Cell differentiation induces TIF1β association with centromeric heterochromatin via an HP1 interaction

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Summary

The transcriptional intermediary factor 1 (TIF1) family protein TIF1β is a corepressor for Krüppel-associated box (KRAB)-domain-containing zinc finger proteins and plays a critical role in early embryogenesis. Here, we examined TIF1β distribution in the nucleus of mouse embryonic carcinoma F9 cells during retinoic-acid-induced primitive endodermal differentiation. Using confocal immunofluorescence microscopy, we show that, although TIF1β is diffusely distributed throughout the nucleoplasm of undifferentiated cells, it relocates and concentrates into distinct foci of centromeric heterochromatin in differentiated cells characterized by a low proliferation rate and a well developed cytokeratin network. This relocation was not observed in isoleucine-deprived cells, which are growth arrested, or in compound RXRα$^{-/-}$/RARγ$^{-/-}$ null mutant cells, which are resistant to RA-induced differentiation. Amino-acid substitutions in the PxVxL motif of TIF1β, which abolish interaction with members of the heterochromatin protein 1 (HP1) family, prevent its centromeric localization in differentiated cells. Collectively, these data provide compelling evidence for a dynamic nuclear compartmentalization of TIF1β that is regulated during cell differentiation through a mechanism that requires HP1 interaction.

Key words: Nuclear compartmentalization, Transcriptional silencing, Endodermal differentiation, Transcriptional intermediary factor 1 β, Heterochromatin protein 1

Introduction

Nuclear compartmentalization is now recognized as an important regulatory device for controlling gene expression during development and differentiation (Lamond and Earnshaw, 1998; Francastel et al., 2000; Gasser, 2001). In interphase nuclei, chromosomes and genes occupy defined positions, which can be modified upon changes in cell function (Strouboulis and Wolffe, 1996; Cremer and Cremer, 2001). Nuclear proteins that participate in the assembly of multimolecular regulatory complexes are also comparmentalized, displaying characteristic spatial and temporal patterns (Misteli, 2001; Hendzel et al., 2001). However, the molecular mechanisms by which such compartmentalization is achieved and regulated according to the functional state of the cell remain to be understood.

Pericentromeric heterochromatin represents a specialized nuclear compartment known to play a role in gene silencing (Henikoff, 2000). When placed next to or within pericentromeric heterochromatin, either by chromosomal rearrangement or trans-recruitment, euchromatic genes usually undergo a stochastic, but clonally heritable, inactivation, leading to a variegated pattern of expression (Wallrath, 1998). Dominant genetic modifiers of this phenomenon, known as position effect variegation (PEV), were isolated in *Drosophila* as mutations that can alter the proportion of cells in which inactivation occurs. One of these modifier genes, *Su(var)2-5*, encodes the heterochromatin-associated protein HP1 (Eissenberg et al., 1990). *Su(var)2-5* suppresses PEV when deleted or enhances PEV when duplicated (Eissenberg et al., 1990; Eissenberg et al., 1992), indicating that HP1 is an essential component of heterochromatin required in a precise stoichiometry to properly set and/or maintain the inactivated state of genes subject to PEV. Three distinct HP1 proteins, HP1α, β and γ, have been described in mammals (Eissenberg and Elgin, 2000). Supporting the notion that these HP1s also play a role in gene silencing, they have been reported (i) to be associated – although not exclusively – with pericentromeric heterochromatin (Nielsen et al., 1999; Minc et al., 2000); (ii) to silence transcription in a deacetylase-dependent manner when directly tethered to DNA (Nielsen et al., 1999); (iii) to cause dose-responsive silencing of centromeric transgenes (Festenstein et al., 1999); (iv) to colocalize in primary B-cells with inactive genes (Brown et al., 1997); and (v) to exhibit conserved heterochromatin targeting and silencing when ectopically expressed in *Drosophila* (Ma et al., 2001). Recent studies have demonstrated that mouse HP1 proteins self-associate and associate with nucleosomal core histones in interphase nuclei (Nielsen et al., 2001). A selective binding to the tail domain of H3 methylated at lysine 9 (Bannister et al., 2001; Lachner et al., 2001) and a direct interaction with several non-histone proteins, including components of the replication machinery, proteins of the nuclear envelope and various transcriptional cofactors have also been described (reviewed in Eissenberg and Elgin, 2000).

Initially identified in a two-hybrid screen for proteins interacting with mouse HP1α (Le Douarin et al., 1996), transcriptional intermediary factor 1 (TIF1) β (also known as KAP-1 or KRIP-1) was reported to function as a bona fide

corepressor for the large family of Krüppel-associated box (KRAB)-domain-containing zinc finger proteins (Friedman et al., 1996; Kim et al., 1996; Moosmann et al., 1996). TIF1β is a member of an emerging family of transcriptional regulators that includes TIF1α and TIF1γ in mammals (Le Douarin et al., 1995a; Venturini et al., 1999) and Bonus in *Drosophila* (Beckstead et al., 2001). All members of this family are composed of a N-terminal RBCC (RING finger, B boxes, coiled coil) motif and a C-terminal bromodomain preceded by a PHD finger. They have all been reported to repress transcription when tethered to DNA through fusion to a heterologous DNA-binding domain. Coimmunoprecipitation experiments showed that TIF1β is associated with HP1α, β and γ in interphase nuclei of various mammalian cell lines (Nielsen et al., 1999; Ryan et al., 1999). In vitro, TIF1β interacts directly with and phosphorylates the HP1 proteins (Nielsen et al., 1999). This interaction is mediated by a conserved PxVxL motif and is required for the repression activity of TIF1β in transiently transfected cells (Nielsen et al., 1999; Ryan et al., 1999). Recently, TIF1β has also been reported to be an intrinsic component of the histone deacetylase N-CoR1 complex (Underhill et al., 2000) and to interact both physically and functionally with the Mi-2α subunit of the nucleosome remodeling and deacetylation (NuRD) complex (Schultz et al., 2001). Taken together, these biochemical data strongly suggest that TIF1β may exert its corepressor function via the organization and/or maintenance of higher-order, heterochromatin-like structures.

Recent genetic studies of TIF1β in mice have provided evidence that TIF1β is a developmental regulatory protein that exerts cellular function(s) essential for early embryogenesis (Cammas et al., 2000). To further investigate the functions of TIF1β in mammalian cells, we have now examined its subnuclear distribution in undifferentiated and differentiated F9 embryonal carcinoma (EC) cells, which represent a well established model system of early embryonic development and cellular differentiation (Hogan, 1977; Hogan, 1983). The F9 EC cells closely resemble the pluripotent inner cell mass (ICM) cells of the early mouse embryo and can be induced to differentiate into primitive endoderm-like cells when grown as monolayer in the presence of retinoic acid (RA) (Strickland et al., 1978). Our data show that, during RA-induced primitive endoderm differentiation, TIF1β is relocated into regions of centromeric heterochromatin. Dissection of the molecular mechanism underlying this differentiation-induced relocation reveals that TIF1β becomes centromere associated through HP1 interaction. We discuss the implications of these data for mechanistic models of TIF1β function in mammalian cells.

Materials and Methods

Plasmids

Details of individual plasmid constructs, which were all verified by sequencing, are available upon request. Mouse cDNAs for TIF1β, HP1α/β/γ and KRAB have previously been described (Le Douarin et al., 1996; Nielsen et al., 1999). The double mutation in the HP1 box of TIF1β (V488A/L490A) was generated by site-directed PCR mutagenesis with the following internal primers (the codon change is shown is underlined): AAU268, 5′-GTGCCACGTGCGAGCGCT-GAACGCCTG-3′, and AAVR1, 5′-CAGGCGTTCAGCGCTCG-CACGTGGCAC-3′. To create FLAG-tagged TIF1β-expressing cell lines, an *Eco*RI fragment containing the coding sequences of TIF1β wildtype or TIF1βV488A/L490A fused in frame to an oligonucleotide coding for the FLAG epitope, MDYKDDDDK, was subcloned into pCX vector (Okabe et al., 1997). In this vector, expression of the TIF1β cDNA is driven by a β-actin promoter plus 200 bp of an enhancer derived from CMV. For yeast two-hybrid assays, DBD and AAD fusions proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3, respectively (Le Douarin et al., 1995b). These plasmids express inserts under the control of the phosphoglycerate kinase (PGK) promoter. pBL1 contains the HIS3 marker and directs the synthesis of epitope (region F of ERα)-tagged ERα DNA-binding domain (DBD) fusion proteins. pASV3 contains the LEU2 marker and a cassette expressing a nuclear-localized VP16 acidic activation domain (AAD) preceding a polylinker and stop codons in all reading frames. For transfection studies in mammalian cells, the indicated cDNAs were cloned into pSG5 (Green et al., 1988).

Antibodies

Monoclonal antibodies (mAbs) used include: mouse anti-TIF1β mAb, 1Tb3, raised against recombinant *E. coli*-expressed mouse TIF1β(123-834) (Nielsen et al., 1999); mouse anti-HP1α mAbs, 2HP1-1H5 for immunocytochemistry and 2HP-2G9 for western blot analysis (Nielsen et al., 1999); mouse anti-RPB1 mAb, 1PB-7C2, raised against the heptad repeat CTD-containing peptide of the RPB1 largest subunit of the human RNA polymerase II (Nguyen et al., 1996); rat anti-BrdU mAb (Becton Dickinson); rat anti-Endo A, TROMA 1 [kindly provided by R. Kemler (Department of Molecular Embryology, Max-Planck Institute, Freiburg, Germany)]; rat anti-laminin B1 (Chemicon, Harrow, UK); mouse anti-FLAG mAb, M2 (Sigma-Aldrich, France); mouse anti-ERα mAb, F3, against the F region of human ERα (Le Douarin et al., 1995b); and mouse anti-VP16 mAb, 2GV-4 (Le Douarin et al., 1995b). The rabbit polyclonal antibodies (pAbs) used include anti-TIF1β pAb, PF64, raised against TIF1β (amino acids 141-155) and anti-fibronectin (a gift of R. Hynes, MIT, Massachusetts).

F9 cell culture and establishment of stably transfected cell lines

Wild-type and mutant F9 cells were grown as monolayers on gelatinized surfaces in Dulbecco's modified Eagle medium (DMEM, Gibco, Invitrogen, France) supplemented with 10% fetal calf serum as previously described (Boylan and Gudas, 1991). To induce differentiation, cells were plated at a density of 10^2 to 10^3 cells/cm^2 and treated with 1.0 μM all-trans retinoic acid (Sigma) or with vehicle (control untreated) for the indicated times, with a change of media every two days. Isoleucine deprivation was performed as described previously (Hosler et al., 1989; Faria et al., 1998). Cells plated 24 hours earlier were incubated with isoleucine-free DMEM supplemented with 10% dialyzed fetal calf serum and grown for 48 or 72 hours. To establish stably transfected cell lines, 5×10^6 exponentially growing F9 EC cells (10^6 per 10 cm plate) were transfected by electroporation with 5 μg of expression plasmids (pCX-FLAG-TIF1βWT or FLAG-TIF1βV488A/L490A) along with 0.1 μg of a plasmid conferring resistance to hygromycin (pPGK-hygro; a gift of D. Metzger, IGBMC, Strasbourg, France). Cells were selected in the presence of 250 μg/ml hygromycin (Euromedex) added to the growth medium 24 hours after transfection over a period of 2 weeks with regular medium changes. Several drug-resistant colonies were subsequently picked in 24-well plates and expanded for western blot and immunofluorescence analysis.

ES cell culture and induction of neuronal differentiation

Mouse ES cells, strain D3, were used in this study. The routine growth and in vitro differentiation of these cells were performed as previously

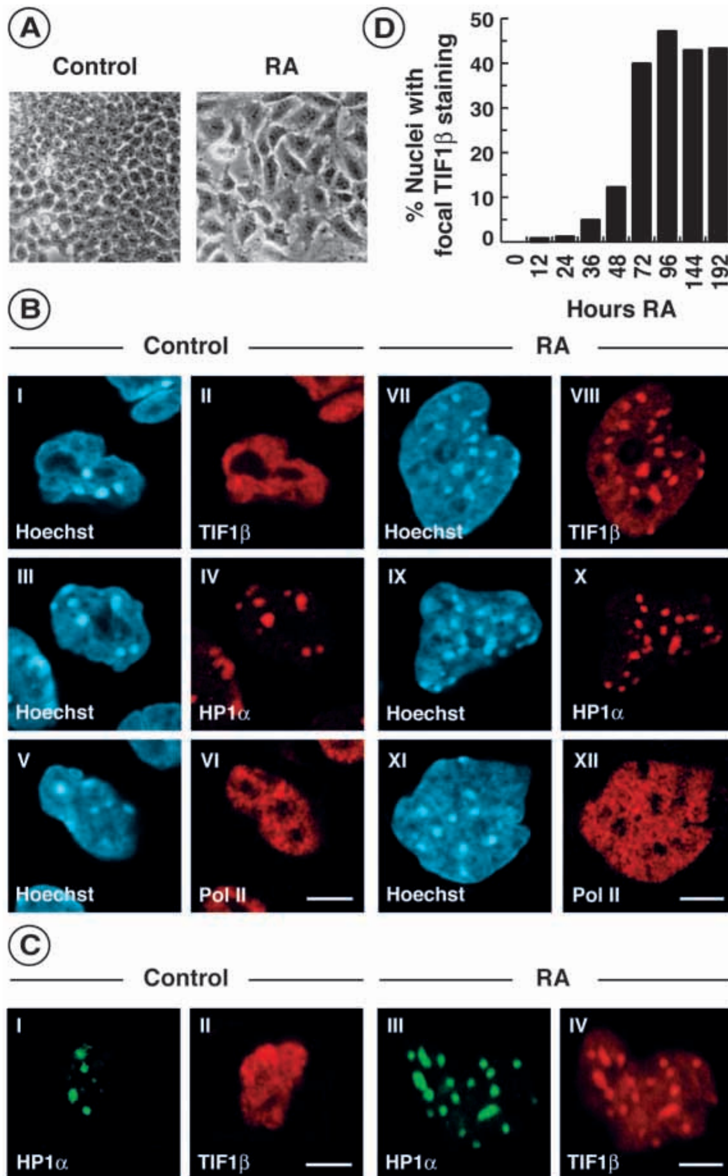

Fig. 1. F9 cell differentiation is accompanied by a specific change in the subnuclear distribution of TIF1β. (A) Phase-contrast photomicrographs of F9 EC cultures showing undifferentiated F9 stem cells (control) and differentiated endoderm-like cells after 4 day exposure to 1 μM retinoic acid (RA). (B) Confocal images of single optical sections through the nucleus of vehicle-treated (control) and RA-treated F9 cells. The left panels show the Hoechst DNA staining, which highlights A/T-rich repeat sequences present in the centromeric heterochromatin (bright blue patches), and the right panels correspond to immunodetection with specific monoclonal antibodies (Abs), as indicated. Identical results were obtained using two different TIF1β-specific Abs (PF64 and 1Tb3) raised against distinct epitopes. Bars, 5 μm. (C) TIF1β colocalizes with all HP1α-containing foci in the nuclei of RA-treated F9 cells. Cells were double-labeled with Abs against HP1α (green) and TIF1β (red). (D) Time course of TIF1β heterochromatin association following RA addition. F9 cells were plated at a density of 10^2 to 10^3 cells/cm^2 in the presence of 1 μM RA and were harvested at the time points indicated. Each coverslip was stained for TIF1β, and cells in ~10 to 20 randomly selected confocal fields were scored for the presence or absence of TIF1β nuclear foci. At least 1000 nuclei were scored per time point. Bars indicate the percentage of nuclei scoring positive for the focal TIF1β staining pattern characteristic of heterochromatin association, plotted versus time after RA addition. The average results from four independent experiments are depicted.

described (Boeuf et al., 2001). In brief, neuronal differentiation was induced by culturing the cells as embryoid bodies for 4 days in the presence of 1.0 μM all-trans RA. After RA treatment, the embryoid bodies were dispersed by trypsinization and transferred to gelatinized tissue culture plates (or glass coverslips for immunocytochemistry) to allow cell attachment and neuronal outgrowth.

Immunofluorescence and confocal microscopy

Cells grown on gelatinized glass coverslips were washed once with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS (pH 7.5) for 10 minutes at room temperature. Samples were then permeabilized twice with 0.1% Triton X-100 in PBS for 5 minutes, washed in PBS and incubated for 16 hours with primary antibodies appropriately diluted in PBS at room temperature. Following two consecutive 5 minute washes in PBS 0.1% Triton X-100, cells were incubated for 1 hour with fluorochrome-conjugated secondary antibodies. Slides were washed twice (5 minutes/wash) in PBS 0.1% Triton X-100, stained for DNA with Hoechst 33258 at 5 μg/ml and mounted in PBS 5% propyl gallate 80% glycerol. Image acquisition was performed using a Leica TCS-4D confocal scanning microscope (Leica Microsystems, Heidelberg, Germany).

Cell cycle analysis

The cell cycle profile of a cell population was determined by measuring cell DNA contents in different phases by flow cytometry. Cells were trypsinized, washed twice with cold PBS and fixed in 70% ethanol for 30 minutes at 4°C. Following two washes in PBS, cells were treated with 1 μg/ml RNase A (Macherey-Nagel, France) for 30 minutes at 37°C, pelleted by centrifugation and resuspended in PBS at a concentration of 10^6 cells/ml. Propidium iodine was added at a final concentration of 15 μg/ml for 30 minutes at room temperature, and the samples were analyzed on a FACScan (Beckton Dickinson, Mountain View, CA) using the CellQuest software.

In situ replication assay

5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co) incorporation was performed by incubating untreated and RA-treated cells in the

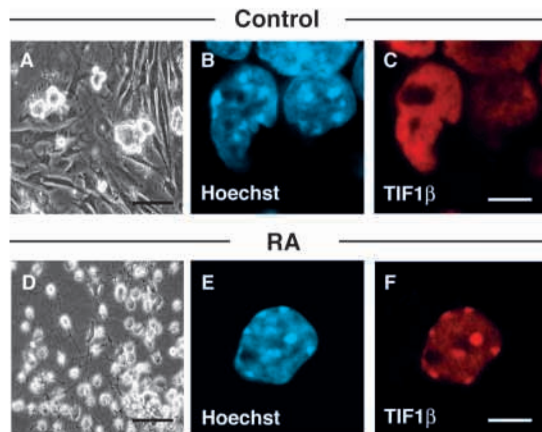

Fig. 2. TIF1β is targeted to centromeric heterochromatin in ES-derived neurons. A and D show representative phase-contrast photomicrographs of undifferentiated ES cells growing on a layer of feeder cells in the presence of LIF (control) and ES-derived neurons taken 4 days after plating of the RA-treated embryoid bodies in defined medium (RA), respectively. Confocal images of single optical sections through the nucleus of undifferentiated and differentiated cells are shown. B and E show the Hoechst DNA staining, and C and F, the TIF1β staining. Bars, 100 μm for (A) and (D) or 5 μm for others.

presence of 40 μM BrdU for 10 minutes. Cells were then washed with PBS, fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized twice with 0.1% Triton X-100 in PBS for 5 minutes. DNA was denatured with 4 M HCl for 10 minutes at room temperature. Neutralization was achieved by three washes with PBS, and the slides were further processed as described above. Note that the HCl treatment did not interfere with the detection and localization of TIF1β.

Immunoprecipitation and western blot analysis

Isolation of whole cell extracts from F9 cells and transfected COS-1 cells, immunoprecipitation and western blot detection were performed as previously described (Chiba et al., 1997; Nielsen et al., 1999).

Yeast two-hybrid interaction assays

Yeast cells grown in YEPD or selective medium were transformed by the lithium acetate procedure (Gietz et al., 1995). Yeast PL3 (Matα *ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::3ERE-URA3*) transformants were grown exponentially for about five generations in selective medium supplemented with uracil. Yeast extracts were prepared and assayed for OMPdecase activity as described previously (Le Douarin et al., 1995b).

Results

Subnuclear relocation of TIF1β upon retinoic acid (RA) treatment of F9 EC cells

The subnuclear distribution of TIF1β in interphase nuclei of RA-treated and untreated F9 cells grown as monolayer cultures was examined by confocal immunofluorescence microscopy. DNA-containing structures were visualized by Hoechst 33258 staining, and endogenous proteins were detected using specific monoclonal antibodies (mAbs) and a Cy3-fluorochrome-conjugated secondary antibody (Fig. 1B). In untreated F9 EC cells, TIF1β was found uniformly distributed throughout the nucleoplasm and largely excluded from nucleoli (Fig. 1BI,II), which is reminiscent of the results obtained in nuclei of peri-implantation embryos (Cammas et al., 2000) and other murine cell lines (Nielsen et al., 1999). After a 4 day treatment with 1 μM RA, most of the F9 cells exhibited the typical morphological features of primitive endoderm-like cells (Fig.

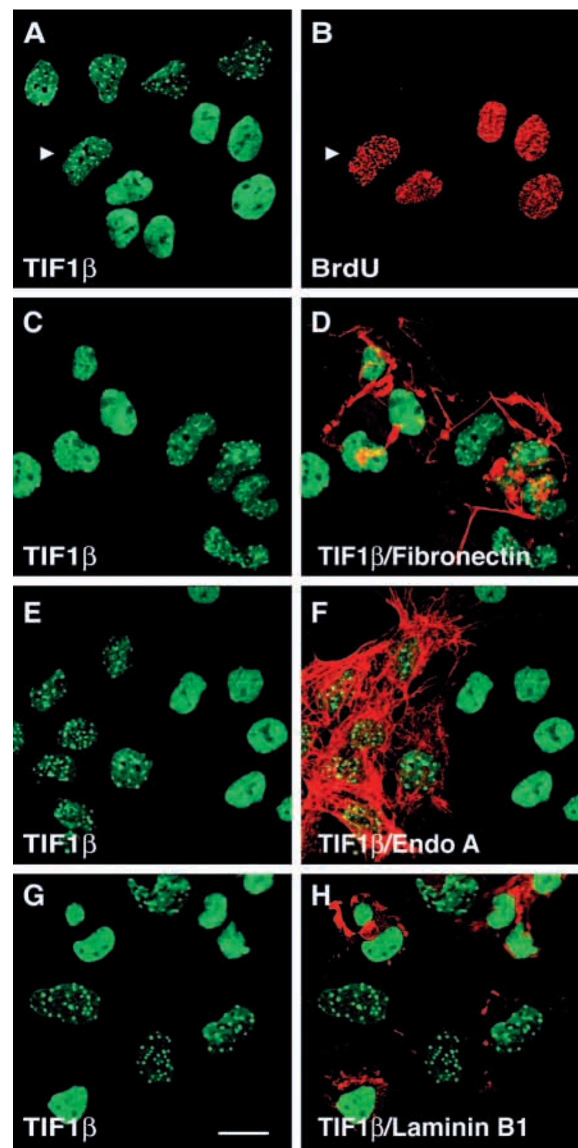

Fig. 3. RA treatment triggers a subnuclear redistribution of TIF1β in a subpopulation of F9 cells exhibiting a low proliferation rate and specific expression patterns for endodermal markers. (A-B) Bromodeoxyuridine preferentially labels the differentiated cells, exhibiting a diffuse nuclear staining of the TIF1β protein. F9 cells treated with 1 μM RA for 6 days were pulse-labeled with BrdU, fixed and stained for TIF1β (green) and BrdU (red) (see the Materials and Methods). The arrowhead points to a rare BrdU-positive cell with a TIF1β nuclear dot pattern. (C-H) Endo A and laminin B1, but not fibronectin, are differentially expressed in the RA-treated F9 cells that exhibit a diffuse or a focal TIF1β staining. F9 cells treated with RA for 6 days were processed for double-label immunofluorescence with antibodies against TIF1β (C to H; green), fibronectin (D, red), Endo A (F, red) and laminin B1 (H, red). Bar, 20 μm.

1A), and in approximately half of these cells, the anti-TIF1β antibody produced a non-uniform pattern of nuclear staining, with between 20 and 30 discrete foci of intense staining against a more diffuse nucleoplasmic distribution (Fig. 1BVIII). These foci enriched in TIF1β protein were indistinguishable in number and appearance from the Hoechst-bright dots (Fig. 1B, compare VII with VIII), which represent centromeric heterochromatin (Brown et al., 1997) and are associated with the heterochromatin-binding protein HP1α (Wreggett et al., 1994) (Fig. 1BIV,X). Importantly, none of the cells in the untreated cultures exhibited similar focal TIF1β staining. Moreover, an antibody against the largest RPB1 subunit of RNA polymerase II did not reveal any significant change in nuclear distribution upon RA treatment; instead, as illustrated in Fig. 1BVI and XII, Pol II showed a granular pattern of staining throughout the nucleoplasm, with little or no colocalization to the heterochromatic regions in both untreated and RA-treated cells. These cells were also processed for double-label immunofluorescence with antibodies against TIF1β and HP1α. Results revealed that in RA-treated cells TIF1β and HP1α both displayed a focal staining which was consistently overlapping (Fig. 1C-III and IV), while in untreated cells a focal staining was observed for HP1α only (Fig. 1C-I and II). Taken together, these results indicate that TIF1β specifically associates to regions of centromeric heterochromatin during RA-induced F9 cell differentiation.

To determine how rapidly RA induced TIF1β heterochromatin association, individual F9 cell nuclei (>1000) were examined for TIF1β staining and Hoechst counterstaining at several time points after RA addition. TIF1β heterochromatic foci were detected in 1% of the nuclei 12 hours of treatment, the earliest time examined in this experiment (Fig. 1D). The percentage of TIF1β-foci-containing nuclei increased progressively to a maximum of ~ 45-50% after 96 hours and remained unchanged at all subsequent time points (Fig. 1D), providing evidence for a stable change in TIF1β distribution. Interestingly, a similar proportion of cells exhibiting heterochromatic TIF1β (44%) was obtained by maintaining the differentiated cultures for up to six days in a RA-free medium after an RA treatment of two days (data not shown). This result indicates that the continued presence of the ligand is not required for maintenance of the heterochromatin association of TIF1β in the differentiated cells.

TIF1β heterochromatin association occurs in response to differentiation

Because differentiating F9 cells divide more slowly than F9 EC cells (Strickland et al., 1980), the RA-induced heterochromatin association of TIF1β could be the result of a decline in growth rate. To test this possibility, the subnuclear distribution of TIF1β was investigated in F9 cells starved of the animo acid isoleucine. Isoleucine starvation has previously been reported to cause partial growth arrest without inducing differentiation (Dean et al., 1986; Hosler et al., 1989). Cell cycle progression of isoleucine-starved F9 cells was determined by flow cytometry, using untreated and RA-treated F9 cells as controls (Table 1). As expected, RA treatment caused a gradual decline in growth rate, indicated by an increase in the percentage of the cell population in the G1 phase from ~20 to 60% at 72

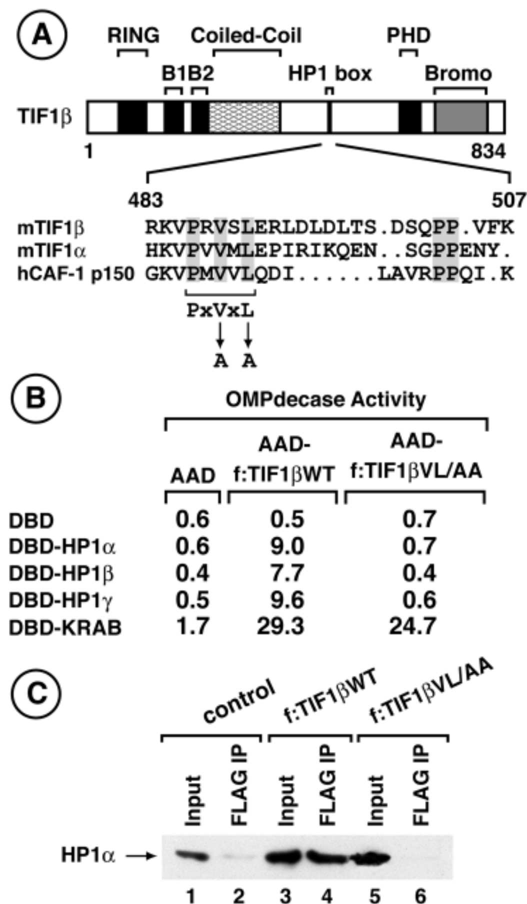

Fig. 4. Identification of two amino-acid residues in the HP1 box of TIF1β, which are critical for the HP1-binding activity of TIF1β. (A) A schematic representation of the conserved domains of TIF1β. Numbers refer to amino-acid positions. An alignment of the HP1 interaction domains from TIF1β, TIF1α and CAF-1 is shown. Invariant amino acids are shaded. Mutations introduced into the conserved hydrophobic residues of the TIF1β HP1 box are indicated below the alignment. Database accession numbers: mouse TIF1β (mTIF1β, X99644); mouse TIF1α (mTIF1α, S78219); human CAF-1 (hCAF-1, XP009408). (B) TIF1βV488A/L490A has no HP1-binding activity in yeast. The indicated FLAG-epitope-tagged TIF1β constructs, f:TIF1β wildtype (f:TIF1βWT) and f:TIF1βV488A/L490A (f:TIF1βVL/AA), were fused with the acidic activation domain (AAD) of VP16 and assayed for interaction with the 'unfused' DNA-binding domain (DBD) of the oestrogen receptor ERα or a DBD fusion containing HP1α, HP1β, HP1γ or the KRAB transcriptional repression domain of KOX-1 in the yeast reporter strain PL3, which contains a *URA3* reporter gene driven by three ERα binding sites (Le Douarin et al., 1995b). Transformants were grown in liquid medium containing uracil. OMPdecase activities determined on each cell-free extracts are expressed in nmol substrate/min/mg protein. The values (±20%) are the average of at least three independent transformants. Note that expression of all fusion proteins was confirmed by western blotting.
(C) TIF1βV488A/L490A has no HP1 binding activity in mammalian cells. Whole cell extracts from COS-1 cells transfected with 5 μg of expression vector for unfused FLAG (control) or FLAG-TIF1β were analyzed by western blotting either directly (input) or following immunoprecipitation with the M2 anti-FLAG antibody (FLAG IP). A western blot probed with a HP1α mAb is shown. Inputs correspond to 1/10 the amount of cell extract used for immunoprecipitation.

hours. A similar decline in growth rate was observed in F9 cells grown for 48 hours in medium without isoleucine (Table 1). However, none of these isoleucine-starved cells exhibited a change in the nuclear distribution of TIF1β when compared to exponentially growing F9 cells (Table 1). Thus, the heterochromatin association of TIF1β observed in RA-treated F9 cells does not result from the slower growth rate associated with differentiation.

Because F9 EC cells are transformed, whereas their differentiated derivatives are not, it was also important to be sure that the RA-induced heterochromatin association of TIF1β in F9 endodermal cells is not due to the loss of their transformed state. To test this possibility, we have investigated the subnuclear distribution of TIF1β in embryonic stem (ES) cells, which are pluripotent stem cell lines derived directly from early mouse embryos without the use of immortalizing or transforming agents. These cells can be propagated as undifferentiated stem cell cultures (Fig. 2A) and can be induced to differentiate into neuronal cells in the presence of RA (Bain et al., 1995) (Fig. 2D). TIF1β was found to be uniformly distributed throughout the nucleoplasm in undifferentiated ES cells (Fig. 2B,C) and concentrated into the large blocks of centromeric heterochromatin in their neuronal derivatives (Fig. 2E,F). It therefore seems clear that the change in TIF1β distribution following F9 cell differentiation reflects a bona fide differentiation response resulting from the change in cell type rather than loss of the transformed state.

To further support this conclusion, we also examined TIF1β localization in compound RXRα$^{-/-}$/RARγ$^{-/-}$ mutant F9 cells, which are refractory to RA-induced differentiation (Chiba et al., 1997). In these mutant cells, no significant change in the staining pattern of TIF1β was observed in the presence of RA (data not shown). Thus, the selective heterochromatin association of TIF1β in the nuclei of RA-treated F9 cells requires that the F9 cells respond to RA.

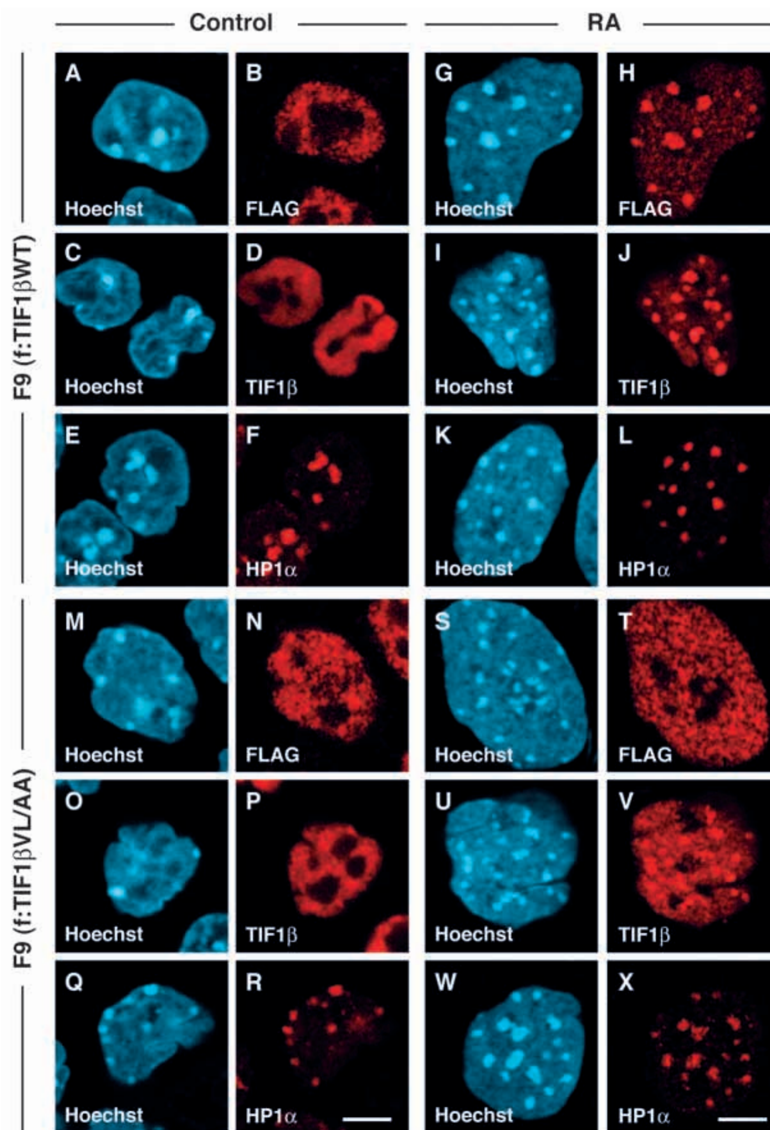

Fig. 5. The double mutation V488A/L490A in the conserved HP1 box residues prevents the RA-induced accumulation of TIF1β in heterochromatin. FLAG-tagged TIF1β-expressing F9 EC-derived cell lines [F9(f:TIF1βWT) and F9(f:TIF1βVL/AA] untreated (control) or treated with 1 μM RA for 4 days (RA) were analyzed by confocal immunofluorescence microscopy. The left panels show the Hoechst DNA staining, and the right panels correspond to immunodetection with specific monoclonal antibodies (Abs), as indicated. Note that the mAb against TIF1β also recognizes the FLAG-tagged proteins. Bars, 5 μm.

TIF1β heterochromatin association correlates with changes in both proliferation and differentiation-specific gene expression

Because only half of the RA-treated F9 cells displayed a change in TIF1β distribution (Fig. 1C), the cell population was further characterized at the single cell level with respect to both proliferation and differentiation. As determined by bromodeoxyuridine (BrdU) pulse-labeling and double-label immunofluorescence, S-phase cells could be detected in both subpopulations of differentiated cells characterized by a diffuse or a focal TIF1β staining pattern (Fig. 3A,B). However, the proportion of BrdU-positive cells was significantly lower in cells exhibiting a focal TIF1β staining as compared with those in which TIF1β distribution was diffuse (24% versus 43%), indicating that cells containing heterochromatin-associated TIF1β have a decreased proliferative activity. To monitor their differentiation state, cells were double-labeled with antibodies to TIF1β and a panel of specific endodermal markers. After a 6 day treatment with 1 μM RA, similar proportions of fibronectin-positive cells exceeding 80% were observed in the two TIF1β cell subpopulations (Fig. 3C,D). By contrast, the proportion of cells positive for the cytokeratin filament EndoA was much higher in cells containing heterochromatic TIF1β as compared with those in which TIF1β was localized in euchromatic areas (80% versus 10%) (Fig. 3E,F). By contrast, most of the cells with unfocused TIF1β staining were found to produce large amounts of laminin B1, whereas the cells containing

heterochromatic TIF1β showed barely detectable amounts of this marker (Fig. 3G,H). Taken together, these results confirm the notion that RA-induced F9 cell cultures are heterogeneous with respect to both proliferation and differentiation (Moore et al., 1986; Kurki et al., 1989) and furthermore indicate that two types of differentiated cells can also be characterized with respect to TIF1β localization in euchromatin versus heterochromatin.

Targeting TIF1β to centromeric heterochromatin requires HP1 interaction

TIF1β interacts with HP1α, β and γ through a 25 amino-acid segment containing the conserved PxVxL motif (Nielsen et al., 1999; Smothers and Henikoff, 2000; Lechner et al., 2000) (see also Fig. 4A). To investigate whether an interaction with the HP1s is required for the differentiation-induced heterochromatin association of TIF1β, we generated a stable F9 EC-derived cell line expressing a FLAG epitope-tagged TIF1β mutant, f:TIF1βVL/AA, in which the conserved hydrophobic residues V488 and L490 in the PxVxL motif were replaced by Alanine residues (Fig. 4A).

The f:TIF1βVL/AA mutant was initially characterized for HP1 interaction using the yeast two-hybrid system. In contrast to FLAG-tagged TIF1β wild-type (f:TIF1βWT), f:TIF1βVL/AA did not interact with HP1α, HP1β and HP1γ, and binding to the KRAB domain of KOX1 was not affected (Fig. 4B). The effect of the double mutation VL/AA on TIF1β-HP1 interaction in mammalian cells was also examined. Expression vectors encoding HP1α and either f:TIF1βWT or f:TIF1βVL/AA were transiently cotransfected in COS-1 cells. The localization of the FLAG-tagged TIF1β proteins in nuclei was confirmed by immunocytofluorescence (data not shown). When these proteins were immunoprecipitated from the respective whole cell extracts with an immobilized monoclonal antibody directed against the FLAG epitope, HP1α was found in the f:TIF1βWT immunoprecipitate (Fig. 4C, lane 4), but not in the f:TIF1βVL/AA immunoprecipitate (Fig. 4C, lane 6), indicating that, as observed in yeast, f:TIF1βVL/AA does not stably associate with HP1α in mammalian cells.

The subnuclear distribution of f:TIF1βVL/AA was then compared to that of the wild-type protein, f:TIF1βWT, using F9 EC-derived cell lines that stably express similar amounts of each FLAG-tagged protein (see Materials and Methods). On the basis of morphological criteria, both f:TIF1βWT- and f:TIF1βVL/AA-expressing cells differentiated into primitive endoderm-like cells in response to RA treatment to the same extent as the parental F9 EC cells (data not shown). As revealed by confocal immunofluorescence microscopy using the anti-FLAG antibody, the f:TIF1βWT protein exhibited in nearly 50% of the RA-treated cells the focal staining pattern characteristic of centromeric localization (Fig. 5H), similar to that observed for the endogenous TIF1β protein (Fig. 5J). By contrast, in both induced and uninduced f:TIF1βVL/AA-expressing cells, the f:TIF1βVL/AA mutant protein displayed a granular distribution pattern throughout the nucleoplasm, with little or no colocalization to the heterochromatic regions (Fig. 5N,T). It should be noted that, in these f:TIF1βVL/AA-expressing cells, neither the average number of HP1α heterochromatic foci (Fig. 5R,X) nor the distribution and dynamic behavior of endogenous TIF1β in response to RA

Table 1. Effects of RA treatment and isoleucine starvation on the F9 cell cycle and the subnuclear distribution of TIF1β

Cells	Time (hours)	DNA content (%)*			TIF1β focal staining (%)†
		G1/G0	S	G2/M	
Control	48	29	32	39	0
	72	21	45	34	0
RA treated	48	50	23	27	15
	72	61	19	20	40
40 Isoleucine starved	24	50	21	29	0
	48	59	19	22	0

*Measured by FACS analysis as an estimate of the percentage of cells in each phase of the cell cycle at specified times.

†Measured as the proportion of cells with a focal TIF1β staining pattern characterstic of heterochromatin association. At least 500 nuclei were analyzed for each experiment.

treatment (Fig. 5, compare P with V) was altered. Taken together, these results indicate that the PxVxL motif of TIF1β is essential for centromeric targeting in RA-induced F9 cells and support the hypothesis that during differentiation TIF1β is targeted to centromeric heterochromatin through HP1 interaction.

Discussion

TIF1β has previously been reported to localize predominantly, although not exclusively, in the euchromatic compartment of several mammalian cell lines (Nielsen et al., 1999; Ryan et al., 1999; Matsuda et al., 2001). A preferential euchromatic localization was also observed in the nuclei of pre-implantation embryos, in both inner cell mass and trophectoderm (Cammas et al., 2000). By contrast, TIF1β was found preferentially associated with regions of heterochromatin in the Sertoli and spermatogenic cells (Weber et al., 2002), indicating that TIF1β has a cell-specific pattern of nuclear compartmentalization. The data presented here provide evidence for a dynamic relocation of TIF1β into the regions of centromeric heterochromatin during RA-induced primitive endodermal differentiation of F9 cells. This selective centromeric targeting of TIF1β is an integral part of the RA response and was also observed in differentiating ES cells.

As measured by the number of nuclei in which TIF1β is localized at centromeric heterochromatin in the differentiated F9 cells, we found that induction of differentiation by a single application of RA was as effective as continuous treatment in stimulating TIF1β redistribution. This result is in full agreement with the hypothesis that this redistribution corresponds to an event related to the differentiation program in which RA triggers the first step of the differentiation process. However, our finding that RA caused a significant change in TIF1β distribution (>10%) only at late times (~48 hours) strongly suggests that relocation of TIF1β does not represent a primary response to RA. It is noteworthy that a similar time course was obtained by using cells synchronized in different phases of the cell cycle (F.C. and R.L., unpublished), indicating that the ability of RA to induce TIF1β relocation may not be cell-cycle dependent. Although cell cycle regulation may occur during the process of differentiation, our present data suggest other levels of regulation. Indeed, we found that RA triggers a nuclear

redistribution of TIF1β only in a subpopulation of cells exhibiting specific changes in both proliferation and differentiation. Most cells with heterochromatic TIF1β were found to contain a well developed cytokeratin intermediate filament system, unlike the cells with euchromatic TIF1β. Moreover, the cells containing heterochromatic TIF1β were characterized by a decreased expression of laminin and a low proliferation rate. On the basis of these criteria, these cells strongly resemble the DifB cells, which have previously been described in the differentiating F9 cell cultures treated with RA (Moore et al., 1986; Kurki et al., 1986). Interestingly, these DifB cells were also shown to differ from the other cells, referred to as DifA cells, by their inability to undergo further differentiation in response to cAMP analogs (Moore et al., 1986). Thus, cells with either heterochromatic or euchromatic TIF1β – although exhibiting both a primitive endoderm-like morphology – may be the product of two alternative differentiation pathways, which suggests that changes in TIF1β distribution are related to specific differentiated cell types. Supporting this hypothesis, an heterochromatin association of TIF1β was observed in the outer layer of visceral endoderm generated in RA-treated F9 embryoid bodies but not in parietal endodermal cells derived from monolayer F9 cell cultures upon treatment with RA and dibutyryl-cAMP (F.C. and R.L., unpublished).

The finding that the PxVxL motif of TIF1β is essential for both HP1 interaction and centromeric localization provides evidence that a major mechanism by which TIF1β is targeted to centromeric heterochromatin is through HP1 interaction. We have shown that TIF1β colocalizes with the heterochromatic HP1α protein in F9 differentiated cells but not in F9 stem cells. Thus, the centromeric targeting of TIF1β following F9 cell differentiation may be a consequence of its direct interaction with the HP1α isoform. Note, however, that in addition to HP1α, F9 cells express HP1β and HP1γ (see the references in the Introduction), to which TIF1β also associates (Nielsen et al., 1999). HP1β, like HP1α, localizes predominantly into the regions of centromeric heterochromatin (Wreggett et al., 1994; Nielsen et al., 1999; Minc et al., 1999), whereas HP1γ is present in both euchromatin and heterochromatin (Nielsen et al., 1999; Nielsen et al., 2001; Minc et al., 2000). These data, together with the recent finding that HP1α, HP1β and HP1γ are capable of mediating self-association in vivo (Nielsen et al., 2001), suggest another model of TIF1β centromeric targeting, which is consistent with our data demonstrating that one important criterion for targeting is the HP1 binding capability. In this alternative model, TIF1β, as a complex with euchromatic HP1γ, is targeted to regions of centromeric heterochromatin through an association of HP1γ with the other heterochromatic HP1 proteins.

Centromeric localization has recently been reported for a number of trans-acting factors (Francastel et al., 2000). For instance, Ikaros, a sequence-specific transcriptional factor required for proper lymphoid development, has been shown to localize to areas of centromeric heterochromatin in B lymphocytes (Brown et al., 1997; Brown et al., 1999). The CCAAT/enhancer-binding proteins α and β (C/EBPα/β) associate with centromeric heterochromatin during hormone-induced differentiation of pre-adipocytes (Tang and Lane, 1999). A dynamic redistribution from heterochromatin to euchromatin has recently been described for the subunit NF-E2p18 of the transcriptional activator NF-E2 during MEL cell differentiation (Francastel et al., 2001). Other proteins involved in gene silencing have also been shown to localize with centromeric heterochromatin (for review, see Francastel et al., 2000). These include DNA methyltransferases [Dnmts (Bachman et al., 2001)], methyl-DNA-binding domain proteins [MBDs (Hendrich and Bird, 1998)], histone methyltransferases [SUV39H (Aagaard et al., 1999)], histone deacetylases [HDAC1 (Kim et al., 1999; Francastel et al., 2001)], and chromatin remodeling factors [Mi2 and ATRX (Kim et al., 1999; McDowell et al., 1999)]. In the case of Ikaros, centromeric targeting has been demonstrated to take place concomitantly with that of several genes that become silenced during lymphocyte differentiation (Brown et al., 1997; Brown et al., 1999). Upon MEL cell differentiation, a concomitant relocation away from subnuclear compartments enriched in heterochromatin has also been observed for the human *β-globin* locus and NF-E2p18 (Francastel et al., 2001), and this relocation results in transcriptional activation (Schübeler et al., 2000). By analogy, it is tempting to speculate that TIF1β, when targeted to centromeric heterochromatin, may mediate cell-type-specific gene silencing by recruiting target genes to this transcriptionally inert compartment. Consistent with this hypothesis, two KRAB-containing zinc finger proteins, KRAZ1 and KRAZ2, have recently been demonstrated to repress transcription and to be targeted to foci of centromeric heterochromatin through TIF1β interaction in transiently transfected NIH3T3 cells (Matsuda et al., 2001). Alternatively, the centromeric heterochromatin compartments in which TIF1β accumulates during cell differentiation may represent storage sites that regulate the nucleoplasmic concentration of TIF1β. From our immunocytofluorescence data, it is clear that TIF1β is not limited to regions of centromeric heterochromatin in differentiated F9 cells, as it also binds to numerous diffuse sites dispersed throughout the nucleoplasm. Thus, it is conceivable that the differentiation-induced accumulation of TIF1β into regions of centromeric heterochromatin may control TIF1β levels within the nucleus and to create compartments, in which TIF1β may exert differential function(s). In this respect, it is noteworthy that TIF1β has recently been shown to associate with and to act as a transcriptional coactivator for C/EBPβ (Rooney and Calame, 2001), indicating that TIF1β can mediate both activation and repression of transcription. Whether TIF1β has both functions in the same nucleus, depending on its localization with respect to centromeric heterochromatin, is presently unknown and will require further biochemical and genetic studies to be determined.

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