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Chromatin states of developmentally-regulated genes revealed by DNA and histone methylation patterns in zebrafish embryos

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ABSTRACT Embryo development proceeds from a cascade of gene activation and repression events controlled by epigenetic modifications of DNA and histones. Little is known about epigenetic states in the developing zebrafish, despite its importance as a model organism. We report here DNA methylation and histone modification profiles of promoters of developmentally-regulated genes *(pou5f1, sox2, sox3, klf4, nnr, otx1b, nes, vasa)*, as well as *tert* and *bactin2*, in zebrafish embryos at the mid-late blastula transition, shortly after embryonic genome activation. We identify four classes of promoters based on the following profiles: (i) those enriched in marks of active genes (H3K9ac, H4ac, H3K4me3) without transcriptionally repressing H3K9me3 or H3K27me3; (ii) those enriched in H3K9ac, H4ac and H3K27me3, without H3K9me3; one such gene was *klf4*, shown by *in situ* hybridization to be mosaically expressed, likely accounting for the detection of both activating and repressive marks on its promoter; (iii) those enriched in H3K4me3 and H3K27me3 without acetylation; and (iv) those enriched in all histone modifications examined. Culture of embryo-derived cells under differentiation conditions leads to H3K9 and H4 deacetylation and H3K9 and H3K27 trimethylation on genes that are inactivated, yielding an epigenetic profile similar to those of fibroblasts or muscle. All promoters however retain H3K4me3, indicating an uncoupling of H3K4me3 occupancy and gene expression. All non-CpG island developmentally-regulated promoters are DNA unmethylated in embryos, but hypermethylated in fibroblasts. Our results suggest that differentially expressed embryonic genes are regulated by various patterns of histone modifications on unmethylated DNA, which create a developmentally permissive chromatin state.

KEY WORDS: *Danio rerio, DNA methylation, embryo, gene expression, histone modification, zebrafish*

Introduction

Vertebrate embryo development proceeds from a cascade of gene activation and repression events in response to extracellular signals and local determinants. These transcriptional changes result from a pre-determined differentiation program and regulate differentiation. Within the embryo, coordination of transcription in specific blastomeres requires intricate gene regulatory networks (Levine and Davidson, 2005; Chan *et al.*, 2009; Morley *et al.*, 2009).

Binding of transcriptional regulators to specific genomic sites, the *raison d'être* of gene regulatory networks, is itself modulated by modifications of DNA and chromatin. Among these, methyla-

Abbreviations used in this paper: ac, acetylated; ChIP, chromatin immunoprecipitation; MBT, mid-blastula transition; MBT+, mid-late blastula transition; me3, trimethylated; PTM, post-translational modification; RT-PCR, reverse transcription polymerase chain reaction; qPCR, quantitative PCR; TSS, transcription start site.

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tion of cytosines within CpG dinucleotides is generally associated with gene repression, notably during development and differentiation (Jaenisch and Bird, 2003). In addition, post-translational modifications (PTMs) of core histones modulate their interaction with nucleosomal DNA and targeting of transcriptional regulators (Kouzarides, 2007). In particular, trimethylation of lysine (K) 9 of histone H3 (H3K9me3) marks promoters of inactive genes in a heterochromatin context. Temporarily inactive genes, such as in undifferentiated cells, are marked by H3K27me3, a mark of facultative heterochromatin, with or without the transcriptionally permissive H3K4me3 (Azuara *et al.*, 2006; Bernstein *et al.*, 2006; Cui *et al.*, 2009). In contrast, promoters of active genes are associated with H3K4me3 and acetylated H3K9 (H3K9ac) or H4. Little is known on histone PTMs associated with developmentally-regulated genes in embryos, and only recently have histone PTM mapping data emerged in the mouse embryo (O'Neill *et al.*, 2006; Vermilyea *et al.*, 2009; Dahl *et al.*, 2010).

Despite the importance of zebrafish (*Danio rerio*) as a model organism for studying vertebrate embryogenesis (Aleström *et al.*, 2006) and developmental regulatory networks (Chan *et al.*, 2009), little is known on histone PTMs associated with developmentally-regulated genes in this organism. In zebrafish, chromatin immunoprecipitation (ChIP) (Collas and Dahl, 2008) has been used to investigate the association of H4ac and c-Myc on specific promoters (Havis *et al.*, 2006), map H3K4me3 sites in gastrula-stage embryos (Wardle *et al.*, 2006) and investigate the role of specific transcription factors in hematopoiesis (Hart *et al.*, 2007). Of note, these ChIP protocols relied on protease treatment of embryos to remove the chorion prior to isolating chromatin. By re-assessing each step of the ChIP assay, we recently reported that proteases are detrimental to ChIP efficiency in zebrafish embryos, and subsequently improved the procedure (Lindeman *et al.*, 2009).

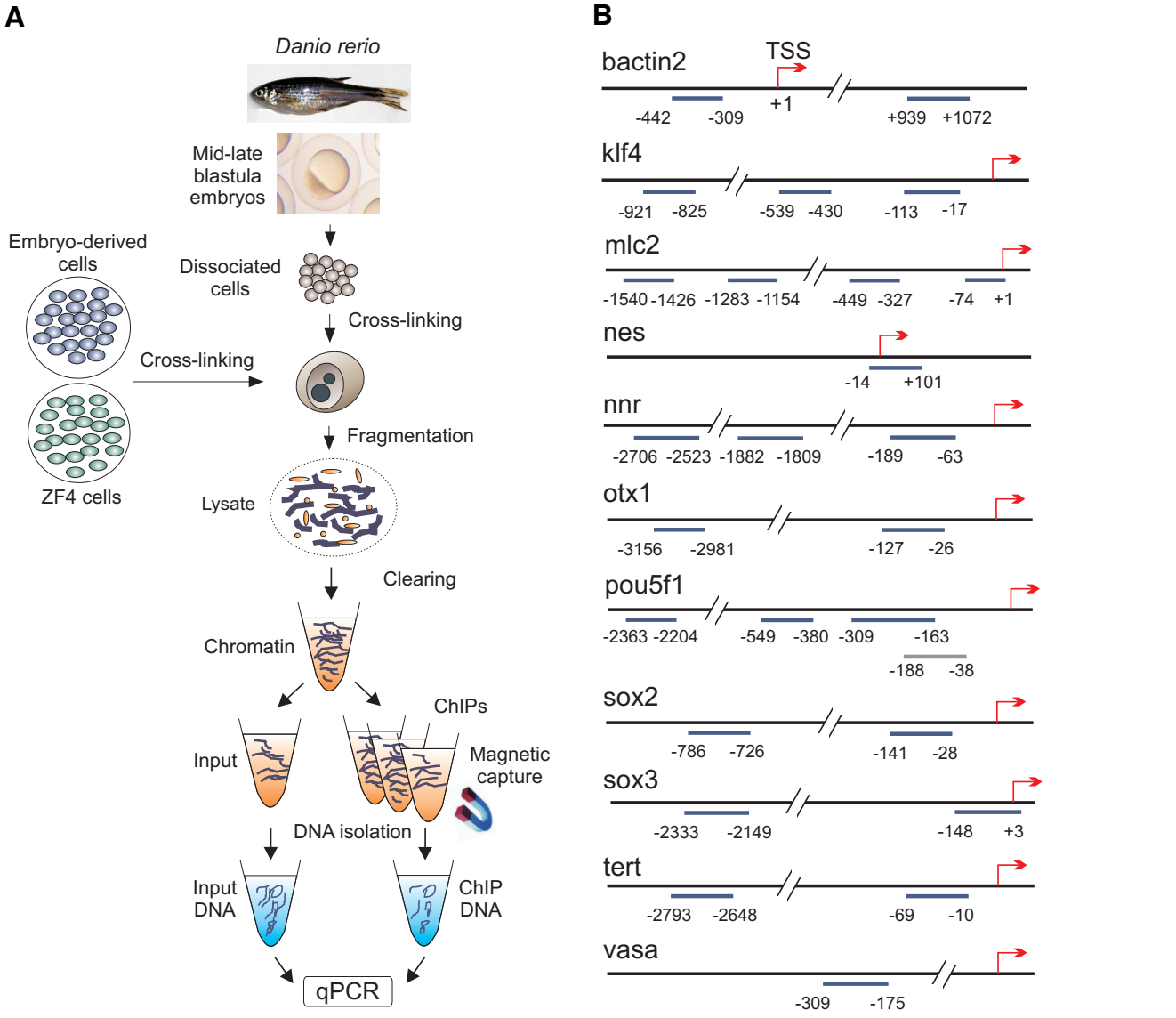

Fig. 1. Chromatin immunoprecipitation (ChIP) assay and map of genomic regions examined in this study. (A) *Post-translational histone modifications in MBT⁺ stage embryos, ZF4 cells and MBT⁺ embryo-derived cultured cells were examined by ChIP-qPCR.* **(B)** *Genomic regions examined by ChIP on indicated genes. Numbers indicate the position of amplicons relative to the TSS (+1).*

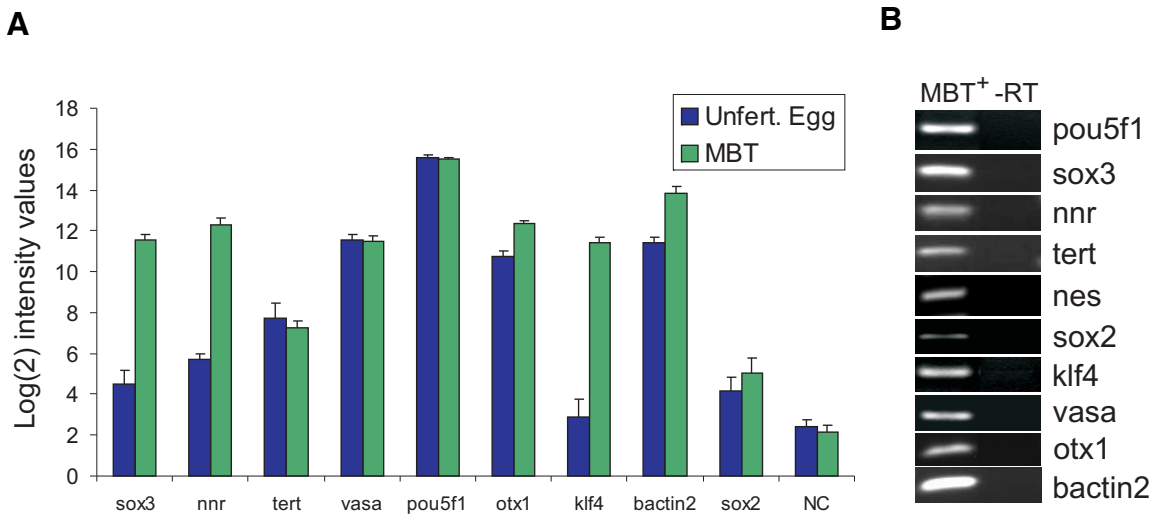

Fig. 2. Expression pattern of selected genes in zebrafish embryos. (A) *Data extracted from Agilent microarray analyses of gene expression in unfertilized eggs and MBT embryos. Data was generated from 3 biological replicates for both stages, with 3 and 4 arrays for unfertilized egg and MBT stages, respectively. Signals were quantile-normalized, multiple probes for each gene were aggregated using the median, and mean±SD calculated based on log_2-transformed values. Negative controls are the mean of the replicates of the median of 150 different negative control probes scattered throughout the array.* **(B)** *RT-PCR analysis of expression of indicated genes in MBT⁺ embryos; -RT, PCR without reverse transcription.*

We define here DNA methylation patterns at single-nucleotide resolution, and the occupancy of transcriptionally activating and repressing histone PTMs on the promoters of several developmentally-regulated genes in AB strain zebrafish embryos at the mid-late blastula transition (3.5 h post-fertilization), shortly after embryonic gene activation. Our results reveal complex epigenetic patterns at this critical developmental time point. These do not necessarily correlate with promoter activity but are suggestive of a developmentally permissive chromatin state.

Results

Gene-specific post-translational histone modifications in embryos at the mid-late blastula transition

Aiming at understanding epigenetic states during zebrafish development, we recently optimized a ChIP assay for zebrafish embryos, resulting in minimal chromatin loss and epitope degradation (Lindeman *et al.*, 2009). We applied this ChIP assay to determine histone PTM enrichment profiles on the promoters of a subset of developmentally-regulated genes in embryos at the mid-late blastula transition (referred to as MBT⁺), i.e. minutes after activation of the embryonic genome (Fig. 1A). The genes included *pou5f1* (Pou-domain class 5 transcription factor 1, formerly named *pou2* and also called *oct4*; GenBank accession number NM_131112), *sox2* (SRY-box containing gene 2, NM_213118) and *sox3* (SRY-box containing gene 3, NM_001001811) – two members of the SRY-related high-mobility group box family of transcription factors implicated in cell fate, *klf4* (Krüppel-like factor 4, NM_131723; a Krüppel-like family transcription factor with a role in cell proliferation and differentiation), *nnr* (nanor; NM_001029947; a gene with a potential role in transcriptional regulation), *vasa* (NM_131057; a maternally-expressed gene with a role in germplasm formation) and *otx1b* (orthodenticle homolog 1b, NM_131250; encoding a bicoid subfamily homeodomain-containing transcription factor involved in brain development). We also examined *tert* (telomerase; NM_001083866), *nes* (nestin; XM_001919887; an early neuronal marker) and the ubiquitously expressed gene *bactin2* (beta-actin 2; NM_181601). Promoter areas examined by ChIP are shown in Fig. 1B. Histone PTMs examined were three modifications associated with transcriptionally active genes (H3K9ac, H4ac and H3K4me3) and two associated with repressed genes (H3K9me3 and H3K27me3).

Before setting out to assess epigenetic states in embryos, we first determined gene expression profiles in unfertilized eggs and in MBT embryos (3 biological replicates each) using custom-designed Agilent microarrays. The data revealed maternal *vasa*, *pou5f1*, *otx1b*, *bactin2* and at a lower level, *tert* transcripts (Fig. 2A). In contrast, *sox3*, *nnr* and *klf4* transcripts were strongly upregulated at MBT, from very low levels in the egg, while *sox2* was slightly upregulated and remained expressed at low level (Fig. 2A). Reverse transcription (RT)-PCR data confirmed transcript detection at the MBT⁺ stage (Fig. 2B).

The genes examined could be categorized into four groups according to their histone PTM profile on promoters (Fig. 3). A first group consisting of *pou5f1*, *sox3*, *nnr*, *tert*, *nes* and the housekeeping gene *bactin2* were enriched in H3K9ac, H4ac and H3K4me3 with no or little repressive H3K9me3 or H3K27me3, consistent with their expression (Fig. 3A). Enrichment levels seemed to vary along the promoter regions assessed, in a gene-specific manner. These differences were not due to varying quantitative (q)PCR efficiencies because opposite patterns could be observed for the same genomic fragments associated with other PTMs (e.g., H3K4me3 on *pou5f1* or *nnr*; Fig. 3A). A second group harbored activating PTMs (H3K9ac, H4ac, H3K4me3) and repressing H3K27me3 (*sox2*, *klf4*; Fig. 3B). A third PTM profile was detected on *otx1b*, with essentially no acetylation but high levels of H3K4me3 and H3K27me3 (Fig. 3C). This combination

was reminiscent of the co-enrichment of these marks on so-called "bivalent" promoters in embryonic stem (ES) cells (Bernstein *et al.*, 2006; Pan *et al.*, 2007). A fourth profile was observed on *vasa*, which harbored, in addition to activating PTMs, H3K27me3 together with H3K9me3 (Fig. 3D). Thus, a subset of expressed developmentally-regulated genes in MBT$^+$ stage embryos is acetylated on H3K9 and H4 with no trimethylation on H3K9 or H3K27, while another subset appears to harbor a combination of activating and repressing PTMs. All promoters are enriched in H3K4me3, corroborating the view that H3K4me3 marks most promoters, at least in undifferentiated cells (Zhao *et al.*, 2007), including in developing zebrafish (Wardle *et al.*, 2006), regardless of expression.

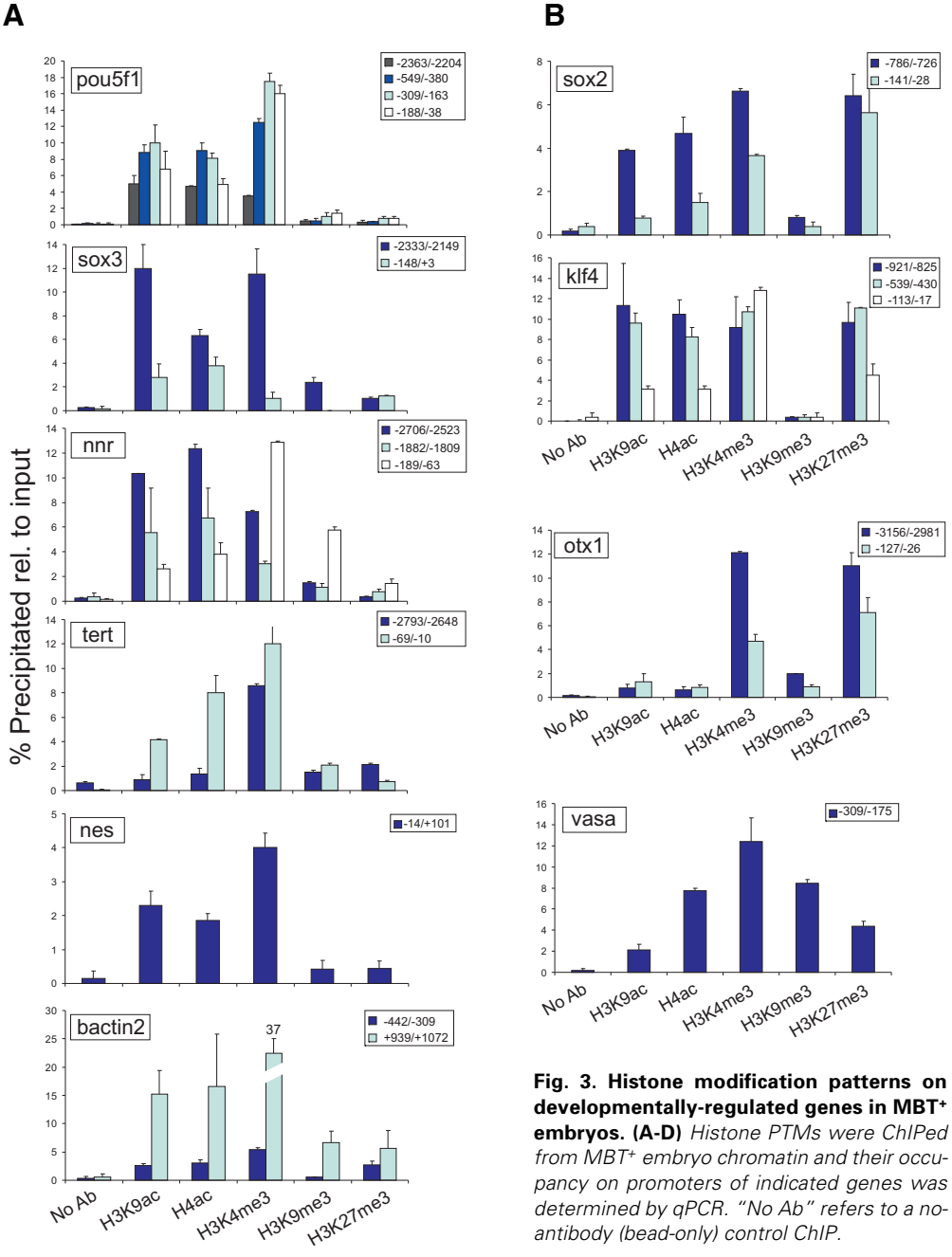

Fig. 3. Histone modification patterns on developmentally-regulated genes in MBT$^+$ embryos. (A-D) *Histone PTMs were ChIPed from MBT$^+$ embryo chromatin and their occupancy on promoters of indicated genes was determined by qPCR. "No Ab" refers to a no-antibody (bead-only) control ChIP.*

Embryonic genes are associated with repressing histone PTMs in fibroblasts and in primary tissue

How specific to embryos were the histone PTM profiles detected was determined bynexamining the zebrafish fibroblast cell line, ZF4 (Driever and Rangini, 1993). All embryonic gene promoters showed strong deacetylation on H3K9 and H4 relative to H3K4me3 (Supplementary Fig. 1A). Deacetylation was consistent with the lack of, or barely detectable, expression of these genes (Supplementary Fig. 1B). In contrast, *tert*, *nes* and *bactin2*, expressed in ZF4 cells, retained acetylated epitopes, illustrating the specificity of the deacetylated state of embryonic genes in these somatic cells.

The distinct histone PTM profiles detected in embryos and in ZF4 cells may reflect an embryonic vs. differentiated state, or alternatively, the fact that ZF4 is an immortalized cultured cell line with altered epigenetic states, as opposed to being a primary somatic cell type. To distinguish between these possibilities, we determined histone PTM profiles in adult zebrafish uncultured muscle biopsies. ChIP data indicate that *pou5f1*, *klf4*, *sox2* and *vasa,n*none of which were expressed in muscle (Fig. 4A), displayed PTMs of repressed genes, namely no H3K9ac or H4ac, but rather, enrichment in H3K4me3 in combination with trimethylated H3K9 and H3K27 (Fig. 4B). In contrast, the expressed *bactin2* and *mlc2* (myosin light chain 2, also called *myl10*; a myogenic marker) promoters were occupied by H3K9ac and H4ac (Fig. 4C). Interestingly, the *mlc2* promoter was also strongly enriched in H3K27me3, suggesting a mosaic epigenetic state with acetylated histones on certain alleles and trimethylated H3K27 on others. Alternatively, this finding suggests a mosaic *mlc2* expression pattern in muscle tissue, as anticipated from heterogeneous cell types in a muscle biopsy. The distinct histone PTM enrichment patterns identified in embryos and in muscle, therefore, respectively reflect the epigenetic states of embryonic cells vs. adult somatic tissue.

Heterochromatinization of embryonic genes in primary cell cultures derived from embryos

To ensure that differences in PTM profiles between embryos and ZF4 cells were not due to fish strain (and thus genotypic) differences, we iso-

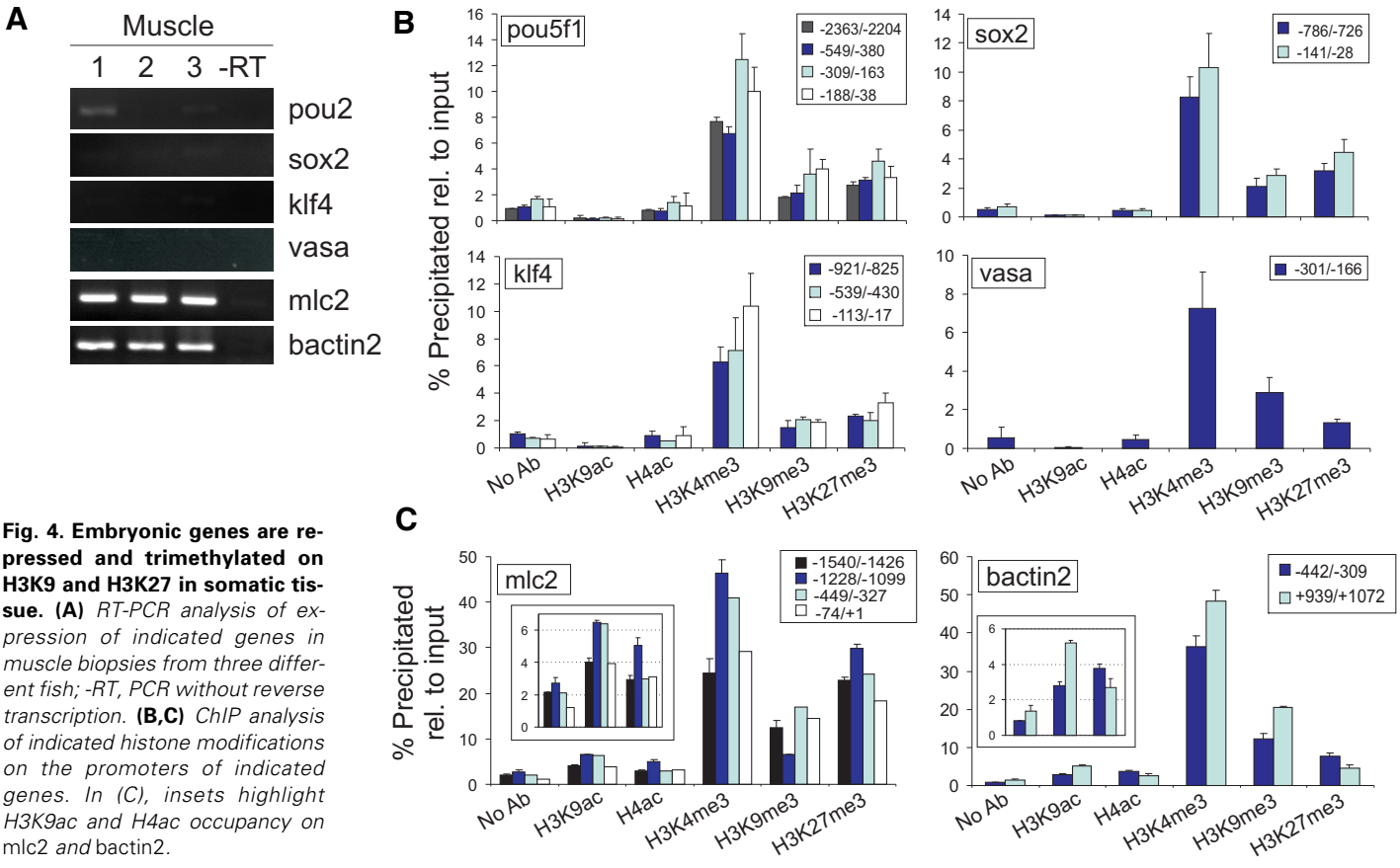

Fig. 4. Embryonic genes are repressed and trimethylated on H3K9 and H3K27 in somatic tissue. (A) *RT-PCR analysis of expression of indicated genes in muscle biopsies from three different fish; -RT, PCR without reverse transcription.* **(B,C)** *ChIP analysis of indicated histone modifications on the promoters of indicated genes. In (C), insets highlight H3K9ac and H4ac occupancy on* mlc2 *and* bactin2.

lated individual cells from MBT+ embryos and seeded them in 6-well plates for 24 h before gene expression and ChIP analysis. As in ZF4 cells, embryonic genes were deacetylated on H3K9 and H4 relative to H3K4me3 (Fig. 5A). Deacetylation was accompanied by enrichment in H3K9me3 and H3K27me3, including on promoters not harboring these marks in the embryo (*pou5f1*, *sox3*, *nnr*, *nes*). Moreover, promoters enriched in H3K27me3 in embryos acquired trimethylation on H3K9 (*sox2*, *klf4*, *otx1b*). *bactin2* retained acetylated marks, consistent with its expression in embryo-derived cells (Fig. 5 A,B).

These results are indicative of heterochromatinization of embryonic genes upon culture of embryo-derived cells under conditions which promote spontaneous differentiation. Indeed, all genes (except *bactin2*) were repressed upon culture (Fig. 5B). The detection of transcripts on day 1 of culture likely reflected the persistence of embryonic mRNAs rather than actual transcription, which would not be permitted by H3K9 and H3K27 hyper-trimethylation. Disappearance of these mRNAs over time supported this view (Fig. 5B, Day 5), and ChIP data on day 5 cultures confirmed the stability of heterochromatinization taking place on the embryonic genes (data not shown).

Mosaic klf4 expression in embryos correlates with co-detection of H3K4me3 and H3K27me3 on the promoter

The co-detection of H3K4me3 and H3K27me3 on a number of embryonic gene promoters suggests that these PTMs may be co-enriched on these promoters in a manner reminiscent of the trimethylated H3K4/H3K27 "bivalency" of developmentally-regulated promoters in'embryonic stem cells (Bernstein *et al.*, 2006). Such bivalency would be assessed by sequential ChIP of H3K4me3 and H3K27me3 and *vice versa*. However, such sequential ChIPs from MBT+ embryo chromatin to assess co-occupancy on *sox2*, *klf4* and *otx1b* was inconclusive (data not shown).

H3K4/K27me3 co-detection could also reflect mosaic expression of these genes in the embryo at the MBT+ stage. Mosaic gene expression is a hallmark of embryonic development in notably mouse (Rossant and Tam, 2009) and zebrafish (Sprague *et al.*, 2003; Lunde *et al.*, 2004; Sprague *et al.*, 2006) and may be at the origin of lineage specification. Whole-mount *in situ* hybridization of MBT+ embryos showed that *sox3* was ubiquitously expressed (Fig. 6), consistent with the activating histone PTMs detected on the promoter. *otx1b* mRNA was also detected in all blastomeres despite its enrichment in H3K4me3 and H3K27me3 (Fig. 6); nevertheless this was consistent with *otx1b* being maternally expressed. In contrast to *sox3* however, *klf4* displayed a striking mosaic expression pattern between blastomeres (Fig. 6). We infer from these results that H3K4/K27me3 co-detection by ChIP on *klf4* reflects a mosaic expression pattern, although this may not be the only explanation (see Discussion).

Embryonic genes are CpG unmethylated in embryos, but strongly methylated in fibroblasts

To provide additional insight on epigenetic states of embryonic genes in MBT+ embryos, we next assessed DNA methylation at

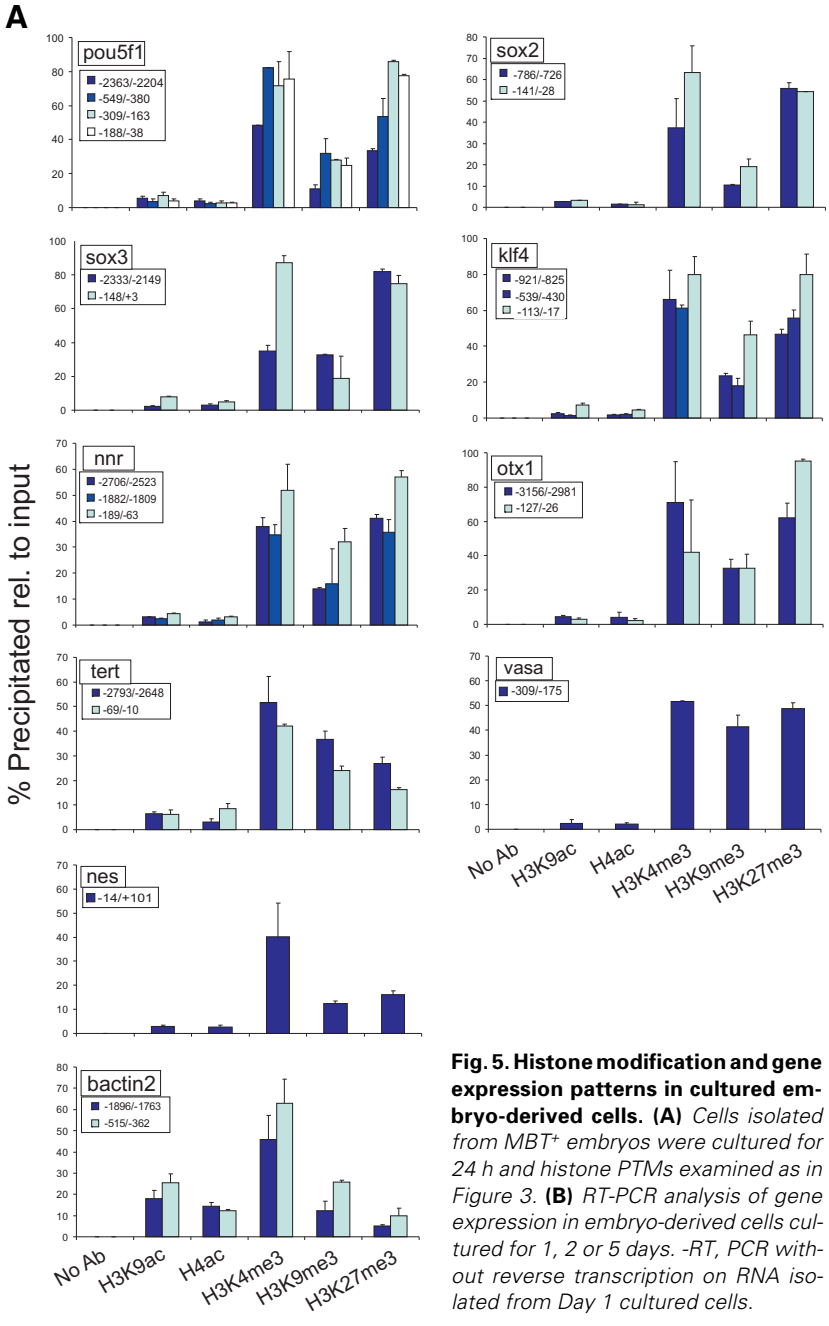

Fig. 5. Histone modification and gene expression patterns in cultured embryo-derived cells. (A) *Cells isolated from MBT+ embryos were cultured for 24 h and histone PTMs examined as in Figure 3.* **(B)** *RT-PCR analysis of gene expression in embryo-derived cells cultured for 1, 2 or 5 days. -RT, PCR without reverse transcription on RNA isolated from Day 1 cultured cells.*

single-base resolution by bisulfite genomic sequencing. We found that embryonic promoters (*sox2*, *pou5f1*, *klf4*, *sox3*, *otx1b* and *vasa*) were unmethylated, whereas *tert* was strongly methylated (Fig. 7). In contrast, in ZF4 cells, *pou5f1*, *sox3*, *klf4*, *otx1b* and *vasa* were strongly methylated (Fig. 7), in agreement with their repressed state. Furthermore, *tert* was also strongly methylated except for two CpGs at position -1157 and -1150 relative to the transcription start site (TSS) (Fig. 7), suggesting that these CpGs are important for telomerase expression in ZF4 cells (Supplementary Fig. 1B). *sox2* was, as in embryos, largely unmethylated in ZF4, which could be explained by its location within a CpG island. These results indicate that embryonic gene promoters are unmethylated at this stage of development, providing a chromatin structure permissive for gene expression.

Discussion

Complex histone PTM enrichment profiles on promoters of early developmentally-regulated genes

We have mapped by ChIP histone PTMs on promoters of early zebrafish developmentally-regulated genes shortly after activation of the embryonic genome. We identify four histone PTM profiles over a background of unmethylated DNA: (i) H3K4me3 and acetylated H3K9 and H4 with no or barely detectable H3K9me3 and H3K27me3 (*pou5f1*, *sox3*, *nnr*, *nes*); (ii) H3K4me3, H3K9ac and H4ac together with repressive H3K27me3 (*sox2*, *klf4*); (iii) H3K4me3 and H3K27me3 without significant acetylation (*otx1b*), a profile reminiscent of the so-called "bivalent" state of repressed or weakly expressed developmentally regulated gene promoters in embryonic stem cells (Azuara *et al.*, 2006; Bernstein *et al.*, 2006); and (iv) H3K4me3, acetylated H3K9 and H4, and trimethylated H3K9 and H3K27 (*vasa*). The diversity of these profiles indicates no simple relationship between promoter occupancy by activating or repressing histone PTMs and the presence of transcripts in the zebrafish embryo at this critical stage of development. This view extends recent findings at the single gene level (O'Neill *et al.*, 2006; Vermilyea *et al.*, 2009) and on a genome-scale (Dahl *et al.*, 2010) in mouse embryos.

Epigenetic states of embryonic genes in relation to developmental dynamics

The complexity of histone PTM profiles reported here reflects the diversity of developmental programs within the embryo, such that not all embryonic genes are expressed in all blastomeres (Rossant and Tam, 2009; Chan *et al.*, 2009). The salt-and-pepper distribution of *klf4* transcripts illustrates the differential programming of individual cells and would account for the apparent co-

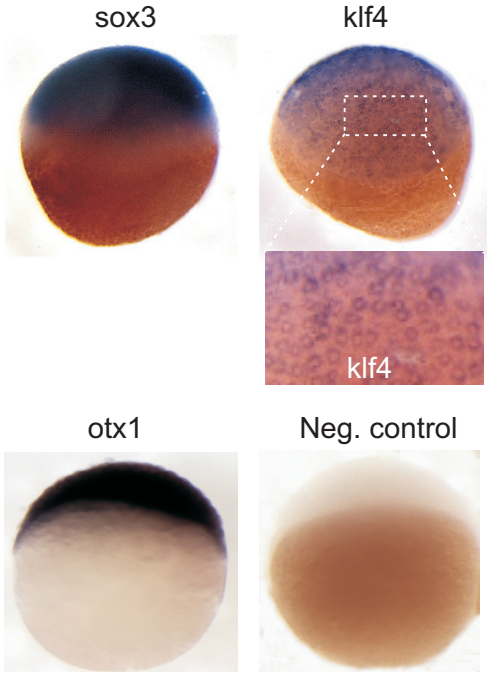

Fig. 6. Whole-mount *in situ* hybridization analysis of expression of indicated genes in MBT⁺ stage embryos. *Enlargement of* klf4 *hybridization pattern (boxed area) shows mosaic expression between blastomeres. A negative control* otx1b *hybridization using a sense* otx1b *probe is also shown.*

enrichment of *klf4* in H3K4me3, H3K9ac – and H3K27me3. *klf4* may be enriched in H3K27me3 (conceivably with K4me3) only in cells not expressing the gene, while expressing cells display H3K4me3 (without K27me3).

This however does not exclude the possibility of true co-enrichment in H3K4/K27me3 within a single cell (albeit possibly on different alleles) or on a single *klf4* promoter sequence, in cells not expressing *klf4*. Based on genome-wide data from mouse and human embryonic stem cells (Azuara *et al.*, 2006; Bernstein *et al.*, 2006; Pan *et al.*, 2007), as well as in hematopoietic progenitors (Cui *et al.*, 2009), such H3K4/K27me3 "bivalent" state would predict that non-*klf4* expressing cells are programmed for later upregulation of the gene. Indeed, *klf4* is strongly upregulated post-MBT (Sprague *et al.*, 2003; Sprague *et al.*, 2006; O'Boyle *et al.*, 2007). Interestingly, recent genome-wide H3K4me3 and H3K27me3 promoter mapping data in mouse embryos reveals co-enrichment of these marks on developmentally-regulated genes (Dahl *et al.*, 2010). Collectively, these and our findings support the view of transcriptional "priming" of developmentally important genes by H3K4/K27me3 co-enrichment (Azuara *et al.*, 2006; Bernstein *et al.*, 2006). Later in development, *klf4* may be upregulated in more cells or in all cells, an event that would be associated with H3K27 demethylation. Demethylation of H3K27me3 may de-repress developmentally important genes not only during embryonic stem cell differentiation (Azuara *et al.*, 2006; Bernstein *et al.*, 2006) but also during development. Alternatively, *klf4* may remain mosaically expressed post-MBT and

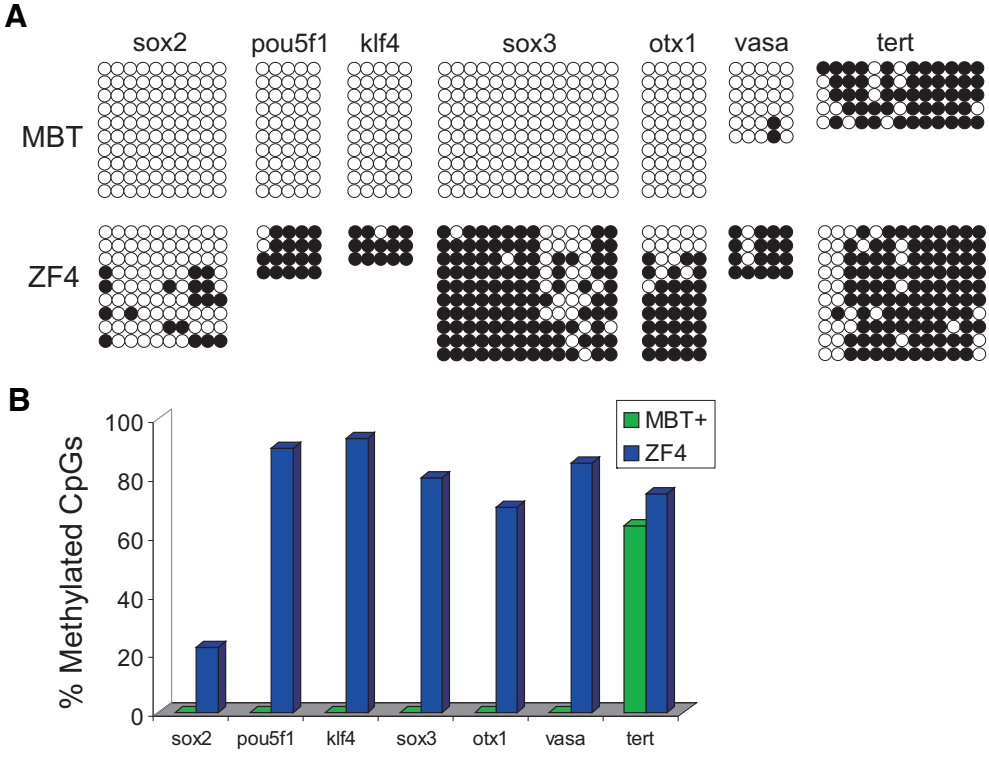

Fig. 7. Embryonic genes are DNA unmethylated in MBT⁺ stage embryos. (A) *Bisulfite sequencing analysis of CpG methylation in the promoter of indicated genes in MBT⁺ embryos and in ZF4 cells. Each circle represents one CpG and methylation is shown in the 5′ to 3′ orientation (left to right); (○) unmethylated CpG; (●) methylated CpG. Three to ten sequenced clones of PCR products are shown to provide quantification (rows).* **(B)** *Percentage of methylated CpGs for each gene, determined from data in (A).*

retain H3K27me3 in a subset of blastomeres.

sox3 and *nnr*, which are not maternally expressed, are upregulated at the MBT and at least for *sox3*, ubiquitously in the embryo. The absence of H3K27me3 on these promoters argues that this transcriptional brake has been released to enable expression throughout the embryo in subsequent stages. Moreover, *sox2* transcripts are only moderately detected at the mid-late MBT, consistent with moderate H3K9 and H4 acetylation on the promoter. Further, histone PTM profiles of *pou5f1* and *tert* would be consistent with some transcription level taking place in the embryo, while the *sox2* profile suggests a repressive effect of H3K27me3 despite promoter occupancy by acetylated H3K9 and H4; indeed, *sox2* is upregulated post-MBT (Mathavan *et al.*, 2005; Sprague *et al.*, 2006) (our unpublished data), i.e. later than *klf4*, *sox3* or *nnr*.

DNA hypomethylation on developmentally-regulated promoters reflects an underlying transcriptionally permissive state

A transcriptionally permissive chromatin organization of developmentally-regulated genes in the embryo is supported by the unmethylated state of DNA in the promoters examined. CpG unmethylated promoters are not necessarily expressed (Weber *et al.*, 2007), in consistency with our findings; nonetheless, the absence of DNA methylation is likely to provide a flexible chromatin configuration prone to timely regulation by other mechanisms (e.g., histone PTMs or transcription factor binding). This relationship between promoter DNA methylation and potential for gene expression is reminiscent of that in embryonic or mesenchymal stem cells: whereas strong CpG methylation is transcriptionally restrictive, no or weak methylation constitutes a permissive state (Azuara *et al.*, 2006; Sørensen *et al.*, 2009).

In contrast to the embryo, all genes except *sox2*, embedded in a CpG island presumably protected from methylation (Weber *et al.*, 2007), are hypermethylated in ZF4 fibroblasts. This is in accordance with their transcriptional repression and reflects the differentiated state of these cells (Driever and Rangini, 1993). Interestingly however, CpG -1157 in the *tert* promoter remains unmethylated while CpG -1150 is demethylated in ZF4 cells. CpG -1150 is flanked by two binding sites for elongation factor 2 (E2F) (www.cbrc.jp/research/db/TFSEARCH.html), a transcription factor implicated in cell cycle regulation (Chen *et al.*, 2009). CpG demethylation at this site may enable E2F binding and induction of telomerase expression in this immortalized cell line. This suggests that *tert* expression is regulated by DNA methylation.

Differentiation events taking place during lineage specification result in cell type-specific gene expression patterns and epigenetic states. The dynamics of histone PTMs during zebrafish development remains to be determined. Asymmetry of genome-wide promoter distribution of, in particular, H3K27me3 between the inner cell mass and trophectoderm (the first two developmental lineages) in mouse blastocysts suggests that gene expression patterns can be promptly modulated in response to developmental decisions (Dahl *et al.*, 2010). Rapid development of zebrafish embryos relative to mammals requires fast and accurate mechanisms of histone deposition and removal, presumably from a pool of maternally stored histones which can be modified, as recently shown in *Xenopus* embryos (Shechter *et al.*, 2009).

Materials and Methods

Embryos and cells

Mid-late blastula stage embryos

Mid-late blastula (referred here to as MBT$^+$) AB strain zebrafish embryos were collected at 3.5 h post-fertilization. For RNA isolation, embryos were immediately snap-frozen in ethanol/dry ice. For ChIP, embryos were processed as described below.

Unfertilized eggs

Unfertilized eggs were collected immediately after spawning, prior to fertilization, washed, snap-frozen in ethanol/dry ice and stored at -80°C prior to RNA extraction.

MBT$^+$ embryo-derived cells

MBT$^+$ embryos (n=500-1000) in system water were transferred to a sterile 100 μm sieve (BD Biosciences; www.bdbiosciences.com). Embryos were rinsed twice for 10 sec in a well containing system water, washed once in 70% ethanol for 15 sec, twice in water for 30 sec, and bleached 3 times in 0.025 % chlorine with 10-sec washing steps in LDF medium (Leibowitz´s L15/Delbecco´s MEM/Hams´s F12 in proportions of 50:35:15). Embryos were treated with 1 mg/ml pronase for 15 min in Hanks solution (Invitrogen; www.invitrogen.com), trypsinized for 5 min in TryLE Express (Invitrogen) and gently pipetted with a transfer pipette while inside the sieve to assist in dechorionation and cell dissociation. Dissociated cells were collected in a Petri dish, leaving chorions in the sieve. Cells were transferred to a tube of fetal bovine serum using a new transfer pipette and sedimented at 300 g for 5 min. Cells were then seeded at 10^5 cells per well in 6-well plates (Nunc, delta-surface; www.nuncbrand.com) in growth medium (LDF supplemented with 2.5% fetal calf serum, 5% zebrafish embryo extract (Fan *et al.*, 2004) at 25 μg/ml protein, 1% heat inactivated SeaGrow trout serum (East Coast Biologics; www.eastcoastbio.com), 10 μg/ml bovine insulin, 50 ng/ml bovine FGF, 50 ng/ml EGF and 31.5% conditioned medium from RTS34 cells). RTS34 cells were cultured in 70% Leibowits's L15 media/30% fetal calf serum and antibiotics (Fan *et al.*, 2006). Cells were cultured at 28°C for 24 h and those forming aggregates were transferred to new wells for further culture.

ZF4 cells

The ZF4 zebrafish fibroblastoid cell line (ATCC, CRL-2050; www.atcc.org) was cultured as described (Driever and Rangini, 1993).

ChIP antibodies

Antibodies used were anti-H3K4me3 (Abcam Ab8580-100, www.abcam.com; or Diagenode pAb-003-050; www.diagenode.com), anti-H3K9me3 (Diagenode pAb-056-050), anti-H3K27me3 (Abcam Ab60052-100; Upstate 07-449, www.upstate.com; or Millipore 07-449, www.millipore.com), anti-H3K9ac (Upstate 06-942), and anti-H4ac (Upstate 06-866).

Chromatin preparation

MBT$^+$ embryos

Chromatin from MBT$^+$ embryos was prepared as described (Lindeman *et al.*, 2009). In short, 500 embryos in water were transferred to a 5-ml syringe, water was replaced with PBS/20 mM Na-butyrate and embryos forced through a 21 gauge needle into a 1.5 ml tube. Isolated cells were cross-linked with 1% formaldehyde in PBS/butyrate for 8 min, the reaction stopped with 125 mM glycine, cells washed in PBS/butyrate, sedimented and snap-frozen in ethanol-dry ice. Cells were lysed in 250 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors and 20 mM Na-butyrate) and sonicated in two lots of 125 μl for 6x30 sec on ice using a Sartorius Labsonic M sonicator with a 3-mm diameter probe at

setting 0.5 cycle and 30% power (Sartorius AG; www.sartorius.com). Fragmented chromatin (~400 bp fragments on average, determined by agarose gel electrophoresis) was diluted in RIPA ChIP buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, protease inhibitors, 20 mM Na-butyrate) to 0.2 U A_{260} before immunoprecipitation.

Muscle biopsy

Muscle biopsies (2 mm^3) from adult fish (n=2 for ChIP; n=3 for RT-PCR) were snap-frozen in liquid nitrogen. Upon thawing, biopsies were crosslinked for 8 min in 1% formaldehyde in PBS/20 mM Na-butyrate and washed 3 times in PBS/butyrate. After adding 300 μl lysis buffer, samples were sonicated for 5x30 sec and sedimented at 10,000 g. The supernatant was removed to a clean tube. Lysis buffer was added to the pellet up to 300 μl, the sample sonicated for another 5x30 sec, sedimented and the supernatant pooled with the first one. The pooled chromatin fraction (containing 400 bp average fragments) was diluted in RIPA ChIP buffer to 0.2 U A_{260}.

ZF4 cells

ZF4 cells ($15x10^6$) were crosslinked in suspension for 8 min in 1% formaldehyde in PBS/butyrate and the reaction stopped with 125 mM glycine. Cells were lysed in 300 μl lysis buffer and sonicated 8x30 sec to an average of 400 bp. Chromatin was diluted in RIPA ChIP buffer before immunoprecipitation.

Embryo-derived cells

Cells which formed aggregates on day 1 of culture were collected and transferred to a 50 ml tube containing LDF medium and 20 mM Na-butyrate. Cells were sedimented, transferred to a 0.6 ml tube and crosslinked for 8 min as above. After quenching and washing, cells were lysed and sonicated for 5x30 sec. Chromatin was diluted in RIPA ChIP buffer to 0.2 U A_{260} before immunoprecipitation.

Chromatin immunoprecipitation and PCR analysis

Chromatin at concentrations indicated above, in 100 μl RIPA ChIP buffer/butyrate, was mixed with 10 μl antibody-Dynabeads Protein A (Invitrogen) complexes overnight at 4°C. Immune complexes were washed three times in RIPA and once in Tris-EDTA (TE) buffer. The cross-link was reversed and DNA eluted in a single step for 2 h at 68°C in 150 μl of 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, 50 mM NaCl, 1% SDS and 50 μg/ml proteinase K. Eluted DNA was purified by phenol-chloroform isoamylalcohol extraction and ethanol precipitation and dissolved in TE buffer or MilliQ H_2O. ChIP DNA was amplified using the Whole Genome Amplification WGA4 kit (Sigma-Aldrich; www.sigma.com) using only 10 amplification cycles. Amplification products were cleaned up using the QiaQuick PCR Purification Kit (Qiagen), eluted in 30 μl TE and diluted further to 150 μl in TE. For all ChIPs, 5 μl DNA was used for quantitative (q)PCR. ChIPs were done from at least four independent chromatin preparations per histone PTM.

ChIP DNA was analyzed by duplicate qPCR on a MyiQ Real-time PCR Detection System using IQ SYBR® Green (BioRad; www.biorad.com). ChIP PCR primers are listed in Supplementary Table 1. PCR conditions were 95°C for 3 min and 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. ChIP Data are presented as mean±SD percent precipitated DNA relative to input.

Bisulfite sequencing

Genomic DNA was purified from MBT^+ embryos and ZF4 cells by double phenol-chloroform extraction and ethanol precipitation, and bisulfite-treated using MethylEasy™ (Human Genetic Signatures; www.geneticsignatures.com) as described (Noer *et al*., 2006). Converted DNA was amplified by PCR using primers designed with Methprimer (www.urogene.org/methprimer/index1.html) and positioned relative to the transcription start site (TSS; www.ensembl.org/Danio_rerio/) as shown in Supplementary Table 2. PCR conditions were 95°C for 7 min and 40 cycles of 95°C for 1 min, 54°C for 2 min and 72°C for 2 min, followed by 10 min at 72°C. PCR products were cloned into *E. coli* by TOPO TA cloning and sequenced.

RNA isolation

Unfertilized eggs and MBT^+ embryos were snap-frozen as described above and kept at -80°C. RNA was prepared from batches of 100 embryos. These were crushed in a mortar with liquid nitrogen, 2 ml Trizol (Invitrogen) was added and the content was transferred to a 15-ml tube and incubated for 5 min at room temperature. Chloroform (400 μl) was added and embryos were shaken by hand for 15 sec and further incubated for 2-3 min. The lysate was centrifuged (10,000 g, 10 min, 4°C), the upper phase was transferred to a new tube, 1 μl glycogen added and RNA was precipitated with 1 ml isopropanol for 10 min at room temperature before centrifugation as above. The pellet was washed with 75% ice-cold ethanol and stored at -80°C or dissolved for DNase treatment and clean-up using the RNeasy Kit (Qiagen). For embryo-derived and ZF4 cells, ~10^6 snap-frozen cells were suspended in 2 ml Trizol, pipetted 2-3 times and incubated for 5 min at room temperature. Chloroform (400 μl) was added, cells were shaken for 15 sec, incubated for 2-3 min, aliquoted into two 1.5-ml tubes and sedimented as above. The rest of the procedure was as above. RNA samples were cleaned up, DNase I-treated and RNA was stored at -80°C until use for microarray processing or RT-PCR.

Reverse-transcription-PCR

RT-PCR was performed from 0.5 μg total RNA (Qiagen RNeasy; www.qiagen.com), using the Iscript cDNA synthesis kit (BioRad). PCR conditions were 95°C for 3 min and 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. RT-PCR primers used are listed in Supplementary Table 3. Products were resolved by agarose gel electrophoresis and stained with ethidium bromide.

Microarray analysis

Microarray techniques were according to Agilent's One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling) manual Version 5.7 (www.agilent.com). Briefly, ~400 ng total RNA and spike-in RNA were used to prepare cDNA and subsequently Cy3-labeled cRNA. Labeled cRNA was fragmented prior to hybridization. Arrays were pre-hybridized, hybridized at 65°C for ~17 h, and washed as per Agilent's protocol. The arrays used contained 44K probes representing all known genes and one probe for each UniGene clusters (non-redundant). Arrays also contained 153 different negative control probes scattered throughout. Array format was 44Kx4 and was custom-designed by Agilent for this study.

Arrays were scanned using an Agilent DNA Microarray Scanner at 5 μm resolution. Using the 16-bit tif images generated, feature extraction was performed using Feature Extraction Software 9.5.3 (Agilent) with background detrend (FeatNCRange, LoPass) and Multiplicative Detrend. Processed signals from feature extraction were imported into the R environment (http://cran.r-project.org/). Data were normalized using quantile normalization (Bolstad *et al*., 2003). This improved correlation slightly, to 0.95-0.99 between biological replicates (n=3 for unfertilized eggs; n=3 for embryos). Genes of interest were extracted from the dataset. Where there were multiple probes for a single transcript or replicate probes, these were aggregated using the median value. These merged intensity values were then log_2-transformed and mean and standard deviation calculated. Microarray expression data are available at NCBI under GEO accession number GSE20137.

Whole-mount in situ hybridization

In situ hybridization was performed using digoxygenin (DIG)-labeled riboprobes as described (Korzh *et al*., 1998). In short, antisense and negative control sense probes were synthesized by *in vitro* transcription using as templates linearized plasmids with the sequence of interest,

DIG-labeled ribonucleotides (Roche; www.roche.com) and the appropriate RNA polymerase (Ambion; www.ambion.com). Products were purified using the RNeasy Mini Kit (Qiagen). Embryos collected at 3.5 h post-fertilization were dechorionated, fixed overnight in 4% paraformaldehyde/PBS and prehybridized overnight at 68°C in hybridization buffer (50% formamide, 5x SSC, 50 mg/ml heparin, 500 mg/ml tRNA, 0.1% Tween-20). Hybridization was done in hybridization buffer containing 50 ng to 100 ng probe overnight at 68°C. Embryos were washed at 68°C for 15 min in hybridization wash solution (1x HWS; 50% formamide, 5x SSC, 0.1% Tween-20), 15 min in 75% HWS/25% 2x SSC, 15 min in 50% HWS/50% 2x SSC, 15 min in 25% HWS/75% 2x SSC, 15 min in 2x SSC, and twice 30 min in 0.2x SSC. For antibody detection, embryos were incubated for 1 h in 2% blocking reagent (Roche) in maleic acid buffer (0.15 M maleic acid, 0.1 M NaCl, pH 7.5), and incubated overnight at 4°C with preadsorbed anti-DIG-AP antiserum (Roche) at a 1:5,000 dilution in 2% blocking reagent in maleic acid buffer. Embryos were washed 6 times 15 min in PBS. Detection was performed in alkaline phosphatase reaction buffer (0.1 M Tris-HCl, pH 9.5, 50 mM NaCl, 0.1 % Tween-20) containing Nitroblue Tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate (Roche). After development of staining to desired intensity, embryos were washed in PBS and preserved in 4 % paraformaldehyde/PBS at 4°C.

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