**Fig. 6** Age-related DNA fragmentation occurred in somatic cells, but not in sperm. The undigested genomic DNAs of sperm, embryos (h; hours post fertilization), and adult muscle of the indicated ages were run on a 1 % agarose gel and stained with ethidium bromide (**a**). In addition to a high molecular weight DNA band (*arrowhead*), a small DNA fragment was seen in an aged fish (*arrow*). Small DNA fragments in the tissues of an aged zebrafish were visualized by ethidium staining (**b**) and by the end-labeling

method we developed (**c**). The same procedure was employed to reveal the stage at which small DNA fragments appeared (**d–f**). The times for exposure to X-ray films were 20 and 60 s for **e** and **f**, respectively. Similar to DNA hypomethylation, this age-related phenomenon was observed in all of the somatic cells examined, but not in male germ cells. The fragments showed a 180–200-bp periodicity, resembling the DNA cleavage observed in apoptosis. Lane M, 1 kb or 100 bp DNA ladder

which some specific DNase digested genomic DNA at nucleosome intervals of 180 bp (Enari et al. 1998).

To increase the sensitivity for detecting small DNA fragments, we developed a method that utilized DIG to end-label the free 3' ends of DNA fragments. This method revealed some DNA bands that were hardly detectable by EtBr staining (Fig. 6c). To investigate the stage at which the fragments first appeared in muscle DNA in the lifetime of zebrafish, we used the end-labeling method for the genomic DNA of different stages. Although small bands were hardly detectable even in 12- and 18-month-old zebrafish DNA by EtBr staining (Fig. 6d), after exposure to X-ray films, we found that small DNA fragments already existed in 1-month-old zebrafish muscle and they increased after 12 months old (Fig. 6e, f). No small DNA fragments were detected in sperm or in d6 larvae with this method.

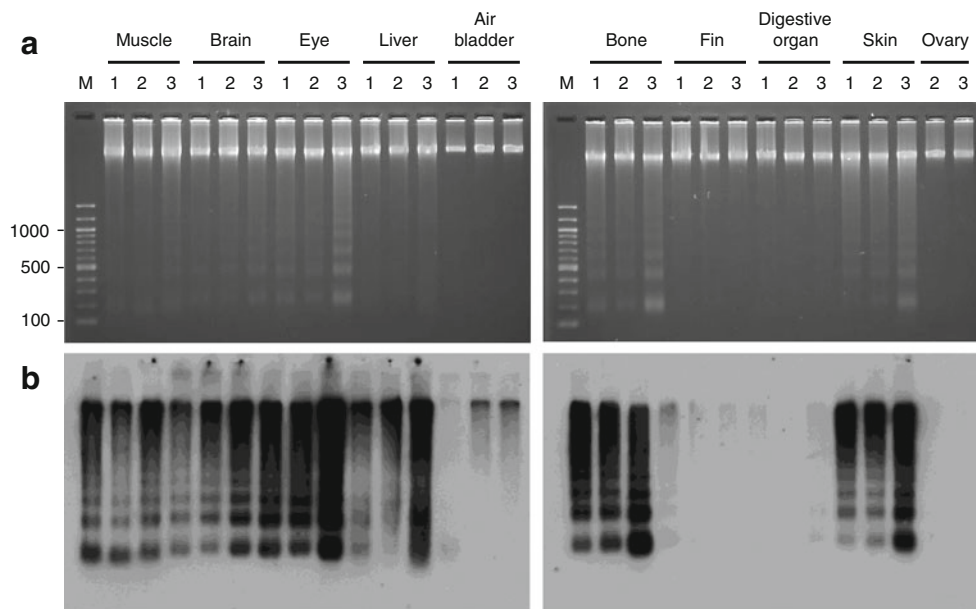
To examine if the generation of small DNA fragments varied among individual fish, we performed the same analysis on various tissues of three 15-month-old fish (Fig. 7a, b). We found that not only the presence or absence of ladders, but also ladder patterns appeared to be very similar among the same tissues, indicating that there was little interindividual variance in DNA fragmentation among the same tissues. The liver was the only organ in which the degradation of genomic DNA

occurred without periodicity, and even this pattern was common among the three fish.

There were two clear differences in DNA fragmentation between the tissues of 15-month-old fish and those of 30-month-old fish. First, the DNA ladder was hardly visible from the air bladder and fin of a 15-month-old fish (Fig. 7b), but appeared in these tissues in a 30-month-old fish (Fig. 6c). This result suggested that each tissue has its own stage at which the level of DNA fragmentation increases. Second, a monomer-sized DNA band (200 bp) was predominant in the DNA ladders of all the tissues from a 30-month-old fish, except for the sperm (Fig. 6c); however, this was not the case for the 15-month-old fish (Fig. 7b). The activity of the enzyme(s) involved in DNA fragmentation may possibly be higher in aged cells than in younger cells; therefore, the genome may be more thoroughly cleaved in aged cells. Alternatively, the genome in aged cells may be more susceptible to such enzyme(s).

## Discussion

In this study, we detected the loss of methylation in the zebrafish genome with aging at borders between CpG island and non-CpG island regions, known as CpG



**Fig. 7** Interindividual variations in DNA fragmentation in zebrafish tissues. Ethidium bromide staining (**a**) and the end-labeling method (**b**) were employed to compare DNA fragmentation

patterns in the tissues of three 15-month-old zebrafish: one male (*lane 1*) and two female fish (*lanes 2 and 3*). These fish were raised from the same batch of embryos. *Lane M*, 100 bp DNA ladder

island shores (Irizarry et al. 2009). In contrast, no significant methylation changes were observed in the repetitive sequences surveyed. In mammals, regions corresponding to CpG island shores have also been shown to be vulnerable to age-related hypomethylation (Ono et al. 1989; Alisch et al. 2012; Heyn et al. 2012; Pirazzini et al. 2012). Thus, the CpG island shore is the region where age-related hypomethylation changes are more likely to occur in common beyond species and, hence, whose methylation changes may be directly involved in the senescence of vertebrates as we suspected. Since the number of loci we investigated in this study was limited, it is unknown if global hypomethylation detected by enzymatic digestions can be solely explained by hypomethylation at CpG island shores. However, judging from the high probability of age-dependent hypomethylation at CpG island shores, decreases in methylation at CpG island shores should markedly influence global methylation levels during zebrafish aging.

It is currently unclear how hypomethylation preferentially occurs at CpG island shores in the somatic cells of aging zebrafish. It is not likely to be caused by the reduced activity of the maintenance methyltransferase Dnmt1 as it results in global, not focal hypomethylation (Shimoda et al. 2005). Since global methylation levels began to decrease well before maturation and were prominent at the juvenile stage when cellular proliferation was active, the fidelity of methylation maintenance at replication forks may somehow be reduced at CpG island shores. Alternatively, age-related hypomethylation at CpG island shores may be executed by the extended action of the Tet1 protein or a protein complex of MBD4, AID, and Gadd45, both of which eliminate unwanted methylation in CpG islands and maintain the unmethylated status of CpG islands (Rai et al. 2008; Wu and Zhang 2011).

How sperm DNA is exempt from age-dependent hypomethylation has also not been elucidated yet. This is not explained by the exceptional expression of any de novo methyltransferases in the testes because all zebrafish paralogues of the mouse de novo methyltransferase genes *dnmt3a* and *dnmt3b* are expressed not only in the testes, but also in somatic tissues such as the brain and liver (Campos et al. 2012). It was previously demonstrated that the zebrafish sperm genome, relative to the fibroblast genome, was packaged in chromatin characterized by abundant linker histones and the absence of H4K16ac (Wu et al. 2011).

The distinctive configuration of the sperm genome may allow for a de novo methyltransferase(s) accessible to CpG island shores or prevent the demethylation activities described above.

DNA fragmentation is another phenomenon that we observed in the somatic cells of aging zebrafish. Similar to methylation changes, it has also been observed in aged mammals (Tagliatalata et al. 1996; Itzhaki et al. 2003). An increase in DNA fragmentation started in zebrafish muscle at the age of 12 months. In our fish facility, this is around the age at which fecundity begins to drop. Since the decline in reproductive activity is a hallmark of zebrafish senescence (Tsai et al. 2007), the increase in DNA fragmentation observed in the present study may be related to the onset of senescence in zebrafish.

The periodic appearance and length of the periodicity of DNA fragmentation strongly suggested that it was generated by apoptosis because a prominent biochemical event in apoptosis is the internucleosomal cleavage of genomic DNA, initially producing 50 to 200 kb segments and then fragments in multiples of approximately 185 bp (Wyllie 1980; Oberhammer et al. 1993). Apoptosis provides an efficient mechanism for excluding cells that are potentially harmful to the animal. Since global hypomethylation affects genome integrity and gene regulation, cells with hypomethylated genomes in the zebrafish, mouse, and *Xenopus* were shown to be eliminated by apoptosis (Jackson-Grusby et al. 2001; Stancheva et al. 2001; Chen et al. 2007; Koji et al. 2008; Anderson et al. 2009). This, together with the observations of age-related increases in apoptotic cells in mammals (Jara et al. 2004; Leeuwenburgh et al. 2005; Whitman et al. 2005; Rice and Blough 2006), makes it tempting to hypothesize that, once a continued methylation change in the somatic cells of vertebrates exceeds a critical threshold, cells die by apoptosis due to inadequate expression profiles, which in turn causes a functional decline in tissues, the prominent feature of senescence. If this is the case, somatic cells need to begin with excess DNA methylation so that they can lose a certain amount before they are phenotypically affected (Cooney 1993). This is in line with the observation that the de novo methylation of some genes occurred in fertilized eggs (Fig. 5). The identification of chemicals or genetic mutations that accelerate or delay age-related hypomethylation at CpG island shores in the somatic cells of zebrafish should help to examine this hypothesis experimentally.

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