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ORIGINAL ARTICLE

Transcriptional Activity and DNA Methylation Dynamics of the Gal4/UAS System in Zebrafish

Shao-Chen Pang[1] · **Hou-Peng Wang**[1] · **Zuo-Yan Zhu**[1] · **Yong-Hua Sun**[1]

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Abstract The Gal4/upstream activating sequence (UAS) system is a powerful genetic tool for the temporal and spatial expression of target genes. In this study, the dynamic activity of the Gal4/UAS system was monitored in zebrafish throughout the entire lifespan and during germline transmission, using an optimized Gal4/UAS, KalTA4/4xUAS, which is driven by two muscle-specific regulatory sequences. We found that UAS-linked gene expression was transcriptionally amplified by Gal4/UAS during early developmental stages and that the amplification effects tended to weaken during later stages and even disappear in subsequent generations. In the F2 generation, the transcription of a UAS-linked enhanced green fluorescent protein (EGFP) reporter was transcriptionally silent from 16 days post-fertilization (dpf) into adulthood, yet offspring of this generation showed reactivation of the EGFP reporter in some strains. We further show that the transcriptional silencing and reactivation of UAS-driven EGFP correlated with the DNA methylation levels of the UAS regulatory sequences. Notably, asymmetric DNA methylation of the 4xUAS occurred in oocytes and sperm. Moreover, the paternal and maternal 4xUAS sequences underwent different DNA methylation dynamics after fertilization. Our study suggests that the Gal4/UAS system may represent a powerful tool for tracing the DNA methylation dynamics of paternal and maternal loci during zebrafish development and that UAS-specific DNA methylation should be seriously considered when the Gal4/UAS system is applied in zebrafish.

Keywords Gal4/UAS · Transcriptional activity · Transcriptional silencing · DNA methylation · Zebrafish

Introduction

The Gal4/upstream activating sequence (UAS) system is a powerful genetic tool for the temporal and spatial expression of genes in *Drosophila* (Duffy 2002), mouse (Lewandoski 2001) and zebrafish (Halpern et al. 2008; Baier and Scott 2009). In general, the Gal4/UAS technique is based on two transgenic strains: an activator strain expressing Gal4 under the control of a temporally and spatially regulated promoter or enhancer and an effector strain carrying a gene of interest regulated by a repeated UAS and a mini-promoter. The Gal4 protein targets the UAS and activates the expression of any genes downstream of the UAS. In zebrafish, the Gal4/UAS system has been extensively employed in cell tracing, cell ablation, and genetic manipulation of specific cells due to its advantages for achieving temporal and spatial regulation of gene expression (Baier and Scott 2009). Another remarkable advantage of the Gal4/UAS system is its ability to act as a transcriptional enhancer. The Gal4-VP16 fusion protein and its variant KalTA4 have been shown to be potent transcriptional enhancers that can amplify the expression of UAS-linked genes, especially when a weak promoter is used (Iyer et al. 2001; Distel et al. 2009; Xiong et al. 2013).

It has been suggested that the efficiency of the Gal4/UAS system is strongly affected by the methylation status of the UAS regulatory sequence (Goll et al. 2009; Akitake et al. 2011). It is commonly accepted that DNA methylation-

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✉ Yong-Hua Sun
yhsun@ihb.ac.cn

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

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mediated gene silencing is crucial for development and differentiation, and DNA methylation profiles generally undergo extensive and dynamic changes during these processes (Meissner 2010). For example, several pluripotency factors, including *Nanog* in mice (Hattori et al. 2007; Farthing et al. 2008) and *klf4* and *pou5f1* in zebrafish (Andersen et al. 2012), are demethylated during early development and methylated in differentiated cells. Additionally, DNA methylation profiles vary significantly in different cell types (Suzuki and Bird 2008). The epigenetic states of sperm and oocytes are highly asymmetric in mice (Cedar and Bergman 2012) and zebrafish (Mhanni and McGowan 2004). Recently, two independent studies of zebrafish genome-wide DNA methylation profiles revealed that the paternal methylomes are stably inherited without significant changes from fertilization into the sphere stage; by contrast, maternal methylomes are reprogrammed such that their epigenetic states match the paternal methylomes after fertilization (Potok et al. 2013; Jiang et al. 2013). These findings suggest that the paternal DNA and maternal DNA undergo different dynamic changes.

In our recent study, we found that the Gal4/UAS system could induce highly efficient transcriptional amplification of primordial germ cell (PGC)-specific gene expression (Xiong et al. 2013). We then asked whether this system could be used to enhance the transcription of other tissue-specific genes. Here, we utilized a single bicistronic construct containing an optimized Gal4/UAS system regulated by the muscle-specific *mylpfa* or *tpma* regulatory regions. We monitored the dynamic transcriptional effects of the Gal4/UAS system throughout development and germline transmission and further show that the transcriptional activation and silencing of the Gal4/UAS correlated with the differential DNA methylation profiles of the UAS in zebrafish. Moreover, we observed asymmetric DNA methylation of the 4xUAS sequence in oocytes and sperm, as well as distinct DNA methylation dynamics in the paternal and maternal 4xUAS during early development. The exogenous UAS makes it easy to distinguish and monitor the DNA methylation profiles of maternal and paternal loci, suggesting that the Gal4/UAS system can be utilized to study the dynamics and mechanisms of DNA methylation in zebrafish.

Materials and Methods

Zebrafish

Zebrafish embryos of the *AB* genetic background were obtained from the China Zebrafish Resource Center (http://zfish.cn; Wuhan, China), raised at 28.5 °C, and staged as described previously (Kimmel et al. 1995). The experiments involving zebrafish were performed under the approval of the Institutional Animal Care and Use Committee of the Institute of Hydrobiology at the Chinese Academy of Sciences.

DNA Constructs and 5′ Upstream Regulatory Region Cloning

To generate the mylpfa:EGFP transgenic construct, the *mylpfa* regulatory region was subcloned into the 4xKGFP plasmid (Distel et al. 2009) containing the Tol2 transposon and the EGFP coding sequence using the *Sal*I and *Eco*RI sites. To generate the mylpfa:Gal4 construct, the KalTA4 coding sequence from the 4xKaloop plasmid (Distel et al. 2009) was subcloned into the mylpfa:EGFP construct using the *Eco*RI and *Not*I sites. To generate the mylpfa:Gal4;UAS:EGFP construct, a UAS:EGFP fragment was generated by overlap extension PCR (Heckman and Pease 2007) using the 4xKGFP plasmid as the template and a set of four primers (U1, U2, U3, and U4) (Supplemental Table S1); this fragment was subsequently cloned into the mylpfa:Gal4 construct using the *Bgl*II and *Xho*I sites.

Based on the sequence in GenBank®, accession number NM_131105.2, the 4.0-kb 5′ upstream regulatory region of *tpma* was amplified from zebrafish genomic DNA by PCR using the following primers: 5′-CACCCATACACTTCCATTC-3′ and 5′-CGCACCAAACACTGGACTGA-3′. The PCR products were cloned into the pMD18T vector and sequenced. To generate the tpma:EGFP transgenic vector, the *tpma* 5′ upstream regulatory region was subcloned into the 4xKGFP plasmid using the *Sal*I and *Eco*RI sites. To generate the tpma:Gal4 construct, the KalTA4 coding sequence from the 4xKaloop plasmid was subcloned into the tpma:EGFP construct using the *Bam*HI and *Not*I sites. To generate the tpma:Gal4;UAS:EGFP construct, the 4xUAS:EGFP fragment was amplified by PCR using the mylpfa:Gal4;UAS:EGFP plasmid as a template and a set of two primers (U5 and U6) (Supplemental Table S1); this fragment was then cloned into the tpma:Gal4 construct using the *Xho*I and *Pst*I sites.

Generation of Transgenic Zebrafish

To establish the *Tg(mylpfa:EGFP)*, *Tg(mylpfa:Gal4;UAS:EGFP)*, *Tg(tpma:EGFP)*, or *Tg(tpma:Gal4;UAS:EGFP)* transgenic zebrafish lines, each transgenic DNA construct and Tol2 transposase messenger RNA (mRNA) were co-injected into fertilized embryos at the one-cell stage. The EGFP-expressing embryos were raised, and transgenic lines were generated as described previously (Pang et al. 2014).

Luciferase Assay

Approximately, 1 nL of a DNA mixture containing 100 ng/μL mylpfa:TA2 or 100 ng/μL mylpfa:TA4, 71 ng/μL firefly

luciferase reporter plasmid (4xUAS:Luc), and 10 ng/μL *Renilla* luciferase reporter plasmid were co-injected into fertilized embryos at the one-cell stage. Forty-eight hours after fertilization, sets of 30 embryos were lysed, and the luciferase activity was measured and calculated as described previously (Wei et al. 2014).

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed using digoxigenin (DIG)-labeled antisense RNA probes and an anti-DIG alkaline phosphatase-conjugated antibody as described (Thisse et al. 2004). The *tpma* probe was synthesized using T3 polymerase (Promega) from *tpma* PCR product templates, which were amplified from the complementary DNA (cDNA) of 4 days post-fertilization (dpf) embryos using the following primers: 5′-TTGGGTGTCTTGCGGAACTT-3′ and 5′-GATCCATTAACCCTCACTAAAGGGAAAGGGCCTCGGAGTATTTGTC-3′, which contains a T3 promoter sequence (underlined).

Imaging

Live embryos and larvae were anesthetized with tricaine, mounted in 3 % methyl cellulose, and imaged using a fluorescence stereo-microscope equipped with a GFP filter (Olympus, MVX10). To compare the fluorescent intensities of the *Tg(mylpfa:EGFP)* and *Tg(mylpfa:Gal4;4xUAS:EGFP)* strains or the *Tg(tpma:EGFP)* and *Tg(tpma:Gal4;4xUAS:EGFP)* strains, fluorescent images were captured using the same parameters for of the two strains. Fresh muscle tissues were embedded in OCT compound (Tissue-Tek) and frozen rapidly. Frozen sections (15 μm) were cut using a thermo-cryostat and imaged with a MVX10 microscope.

Bisulfite Sequencing

Total genomic DNA was isolated from muscle tissue, eggs, sperm, and embryos using a tissue DNA kit (Omega). The genomic DNA (0.5 μg) was treated with CT conversion reagent (Invitrogen) at 98 °C for 10 min, 64 °C for 2.5 h, and 4 °C for 16 to 20 h. Amplification of the 4xUAS region was performed with two PCR runs using the bisulfite-treated DNA and the primers 5′-GGAGTTTAAAGTGAGGTTGAGA-3′ and 5′-AAAATATATAAATAAATCRTCRACCT-3′; the PCR program included an annealing temperature of 53 °C and 35 amplification cycles. The PCR products were ligated into the pMD18T vector and sequenced.

Quantitative RT-PCR

The cDNAs of embryos, larvae, and muscle tissue were synthesized as described (Pang et al. 2014). Quantitative RT-PCR was performed using SYBR Mix (Bio-Rad) and a CFX Real-Time PCR System (Bio-Rad). Primers used for *gal4* were 5′-AAGCGAAGTCCACTCACAAG-3′ and 5′-CGAATAAGCCAGTCAAAAGG-3′, those used for *egfp* were 5′-CAGTGCTTCAGCCGCTACCC-3′ and 5′-GGATGTTGCCGTCCTCCTTG-3′, and those used for *β-actin* were 5′-GATGATGAAATTGCCGCACTG-3′ and 5′-ACCAACCATGACACCCTGATGT-3′. The transcript levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method with *β-actin* as the internal control as described previously (Livak and Schmittgen 2001).

Results

Dynamic Activity of an Optimized Gal4/UAS System Driven by the *mylpfa* 5′ Upstream Regulatory Region in Zebrafish

Previously, an optimized Gal4/UAS system was established in zebrafish (Distel et al. 2009). They compared the activator activity and toxic effect of three Gal4-VP16 activators, GalTA4, TA3, and TA2, which are different in the repeats of VP16 trans-activation domain (13-amino acid core sequence), and it was suggested that the TA4 is a relative highly efficient activator with low toxicity compared with TA2. To test the optimized Gal4/UAS system in our study, we generated two constructs of Gal4-activator, mylpfa:TA2 and mylpfa:TA4, both of which were driven by 5′ upstream regulatory region of fast muscle-tissue-specific gene *mylpfa* (Ju et al. 2003), and co-injected equal molecular amounts of them along with 4xUAS:Luc into zebrafish embryos. The equal molecular amount of mylpfa:Luc was injected into embryos as a control. Relative luciferase assays revealed that the transcriptional activity of the TA4/4xUAS driven by the *mylpfa* 5′ upstream regulatory region was significantly higher (1.6-fold) than that of the mylpfa:Luc (Fig. 1a). Although the TA2 activator exhibited higher activation ability (Fig. 1a), malformed embryos (deformed head, trunk, or tail, and even ball shape) were observed among those injected with the TA2 at a dosage of 100 ng/μL (Supplemental Fig. 2c). Therefore, the TA4 activator was used in the subsequent studies.

To analyze the dynamic transcriptional activity of the Gal4/UAS system in zebrafish, we designed two constructs, mylpfa:EGFP and mylpfa:Gal4;UAS:EGFP (Fig. 1b, c), and injected them into zebrafish embryos. Three independent transgenic zebrafish lines of genotype *Tg(mylpfa:EGFP)* were established, and seven germline-transmitted transgenic founders of *Tg(mylpfa:Gal4;UAS:EGFP)* were obtained. The transcriptional activities of the *egfp* reporter in F1 *Tg(mylpfa:EGFP)* and F1 *Tg(mylpfa:Gal4;UAS:EGFP)* embryos from 1 to 8 dpf were analyzed using quantitative RT-PCR (Fig. 1d). At 1 dpf, as expected, the relative transcriptional

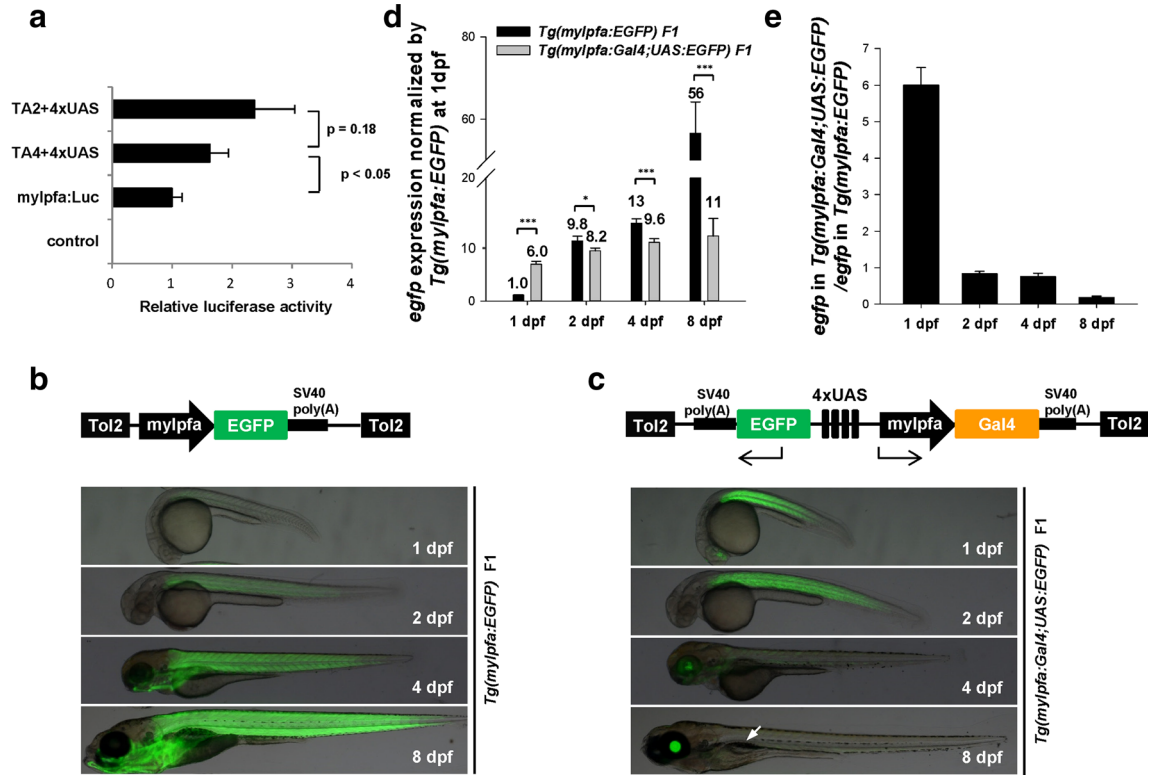

Fig. 1 Dynamic activity of the optimized Gal4/UAS system driven by the *mylpfa* regulatory region in zebrafish. **a** Gene expression driven by the *mylpfa* regulatory region with or without the Gal4/UAS system was compared using in vivo luciferase assays. Two types of Gal4 activator proteins, TA4 and TA2, were used. **b** Schematic representation of the construct used to generate the *Tg(mylpfa:EGFP)* transgenic zebrafish lines and EGFP dynamics in F1 *Tg(mylpfa:EGFP)* embryos at 1, 2, 4, and 8 dpf. **c** Schematic representation of the construct used to generate the *Tg(mylpfa:Gal4;UAS:EGFP)* transgenic zebrafish lines and EGFP dynamics in F1 *Tg(mylpfa:Gal4;UAS:EGFP)* embryos at 1, 2, 4, and 8 dpf. **d** Transcriptional levels of the *egfp* reporter in F1 *Tg(mylpfa:EGFP)* and F1 *Tg(mylpfa:Gal4;UAS:EGFP)* embryos at 1, 2, 4, and 8 dpf were analyzed by quantitative RT-PCR (*$P<0.05$, ***$P<0.005$). **e** Ratio between *egfp* transcripts expressed in F1 *Tg(mylpfa:Gal4;UAS:EGFP)* and *egfp* transcripts expressed in F1 *Tg(mylpfa:EGFP)* at 1, 2, 4, and 8 dpf

level of *egfp* in the *Tg(mylpfa:Gal4;UAS:EGFP)* embryos was 5.0-fold ((6.0−1.0)/1.0) higher than in the *Tg(mylpfa:EGFP)* embryos (Fig. 1d, e). However, from 2 to 8 dpf, relative *egfp* expression in the *Tg(mylpfa:Gal4;UAS:EGFP)* embryos decreased in comparison to the *Tg(mylpfa:EGFP)* embryos, by 16 % ((8.2−9.8)/9.8) at 2 dpf and 80 % ((11−56)/56) at 8 dpf (Fig. 1d, e). In F1 *Tg(mylpfa:EGFP)* embryos, EGFP expression increased gradually from 1 to 8 dpf (Fig. 1b), and the fluorescence of the transgenic adult fish was visible by unaided eyes in daylight (data not shown), as previously described (Gong et al. 2003). In F1 *Tg(mylpfa:Gal4;UAS:EGFP)* embryos, although EGFP expression was stronger than in *Tg(mylpfa:EGFP)* embryos from 1 to 2 dpf, EGFP expression decreased dramatically from 4 to 8 dpf (Fig. 1c). The F1 *Tg(mylpfa:Gal4;UAS:EGFP)* larvae exhibited defects in swim bladder development and food intake (Supplemental Fig. 1) beginning at 6 dpf and survived no more than 20 dpf, possibly because of the toxic effects of the widely expressed KalTA4 protein. Therefore, we could not generate any stable transgenic lines of *Tg(mylpfa:Gal4;UAS:EGFP)*. These results suggest that the Gal4/UAS system driven by the *mylpfa* 5′ upstream regulatory region enhances *egfp* transcription before 2 dpf but that the enhancer effects gradually disappear after 2 dpf.

Cloning and Characterization of the Fast-Muscle-Specific *tpma* 5′ Upstream Regulatory Element

To investigate whether the observed toxic effect was *mylpfa* regulatory region-specific, we cloned another 5′ upstream regulatory element from the fast muscle-tissue-specific gene *tpma*, which encodes tropomyosin in zebrafish. The expression pattern of *tpma* in early development was analyzed by whole-mount in situ hybridization. *tpma* was expressed at the onset of segmentation in the adaxial cells of the three-somite stage (Fig. 2a). By the 15-somite stage, *tpma* expression persisted throughout somitogenesis and was predominantly observed in the formed somites and adaxial cells (Fig. 2b). At 1 dpf and later, *tpma* was strongly expressed throughout the myotome (Fig. 2c); cranial muscles, including the levator arcus palatini (lap) muscle and sternohyoideus (sh) muscle (Schilling and Kimmel 1997); the hypaxial body wall; and the pectoral fin (pf) muscles (Fig. 2d, e). In comparison with

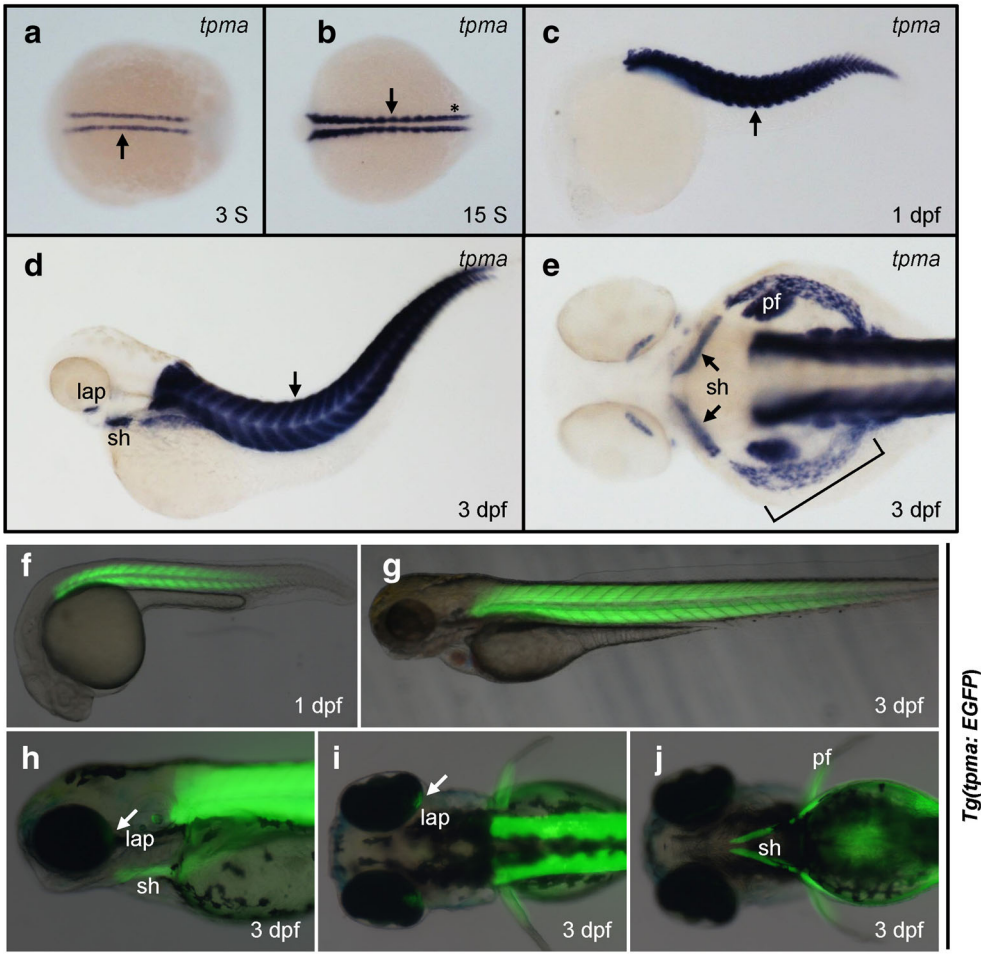

Fig. 2 Cloning and analysis of the *tpma* 5′ upstream regulatory element. **a**–**e** Whole-mount in situ hybridization revealed that zebrafish *tpma* is expressed in muscle cells: adaxial slow muscle precursor cells (*arrow* in **a**); presomitic mesoderm (*asterisk* in **b**); formed somites (*arrow* in **b**, **c**, **d**); cranial muscles, including the levator arcus palatini (*lap*) muscle (**d**) and sternohyoideus (*sh*) muscle (**d**, **e**); pectoral fin (**e**) muscle; and hypaxial body wall (*bracket* in **e**). **f**–**j** EGFP was faithfully expressed in the muscle cells of *Tg(tpma:EGFP)* transgenic zebrafish: formed somites (**f**, **g**); cranial muscles, including the levator arcus palatini (*lap*) muscle (**h**, **i**) and sternohyoideus (*sh*) muscle (**h**, **j**); and pectoral fin (*pf*) muscle (**j**). Embryos in **a**, **b**, **e** and **i** were in dorsal view with anterior to the left; embryos in **j** was in ventral view with anterior to the left; and embryos in **c**, **d**, **f**, **g**, and **h** were in lateral view with anterior to the left

mylpfa (Ju et al. 2003), *tpma* is less expressed in the cranial muscles.

A 4.0-kb upstream regulatory region of *tpma* was cloned from zebrafish genomic DNA and sequenced. Sequence analysis showed that there were nine putative binding sites for the transcription factor MyoD and one putative binding site for the transcription factor Pax4 in the 4.0-kb potential 5′ upstream regulatory region (Supplemental Fig. 3). The construct of tpma (4.0 kb):EGFP was generated and injected into zebrafish embryos, in which EGFP was expressed strongly in the trunk muscles (Supplemental Fig. 4a). Furthermore, a series of *egfp* and firefly luciferase gene constructs containing progressive 5′ deletions of the *tpma* 5′ upstream regulatory region, starting between position −3728 and position −423 and extending through position + 160, were generated. Analysis of transient *egfp* expression and dual-luciferase assays revealed that the 4.0-kb *tpma* 5′ upstream regulatory element possessed the strongest transactivation capability (Supplemental Fig. 4). Therefore, we utilized this 4.0-kb *tpma* 5′ upstream regulatory element to generate the transgenic zebrafish in the subsequent study.

A transgenic tpma:EGFP construct (Fig. 3a) was generated and injected into zebrafish embryos, and two independent *Tg(tpma:EGFP)* transgenic zebrafish lines were established. Examination of transgenic *Tg(tpma:EGFP)* larvae by fluorescence microscopy revealed that the skeletal muscles (Fig. 1c) and cranial muscles, including the levator arcus palatini (lap) muscle, sternohyoideus (sh) muscle, and pectoral fin (pf) muscle, expressed EGFP (Fig. 2f–j). The EGFP expression pattern of the *Tg(tpma:EGFP)* embryos was consistent with the RNA in situ hybridization of *tpma* in zebrafish embryos (Fig. 2c–e). This result demonstrates that the cloned 4.0-kb *tpma* 5′ upstream regulatory element drives faithful EGFP expression in zebrafish embryos.

Dynamic Activity of the Optimized Gal4/UAS System Driven by the *tpma* 5′ Upstream Regulatory Region in Zebrafish

The transgenic construct of tpma:Gal4;4xUAS:EGFP (Fig. 3b) was generated and injected into zebrafish embryos, and three independent *Tg(tpma:Gal4;UAS:EGFP)* transgenic zebrafish lines were established. Dynamic EGFP expression was observed in F1 *Tg(tpma:EGFP)* and *Tg(tpma:Gal4;UAS:EGFP)* embryos and compared across the early developmental stages.

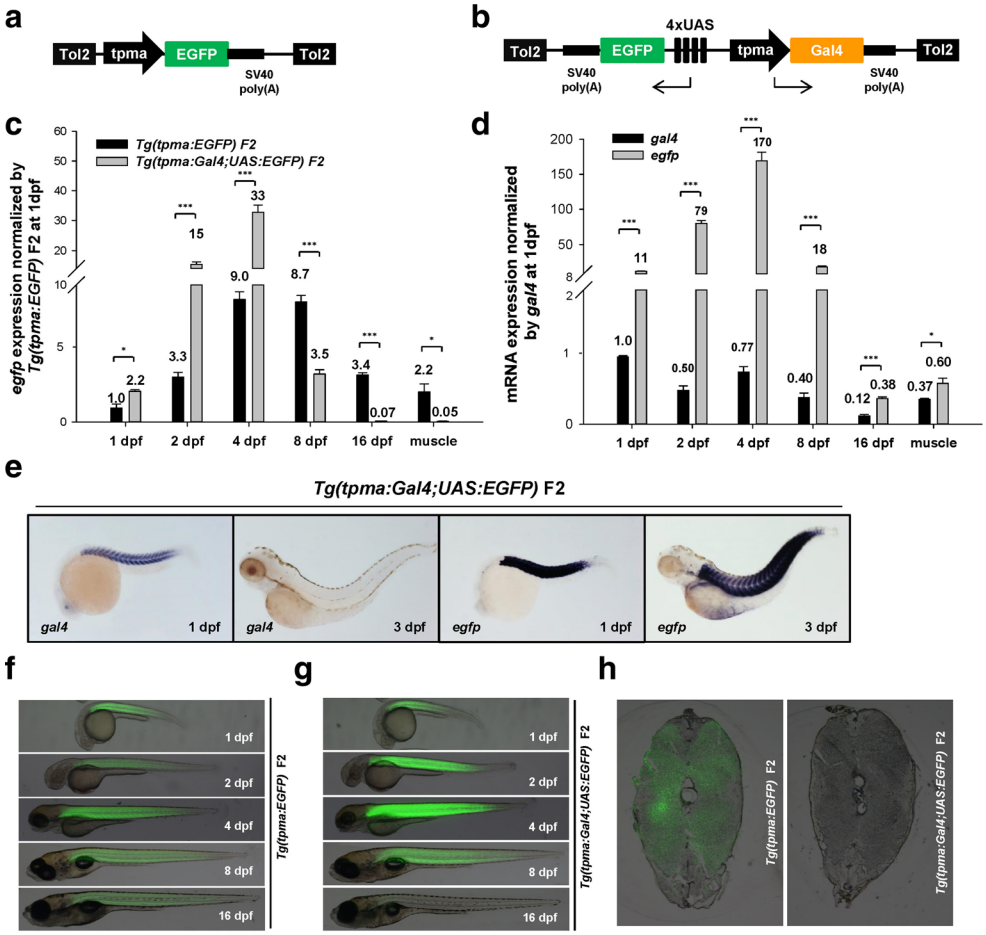

Fig. 3 Transcriptional amplification and silencing of the Gal4/UAS system driven by the *tpma* regulatory element in *Tg(tpma:Gal4;UAS:EGFP)* zebrafish. **a** Schematic representation of the construct used to generate the *Tg(tpma:EGFP)* transgenic zebrafish lines. **b** Schematic representation of the construct used to generate the *Tg(tpma:Gal4;UAS:EGFP)* transgenic zebrafish lines. **c** Transcript levels of the *egfp* reporter in F2 *Tg(tpma:EGFP)* and F2 *Tg(tpma:Gal4;UAS:EGFP)* embryos at 1, 2, 4, and 16 dpf and in adult muscle tissue were analyzed by quantitative RT-PCR (*$P<0.05$, ***$P<0.005$). **d** Transcript levels of the *gal4* and *egfp* in F2 *Tg(tpma:Gal4;UAS:EGFP)* embryos at 1, 2, 4, and 16 dpf and in adult muscle tissue were analyzed by quantitative RT-PCR (*$P<0.05$, ***$P<0.005$). **e** Transcript levels of the *gal4* and *egfp* in F2 *Tg(tpma:Gal4;UAS:EGFP)* embryos at 1 and 3 dpf were analyzed by whole-mount in situ hybridization. **f** EGFP expression in F2 *Tg(tpma:EGFP)* embryos at 1, 2, 4, 8, and 16 dpf. **g** EGFP expression in F2 *Tg(tpma:Gal4;UAS:EGFP)* embryos at 1, 2, 4, 8, and 16 dpf. **h** EGFP expression in the adult muscle tissue of F2 *Tg(tpma:EGFP)* and F2 *Tg(tpma:Gal4;UAS:EGFP)* zebrafish

We found that EGFP expression was higher in *Tg(tpma:Gal4;UAS:EGFP)* embryos than in *Tg(tpma:EGFP)* embryos beginning at 48 hpf, and this difference persisted until 80 hpf (Supplemental Fig. 5a). The *Tg(tpma:Gal4;UAS:EGFP)* transgenic zebrafish did not display any abnormalities and were raised to adulthood. However, when the transgenic fish reached adulthood, the fluorescence in the muscle tissue of the F1 *Tg(tpma:Gal4;UAS:EGFP)* fish was weaker than that in the F1 *Tg(tpma:EGFP)* fish (Supplemental Fig. 5b).

Next, we investigated the transcription and translation of the *egfp* reporter in *Tg(tpma:Gal4;UAS:EGFP)* embryos from the F2 generation. During early development, *egfp* transcript levels were higher at 1, 2, and 4 dpf in the *Tg(tpma:Gal4;UAS:EGFP)* embryos than in the *Tg(tpma:EGFP)* embryos by 1.2-fold ((2.2−1.0)/1.0), 3.5-fold ((15−3.3)/3.3), and 2.7-fold ((33−9.0)/9.0), respectively (Fig. 3c). However, the transcriptional effects that predominated due to the presence of the Gal4/UAS system gradually decreased from 8 dpf into adulthood, and only low levels of *egfp* transcripts were detected in the muscle tissue of the adult *Tg(tpma:Gal4;UAS:EGFP)* fish (Fig. 3c). We then analyzed the relative transcriptional enhancer activity of the Gal4/UAS system in the *Tg(tpma:Gal4;UAS:EGFP)* F2 embryos and adults by normalizing the mRNA level of *egfp* to that of *gal4* (*egfp*/*gal4* ratio) at each developmental stage. Although *gal4* expression was relative stable (Fig. 3d), the *egfp*/*gal4* ratios were 11 (11/1.0), 158 (79/0.50), 220 (170/0.77), and 45 (18/0.40) at 1, 2, 4, and 8 dpf, respectively, indicating highly efficient transcriptional enhancement by the Gal4/UAS system. However, the *egfp*/*gal4* ratios dramatically

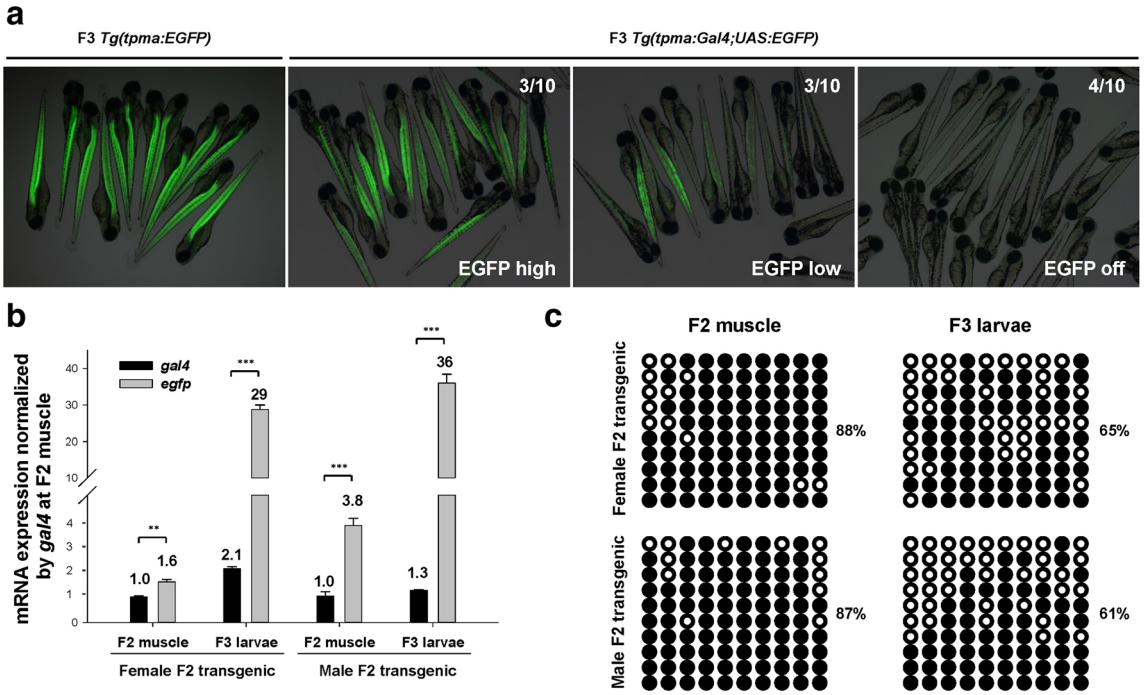

Fig. 4 *egfp* was reactivated during inheritance, and transcriptional silencing of *egfp* was epigenetically regulated. **a** EGFP was consistently expressed in F3 *Tg(tpma:EGFP)* fish, and F3 *Tg(tpma:Gal4;UAS:EGFP)* larvae expressed high levels of EGFP (EGFP high), low levels of EGFP (EGFP low), or no EGFP (EGFP off) at 4 dpf, which were reproduced from different F2 fish. **b** Female or male F2 *Tg(tpma:Gal4;UAS:EGFP)* fish whose offspring expressed high levels of EGFP were crossed with wild-type zebrafish. Transcript levels of the *gal4* and *egfp* in the muscle tissue of F2 female and male adults and in the "EGFP high" F3 larvae were analyzed by quantitative RT-PCR (**$P<0.01$, ***$P<0.005$). **c** The DNA methylation statuses of the *Tg(tpma:Gal4;UAS:EGFP)* F2 adult muscle tissue and F3 larvae were analyzed by bisulfite sequencing. Methylation status of ten sequential CpGs within 4xUAS region was shown on the *horizontal axis*, and ten representative TA clones were shown on the *vertical axis*. *Black circles* indicated methylated CpGs, and *open circles* indicated unmethylated CpGs. *Each line* represents an examined clone

decreased to 3.1 (0.38/0.12) and 1.6 (0.60/0.37) at 16 dpf and adulthood, respectively (Fig. 3d), indicating a decrease in the transcriptional enhancer activity. The strong transcriptional enhancer activity observed before 8 dpf was further confirmed by whole-mount in situ hybridization analysis of *gal4* and *egfp* in the *Tg(tpma:Gal4;UAS:EGFP)* embryos at 1 or 3 dpf, which showed much higher signals for *egfp* staining than *gal4* staining (Fig. 3e).

Next, we analyzed the dynamic EGFP expression from the Gal4/UAS system driven by the *tpma* 5′ upstream regulatory element. In the F2 generation of the *Tg(tpma:EGFP)* line, EGFP expression progressively increased from 1 dpf and peaked at 4 dpf (Fig. 3f). EGFP expression decreased in muscle tissues from 16 dpf to adulthood (Fig. 3f, h). Similarly, EGFP expression in the F2 *Tg(tpma:Gal4;UAS:EGFP)* fish gradually increased from 1 dpf and peaked at 4 dpf (Fig. 3g). From 1 to 4 dpf, EGFP expression was significantly higher in the *Tg(tpma:Gal4;UAS:EGFP)* larvae than in the *Tg(tpma:EGFP)* larvae (Fig. 3f, g), indicating strong transcriptional amplification by the Gal4/UAS system. Then, EGFP expression decreased beginning at 8 dpf in the *Tg(tpma:Gal4;UAS:EGFP)* larvae and was hardly detectable by 16 dpf (Fig. 3g). There was even no visible EGFP expression in the muscle tissue of the *Tg(tpma:Gal4;UAS:EGFP)* adults (Fig. 3h), suggesting the expression silence of the Gal4/UAS system in F2 adults. These results demonstrate that the Gal4/UAS system driven by the *tpma* 5′ upstream regulatory element enhanced the transcriptional activity of the *egfp* reporter before 4 dpf in zebrafish and that this effect tended to disappear after 8 dpf.

Silencing and Reactivation of the Transgene Correlated with Epigenetic Modifications in *Tg(tpma:Gal4;UAS:EGFP)* Zebrafish

We continued to observe the expression of the transgenes in the *Tg(tpma:EGFP)* and *Tg(tpma:Gal4;UAS:EGFP)* transgenic lines in subsequent generations. EGFP was consistently expressed in the muscle tissue of F3 *Tg(tpma:EGFP)* larvae after germline transmission (Fig. 4a). The F2 heterozygous *Tg(tpma:Gal4;UAS:EGFP)* adult zebrafish were mated with wild-type zebrafish. Among ten F2 *Tg(tpma:Gal4;UAS:EGFP)* adults, three produced high-level EGFP-expressing offspring (EGFP high), three produced low-level EGFP-expressing offspring (EGFP low), and four produced offspring with no EGFP expression (EGFP off) (Fig. 4a).

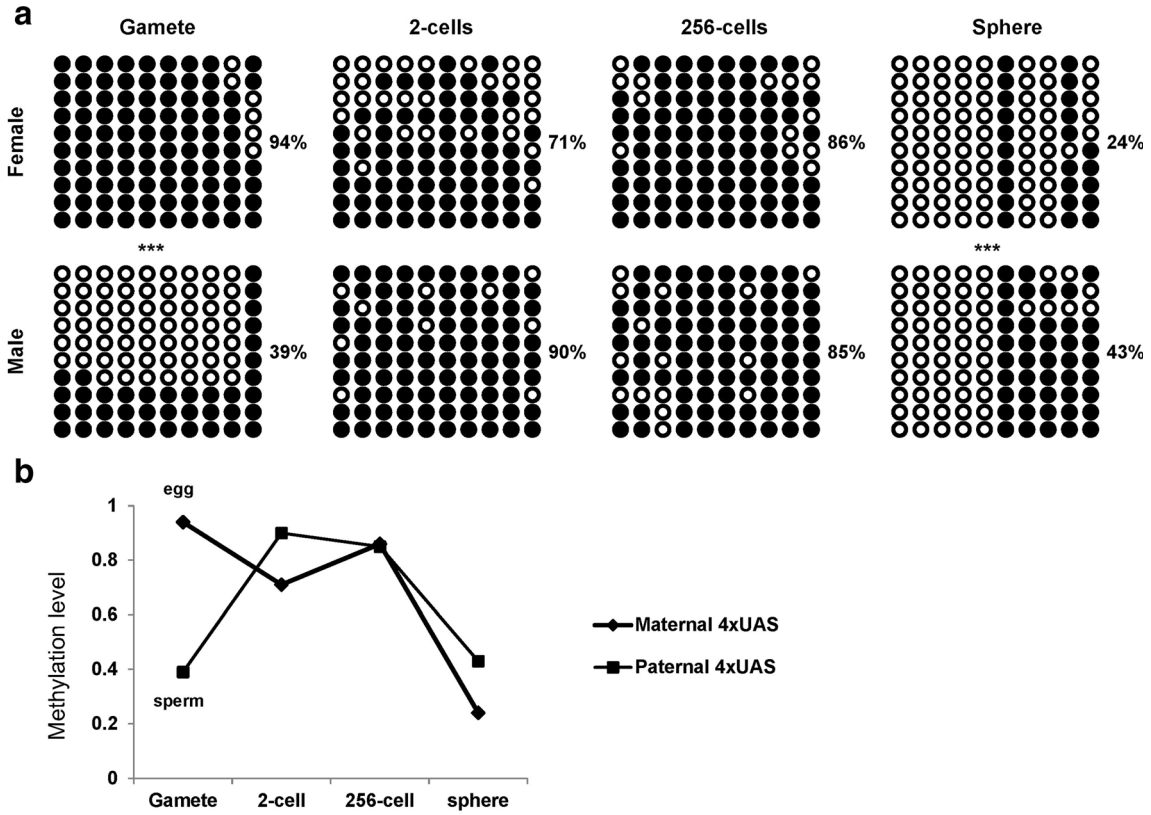

Fig. 5 DNA methylation status of transgenes inherited from the maternal or paternal genome underwent opposite dynamic changes. **a** Bisulfite sequencing analysis of the 4xUAS sequences inherited from the maternal or paternal genomes at the gamete, 2-cell, 256-cell, and sphere stages. Methylation status of ten sequential CpGs within 4xUAS region was shown on the *horizontal axis*, and ten representative TA clones were shown on the *vertical axis. Black circles* indicated methylated CpGs, and *open circles* indicated unmethylated CpGs. *Each line* represents an examined clone. *Stars* indicated the statistic significance between two samples at the same developmental stages (***$P<0.005$). **b** Dynamic DNA methylation levels of the 4xUAS sequences inherited from the maternal or paternal genomes at the gamete, 2-cell, 256-cell, and sphere stages

These results demonstrate that although the *egfp* reporter was transcriptionally silent in the muscle tissue of the F2 *Tg(tpma:Gal4;UAS:EGFP)* generation, it could be transcriptionally reactivated in the F3 *Tg(tpma:Gal4;UAS:EGFP)* larvae.

Next, we analyzed the dynamic EGFP expression of the Gal4/UAS system driven by the *tpma* 5′ upstream regulatory element. Female or male heterozygous F2 *Tg(tpma:Gal4;UAS:EGFP)* fish whose offspring expressed a high level of EGFP were mated with wild-type zebrafish. cDNA was synthesized from the muscle tissue of adult females or males and their offspring larvae (EGFP high). The transcriptional activities of the *egfp* and *gal4* genes in the muscle tissue of F2 *Tg(tpma:Gal4;UAS:EGFP)* fish and the F3 transgenic larvae were analyzed by quantitative PCR. The transcription level of *gal4* in F3 larvae was only 1.1-fold ((2.1 −1.0)/1.0) or 30 % ((1.3−1.0)/1.0) higher than that in the muscle tissues of female or male F2 adult, but *egfp* transcripts in the F3 larvae were increased 17-fold ((29−1.6)/1.6) or 8.5-fold ((36−3.8)/3.8) compared to those in the muscle tissues of female or male F2 adult (Fig. 4b). The *egfp*/*gal4* ratios in the F3 larvae were 14 (29/2.1) or 29 (36/1.3), which was significantly higher than was observed in the muscle tissue of the F2 adults (1.6 [1.6/1.0] or 3.8 [3.8/1.0]), indicating that the transcription was much more strongly enhanced by the Gal4/UAS system at the larval stage than during adulthood.

Previous studies revealed that *gfp* expression promoted by the Gal4/UAS system is epigenetically regulated in transgenic zebrafish (Goll et al. 2009; Akitake et al. 2011). There was a high obs/exp ratio of CpG dinucleotides (Gardiner-Garden and Frommer 1987) (0.97) in the 4xUAS sequence used in our study, indicating that this sequence is highly sensitive to regulation by CpG methylation. To explain the observed GFP silencing and reactivation in our Gal4/UAS system, we examined the DNA methylation status of the 4xUAS sequence in the muscle tissue and larvae (4 dpf) of our transgenic zebrafish. Female or male heterozygous F2 *Tg(tpma:Gal4;UAS:EGFP)* fish whose offspring expressed high levels of GFP were mated with wild-type zebrafish. DNA from F2 female or male adult muscle tissue and F3 larvae (GFP high) was isolated, bisulfite treated, and sequenced. The bisulfite sequencing analysis showed that the 4xUAS sequence was

hypermethylated in female (average of 82 % CpG methylation) and male muscle tissues (average of 89 % CpG methylation) and moderately methylated in the F3 larvae either from F2 male (average of 62 % CpG methylation) or from F2 female (average 63 % CpG methylation) (Fig. 4c). Another independent experiment was conducted to further confirm the dynamic changes of CpG methylation profiles from F2 adults to F3 larvae (Supplemental Fig. 6). These results suggest that the transcriptional silencing and reactivation observed in our Gal4/UAS system driven by the *tpma* 5′ upstream regulatory element correlate with the DNA methylation status of the 4xUAS sequence.

The Maternal and Paternal 4xUAS Sequences Underwent Different Dynamic Changes in DNA Methylation During Early Development

The 4xUAS sequence was hypermethylated in adult muscle tissue but demethylated in the offspring larvae, indicating that methylation status was reset during germline transmission. We were interested in the dynamic changes in DNA methylation of the 4xUAS sequence. Female or male heterozygous F2 *Tg(tpma:Gal4;UAS:EGFP)* fish whose offspring expressed high levels of GFP were mated with wild-type zebrafish. DNAs from 2-cell, 256-cell, and sphere embryos, as well as eggs and sperm, were isolated, bisulfite treated, and sequenced. Interestingly, the 4xUAS sequence was hypermethylated in eggs (average of 94 % CpG methylation) (Fig. 5a, b), and this level of DNA methylation was even higher than that of the adult muscle tissue (88 % CpG methylation). However, the 4xUAS sequence was hypomethylated in sperm (average of 39 % CpG methylation) (Fig. 5a, b). These results suggest that the transgene derived from the maternal genome possessed a markedly different epigenetic status from that of the paternal genome. Notably, the maternal 4xUAS was rapidly demethylated, with a decreased DNA methylation level (71 %) in the two-cell stage (Fig. 5a, b). The demethylation of the 4xUAS was consistent in the sphere stage, although slightly higher levels of DNA methylation were observed at the 256-cell stage (86 %) (Fig. 5a, b). In contrast, after fertilization, the paternal 4xUAS was methylated rapidly, with increased DNA methylation levels of 90 and 85 % at the 2-cell and 256-cell stages, respectively (Fig. 5a, b). Later, the paternal 4xUAS began to be demethylated, with a decreased DNA methylation level of 43 % (Fig. 5a, b). These results demonstrate that the maternal 4xUAS underwent rapid demethylation after fertilization; in contrast, the paternal 4xUAS underwent rapid de novo methylation after fertilization and was then demethylated from the 256-cell stage to the sphere stage. Demethylation of the 4xUAS at the sphere stage enabled transcription of the UAS-linked gene in later larval stages.

Discussion

The Gal4/UAS system, a binary system providing temporal and spatial manipulation of gene expression, has been extensively employed in zebrafish (Halpern et al. 2008; Baier and Scott 2009). An optimized Gal4/UAS system, the KalTA4/4xUAS system, was developed to balance transcriptional activity with toxicity (Distel et al. 2009). In our previous study, we reported that this optimized Gal4/UAS system efficiently enhanced primordial germ cell (PGC)-specific gene expression (Xiong et al. 2013). Next, we asked whether the enhancer activity of this Gal4/UAS system is maintained in other tissues. In the present study, the dynamic activity of the optimized Gal4/UAS system, KalTA4/4xUAS, driven by two muscle-tissue-specific 5′ upstream regulatory regions, *mylpfa* and *tpma*, was investigated during early zebrafish developmental stages, into adulthood and to subsequent generations. We found that this optimized Gal4/UAS system driven by the *mylpfa* or *tpma* 5′ upstream regulatory regions efficiently enhanced transcriptional expression of the UAS-linked gene in early development. However, the Gal4/UAS system driven by the muscle-tissue-specific upstream regulatory regions was susceptible to transcriptional silencing in adult tissues and in subsequent generations. The EGFP reporter was silent in the F1 *Tg(mylpfa:Gal4;UAS:EGFP)* larvae and in the muscle tissue of F2 *Tg(tpma:Gal4;UAS:EGFP)* adults. These results were very different from what we observed in our previous study. In that study, without the Gal4/UAS system, a GFP reporter regulated by a PGC-specific promoter (*kop*) was not detectable until the somite stage, and the GFP expression was not observed after 9 dpf. In the presence of the Gal4/UAS system, expression of the GFP reporter was detectable in the PGC at shield stage and was observed consistently until 25 dpf. Moreover, transcriptional silencing of the PGC-specific gene expression was not observed during germline transmission (Xiong et al. 2013). We suggest that the steady enhancer activity of the Gal4/UAS system was dependent on a weakly active tissue-specific regulatory region, whereas a strong regulatory region was prone to transcriptional silencing in later development and subsequent generations. Additionally, in mammals, PGCs undergo global demethylation and maintain a low level of DNA methylation (Seisenberger et al. 2013). We suggest that transcriptional silencing of the UAS-linked gene was not observed in zebrafish PGCs after germline transmission because of this demethylation of DNA in the PGCs, which is significantly different from the process in somatic cells.

Tandem repetitive sequences frequently attract DNA methylation, which always causes transcriptional silencing of genes in vertebrates. In this study, we found that the 4xUAS was highly methylated in the muscle tissue of the F2 *Tg(tpma:Gal4;UAS:EGFP)* adults in which the UAS-linked gene was transcriptionally silent (Fig. 4c). Similar results were

observed in previous work in which DNA methylation occurred in both 14 tandem copies of the same upstream activator sequence (14xUAS) (Goll et al. 2009) and four distinct upstream activator sequences arrayed in tandem (4xnr UAS) (Akitake et al. 2011) in zebrafish. Interestingly, the silent *egfp* was reactivated in the later generation, and the increased transcriptional activity of the *egfp* gene was accompanied by decreased DNA methylation of the 4xUAS sequence (Fig. 4b, c). These results suggest that transcriptional silencing of the UAS-linked gene is correlated with the increase in DNA methylation of the 4xUAS in *Tg(tpma:Gal4;UAS:EGFP)* fish. However, the F3 *Tg(tpma:Gal4;UAS:EGFP)* larvae showed lower EGFP expression than in the *Tg(tpma:EGFP)* larvae, and 40 % of the F2 *Tg(tpma:Gal4;UAS:EGFP)* fish even produced larvae with no EGFP expression (Fig. 4a). These results suggest that the transcriptional repression effect of the epigenetic modification may persist in later generations.

Strikingly, asymmetric DNA methylation profiles of the 4xUAS sequences were observed in the germ cells of the *Tg(tpma:Gal4;UAS:EGFP)* fish. The 4xUAS was hypermethylated in oocytes but hypomethylated in sperm. It is also notable that the methylation profiles in different sperm samples are quite diverse: the ten sequential CpG sites were either highly methylated or highly de-methylated. In view of all the samples that we have assayed, it seems that the methylation of the first three CpG islands is correlated to the hypermethylation of all the potential CpG islands in the UAS sequence, therefore leading to the striking difference in the same sample. Similar asymmetric DNA methylation profiles were reported in a previous study in which a transgene locus (pUSVCAT) generated by the injection and integration of supercoiled plasmids as repetitive multicopy transgene concatemers was hypomethylated in sperm and hypermethylated in unfertilized eggs (Martin and McGowan 1995). After fertilization, the maternal and paternal 4xUAS sequences underwent obviously different DNA methylation reprogramming. The maternal 4xUAS was rapidly demethylated and reached a low DNA methylation state at the sphere stage. By contrast, the paternal 4xUAS underwent rapid de novo methylation and exhibited increased DNA methylation from the 2-cell stage to the 256-cell stage but was then demethylated from the 256-cell stage to the sphere stage (Fig. 5b). Recent research studying methylation in early zebrafish development using whole-genome bisulfite sequencing has revealed a cluster of differentially methylated regions (DMRs) that are hypermethylated in the egg, hypomethylated in the sperm, moderately methylated from the 2-cell to 256-cell stage, and hypomethylated at the sphere stage (Potok et al. 2013; Jiang et al. 2013). This cluster of loci has a relatively high CpG obs/exp ratio (~1.10) which is close to the obs/exp ratio (0.97) of the 4xUAS in our study. Our results suggest that the transgenic 4xUAS is subject to similar DNA methylation reprogramming because of its high obs/exp ratio (Potok et al. 2013). However, in our study, the paternal 4xUAS underwent both rapid de novo methylation and demethylation, which differs from the results of previous whole-genome methylation profile analyses. In those studies, the methylation statuses of 22 DMRs between the paternal and maternal loci were analyzed only in 8-cell stage embryos (Jiang et al. 2013) or in a mixture of 2- to 16-cell stage embryos (Potok et al. 2013).

Taken together, our results suggest that the Gal4/UAS system driven by a strong regulatory region enhances transcriptional activity during early development (approximately 1–4 dpf) but is also subject to transcriptional silencing through DNA methylation of the UAS. Thus, UAS-specific DNA methylation should be seriously considered when strong regulatory regions such as muscle-tissue-specific regulatory regions or ubiquitous regulatory regions are used. In addition, the exogenous transgenic 4xUAS in our studies enabled us to easily distinguish and analyze the dynamic DNA methylation changes at the paternal and maternal loci in two sets of embryos. We suggest that the Gal4/UAS system could be used as a model to investigate the mechanisms of asymmetric DNA methylation in germ cells and the DNA methylation reprogramming of paternal and maternal loci.

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