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Disruption of the Interaction between Transcriptional Intermediary Factor 1β and Heterochromatin Protein 1 Leads to a Switch from DNA Hyper- to Hypomethylation and H3K9 to H3K27 Trimethylation on the MEST Promoter Correlating with Gene Reactivation

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Here, we identified the imprinted mesoderm-specific transcript (MEST) gene as an endogenous TIF1β primary target gene and demonstrated that transcriptional intermediary factor (TIF) 1β, through its interaction with heterochromatin protein (HP) 1, is essential in establishing and maintaining a local heterochromatin-like structure on MEST promoter region characterized by H3K9 trimethylation and hypoacetylation, H4K20 trimethylation, DNA hypermethylation, and enrichment in HP1 that correlates with preferential association to foci of pericentromeric heterochromatin and transcriptional repression. On disruption of the interaction between TIF1β and HP1, TIF1β is released from the promoter region, and there is a switch from DNA hypermethylation and histone H3K9 trimethylation to DNA hypomethylation and histone H3K27 trimethylation correlating with rapid reactivation of MEST expression. Interestingly, we provide evidence that the imprinted MEST allele DNA methylation is insensitive to TIF1β loss of function, whereas the nonimprinted allele is regulated through a distinct TIF1β–DNA methylation mechanism.

INTRODUCTION

Cell functions result from the interpretation of genetic and epigenetic information established by a large cohort of complexes acting at the chromatin level (reviewed in Mellor, 2006). Gene silencing, in particular, plays essential roles during development and cell differentiation that require progressive extinction of pluripotent genes and specific cell lineage genes (reviewed in Rajasekhar and Begemann, 2007). Several histone modifications, believed to establish a “histone code,” are thought to be essential in this silencing program; these modifications include H3K9 trimethylation (3meH3K9) and H4K20 trimethylation (3meH4K20), two modifications well known to be associated with heterochromatin structures (Schotta et al., 2004; Grewal and Jia, 2007); H3K27 trimethylation (3meH3K27), a mark assumed to be associated with facultative heterochromatin and to be particularly enriched in polycomb response elements (Bracken et al., 2006). Moreover, recent studies indicate that different combinations of these and other histone marks can lead to different outputs that are difficult to anticipate considering the complexity of this combinatorial code (reviewed in Hirose, 2007; Reik, 2007). Another key epigenetic modification is DNA methylation that has well-characterized functions in genomic imprinting, X chromosome inactivation, silencing of tumor suppressor genes, and repression of viral elements (reviewed in Latham et al., 2008). It has been predicted that ~6% of CpG islands display tissue- and or developmental stage-specific DNA methylation pattern (Song et al., 2005; Weber et al., 2007). Although DNA methylation correlates with gene repression, the functional relevance of this level of regulation remains largely to be established in vivo.

Among complexes known to be involved in establishing gene silencing are those containing the transcriptional corepressor, TIF1β (Transcriptional Intermediary factor 1β), that plays essential roles during early embryonic development and terminal cell differentiation (Cammas et al., 2000; 2004). TIF1β (Le Douarin et al., 1996) (also known as KAP-1, Friedman et al., 1996; or TRIM28) is the universal corepressor for the Krüppel-associated box domain containing zinc-finger proteins (KRAB-ZFP) family of transcription factors that constitutes the largest family of repressors in mammals (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996; Abrink et al., 2001). TIF1β is an intrinsic component of two chromatin remodeling and histone deacetylase complexes, N-CoR1 and NuRD (Underhill et al., 2000; Schultz et al., 2001) and directly interacts with the histone methyltransferase SETDB1, which specifically methylates H3K9 preferentially within euchromatin (Schultz et al., 2002). TIF1β also interacts with members of the heterochromatin protein (HP) 1 family through a specific pentapeptide
TIF1-H1 Association Functions in MEST Repression

Immunoprecipitation and Western Blot Analysis
Isolation of whole cell extracts from F9 cells and Western blot detection were performed as described previously (Chiba et al., 1997; Nielsen et al., 1999).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
RNA extraction was performed with TRIzol reagent (Invitrogen). MEST cDNA was amplified with AHY249 (5'-GAATTCCTGAAGAGCCTGGG-3') and AHYN102 (5'-CTTTCCAAAGATCTGGATC-3'); PEG3 cDNA with BB400 (5'-CTTCTGGAACGCCGATTATG-3') and BB401 (5'-CTTCTGGAACGCCGATTATG-3'); PEG10 cDNA with BB402 (5'-CGAATTCGCTTTATATTCGC-3') and BB403 (5'-CGAATTCGCTTTATATTCGC-3'); COPG2 cDNA with BB400 (5'-GATTTCGCAAGCAGCTCTC-3') and BB401 (5'-GATTTCGCAAGCAGCTCTC-3').

Materials and Methods
Details on individual plasmid constructs, which were all verified by sequencing, are available upon request.

Antibodies
Antibodies used in this study were as follows: mouse anti-TIF1β monoclonal antibody (mAb); THCs raised against recombinant Escherichia coli expressed mouse TIF1β (123-834) (Nielsen et al., 1999); rabbit anti-TIF1β polyclonal antibody (pAb); PF64, raised against TIF1β (amino acids 141–155; Cammas et al., 2002); mouse anti-FLAG mAb 2FLB11; anti-HFp1 mAb 2HF-2C9; and anti-HFp1 mAb 1M0D-1A9; anti-HFp1 mAb 2 M0D-1A6 (Nielsen et al., 1999). The antibodies specific for the different histone modifications were purchased from Millipore (Billerica, MA).

Cell Culture
Wild-type (WT) and mutant F9 cells were grown as monolayers in DMEM (Invitrogen, France) supplemented with 10% fetal calf serum as previously described (Boylan and Gudas, 1991). Cells were counted with a particle counter (Coulter Z2).

DNA Fluorescence in Situ Hybridization (FISH)
Wild-type and mutant F9 cells were grown on gelatin-coated coverslips for 72h washed for 5 min in 1× PBS, fixed in 2% paraformaldehyde 10 min at room temperature (RT). Coverslips were treated with 0.1 M Tris-Cl, pH 7.2, for 10 min at RT and washed in 1× phosphate-buffered saline (PBS) for 5 min. Cells were permeabilized for 10 min at RT with 1× PBS; 0.1% Triton X-100, and 0.1% saponin, and then they were incubated in 20% glycerol, 1× PBS, for 10 min at RT and washed in 1× PBS for 5 min. Cells were then fixed in 1× PBS, 4°C, for 10 min on a cold plate to bring the chromosome arms to a single plane for better visualization. Coverslips were permeabilized with 1% formaldehyde for 10 min at 37°C resuspended in lysis buffer (1× PBS, 50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% Na-deoxycholate) at a final concentration of 12.5 × 10⁶ cells/500 μl, incubated on ice for 10 min, and sonicated to average fragment size of 200–300 base pairs. The clarified solubilized chromatin was diluted fivefold in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, and 1.1% Triton X-100). Immunoprecipitation was performed with 8 μl of mAb (TIF1β and HP1α) and 0.1 μl of pAb (TIF1β). The beads were washed sequentially once with low salt buffer (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100), high salt buffer (20 mM Tris-HCl, pH 8.1, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, and 1% Triton X-100), LiCl buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, and 1% deoxycholate) and twice with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Immunecomplexes were eluted twice with 250 μl of elution buffer (1% SDS and 0.1 M NaHCO₃) for 15 min at RT. Eluates and input chromatin were heated at 65°C overnight in the presence of 0.2 M NaCl. ChIP DNA was quantified by real-time PCR using the QuantiTect SYBR Green Kit (Qiagen, Hilden, Germany), and the final results for each sample were normalized to the inputs. PCR reactions were performed in triplicate in a LightCycler (Roche Diagnostics, Mannheim, Germany) with 3 μl of cDNA. Primer sequences for the MEST promoter were as follows: forward, 5'-CACGAGCTTCTGGGATTGG-3' and reverse, 5'-AACCCTGACAT-TCTAGTGAAC-3'; for the region 5' 10 kb upstream of the MEST promoter: forward, 5'-TGTTGCGAGAATCAGCTTGAAG-3' and reverse, 5'-GGAGAAGT-GACCACCACTCTG-3'; for the motif 5' 4 kb upstream of the MEST promoter: forward, 5'-ATTCGTTGATAGCT-3' and reverse, 5'-ATCGCTTACCTG-3'; for the region 3' 5 kb downstream of the MEST promoter: forward, 5'-TTCTCCTGACACCTG-3' and reverse, 5'-GGAGAAGT-GACCACCACTCTG-3'; for the major satellites: forward, 5'-TTATCGGGAATCCTG-3' and reverse, 5'-AAGGAGCTTGCGC-3'; and for the minor satellites: forward, 5'-GAGCAGTCGAAAAAT- GACGAGAAC-3' and reverse, 5'-CATATCCAGCTTCACTG-3'.

DNA Fluorescence in Situ Hybridization (FISH)
TCS-SP5 confocal scanning microscope (Leica Microsystems, Mannheim, Germany) that was equipped with an HCX PLAPO 63 x 1.4 oil immersion objective lens. Images of 20–30 serial optical sections, spaced by 0.25 μm, were acquired per cell nucleus. Approximately 250 nuclei were analyzed per cell type by using an "in-house" software (TIMT) that calculates the minimal distance though the three dimensions between heterochromatin and the FISH signal. To this end, the distribution of the heterochromatin and euchromatin was measured through the whole image stacks and normalized to the surface area. The nearest heterochromatin domain was then searched and marked on the projection of all recorded sections by using Photoshop.

**RESULTS**

**Identification of MEST as a TIF1B Primary Target Gene**

To identify TIF1B endogenous target genes, we compared the transcriptome of WT F9 cells with that of the previously described TIF1B<sup>HP1box/+</sup> F9 cells expressing a mutated TIF1B protein (TIF1B<sup>HP1box+</sup>) unable to interact with HP1 (Cammas et al., 2004). The MEST gene displayed one of the highest -fold induction in mutant cells compared with WT cells (data not shown). To validate this microarray analysis, we performed quantitative (q)RT-PCR analysis with MEST-specific primers on RNA prepared from TIF1B<sup>+/−</sup> and TIF1B<sup>HP1box/−</sup> cells (Figure 1A). MEST expression is undetectable in TIF1B<sup>+/−</sup> cells, whereas it is highly expressed in TIF1B<sup>HP1box/−</sup> cells, confirming the microarray analysis and demonstrating that MEST repression requires the interaction between TIF1B and HP1 within F9 EC cells (Figure 1A).

**Bisulfite Conversion**

Five micrograms of genomic DNA was digested with EcoRI for 1 h at 37°C, purified, and denatured with 0.3 M NaOH for 10 min at 42°C. DNA was converted by adding a solution containing 0.55 mg of hydroquinone (Sigma Chemie, Deisenhofen, Germany) and 0.202 g of sodium hydrogen sulfite (Aldrich, Germany) at pH 5.05 for 15 h at 54°C. Converted DNA was purified (Chemie, Deisenhofen, Germany) and 0.202 g of sodium hydrogen sulfite converted by adding a solution containing 0.55 mg of hydroquinine (Sigma Aldrich, Germany) was ethanol precipitated in presence of glycochle (Invitrogen) and cloned in blunt-ended pBluescript.

Figure 1. MEST is a TIF1B primary target gene. (A) Expression of MEST (PEG1), PEG3, PEG10, MEG3, IGF2R, and COPG2 was assessed in TIF1B<sup>+/−</sup> and TIF1B<sup>HP1box/+</sup> F9 cells by RT-PCR analysis. HPRT was used as a control house-keeping gene. (B) Schematic representation of the MEST gene with the black boxes representing the exons. The arrow labeled +1 represents the transcription start site described in Lelebvre et al. (1997). The different regions amplified for the ChIP analysis are shown. (C and D) RA-inducible expression of MEST is independent of TIF1B–HP1 interaction. TIF1B<sup>+/−</sup> (C) and TIF1B<sup>HP1box/−</sup> (D) cells were treated for 96 h with either ethanol (no) or 1 μM all-trans RA (RA). MEST expression was measured by qRT-PCR and normalized with HPRT expression. (E) ChIP assay with two anti-TIF1B antibodies (p, PF64 pAb; m, 1TB3 mAb) was performed in WT EC F9 cells. The three positions analyzed on the MEST gene are shown in B, and the HPRT promoter region was also analyzed. Results are the average of at least three independent experiments. *p < 0.05 and **p < 0.005.
in TIF1β<sup>HP1box/−</sup> compared with TIF1β<sup>+/−</sup> cells was validated by qRT-PCR (Figure 1A). These results indicate that TIF1β regulates either directly or indirectly the expression of a large number of genes, including a significant proportion of imprinted genes. We also measured the expression of COPG2 whose 3′-untranslated region (UTR) overlaps MEST 3′-UTR on the mouse chromosome 6 (Lee et al., 2000). This gene is expressed at a slightly, although not statistically significant, higher level in TIF1β<sup>HP1box/−</sup> compared with TIF1β<sup>+/−</sup> cells, implicating that TIF1β-HP1 is specifically involved in the regulation of MEST gene expression within this chromosomal locus (Figure 1A). To assess the regulation of MEST gene expression during differentiation, TIF1β<sup>+/−</sup> and TIF1β<sup>HP1box/−</sup> cells were treated for 4 d with 1 μM RA to induce PrE. qRT-PCR analysis shows that MEST expression is equivalently induced in both genetic backgrounds and remains 10<sup>4</sup> lower in TIF1β<sup>+/−</sup> cells than in TIF1β<sup>HP1box/−</sup> cells (Figure 1, C and D). These results indicate that MEST expression is inducible during PrE differentiation through a TIF1β-HP1 interaction-independent mechanism. Therefore, MEST gene expression regulation was only analyzed in noninduced conditions.

To establish whether MEST is a direct TIF1β target gene, we performed ChIP in WT F9 cells with two independent TIF1β antibodies, the monoclonal 1TB3 (Nielsen et al., 1999; Figure 1E, m) and the polyclonal PF64 (Cammas et al., 2002; Figure 1E, p) followed by real-time PCR. Results are represented as (signal with specific antibodies − signal with no antibody) normalized with the initial materials (input). TIF1β is significantly enriched on the MEST promoter region compared with regions 3.9 kb upstream and 6.3 kb downstream of this MEST promoter region as well as to the promoter region of the constitutively active HPRT gene (10.5; 17.4- and 12.5-fold, respectively, with the mAb; Figure 1E). These data strongly suggest that, in EC F9 cells, MEST is a primary target of TIF1β that maintains repression of this gene via a mechanism requiring its interaction with HP1.

### The Interaction between TIF1β and HP1 Is Essential for Stable Recruitment of Both TIF1β and HP1 to MEST Promoter

To investigate the recruitment of HP1 to an endogenous TIF1β target gene, we performed ChIP with antibodies specific of the three HP1 isoforms, HP1α, HP1β, and HP1γ, in TIF1β<sup>+/−</sup> F9 cells. All three HP1 isoforms are significantly enriched on the MEST promoter region, whereas they are not significantly enriched in the 5′ upstream or 3′ downstream positions (Figure 2, A–C, Het). None of the HP1 isoforms are significantly recruited to the promoter of the HPRT gene, whereas all of them are, as expected, recruited to the constitutive heterochromatic major satellites, confirming that the signals detected on MEST promoter correspond to specific recruitment of the three HP1 isoforms (Figure 2, A–C). Furthermore, to determine whether the interaction between TIF1β and HP1 is required for HP1 recruitment to the MEST promoter, the same ChIPs were performed in TIF1β<sup>HP1box/−</sup> cells. As illustrated on Figure 2, A–C (Mut), no HP1 is significantly detected at any position of the MEST gene after ChIP with HP1α, HP1β, or HP1γ antibodies within TIF1β<sup>HP1box/−</sup> cells. In contrast, all three HP1 isoforms are still clearly detectable on the major satellites at a level equivalent to that observed in TIF1β<sup>+/−</sup> cells (Figure 2, A–C). This demonstrates that the interaction between TIF1β and HP1 is essential for specific HP1 recruitment to the MEST promoter, whereas it is not involved in the recruit-
ment of these proteins to the constitutive heterochromatic major satellites.

To determine whether the interaction between TIF1β and HP1 is also involved in the recruitment of TIF1β to the MEST promoter, ChIPs with the two TIF1β Abs, was performed in TIF1β+/− and TIF1βHP1box−/− cells. Surprisingly, TIF1βHP1box recruitment to MEST promoter is drastically decreased compared with the recruitment of TIF1β in TIF1β+/− cells, although it remains significantly above the signal detected on the HPRT promoter and on the major satellites sequences (Figure 2D; data not shown). This strongly suggests that the interaction between TIF1β and HP1 is not only essential for the recruitment of HP1 to the MEST promoter, but also for efficient TIF1β recruitment and/or stabilization to this region. Altogether, these data indicate that TIF1β and HP1 are recruited to MEST promoter through a mechanism that requires their mutual interaction to maintain gene repression within F9 EC cells.

**TIF1β, through Its Interaction with HP1, Maintains a Heterochromatin-like Structure on the MEST Promoter**

MEST is an imprinted gene that possesses a CpG island in its promoter region (Lefebvre et al., 1997; Figure 1B). It was therefore important to assess the DNA methylation status of this MEST CpG island in TIF1β+/− and TIF1βHP1box−/− cells. To this end, we performed bisulfite sequencing and analyzed the methylation status of the MEST gene from position −455 base pairs to +269 base pairs comprising 56 CpG dinucleotides. This region of the MEST gene is fully methylated on both alleles in TIF1β+/− cells (Figure 3A). A notable exception to this full methylation pattern is the first position upstream to the transcription start site (at −33 base pairs) that is methylated in only 60% of MEST alleles (Figure 3A). In contrast to TIF1β+/− cells, only ~50% of MEST alleles are methylated within the CpG island in TIF1βHP1box−/− cells (Figure 3A). This loss of MEST gene methylation is not a consequence of a general demethylation process within TIF1βHP1box−/− cells, because digest of TIF1β+/− and TIF1βHP1box−/− genomic DNA by the methylation-sensitive enzyme HpaII shows the same pattern on agarose gel (data not shown).

To establish the profile of histone modifications induced by TIF1β–HP1 recruitment to MEST promoter, we performed ChIP with antibodies specific of different histone modifications in TIF1β+/− cells. This analysis reveals that MEST promoter is highly enriched in 3meH3K27 and 3meH4K20, two modifications specific in the constitutive heterochromatic major satellite sequences demonstrating the specificity of the ChIP (Figure 3B). Furthermore, acetylation of H3K9 (AcH3K9), a mark associated with gene expression, is not significantly enriched on MEST promoter region of TIF1β+/− cells, whereas it is highly enriched on the promoter region of the constitutively transcribed HPRT (Figure 3B). As expected, this mark is also absent at the silent heterochromatin major satellites. Last, we analyzed the enrichment in 3meH3K27. 3meH3K27 is not significantly enriched within neither the MEST promoter, nor the HPRT promoter or the heterochromatic major satellite sequences (Figure 3B). It is noteworthy that AcH3K9 and 3meH3K27 are slightly, although significantly, enriched 4 and 10 kb upstream the MEST promoter region within TIF1β+/− cells (Figure 3B). To demonstrate the specific role of TIF1β interaction with HP1 in the maintenance of this chromatin structure, the same ChIP experiments were performed in TIF1βHP1box−/− cells. As expected, considering the loss of MEST repression in this cell line, 3meH3K9 and 3meH4K20 are lost from the MEST gene in TIF1βHP1box−/− cells, with a concomitant enrichment of AcH3K9. More surprisingly, 3meH3K27 becomes highly enriched in the MEST promoter region and at 4 kb upstream this region (11.9- and 6-fold, respectively, compared with the signal in TIF1β+/− cells). This phenomenon is specific of the MEST promoter and its 4 kb upstream region since none of the histone modifications presented above are significantly altered at 10 kb upstream the MEST promoter region nor within the HPRT promoter nor the major satellite sequences in TIF1βHP1box−/− compared with TIF1β+/− cells. To find out whether the 3meH3K27 mark that occurs specifically in TIF1βHP1box−/− cells displays any preferential association with the methylated or unmethylated MEST allele, we performed bisulfite sequencing after a 3meH3K27 ChIP in TIF1βHP1box−/− cells. As illustrated on Figure 3C, 3meH3K27 is exclusively associated with the unmethylated MEST allele.

**MEST Preferentially Associates with Pericentromeric Heterochromatin in a TIF1β–HP1 Interaction-dependent Manner**

It has been suggested that nuclear organization and in particular the localization of specific genomic loci close to heterochromatin has important implications for gene silencing (Brown et al., 1999). We therefore assessed the localization of the MEST gene within TIF1β+/− and TIF1βHP1box−/− cells by FISH and confocal microscopy. The distance between the spots corresponding to the hybridization of the MEST gene to the nearest heterochromatin domain defined as bright spots of Hoechst staining was determined throughout the three dimensions of the nucleus using an in-house (TIMT) software. The distance was normalized by the surface of the nucleus. In both cell lines, three types of nuclei were observed: 1) both MEST alleles associated with heterochromatin (2HC), 2) both MEST alleles excluded from heterochromatin (2EU), and 3) one MEST allele within heterochromatin and one excluded from this compartment (1HC + 1EU) (Figure 3D). Interestingly, the proportion of the (1EU + 1HC) and (2EU) populations are significantly different within TIF1β+/− and TIF1βHP1box−/− cells lines according to the statistical chi-square test (χ² < 0.01), whereas the (2HC) populations are equivalently represented in both cell lines (7.1 and 10.2% in TIF1β+/− and TIF1βHP1box−/− cells, respectively). The (1EU + 1HC) population represents 46.5% of TIF1β+/− cells, whereas it is found in only 24% of TIF1βHP1box−/− cells (Figure 3D). Furthermore 43.3% of TIF1β−/− cells are (2EU) compared with 68.9% for TIF1βHP1box−/− cells (Figure 3D). These changes are unlikely to result from a global difference of heterochromatin distribution between the two cell lines because no obvious difference is observed by Hoechst staining. Finally the average distance between the “EU” spots and the nearest heterochromatin domain is equivalent in both genetic backgrounds (0.952 and 0.895 μm in TIF1β+/− and TIF1βHP1box−/− cells, respectively) (Figure 3D). Furthermore, we analyzed the subnuclear localization of the chromosome X-associated gene, HPRT, and we found that this gene is euchromatic in 84.4 and 85.9% of TIF1β+/− and TIF1βHP1box−/− nuclei, respectively (data not shown). These data strongly suggest that the association of TIF1β with HP1 facilitates the anchorage of the MEST locus within pericentromeric heterochromatin.
Is Permanently Required on MEST Promoter to Maintain Transcriptional Repression

To address the kinetic of loss of TIF1β/HP1 interaction, we established a cell line allowing hydroxytamoxifen (OHT)-inducible disruption of the interaction between these two proteins. This cell line (TIF1β<sup>L2/HP1box/Cre-ERT2</sup>) carries 1) one floxed TIF1β allele (TIF1β<sup>L2/</sup>), 2) the other TIF1β allele with the HP1box motif mutated by homologous recombination (TIF1β<sup>HP1box/</sup>), and 3) an expression vector driving stable expression of the OHT-inducible CreERT2 recombinase (Indra et al., 1999). These cells were treated for different

TIF1β Is Permanently Required on MEST Promoter to Maintain Transcriptional Repression

Figure 3. TIF1β, through its interaction with HP1, maintains a heterochromatin-like structure on the MEST promoter. (A) DNA methylation of the MEST CpG island from position –455 base pairs to position 269 base pairs was analyzed in TIF1β<sup>+/+</sup> (black square) and TIF1β<sup>HP1box/</sup> F9 cell lines (open triangles) by bisulfite sequencing. (B) The histone modification status of MEST promoter and of its surrounding regions, of the HPRT promoter and of the major satellite sequences were analyzed by ChIP in nontreated TIF1β<sup>+/+</sup> (black bars; Het) and TIF1β<sup>HP1box/</sup> F9 cell lines (white bars; Mut). As annotated trimethylation of H3K9 (3meH3K9), H4K20 (3meH4K20), H3K27 (3meH3K27) and acetylation of H3K9 (AcH3K9) were assessed. (C) The DNA methylation status of the MEST promoter region associated with 3meH3K27 was assessed by bisulfite sequencing after ChIP with an anti-3meH3K27 pAb in TIF1β<sup>HP1box/</sup> cells. The open circles represent the unmethylated CpG and the black circles the methylated CpG. (D) DNA FISH analysis was performed using a MEST gene specific probe in TIF1β<sup>+/+</sup> and TIF1β<sup>HP1box/</sup> F9 cell lines (red dots). Pericentromeric heterochromatin was labeled by Hoechst staining and occurs as bright blue spots. Representative cells with two spots within heterochromatin (A and B), one spot within euchromatin and one spot within heterochromatin (C and D) or two spots within euchromatin (E and F) are shown. The percentages represent the analysis of >250 cells for each genotype. *p < 0.05, **p < 0.005, and ***p < 0.0005.
times with OHT and collected for genotyping and analysis of MEST expression. Excision of the floxed TIF1β-2 allele was detectable as early as 12 h of OHT treatment and 48 h of OHT treatment were sufficient to detect MEST expression, which was further increased by 122-fold until 9 d of OHT treatment (Figure 4, A and B). It is noteworthy, that, although MEST expression increases with time after OHT treatment, it only reaches 10% of the level obtained in TIF1β<sup>Hp1box</sup>-/- cells (data not shown). These results indicate that MEST repression is rapidly lost upon disruption of the interaction between TIF1β and HP1 and strongly suggest that the interaction between these two proteins is permanently required to maintain MEST repression.

**TIF1β Is Sufficient to Establish the Heterochromatin-like Structure on MEST Gene**

As described above, TIF1β, through its interaction with HP1, is essential to maintain MEST repression. A key question that remained to be answered was whether TIF1β is also involved for the establishment of MEST repression. To address this question, we used the previously established cell line TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β by doxycycline (Dox) treatment within TIF1β<sup>Hp1box</sup>-/- cells (Cammas et al., 2004). f.TIF1β expression was verified by Western using the anti-FLAG mAb in TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β cells. As expected, f.TIF1β is expressed only in presence of Dox; however, this does not lead to any significant increase in the total level of TIF1β in these cells, strongly suggesting that f.TIF1β level is very low compared with endogenous TIF1β (Figure 5A). f.TIF1β expression was also verified by immunofluorescence and found to be homogenous in each Dox-treated TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β cell (data not shown). f.TIF1β recruitment to MEST promoter was then assessed by ChIP with the anti-FLAG antibody. As illustrated on Figure 5B, f.TIF1β is efficiently recruited to MEST promoter only in presence of Dox.

MEST expression was quantified in presence or absence of Dox treatment. Dox induces a twofold decrease of MEST expression compared with nontreated cells (Figure 5C), demonstrating that f.TIF1β is able to partially restore MEST repression. This twofold decrease in MEST expression was maintained even after 15 passages, strongly suggesting that these cells are refractory to further repression (data not shown), most likely due in part to the low level of f.TIF1β expression. It was then essential to determine whether f.TIF1β could reestablish the heterochromatin-like structure observed in TIF1β<sup>+</sup> cells. To this end, we analyzed specific histone modifications in TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β cells in presence or absence of Dox treatment. ChiP experiments show that 3meH4K20 and 3meH3K9 are increased by 3.6- and 3.1-fold, respectively, in the promoter region of Dox-treated cells compared with nontreated cells, whereas 3meH3K27 and AcH3K9 are both decreased by 1.7-fold in these same conditions (Figure 5D). Although the amplitude of these changes is relatively limited, they demonstrate that f.TIF1β is able to reestablish to some extent the histone modification profile observed in TIF1β<sup>+</sup> cells. As mentioned above, the level of f.TIF1β is extremely low compared with that of the endogenous protein and might be a limiting factor for detection of DNA methylation by bisulfite sequencing. We therefore analyzed DNA methylation of the -176- to -57-bp region upstream the MEST transcription start site after immunoprecipitation of Dox-treated TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β chromatin with the anti-FLAG mAb. As control, total DNA before ChIP (input) was also analyzed. As shown on Figure 5E, ~100% of the MEST alleles associated with f.TIF1β are methylated, whereas only ~50% are methylated in the input. This demonstrates that f.TIF1β is able to establish DNA methylation at the MEST promoter; however, most likely because of the low level of f.TIF1β expression, this rescue is undetectable on total TIF1β<sup>Hp1box</sup>-/-/rTA-f.TIF1β cell DNA.

**MEST Proximal Promoter Region Is Highly Methylated in Liver**

It has been suggested that several imprinted loci become aberrantly methylated on both alleles in F9 cells (Yeivin et al., 1996). To decipher whether the full methylation of MEST observed in our F9 model corresponds to any physiological condition, we tested MEST expression in different adult mouse tissues. As shown previously, MEST expression is very low in all tissues and in particular in liver in which MEST expression is 19-fold lower than in TIF1β<sup>Hp1box</sup>-/- F9 cells (Figure 6A; data not shown; Lui et al., 2008). We analyzed the methylation status of the MEST proximal promoter region in adult liver. As illustrated on Figure 6B, bisulfite sequencing analysis shows that the MEST promoter region between -107 and -57 bp is methylated on 90% of the alleles. These results strongly suggest that, as in F9 EC cells, MEST proximal promoter region is methylated on both alleles.

**DISCUSSION**

In this study, we identified the MEST gene as an endogenous TIF1β primary target gene in embryonal carcinoma F9 cells. We demonstrate that the interaction between TIF1β and HP1 proteins is critical for MEST repression, being essential for mutual recruitment and/or stabilization of both proteins on the promoter region. We further show that TIF1β–HP1 interaction is critical for the maintenance and the establishment of a local heterochromatin-like structure characterized by H3K9 trimethylation and hypoacetylation, H4K20 trimethylation, DNA hypermethylation, and enrich-
The interaction between TIF1 of a heterochromatin-like structure and is dependent upon methylation, but of which, only one involves the formation of these results indicate that the two MEST alleles: 1) although both alleles are fully methylated in TIF1β+/− EC F9 cells, only 50% of the MEST fragments sequenced are methylated in TIF1β+/−box/−, strongly suggesting that only one allele is demethylated upon disruption of the interaction between TIF1β and HP1; 2) only one of the two alleles seems to be significantly associated with pericentromeric heterochromatic foci, a localization that requires the interaction between TIF1β and HP1; and 3) upon disruption of the interaction between TIF1β and HP1, there is a switch from high levels of 3meH3K9 and 3meH4K20 and low level of 3meH3K27 to low levels of 3meH3K9 and 3meH4K20 and high level of 3meH3K27 that is exclusively associated with the demethylated MEST allele. These results indicate that the two MEST alleles are clearly silenced by two different mechanisms that both rely on DNA methylation, but of which, only one involves the formation of a heterochromatin-like structure and is dependent upon the interaction between TIF1β and HP1. Because 50% of MEST methylation is refractory to TIF1β loss of function, it is very likely that TIF1β does not regulate the allele of maternal origin that is imprinted but rather the paternal nonimprinted allele. This strongly suggests that the molecular mechanisms of gene repression described in this study apply to both imprinted and nonimprinted genes, a conclusion in line with the large number of imprinted and nonimprinted genes up-regulated upon loss of interaction between TIF1β and HP1. Very interestingly, we show in liver that the MEST proximal promoter region between −107 and −57 base pairs is methylated on 90% of the alleles, which correlates with a very low level of MEST expression. This result is in striking contrast with the observation made on the MEST distal promoter region (−1001 to −792 bp) that is methylated only on 50% of MEST alleles (Lui et al., 2008). The different methylation status between these two MEST promoter regions strongly suggests that there is a boundary element between −792 and −107 base pairs that inhibits DNA methylation spreading on the nonimprinted allele but not on the imprinted alleles that is methylated, even on the MEST distal promoter. Together, these results highlight the existence of different mechanisms of DNA methylation establishment and/or maintenance. The marks that allow the preferential association of TIF1β with the nonimprinted allele and the establishment and/or maintenance of DNA methylation on specific gene area remain to be elucidated, but most likely they involve a complex combinatorial presence of histone modifications on each MEST allele. This is the first demonstration that the nonimprinted allele of an imprinted gene is regulated by DNA methylation in vivo.

Concerning the molecular mechanism underlying TIF1β functions as a corepressor, it is interesting to note that, although 3meH3K9 and to a lesser extent 3meH4K20 extend around 4 kb upstream the MEST transcription start site, HP1 binding is limited to the proximal MEST promoter region, indicating that, in contrast to constitutive heterochromatin, HP1 is not spreading to surrounding regions and that 3meH3K9 and 3meH4K20 on their own are not sufficient for HP1 recruitment. This conclusion is in agreement with the
finding that in heterochromatin, HP1 association to chromatin requires both H3K9 trimethylation and direct association with Suv39H1 (Stewart et al., 2005). It is very likely that, here, HP1 chromatin association necessitates both H3K9 trimethylation and direct binding to TIF1β. Furthermore, we find that MEST repression is rapidly lost upon disruption of the interaction between TIF1β and HP1 strongly suggesting that this repression requires permanent loading of TIF1β on MEST promoter region. This is in contrast with studies using integrated artificial TIF1β target genes, in which the authors concluded that a single pulse of TIF1β expression allowed transgene silencing for several passages (Sripathy et al., 2006). These data clearly demonstrate that HP1 loading on MEST promoter for repression can rapidly be displaced for gene activation. The loss of HP1 on MEST promoter is accompanied by loss of 3meH3K9, 3meH4K20, DNA methylation and a gain of 3meH3K27, demonstrating that all these chromatin modifications are very dynamic and interdependent as previously suggested in other systems (Freitag et al., 2004; Smallwood et al., 2007; Wu et al., 2008). This conclusion is also in line with studies indicating that HP1 distribution within the nucleus is highly dynamic (Cheutin et al., 2003) and that DNA methylation is not always as stably established as assumed previously (Kangaspeska et al., 2008; Métivier et al., 2008). It is also intriguing that in most studies 3meH3K27 is associated with gene silencing, whereas in the present study, 3meH3K27 is associated with gene activation in TIF1βHP1−/− cells. This association of 3meH3K27 with gene activation has, however, been observed in some cases and often correlates with loss of DNA methylation, suggesting that 3meH3K27 could be a “default mark” when DNA methylation is lost on genes normally regulated by the formation of a heterochromatin-like structure (Peters et al., 2003; Mathieu et al., 2005; McCarvey et al., 2006; Papp and Müller, 2006).

In conclusion, we have demonstrated that the expression of the imprinted MEST gene expression is tightly regulated by a complex interplay of epigenetic modifications and in particular by two distinct DNA methylation-dependent mechanisms of which, only one is dependent upon TIF1β.

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TIF1β–HP1 Association Functions in MEST Repression


