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Chromatin dynamics of the developmentally regulated *P. lividus* neural alpha tubulin gene

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ABSTRACT Over 40 years ago, Allfrey and colleagues (1964) suggested that two histone modifications, namely acetylation and methylation, might regulate RNA synthesis. Nowadays it is universally accepted that activation of gene expression strictly depends on enzymatic mechanisms able to dynamically modify chromatin structure. Here, using techniques including DNaseI hypersensitive site analysis, chomatin immunoprecipitation and quantitative PCR analysis, we have analyzed the dynamics of histone post-translation modifications involved in developmentally/spatially controlled activation of the sea urchin *PlTalpha2 tubulin* gene. We have demonstrated that only when the PlTalpha2 core promoter chromatin is acetylated on H3K9, tri-methylated on H3K4 and not di-methylated on H3K27, RNA pol II can be enrolled. In contrast, we have shown that when chromatin is methylated both on H3K9 (me2/3) and H3K27 (me2) and mono methylated on H3K4 the promoter is not accessible to RNA pol II. Our results suggest that, during *P. lividus* embryogenesis, both HAT/HDAC and HMT/HDM activities, which are able to regulate accessibility of the PlTalpha2 basal promoter to RNA polymerase II, are coordinately switched-on.

KEY WORDS: *Paracentrotus lividus, chromatin modification, epigenetic reprogramming, nervous system*

Introduction

Once considered a mere DNA packaging device, chromatin is now viewed as a highly dynamic structure that affects all DNA transactions within the nucleus, namely transcription, replication, repair, recombination and transposition, as well as chromosome segregation. The basic unit of chromatin is the nucleosome, which is composed of 147 bp of DNA wrapped around a protein core of two molecules of each of H2A, H2B, H3 and H4 histones. Covalent modifications of the nucleosome core, incorporation of histone variants, DNA methylation and other factors such as chromatin remodeling enzymes or small RNAs are thought to define distinct chromatin states and modulate the accessibility of DNA, either directly or indirectly through the recruitment of additional effectors (Rando and Ahmad, 2007; Campos and Reinberg, 2009; Li *et al.,* 2007).

Over the past decade, studies have shown that histones can undergo to a vast range of post translational modifications (PTMs), either before or after their incorporation into nucleosomes. PTMs, that are particularly numerous on the unstructured N-tails of histones H3 and H4, include acetylation, methylation, ubiquitination or sumoylation of lysine residues, methylation of arginine residues and phosphorylation of serine and threonine residues. Moreover, up to two and three methyl groups can be respectively added to arginine and lysine residues, further enriching the repertoire of histone PTMs. Finally, specific combinations of chromatin modifications constitute the epigenetic marks of eukaryotic genome and identify different chromatin functional states, that can also be inherited (Kouzarides, 2007; Rando and Ahmad, 2007; Feil, 2008; Mellor *et al.,* 2008; Vaquero, 2009)

Eukaryotes have evolved regulatory mechanisms to induce structural changes to chromatin in response to environmental and cellular cues, accomplished by specific enzymatic activities (mainly HAT/HDAC and HMT/HDM) and able to modulate chromatin packaging and regulator binding, thus affecting the recruitment of specific proteins to DNA. One of the emerging challenges in the study of chromatin covalent modifications is to understand the role played by these marks in different chromatin functional contexts as well as to characterize the factors regulating and targeting these enzymatic activities to chromatin.

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; HAT, histone acetyl transferase; HDAC, histone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase; PTMs, post-translational modifications.

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Thus, in general, histone acetylation is thought to be associated with active transcription, regardless of the site of acetylation. In contrast, histone methylation has proven to be a far more complex entity: not only do distinct histone lysine residues have different, sometimes opposing, functions when methylated, but different degrees of methylation (mono-, di-, or tri-methylation) on the same residue may have vastly differing functions (Campos and Reinberg, 2009; Robertson *et al.,* 2008; Mosammaparast and Shi, 2010).

All echinoderm larvae have a nervous system that appears to be centered on the ciliary band with what may be sensory ganglia (apical, oral and lateral ganglia), and that control swimming and feeding. In particular the animal plate of the sea urchin embryo becomes the apical organ, a sensory structure of the larva, while the neurogenic ciliated band becomes morphologically distinct after gastrulation between the oral and aboral ectoderm territories with lineage contributions from both (Nakajima *et al.,* 2004; Yaguchi *et al.,* 2006).

We previously identified and characterized a specific alpha tubulin mRNA (alpha2) of the sea urchin *Paracentrotus lividus*. Its expression starts from the hatching blastula stage in the apical plate and progressively increases during embryogenesis (Gianguzza *et al.,* 1989), maintaining its localization restricted to the larval nervous system (apical, oral and lateral ganglia and ciliary band; Gianguzza *et al.,* 1995). Accordingly, the deduced encoded protein is a specific terminal product of neural differentiation: it is indeed an axonal glutamylated tubulin isotype, homologous to the corresponding neural mammalian isotype (Casano *et al.,* 1996; Tuszynski *et al.,* 2006). We also isolated the corresponding PlTalpha2 gene, consisting of three exons and two introns and the regulatory sequences involved both in its basal and in its spatio-temporal transcription regulation. These regulatory sequences are in fact able to drive a proper spatio-temporal expression profile of a reporter gene when microinjected into the embryos. Finally, gene transfer experiments show that sequences required for proper expression are localized in a region from -4.5 to +0.8 Kb (5' upstream and first intron sequences; Costa *et al.,* 2004; Ragusa *et al.,* in press) and that the minimal promoter sequence is between –50 and +150 bp (Gianguzza *et al.,* unpublished data).

In this study we used chromatin immunoprecipitation (ChIP) to demonstrate that this core promoter region of the PlTalpha2 gene (now re-named PlTuba1a - Ragusa *et al.,* in press) gains transcriptional competence depending on specific and developmentally controlled histone tails modifications.

Results

Core promoter accessibility

Since transcription of the PlTalpha2 gene is developmentally regulated (Gianguzza *et al.,* 1989), we supposed that its core promoter could be differentially accessible to RNA polymerase during sea urchin development. In order to investigate this aspect, nuclei from *P. lividus* embryos at both morula (when the gene is off) and gastrula stage (when the gene is switched-on) were treated with increasing amounts of DNaseI and analyzed by Southern blot hybridization.

To identify DNaseI hypersensitive sites both upstream and downstream of TSS (Transcription Start Site), we digested DNA with SalI restriction enzyme that releases a genomic fragment of 2.5 Kb, including a reverse CAAT box, a canonic TATA box the TSS and the first and second intron, and hybridized it to a radioactive 100 bp probe complementary to the 5' end of the 2.5 Kb SalI fragment. Numerous hypersensitive DNAseI sites were observed only at the gastrula stage (see scheme at Fig. 1) at level of core promoter, first intron and second intron. This observation suggested that a different chromatin structure, permissive for transcription, was acquired, during the development, by the PlTalpha2 gene and particularly by its core promoter at gastrula stage, in agreement with its developmentally regulated transcriptional activation at this stage (Gianguzza *et al.,* 1989).

Post translational modifications (PTMs) and PlTalpha2 *gene activation*

Using ChIP and conventional PCR, we preliminarly investigated whether transcriptional regulation was linked to a modification of H3 histone acetylation and methylation pattern both in core promoter and in the first intron of PlTalpha2 gene. The results (not shown) demonstrated a tight correspondence between PlTalpha2 gene transcriptional activity at gastrula stage and the general lysine acetylation of H3 tails in both analyzed regions, while very low association of H3K9me2 to the analyzed regions was found. Conversely, transcriptional silencing at morula stage correlates, as expected, with a reciprocal pattern of histone modifications, i.e. H3K9 dimethylation, and a remarkably reduced H3 acetylation.

The core promoter lies at the center of the transcription process, therefore it is often an overlooked component in the regulation of gene expression. Moreover both core promoter accessibility and

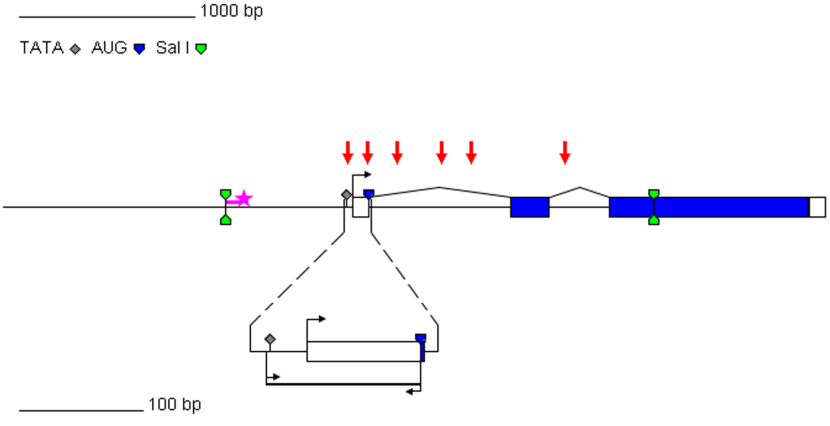


Fig. 1. Map of the *Pltalpha2* gene and schematic representation of the DNase I-hypersensitive sites observed at gastrula stage. *The gene structure was already described by Costa* et al., *(2004). Solid boxes represent exon regions (blue: coding regions; white: 5' and 3' UTRs). Downward arrows indicate the centers of DNaseI cutting sites at the gastrula stage. Upward-downward (double green) arrows show positions of SalI restriction sites. The position of the probe sequence (thick pink line with star) is also shown. Positions of TATA box and TSS (grey diamond and black arrow, respectively) are indicated. An enlarged view of the 5'-flanking region of the PlTalpha2 gene is drawn under the main map. The position of the qPCR fragment used for the ChIP analysis of the promoter is shown underneath (between arrows). The upper bar corresponds to 1000 bp of the main map, while the lower bar corresponds to 100 bp of the enlarged view.*

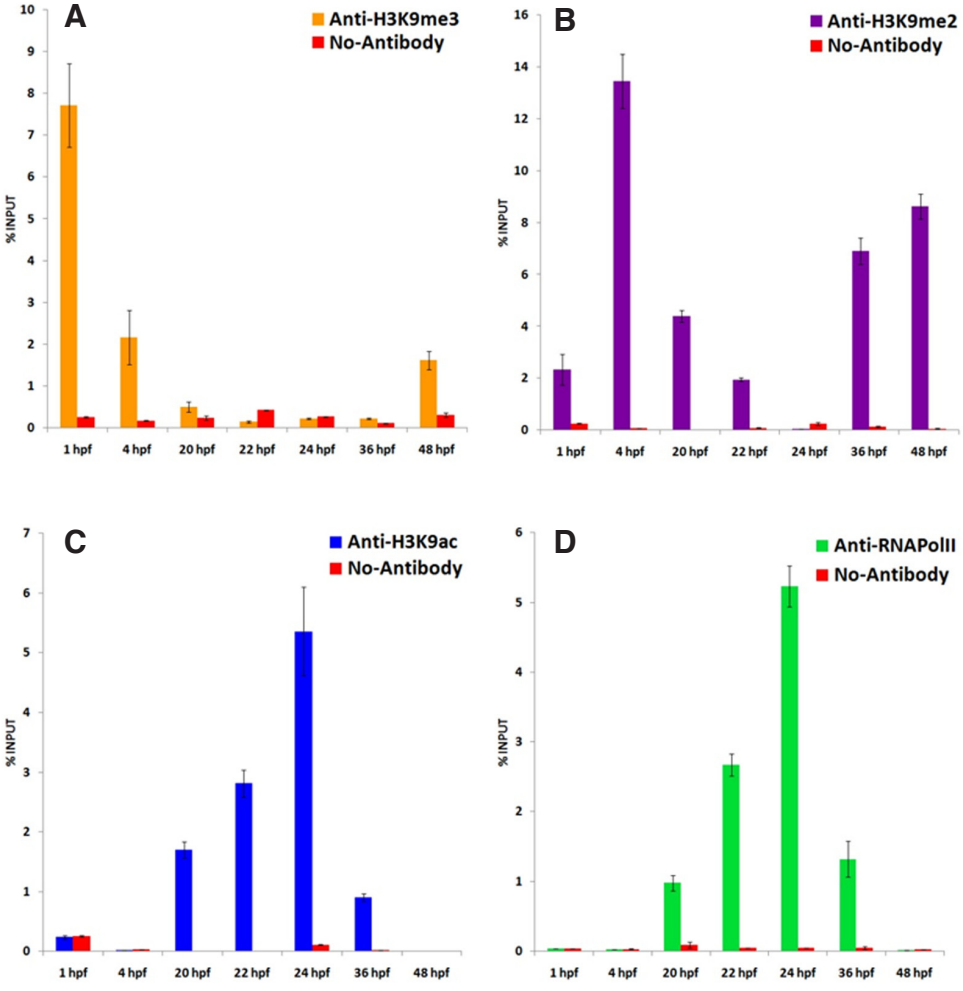


Fig. 2. Quantitative chromatin immunoprecipitation (qChIP) analysis of the *PlTalpha2* gene core promoter. *Input chromatin was isolated from sea urchin embryos at different developmental stages (from 1 to 48 hour post fertilization, hpf). Chromatin was immunoprecipitated using antibodies against H3K9me3* **(A)**, *H3K9me2* **(B)**, *H3K9ac* **(C)**, *RNA pol II* **(D)**, *and analyzed by real-time PCR. Bars represent the mean (±SD) of three independent experiments. Red bars: control; colored bars: abundance of modified histones or RNA pol II. Data were normalized with respect to the standard curve and to 1% input values. Real-time PCR primers were designed to amplify a 120bp region containing the TATA box and the TSS. qChIP experiments were carried out as described in Material and Methods.*

core-promoter recognition complexes (both prototypic and non-prototypic) may play a critical regulatory role in driving cell specific programs of transcription during development and in maintaining active gene expression across cell divisions (Goodrich and Tjian, 2010; Juven-Gershon and Kadonaga, 2010).

Then to better understand the relationship between chromatin modifications and PlTalpha2 transcriptional activation during sea urchin development we decided to carry out time course ChIP-qPCR assays just on PlTalpha2 core promoter sequences, using a set of antibodies directed against different PTMs of H3 tails (see M&M).

The results of these experiments clearly showed that the H3K9 of the nucleosomes present on the core promoter are strongly 3-methylated at early developmental stages (1 - 4 hrs) (Fig. 2A) they are then de-methylated (Fig. 2B) and increasingly acetylated (Fig. 2C) with a peak of acetylation at 24 hrs of development. This dynamics of the PTMs is in agreement with the expression profile of the PlTalpha2 tubulin gene during development, in fact the alpha2 transcript appears only from blastula stage (about 15 hrs of development), accumulating during late developmental stages (Gianguzza *et al.*, 1989).

To strongly correlate these PTMs with the developmental regulation of transcription, we performed time course ChIP-qPCR experiments also with anti-RNA pol II antibodies.

The results (Fig. 2D) showed that RNA pol II is engaged on the PlTalpha2 core promoter between 20 and 36 hrs of development, in concomitance with the acquirement of the acetylated state by H3K9.

Interestingly we also found evidence that around 36 hrs of development a new H3K9 methylation wave occurs. H3K9 is indeed clearly deacetylated and progressively re-methylated, regaining a tri-methylated state at 48 hrs (Fig. 2 A-C). These results are in agreement with previous evidence suggesting that tubulin genes are transcriptionally regulated up to the gastrula stage and post-transcriptionally regulated thereafter. The accumulation of tubulin mRNA after gastrulation has been indeed attributed to an ontogenetic increase in tubulin RNA stability, assayed by actinomycin D chase and RNA gel blot hybridization (Gong and Brandhorst, 1988 a, b).

A leading role in terms of histone code and transcriptional regulation is also played by methylation of H3K4 (Robertson *et al.,* 2008; Rudolph *et al.,* 2007). For H3K4 methylation a dual role has been highlighted: when H3K4 is mono-methylated chromatin structure has been found to be more packed, whereas tri-methylated H3K4 associates with a chromatin open structure, accessible to transcriptional regulatory proteins. Our results (Fig. 3 A-B) performed with anti-H3K4 me1 or anti-H3K4me3 antibodies are in agreement both with bibliographic data and with the previous results of this paper. H3K4 methylation levels in the nucleosomes present in PlTalpha2 core promoter change indeed during development. In particular, during the first hours (up to 4 hrs) of development, when the PlTalpha2 gene is not expressed, we found that H3K4 is mainly mono-methylated. Later in development (from 20 to 36 hrs) it progressively becomes tri-methylated, with a peak at 24 hrs, regaining then a mono-methylated state. From a comparative analysis, a full correspondence of H3K4 methylation and H3K9 methylation/acetylation dynamics emerged, thus confirming a tight association of these PTMs with the acquirement of a transcriptionally active conformation by the PlTalpha2 tubulin gene. Furthermore, there is a tight correspondence between the

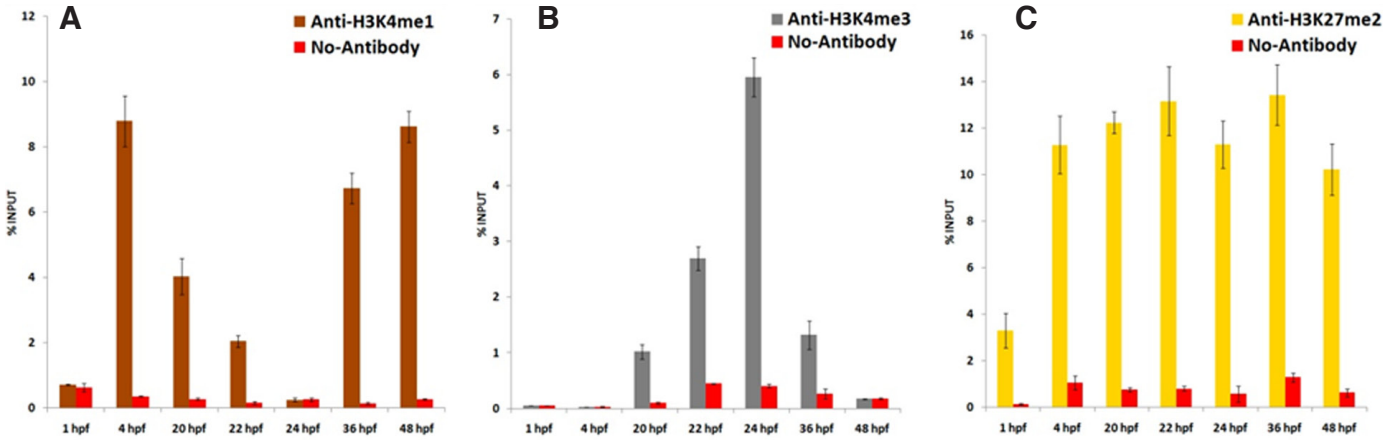
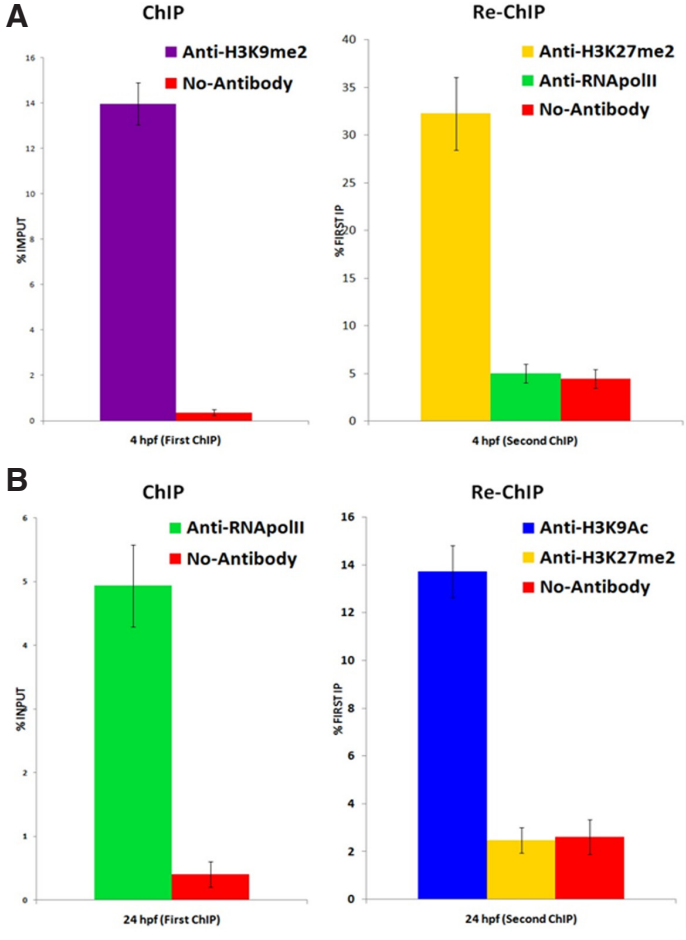


Fig. 3. Quantitative chromatin immunoprecipitation (qChIP) analysis of the *PlTalpha2* gene core promoter. *Chromatin from different sea urchin embryo developmental stages was immunoprecipitated with antibodies against H3K4me1* **(A)**, *H3K4me3* **(B)** *or H3K27me2* **(C)** *and analyzed by real-time PCR (for details see Fig. 2).*

accumulation of K4H3me3 and the maximum association of RNA pol II with the core promoter (Fig. 2D). It is interesting to note that the methylation of H3K4 has been related with the transcriptional activation of genes and is a strong predictor for RNA pol II promoters. In particular, the trimethylated form of H3K4 has been found primarily around the transcription start sites of active genes (van Ingen *et al.,* 2008) as in our case.



Finally, we also analyzed the methylation state of H3K27 by using anti-H3K27me2 antibody. Surprisingly, the results of the time course ChIP-qPCR experiments (Fig. 3C) showed that H3K27 of the nucleosomes present on the core promoter were uniformly methylated at all developmental stages. Since H3K27me2 is not a clear mark of constitutive heterochromatin, we checked whether H3K27me2 histone tail modification was associated with activation or repression of PlTalpha2 gene by sequential chromatin immunoprecipitation (Re-ChIP). This technique has been used to address, in a qualitative manner, whether two proteins can simultaneously co-occupy a stretch of DNA (Furlan-Magaril *et al.,* 2009; Medeiros *et al.,* 2009). We applied the Re-ChIP protocol to analyze chromatin modifications on core promoter of PlTalpha2 gene at two developmental stages: the morula stage, when the gene is not expressed, and the gastrula stage, when the gene is transcriptionally active only in the neural territory (Gianguzza *et al.,* 1995). For the first ChIP performed on chromatin extracted from the morula stage we used the anti-H3K9me2 antibody, and for the Re-ChIP, we used an anti-H3K27me2 antibody or an anti-RNA pol II antibody. For the first ChIP on chromatin extracted from the gastrula stage we used the anti-RNA pol II antibody and for the Re-ChIP anti-H3K27me2 or anti-H3K9Ac antibodies.

Thus we demonstrated the co-presence of H3K9me2 and H3K-27me2 on chromatin of morula stage, when the PlTalpha2 gene is not transcribed in any cells (Fig. 4A).

On the contrary, performing at gastrula stage the first ChIP with the anti-RNA pol II antibody that permit to sort only transcribed chromatin, we observe the presence of H3K9Ac but not of H3K-

Fig. 4. Re-ChIP analysis of the *PlTalpha2* gene core promoter. *The bars show the qPCR results of the ChIP and Re-ChIP pull-downs experiments.* **(A)** *Input chromatin was isolated from sea urchin embryos at morula stage (4 hpf). The first ChIP was performed with Anti-H3K9me2 (left); the second ChIP was performed on first IP with H3K27me2 or RNA pol II antibodies (right).* **(B)** *Input chromatin isolated from gastrula stage (24 hpf). The first ChIP was performed with Anti-RNApolII (left); the second ChIP was performed on first IP with H3K9ac or H3K27me2 antibodies (right). The ChIP results obtained by 3 independent replicate experiments are represented as percentage (±SD) of input chromatin (first ChIP) or as percentage (±SD) of the first IP (second ChIP).*

27me2 in PlTalpha2 core promoter (Fig. 4B).

Discussion

Histone modifications are believed to mark local chromatin for activation or silencing of gene activity. In general terms, hypoacetylation, H4K20me3, H3K9me3 and H3K27me2/3 are typical marks of silenced chromatin, whereas hyperacetylation, H3K4me3 and H3K36me3 are hallmarks of actively transcribed chromatin.

It is, however, becoming increasingly clear that a single histone mark does not proscribe a final outcome with respect to transcription or the local chromatin structure. For example, marks such as H3K9me2/3 and H3K4me2/3, although enriched on either silenced or actively transcribed genes, respectively, are also present in the reciprocal states (Cloos *et al.,* 2008; Mosammaparast and Shi, 2010). Therefore the association between a specific epigenome and its transcriptional status is more complicated (Feil, 2008; Meagher, 2010).

On the other hand, in the sea urchin embryo, the correlation between modification of nucleosomes and transcriptional competence has been poorly investigated and the few reports that in this embryonic system have dealt with chromatin architecture and transcription, concern the histone genes (Di Caro *et al.,* 2007).

All these reasons led us to investigate the existence of developmentally dependent changes of the PlTalpha2 gene epitype by ChIP.

We found that specific histone modifications (H3K9Ac, H3K4me3), which induce an accessible chromatin conformation, occur dynamically at level of core promoter in the same developmental window previously highlighted as the temporal interval in which the PlTalpha2 neural gene is expressed (Gianguzza *et al.,* 1989; Gianguzza *et al.,* 1995).

We found also a constitutive presence of dimethylated H3K27, that is classically implicated in silent chromatin. Recently, bivalent domains that possess both activating and repressive modifications (H3K27me and H3K4me) have been found. The enrichment of these opposing modifications with further PTMs (i.e. H3K9 acetylation/methylation) can dictate distinct functional types of chromatin (Bernstein *et al.,* 2006; Mosammaparast and Shi, 2010; Cloos *et al.,* 2008, Kouzarides, 2007).

We demonstrated that only when the core promoter chromatin is acetylated on H3K9, tri-methylated on H3K4 and not di-methylated on H3K27, RNA pol II can be enrolled, certainly in a specific temporal window and probably only in the neural territory. On the other hand, we showed that when the core promoter chromatin is methylated both on H3K9 (me2/3) and H3K27 (me2) and mono methylated on H3K4 the chromatin is not accessible to RNA pol II. Thus specific and related PTMs certainly limit the developmental temporal window of gene activation and probably contribute in localizing transcription to neural territory, repressing transcription in non neural territories.

In conclusion, our results demonstrate, for the first time in sea urchin, a tight association between the dynamics of specific histone modifications and the defined gene functional outcomes, demonstrating also a high degree of conservation of epigenetic regulation between echinodermata and higher organism during development. Moreover these data could represent the first demonstration that HAT/HDAC and HMT/HDM enzymatic activity are coordinately switched-on during sea urchin development to regulate the expression of a specific gene.

Materials and Methods

Embryo culture

Gametes were collected from gonads of the sea urchin *P. lividus* from the west coast of Sicily. Eggs were fertilized at a concentration of 5000/ml and the embryos were grown under gentle rotation at 18°C in Millipore-filtered seawater (MFSW) containing antibiotics (50 mg/l streptomycin sulfate and 30 mg/l penicillin). Developmental stages were monitored by optical microscopy.

DNaseI hypersensitive site analysis

Embryos, at the morula (4 hours post fertilization, hpf) and gastrula (24 hpf) stages, were immobilized by adding SDS 20% (0,3 ml/l seawater) and allowed to settle. Functionally intact nuclei were isolated according to von Holt et. al. (1989), and stored in liquid nitrogen. After thawing, nuclei were digested with DNaseI according to Sippel *et al.,* (1996). Typically, 0, 3, 6, 12 and 24 units of enzyme were added for 2 x 10^7 nuclei and the nuclei were incubated for 15 min at 4°C. For DNaseI hypersensitive site mapping, the isolated genomic DNA from DNaseI-treated nuclei was digested with SalI restriction enzyme and analyzed by conventional Southern hybridization procedure.

ChIP and qPCR analysis

Embryos (500 μl aliquots) were recovered at the desired stage of development (1, 4, 20, 22, 24, 36 and 48 hpf), and incubated with 1% formaldehyde for 10 min. Crosslinking was stopped by addiction of glycine to a final concentration of 0.125 M, and cross-linked chromatin was sonicated to reduce DNA size to 200-500 base pairs. Sonicated chromatin was then used for the ChIP analysis. All ChIP reactions were made with ChIP-IT™ Express Chromatin Immunoprecipitation Kit (Active Motif #53008), according to the manufacturer's protocols. Immunoprecipitation was carried out with mouse antibodies (previously tested against sea urchin proteins) against RNA pol II (Abcam #ab24759), H3K9ac (Abcam #ab4441), H3K9me2 (Abcam #ab1220), H3K9me3 (Abcam #8898), H3K4me3 (Abcam #ab1012), H3K4me1 (Abcam #ab8895) and H3K27me2 (Abcam #ab24684).

Quantitative real time PCR was performed using the SsoFast EvaGreen Supermix kit (BioRad #172-5201), in a 20 μl QPCR reaction volume, according to the manufacturer's protocols. Primers used were: Real-Prom-1D (5'- GGGGTATAAATACGAGTGCGCCG) and Real-Prom-1R (5'- GATGATACATTATTCGAATTCGAAG) that amplify a fragment including the TATA box and the TSS (from -33 to + 92). The samples were amplified using an BioRad CFX96 Real-Time PCR System, and quantified with a calibration curve obtained with DNA isolated from crosslinked, sonicated chromatin. Data normalization was made as % of Input method.

For the Re-ChIP, precipitated complexes from the first ChIP were eluted by a 10 min incubation with 100 μl of ChIP elution buffer (50 mM Tris–HCl, pH 7.5, 10 mM EDTA and 1%SDS) at 68°C and a second ChIP reaction was carried out with ChIP-IT™ Express Chromatin Immunoprecipitation Kit.

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