The histone subcode: poly(ADP-ribose) polymerase-1 (Parp-1) and Parp-2 control cell differentiation by regulating the transcriptional intermediary factor TIF1β and the heterochromatin protein HP1α

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ABSTRACT Recent advances reveal emerging unique functions of poly(ADP-ribose) polymerase-1 (Parp-1) and Parp-2 in heterochromatin integrity and cell differentiation. However, the chromatin-mediated molecular and cellular events involved remain elusive. Here we describe specific physical and functional interactions of Parp-1 and Parp-2 with the transcriptional intermediary factor (TIF1β) and the heterochromatin proteins (HP1) that affect endodermal differentiation. We show that Parp-2 binds to TIF1β with high affinity both directly and through HP1α. Both partners colocalize at pericentric heterochromatin in primitive endoderm-like cells. Parp-2 also binds to HP1β but not to HP1γ. In contrast Parp-1 binds weakly to TIF1β and HP1 only. Both Parps selectively poly(ADP-ribosyl)ate HP1α. Using shRNA approaches, we provide evidence for distinct participation of both Parps in endodermal differentiation. Whereas Parp-2 and its activity are required for the relocation of TIF1β to heterochromatic foci during primitive endodermal differentiation, Parp-1 and its activity modulate TIF1β-HP1α association with consequences on pericentral endodermal differentiation. Both Parps control TIF1β transcriptional activity. In addition, this work identifies both Parps as new modulators of the HP1-mediated subcode histone. — Quénet, D., Gasser, V., Fouillen, L., Cammas, F., Sanglier-Cianferani, S., Losson, R., Dantzer, F. The histone subcode: poly(ADP-ribose) polymerase-1 (Parp-1) and Parp-2 control cell differentiation by regulating the transcriptional intermediary factor TIF1β and the heterochromatin protein HP1α. FASEB J. 22, 3853–3865 (2008)

Key Words: epigenetics · pericentric heterochromatin · post-translational modification · protein interactions

Dynamic reorganization of heterochromatic compartments and the epigenetic changes that are associated are currently recognized as important regulators of gene silencing that accompanies diverse cellular processes, including cell differentiation (1). This finding is further determined by the compartmentalization of specific protein complexes containing histones and nonhistone proteins, chromatin-remodeling and chromatin modifying enzymes, and by the post-translational modifications of histones defined as the histone code (2–4). However, how these activities are coregulated remains unknown.

A broad distribution of heterochromatin (HC) is observed at pericentromeric regions known to play an important role in gene silencing (4). Its epigenetic nature is defined by methylation of cytosine-phosphate-guanine (CpG), hypoacetylation of histones, and methylation at lysine 9 of histone H3 (meH3K9), which is necessary for the enrichment in heterochromatin protein 1 (HP1) (5). Three distinct mammalian HP1s have been characterized: HP1α and HP1β are primarily found within centromeric HC; whereas HP1γ is enriched at euchromatic sites (6). These proteins participate in chromatin packaging and have a well-established function in HC-mediated silencing. The structure of the HP1 proteins consists of an N-terminal chromodomain (CD) that binds meH3K9 and the histone fold motif of histone H3, a central hinge domain (hinge) that displays RNA/DNA binding activities, and a C-terminal chromoshadow domain (CSD) recognized as a protein-protein interaction domain (7, 8). It has been shown recently that HP1s are also targets of post-translational modifications similar to those described for histones, which define the existence of an HP1-mediated histone subcode (9). Notably, HP1s interact with proteins involved in transcriptional regulation through a specific PxVxL motif called HP1 box, among them the transcription intermediary factor (TIF1β) (10–12). TIF1β functions as a corepressor for the large family of
Krüppel-associated box (KRAB)-domain-containing zinc-finger proteins and acts as a molecular scaffold to coordinate various activities that regulate chromatin structure and dynamics (13–15). In addition, TIF1β exerts essential functions in early embryonic development (16) and spermatogenesis (17).

The mouse embryonal carcinoma F9 cells represent a well-established model of endodermal differentiation that can be induced to differentiate into primitive-endoderm-like (PrE) cells when grown as a monolayer in the presence of retinoic acid (RA) and subsequently in parietal endoderm-like (PE) cells when grown in the presence of RA and dibutyryl cAMP (18). In this system, TIF1β-HP1s association plays an essential role in the relocation of TIF1β from euchromatin to HC and in the progression through differentiation by regulating the expression of endoderm-specific genes (19).

Another regulatory mechanism that controls chromatin structure and integrity is the modification of histones and other nuclear proteins by poly(ADP-ribose) polymers catalyzed by poly(ADP-ribose) polymerases (Parps). Among the 17 members of the Parp family, Parp-1 and Parp-2 heterodimerize, share common binding partners and have been described as active players in the single-strand break/base excision repair process (20). Parp-1- and Parp-2-deficient mice and cells are very sensitive to both ionizing radiation and alkylating agents, thus supporting a role of both Parps in the cellular response to DNA damage (21). Moreover, Parp-1−/−Parp-2−/− embryos die at gastrulation, demonstrating the crucial role of poly(ADP-ribose)ylation during embryonic development (21). Several lines of evidence support the view that Parp-1 and Parp-2 play prominent roles in the maintenance of constitutive and facultative HC integrity, with, however, the emergence of specific functions for Parp-2. Both proteins localize to telomeres (22), centromeres (23, 24), and rDNA (25), where they interact and regulate specific partners. Parp-2−/− cells exhibit DNA damage-induced kinetochore defects; whereas the Parp-1−/−Parp-2−/− background displays specific female embryonic lethality associated with X chromosome instability (21).

Interestingly, both Parp-1 and Parp-2 were also suggested to play critical roles in the progression through differentiation. In the developing mammalian central nervous system, Parp-1 serves roles in transcriptional events required for neuronal differentiation (26). We have recently described the appearance of specific spontaneous defects in differentiation processes, including adipogenesis (27), spermiogenesis (28), and T-lymphocyte maturation (29) in the Parp-2−/− mice. However, the chromatin-mediated molecular mechanisms by which Parp-1 and Parp-2 may control differentiation have not yet been elucidated.

In this work we provide the first evidence for physical and functional selective interactions between Parp-2, Parp-1, TIF1β, and HP1α that have fundamental implications in HC structure and/or function governing endodermal differentiation. We show that Parp-2 physically binds to TIF1β with high affinity both directly and through HP1α. Both proteins relocate to pericentric HC throughout differentiation. We also identified a direct interaction of Parp-2 with HP1β. A weaker but significant direct interaction of Parp-1 with TIF1β and HP1β was also detected. Both Parps selectively poly(ADP-ribosylate) HP1α. Using shRNA approaches, we show that Parp-2-dependent poly(ADP-ribosylat)ion is required for primitive-endodermal differentiation possibly by targeting TIF1β to heterochromatin foci, whereas Parp-1 and its activity participate in the maintenance of TIF1β-HP1 association required for progression through parietal endodermal differentiation. Both Parps control TIF1β-mediated transcriptional activity.

In addition, this work identifies Parp-1 and Parp-2 as new actors of the silencing subcode histone that underlies the histone code.

**MATERIALS AND METHODS**

**Plasmids and antibodies**

Plasmids and antibodies used are detailed in Supplemental Information.

**Cell culture and establishment of stable depleted cell lines**

Wild-type and mutant F9 cells were grown in Dulbecco’s modified Eagle’s medium-4.5 g/L glucose (DMEM; Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FBS; PanBiotech, Aidenbach, Germany) and 1% Gentamicin (Life Technologies, Inc.) at 37°C in 5% CO₂. To induce PrE and PE differentiations, cells were treated, respectively, with 1 μM all-trans RA (Sigma, Lyon, France) alone or in combination with 250 μM dibutyryl cAMP (dbcAMP; Sigma) as described previously (30). To establish stable scr, shParp-1, and shParp-2 F9 cell lines, 5 × 10⁶ exponentially growing F9 cells were transfected with 5 μg of Xmnl-linearized pSuper-scrParp-2, pSuper-shParp-1, or pSuper-shParp-2 vectors together with 250 ng of A/JIII-linearized pGK-Hygro vector. Selection was started by adding 400 μg/ml Hygromycin B (Roche, Basel, Switzerland) to the growth medium 24 h posttransfections for over a period of 2 wk. Several drug-resistant colonies were isolated, expanded, and analyzed for the absolute levels of Parp-1, Parp-2, TIF1β, HP1α, and actin by Western blotting. To establish stable shParp-1-shParp-2 cell lines, 5 × 10⁶ exponentially growing cells of a selected shParp-1 F9 clone were transfected with 5 μg of Xmnl-linearized pSuper-shParp-2 together with 250 ng of A/JIII-linearized pGK-Neo plasmid. Selection was started by adding 600 μg/ml neomycin to the growth medium 24 h posttransfections for over a period of 2 wk. Neomycin-resistant clones were isolated, expanded, and analyzed as mentioned above.

**Immunoprecipitation, mass spectrometry, Western blot analysis, and glutathione S-transferase (GST) pull-down**

For immunoprecipitation in testis cells, 30 testes were collected from 10-wk-old C57/B6 mice (Janvier, Le Genest Saint Isle, France) and homogenized by 20 Dounce (no. 2) strokes in lysis buffer [10 mM Tris-HCl, pH 8; 400 mM NaCl; 1% Nonidet P-40; 2 mM dithiothreitol (DTT); 0.5 mM Pefabloc,
In vitro binding assays

Escherichia coli expression and purification of GST, GST-TIF1β, and GST-HP1 fusion proteins were performed as described previously (10). Equivalent amounts of purified proteins, quantified by Coomassie staining after SDS-PAGE, were incubated with 300 ng of either purified human Parp-1 or murine Parp-2 in binding buffer (20 mM Tris-HCl, pH 7.5; 300 mM NaCl; 0.5 mM Pefabloc; 0.1% Nonidet P-40; and PIC) for 2 h at 4°C. The beads were washed twice with washing buffer (10 mM Tris-HCl, pH 8; 2 mM DTT; 0.5% Nonidet P-40; 0.5 mM Pefabloc; and PIC) containing 500 mM NaCl and twice with washing buffer containing 50 mM NaCl. Final pellets were resuspended in 50 μl of Laemmli buffer and subjected to 10% SDS-PAGE. Coprecipitated proteins were stained by SyproRuby (Molecular Probes, Eugene, OR, USA) according to the manufacturer’s instructions. Nano-liquid chromatography-mass spectrometry/mass spectrometry experiments were performed using a CapLC capillary liquid chromatography system (Waters, Milford, MA, USA) coupled to a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF II, Waters) according to standard protocols (31). For immunoprecipitation in F9 or F9-derived cells, 10^7 cells were lysed by 3 cycles of freezing and thawing in lysis buffer as above. Cleared lysates were quantified by Bradford protein assay. Following treatment with RNase I (1 mg/ml) for 30 min at room temperature, 200 μg of total proteins was incubated with purified anti-Parp-2 pAb, anti-TIF1β mAb, or the control antibody overnight at 4°C and immunoprecipitated using protein A agarose for 2 h at 4°C. Beads were washed with (10 mM Tris-HCl, pH 8; 50 to 500 mM NaCl; 0.1% Nonidet P-40; 2 mM DTT, and PIC), resuspended in Laemmli buffer, and analyzed by 10% SDS-PAGE and immunoblots. Blots were probed with the appropriate specific antibodies followed by peroxidase-conjugated secondary antibodies, and developed using the ECL detection kit (Amersham, Little Chalfont, UK). When indicated, 100 nM of the Parp inhibitor Ku-0058948 (32) was added to the culture medium 2 h before lysis and maintained throughout the experiment. GST pull-down analysis was performed as described previously (22).

Immunofluorescence

Immunofluorescence was performed as described previously (25). Images were captured using a Leica microscope (Leica Microsystems, Heidelberg, Germany) and the capture software OpenLab (Improvision, Perkin Elmer, Inc., Coventry, UK).

In vitro binding assays

Escherichia coli expression and purification of GST, GST-TIF1β, and GST-HP1 fusion proteins were performed as described previously (10). Equivalent amounts of purified proteins, quantified by Coomassie staining after SDS-PAGE, were incubated with 300 ng of either purified human Parp-1 or murine Parp-2 in binding buffer (20 mM Tris-HCl, pH 7.5; 300 mM NaCl; 0.5 mM Pefabloc; 0.1% Nonidet P-40; and PIC) for 2 h at 4°C. The beads were washed twice with washing buffer (10 mM Tris-HCl, pH 8; 2 mM DTT; 0.5% Nonidet P-40; 0.5 mM Pefabloc; and PIC) containing 500 mM NaCl and twice with washing buffer containing 50 mM NaCl. Beads were resuspended in Laemmli buffer and analyzed by Western-blotting.

Heteromodification and noncovalent binding of poly(ADP-ribose) on GST-fusion proteins

E. coli-expressed GST-fused proteins were purified as above and quantified by Coomassie staining on SDS-PAGE. Heteromodification of equivalent amounts of purified proteins by either human Parp-1 or mouse Parp-2 was performed as described previously (33).

Quantitative RT-PCR

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using total F9 RNA samples was performed by using the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions in combination with the Light Cycler Detection System. The PCR products were analyzed with the manufacturer’s software. The following primer sequences were used: Hprt-5’-5’-TGACACTTGCA-AAACAATGCA-3’; Hprt-3’, 5’-GGTCCTTTTCACACGCAATG-3’; HNF4–5’, 5’-CTCCAAAACCTCTGGATAGAC-3; Mest-3’, 5’-GAAAATCAGAGAGCTGG-3; HNF4–3’, ACAGTCCACATGGAAGGT; HNF4–5’, CTCCTTTCATGCCA-GCCC.

RESULTS

Association of Parp-2 and Parp-1 with TIF1β and HP1α in mammalian cells

To identify interacting partners of Parp-2, testis cell extracts were immunoprecipitated with an anti-Parp-2 antibody or a control antibody, and captured proteins were analyzed by mass spectrometry (Fig. 1A). Among the proteins identified, we isolated previously described partners of Parp-2, including Parp-1 and DNA polymerase β (33) and a partner of Parp-1 named macroH2A1.2 (34), thus clearly supporting the validity of the approach used. Interestingly, we isolated 6 unique tryptic peptides from TIF1β. The functional similarities between Parp-2, Parp-1, and TIF1β, with regard to their accumulation on pericentric HC and their role in differentiation pathways, in addition to the previously described interaction of TIF1β with HP1α in mouse embryocarcinoma F9 cells, prompted us to investigate further whether these proteins could be physically associated in this model (Fig. 1B). F9 cell extracts were immunoprecipitated with an anti-Parp-2 antibody or an irrelevant antibody, and immunoprecipitation of TIF1β and HP1α was assessed by Western blotting. We detected significant association of TIF1β and HP1α with Parp-2 in F9 cells (Fig. 1B, lane 2), whereas no association was detected using the control antibody (Fig. 1B, lane 1). In an analog experiment, when F9 cell extracts were immunoprecipitated with an anti-TIF1β antibody or a control antibody, significant fractions of HP1α and Parp-1 were found in the TIF1β immunoprecipitate (Fig. 1B, lane 6) but not in the control immunoprecipitate (Fig. 1B, lane 5). Taken together, these results describe an association of a subset of Parp-1 and Parp-2 with TIF1β and HP1α in mammalian cells.
Parp-2 and Parp-1 interact differentially with HP1 isoforms and TIF1β in vitro

To characterize the complex further, binding assays between Parp-1, Parp-2, TIF1β, and HP1α were performed in vitro using purified recombinant proteins (Fig. 2). To carefully address the specificity of the interaction with members of the HP1 family, we also tested HP1β and HP1γ. GST-HP1 fusion proteins were expressed in *E. coli*, purified on glutathione S-sepharose beads, and batched with purified recombinant Parp-1 or Parp-2. After GST-pull down followed by stringent washes, copurification of Parps were analyzed by Western blotting. As shown in Fig. 2A (top panel), we detected binding of Parp-2 to GST-HP1α (lane 4) and GST-HP1β (lane 6) but not to GST-HP1γ (lane 10) nor GST (lanes 2, 8). In contrast, Parp-1 bound weakly but reproducibly to GST-HP1β only (lane 5).

To identify the region of HP1α to which Parp-2 binds, purified GST fusion proteins expressing various HP1α deletion domains—GST-CD, GST-hinge, and GST-CSD—were tested for interaction with purified Parp-2. As shown in Fig. 2A (bottom panel), Parp-2 interacts with the CSD (lane 3) and hinge domain (lane 2) of HP1α, whereas no interaction was detected with the CD (lane 1). Thus Parp-2 but not Parp-1 can directly interact with HP1α in vitro.

Because TIF1β also interacts directly with HP1α (11), we next addressed whether the association of TIF1β with Parp-2 detected in F9 cells (Fig. 1B) requires HP1α. Flag-tagged proteins expressing either wild-type TIF1β (Flag-TIF1β) or TIF1β with a mutation in the HP1 box that disrupts its interaction with HP1α (Flag-TIF1βHP1box) were coexpressed in Cos1 cells together with GST-Parp-2 or GST alone. To test the role of poly(ADP-ribosylation), the same assay was performed in the presence of the Parp inhibitor Ku-0058948. Coprecipitating proteins were analyzed by GST pull-down experiments and Western blotting. As shown in Fig. 2B, we observed a significantly weaker copurification of Flag-TIF1βHP1box with GST-Parp-2 (lane 4) when compared to Flag-TIF1β (lane 3). Thus, the HP1 box mutation of TIF1β that was previously described to disrupt the interaction between TIF1β and HP1 proteins (19) also impairs the interaction between TIF1β and Parp-2, which suggests that TIF1β-Parp-2 association involves—at least partly—HP1 proteins. No copurification with GST was detected (Fig. 2B, lane 2). The addition of Ku-0058948 also significantly impaired the binding of Flag-TIF1β to GST-Parp-2, thus indicating a role of poly(ADP-ribose) in the association of both partners (Fig. 2B, lanes 1, 3).

We next compared the direct binding efficiency of Parp-1 and Parp-2 to TIF1β (Fig. 2C). Under similar conditions of binding assays as above, we identified an efficient binding of Parp-2 to TIF1β and a weaker but reproducible interaction with Parp-1 (Fig. 2C, top panel). To verify whether Parp-2 and TIF1β also associate independently of HP1 in vivo, nuclear extracts from TIF1βHP1box−/− cells expressing the mutated TIF1βHP1box were immunoprecipitated with an anti-Parp-2 antibody, and the immunoprecipitates were probed for the presence of TIF1β (Fig. 2C, bottom panel). Coimmunopre-
cipation of TIF1β with Parp-2 was detected both in TIF1βHP1box/− cells (Fig. 2C, lane 3) and in the parental F9 cells (Fig. 2C, lane 2), but not in control immunoprecipitate (Fig. 2C, lane 1).

Finally, we found that TIF1β interacts with either the central E or the catalytic F domains of Parp-2 but not the N-terminal DNA-binding domain (data not shown).

Taken together, these results describe an heterochromatic protein network characterized by 1) a selective efficient binding of Parp-2 to HP1α and HP1β but not HP1γ, 2) an association of Parp-2 with TIF1β both directly and through HP1, and 3) a weaker but reproducible direct binding of Parp-1 to HP1β and TIF1β.

Selective poly(ADP-ribosylation) and noncovalent binding of poly(ADP-ribose) to HP1α

To gain further insights into the functional interactions governing this protein network, we evaluated the ability of either Parp-1 or Parp-2 to poly(ADP-ribose)ate HP1 isotypes and TIF1β (Fig. 3). Purified GST-HP1 and GST-TIF1β fusion proteins or GST alone were incubated with either Parp-1 or Parp-2 or no protein, in the presence of α-32P-NAD+ and DNase-I-treated calf thymus DNA. Autoradiography revealed that both Parp-1 and Parp-2 were able to poly(ADP-ribose)ate selectively HP1α but not HP1β, HP1γ, TIF1β, or GST alone (Fig. 3A). To identify the domain of HP1α poly(ADP-ribose)ated, we used the same approach with the purified E. coli-expressed deletion domains of HP1α and found that HP1α is poly(ADP-ribose)ated on its hinge domain by both Parp-1 and Parp-2 (Fig. 3B).

To test whether HP1 isoforms or TIF1β could directly bind to poly(ADP-ribose), similar amounts of purified GST, GST-HP1, and GST-TIF1β fusion proteins were spotted onto nitrocellulose and incubated with radioactive poly(ADP-ribose) (Fig. 3C). Detection of a radioactive signal was observed only for GST-HP1α, thus showing that HP1α binds tightly and stably to the poly(ADP-ribose).

The same conditions were used to identify the domain of HP1α that could bind to poly(ADP-ribose) and showed that PAR binds specifically to the hinge domain (Fig. 3D).

Interaction and colocalization of Parp-2 with TIF1β onto pericentric HC in PrE cells

The above results and the recent advances describing an essential role of TIF1β-HP1 interaction during
endodermal differentiation (19) prompted us to follow the association of Parps and TIF1β in this process. We first analyzed the expression of Parp-1, Parp-2, TIF1β, and HP1α in nuclear extracts of undifferentiated F9 or differentiated PrE and PE cell lines. Whereas the expression of Parp-1 and TIF1β decreased throughout differentiation of F9 to PrE and PE cells, the expression of Parp-2 and HP1α remained constant (Fig. 4A).

We next monitored the colocalization of Parp-1 and Parp-2 with TIF1β throughout the differentiation of F9 cells by indirect immunofluorescence (Fig. 4B). As described previously, Parp-2 (Fig. 4Bb) and Parp-1 (Fig. 4Bh) displayed a nuclear punctate distribution in F9 cells, with, however, a stronger accumulation in nucleoli (25), whereas TIF1β (Fig. 4Ba, j) showed homogeneous nuclear staining and was excluded from nucleoli. Interestingly, the differentiation into PrE induced a dynamic targeting of Parp-2 (Fig. 4Be) and TIF1β (Fig. 4Bd, m) but not Parp-1 (Fig. 4Bn) onto pericentric HC foci where both proteins colocalize. On further differentiation into PE cells, Parp-1 (Fig. 4Bg), Parp-2 (Fig. 4Bh), and TIF1β (Fig. 4Bf, p) exhibited a pattern similar to undifferentiated cells, although with less accumulation of Parps in the nucleoli.

To support this result further, we examined the association of Parp-2 and TIF1β throughout differentiation. Nuclear extracts of F9, PrE, and PE cells were immunoprecipitated with an anti-Parp-2 antibody or an irrelevant antibody, and the immunoprecipitates were probed for the presence of TIF1β (Fig. 4C). The amount of TIF1β coimmunoprecipitated was correlated with the expression of each partner throughout differentiation. Indeed, TIF1β was clearly detected in Parp-2 immunoprecipitates of F9 and PrE cell extracts (Fig. 4C, lanes 2, 3) containing significant amounts of proteins, but not readily in PE cell extracts, due to limited amounts of proteins expressed (Fig. 4C, lane 4). No TIF1β was detected in the control immunoprecipitate (Fig. 4C, lane 1). Together, these results indicate that the interaction of TIF1β with Parp-2 is maintained in the differentiated PrE cells.

Altogether, these data indicate that Parp-2 associates with TIF1β within regions of pericentric HC throughout differentiation of F9 to PrE cells in addition to the protein complex formed in the euchromatin compartment of F9 stem cells (Fig. 1).

Parp-2 is required for the differentiation into PrE cells, whereas Parp-1 is required for terminal differentiation into PE and visceral-endoderm-like (VE) cells

To investigate whether the absence of either Parp-2, Parp-1, or both lead to a defect in the differentiation of F9 cells to endoderm-like cells, we used the shRNA approach to generate stable clones depleted in either Parp-1 (shParp-1), Parp-2 (shParp-2), or both proteins (shParp-1;shParp-2). A Western blot analysis of the selected clones is shown in Fig. 5A. When compared to the expression of a housekeeping protein β-actin, the extent of Parps depletion was estimated to be more than 99% in the selected clones. Noticeably, the depletion of either Parp-1, Parp-2, or both had no effect on the level of TIF1β expression.
We induced each depleted F9 cell line to differentiate and followed differentiation by the morphological features characteristic of PrE and PE cells (30). To evaluate the requirement of Parp-1 or Parp-2 catalytic activity, cells were grown in the absence or in the presence of Ku-0058948 throughout differentiation. As shown in Fig. 5B, when grown in the presence of the Parp inhibitor, control scr-F9 cells displayed a weak reduced capacity to differentiate to both PrE (Fig. 5Be) and PE (Fig. 5Bf), which suggests a potential involvement of poly(ADP-ribosyl)ation in both stages. In contrast, the knockdown of Parp-2 clearly impaired differentiation into PrE (Fig. 5Bh), even though a population of remaining cells was still able to progress to PE (Fig. 5Bi). The addition of Ku-0058948 had no major additional incidence on the differentiation of shParp-2 to PrE (Fig. 5Bh) but significantly disrupted further differentiation to PE (Fig. 5Bi), thus suggesting a role of Parp-1 catalytic activity in the second stage of differentiation. In line with this observation, Parp-1-depleted cells differentiated into PrE (Fig. 5Bn) but did not differentiate further into PE (Fig. 5Bo). The addition of Ku-0058948 significantly impaired the potential of shParp-1 to differentiate to PrE (Fig. 5Bq), in agreement with an essential role of Parp-2-dependent poly(ADP-ribosyl)ation in primitive endodermal differentiation substantially increased in the absence of Parp-1. As expected, the depletion of both Parp-1 and Parp-2 completely inhibited the differentiation to both PrE and PE (Fig. 5Bt, u).

Taken together, these data firmly assign an essential role of Parp-2 and its activity in the differentiation of F9 to PrE, whereas Parp-1 and the associated activity are crucial for terminal differentiation to PE. Expression of Troma-1 in these cells confirmed their differentiated status (data not shown). We also studied the requirement of either Parp-1, Parp-2, or both in an other model of terminal differentiation into VE cells and found an essential role of Parp-1 but not Parp-2 in this process (Supplemental Fig. 1).

**Impaired relocation of TIF1β to pericentric HC in Parp-2-depleted PrE cells but not in Parp-1-depleted PrE cells**

Differentiation to PrE is accompanied by relocation of TIF1β from euchromatin to HC (30). To investigate the role of Parp-1 and -2 and poly(ADP-ribosyl)ation in this process, we compared the dynamic relocation of TIF1β onto pericentric HC during PrE differentiation in each stable depleted cell line and in the absence or in the presence of Ku-0058948 (Fig. 6). In non-treated cells, TIF1β staining was homogeneously distributed within the nucleus of scr, shParp-1, shParp-2, or shParp-1;shParp-2 F9 cells (data not shown). After 4 days of RA treatment, normal targeting of TIF1β to pericentric HC was observed in an average of 33% of shParp-1-PrE compared to 31% in the control scr-PrE, thus indicating that the absence of Parp-1 has no effect on TIF1β relocation. However, when grown in the presence of Ku-0058948, the percentage of control scr-PrE display-
ing focal staining of TIF1β significantly decreased to 12%, reflecting the involvement of poly(ADP-ribosyl)
ation in TIF1β HC targeting. In similar conditions the
differentiation to PrE was only slightly affected (Fig.
5Be). Similarly, the knockdown of Parp-2 clearly im-
paired TIF1β redistribution to the same extent as for
Ku-0058948-treated scr-PrE, thus suggesting an essen-
tial role of Parp-2 and its catalytic activity in this process.
Accordingly, the addition of Ku-0058948 had no major addi-
tional incidence on TIF1β relocation in shParp-2-
PrE but decreased the percentage of shParp-1-PrE with
TIF1β foci.

Taken together, these results reveal an essential
specific role of Parp-2, but not Parp-1, and its catalytic
activity in the TIF1β targeting to heterochromatic foci
during PrE differentiation. Under similar conditions, a
wild-type-like accumulation of both HP1α and tri-
methyl-H3K9 to HC was detected in all cases (Supple-
mental Fig. 2).

**Parp-1 but not Parp-2 controls the interaction
between TIF1β and HP1α**

The TIF1β-HP1 interaction is indispensable for PE
differentiation (19). The absence of PE differentiation
in shParp-1, shParp-1;shParp-2, or shParp-2 cells treated
with Ku-0058948 suggests a role of Parp-1 and its activity
in the association of TIF1β with HP1. To test this hypo-
thesis, we examined the association of both part-
ners by coimmunoprecipitation in early differentiating
PrE, shParp-1-PrE, and shParp-2-PrE in a window of
time in which TIF1β-HP1 association is required for
terminal differentiation (Fig. 7A, left panel). Interest-
ingly, the absence of Parp-1 but not Parp-2 caused a
weak but reproducible decrease in the coimmunopre-
cipitation of HP1α with TIF1β compared to the associ-
ation detected in the control F9 (Fig. 7A; compare
lanes 2, 3 with 1) thus revealing a partial but essential
and specific role of Parp-1 compared to Parp-2 in the
maintenance of TIF1β-HP1α interaction. To examine
further the role of poly(ADP-ribosyl)ation in this asso-
ciation, similar coimmunoprecipitation experiments
were performed in the presence of the Parp inhibitor
(Fig. 7A, right panel). Inhibition of Parp activity also
impaired TIF1β-HP1α coimmunoprecipitation (Fig.
7A; compare lanes 6, 7). Taken together, these results
are in favor of a role of Parp-1 but not Parp-2 and
poly(ADP-ribosyl)ation in the association of TIF1β with
HP1α that controls the progression through terminal
differentiation.

**Both Parps control TIF1β transcriptional activity**

Gene expression analysis in early differentiating PrE cells
has revealed an essential role of TIF1β-HP1 association
for induction of the endoderm-specific gene HNF4 (19)
and repression of the mesoderm-specific transcript Mest
(unpublished data). To investigate the role of Parp-1 and
Parp-2 in TIF1β transcriptional activity, we analyzed the
transcriptional level of both genes in control (scr),
shParp-1, shParp-2, and shParp-1;shParp-2 by qRT-PCR
(Fig. 7B). In agreement with previous data, we detected
a 2.4-fold reduction of HNF4 (19) and a 1.72-fold induction
of Mest in TIF1βHP1box/– cells (data not shown). Surpris-

The TIF1β-HP1 interaction is indispensable for PE
differentiation (19). The absence of PE differentiation
in shParp-1, shParp-1;shParp-2, or shParp-2 cells treated
with Ku-0058948 suggests a role of Parp-1 and its activity
in the association of TIF1β with HP1. To test this hypo-
thesis, we examined the association of both part-
ners by coimmunoprecipitation in early differentiating
PrE, shParp-1-PrE, and shParp-2-PrE in a window of
time in which TIF1β-HP1 association is required for
terminal differentiation (Fig. 7A, left panel). Interest-
ingly, the absence of Parp-1 but not Parp-2 caused a
weak but reproducible decrease in the coimmunopre-
cipitation of HP1α with TIF1β compared to the associ-
ation detected in the control F9 (Fig. 7A; compare
lanes 2, 3 with 1) thus revealing a partial but essential
and specific role of Parp-1 compared to Parp-2 in the
maintenance of TIF1β-HP1α interaction. To examine
further the role of poly(ADP-ribosyl)ation in this asso-
ciation, similar coimmunoprecipitation experiments
were performed in the presence of the Parp inhibitor
(Fig. 7A, right panel). Inhibition of Parp activity also
impaired TIF1β-HP1α coimmunoprecipitation (Fig.
7A; compare lanes 6, 7). Taken together, these results
are in favor of a role of Parp-1 but not Parp-2 and
poly(ADP-ribosyl)ation in the association of TIF1β with
HP1α that controls the progression through terminal
differentiation.

**Both Parps control TIF1β transcriptional activity**

Gene expression analysis in early differentiating PrE cells
has revealed an essential role of TIF1β-HP1 association
for induction of the endoderm-specific gene HNF4 (19)
and repression of the mesoderm-specific transcript Mest
(unpublished data). To investigate the role of Parp-1 and
Parp-2 in TIF1β transcriptional activity, we analyzed the
transcriptional level of both genes in control (scr),
shParp-1, shParp-2, and shParp-1;shParp-2 by qRT-PCR
(Fig. 7B). In agreement with previous data, we detected
a 2.4-fold reduction of HNF4 (19) and a 1.72-fold induction
of Mest in TIF1βHP1box/– cells (data not shown). Surpris-

**Parp-1 but not Parp-2 controls the interaction
between TIF1β and HP1α**

The TIF1β-HP1 interaction is indispensable for PE
differentiation (19). The absence of PE differentiation
in shParp-1, shParp-1;shParp-2, or shParp-2 cells treated
with Ku-0058948 suggests a role of Parp-1 and its activity
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PrE, shParp-1-PrE, and shParp-2-PrE in a window of
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are in favor of a role of Parp-1 but not Parp-2 and
poly(ADP-ribosyl)ation in the association of TIF1β with
HP1α that controls the progression through terminal
differentiation.
ingly, whereas the expression of HNF4 was equivalent in control and shParp-1 cells, we found a significant reduced expression in shParp-2 and shParp-1;Parp-2 cells in comparison with control cells (Fig. 7B, left panel; 8- and 3.2-fold, respectively). In contrast, Mest was up-regulated by 5.9-, 7-, and 3.9-fold in the absence of either Parp-1, Parp-2, or both Parps, respectively (Fig. 7B, right panel). Together, these results clearly indicate a role of both Parps in the expression of TIF1β-regulated specific genes in addition to the critical role of TIF1β-HP1 complex.

DISCUSSION

It was previously shown that TIF1β-HP1 interaction plays a critical role during F9 cell differentiation (19). Here, we provide evidence for physical and functional selective interactions between Parp-1, Parp-2, TIF1β, and HP1 in mammalian cells that provide an additional level of regulation through at least two distinct mechanisms: whereas Parp-2 activity controls TIF1β targeting to pericentric HC, Parp-1 activity essentially acts on TIF1β-HP1 complex.
HP1 association. Both might contribute to TIF1β-dependent gene expression.

Differential association of Parp-2 and Parp-1 with TIF1β and HP1 isotypes

In a proteomic screen of Parp-2 interacting proteins in testis cell extracts, we identified the transcriptional intermediary factor 1β (TIF1β) together with Parp-1. Interestingly, both Parps and TIF1β display several functional similarities in support of a dynamic interplay between these partners: 1) both Parps and TIF1β relocate on pericentric HC, in metaphase cells for Parp-1 (23) and Parp-2 (24) and in differentiated cells for TIF1β (30); 2) Parp-1 and Parp-2 (21) and TIF1β (16) exert central cellular functions during embryonic development; and 3) both Parps function in various differentiation processes, including neurogenesis (26), dendritic cell differentiation (35), spermiogenesis (28), adipogenesis (27), and T-lymphocyte development (29), whereas TIF1β in association with HP1 is essential for F9 cells to differentiate into endoderm-like cells (19). In line with this latter observation, we next confirmed that TIF1β and HP1α can be coimmunoprecipitated with Parp-2 and Parp-1 in F9 cells, thus describing the existence of a protein network involving Parp-1, Parp-2, TIF1β, and HP1α. We further characterized this complex combining in vitro protein-protein interaction assays and GST pull-down experiments and show here that both Parp-2 and Parp-1 interact directly but selectively and with significantly different affinities with both HP1 isoforms and TIF1β. Whereas Parp-2 binds efficiently and with high affinity to HP1α, HP1β, and TIF1β, Parp-1 interacts only weakly but reproducibly with HP1β and TIF1β. The association of Parp-2 with TIF1β is mediated both by a direct interaction of both partners and an indirect interaction through HP1α. Indeed, the PxVxL motif of TIF1β essential for its interaction with HP1 (11) is also required for its association with Parp-2. Together, these data describe Parp-1 and Parp-2 as new components of the TIF1β-HP1-containing heterochromatin network but strongly support the hypothesis that both proteins possibly display distinct functional roles during endodermal differentiation. Accordingly, we observed a dynamic relocation of Parp-2 to centromeric regions during PrE differentiation, where it colocalizes with TIF1β. In addition, Parp-2-TIF1β association is maintained in PrE cells. In contrast, Parp-1 subcellular localization remains unaffected throughout differentiation.

We next evaluated the importance of poly(ADP-ribose)ylation in the complex. Despite the high 80% sequence identity and structural similarities among all three HP1 isotypes, we identified a selective poly(ADP-ribose)ylation of HP1α by both Parp-2 and Parp-1. This result suggests a specific direct role of Parp activity in the modulation of higher-order chromatin structures and molecular interactions involving HP1α. The mechanism by which Parp-2 and Parp-1 control HP1α function involves both a covalent heteromodification and a noncovalent binding of poly(ADP-ribose) to HP1α. By targeting the hinge domain of HP1α, both ways of regulation can help to adjust various hinge-specific functions of HP1α. It is well recognized that the RNA-binding activity of HP1α that resides within its central hinge domain contributes to its recognition of pericentric HC (36, 37). Thus, the effect of HP1α poly(ADP-ribose)ylation would be basically to modulate its ability to bind HC, owing to electrostatic repulsion of the negatively charged ADP-ribose polymers present on HP1α from RNA. The normal accumulation of HP1α on pericentric HC observed in shParp-2 and shParp-1 cells could then be assigned to the compensating meH3K9-binding property of the CD of HP1α. Alternatively, the modification of the hinge domain could modulate the contact of HP1α with selective partners inside the Parp-1-Parp-2-TIF1β-HP1 complex that would reflect a dynamic equilibrium and important regulatory events occurring in this heterochromatic protein network. Accordingly, we found reduced communoprecipitation of both Parp-2 and HP1α with TIF1β in the presence of the Parp inhibitor. Altogether it is tempting to propose Parp-1, Parp-2, and poly(ADP-ribose) as auxiliary factors that contribute to the dynamic nature of HP1 either 1) by facilitating its association/dissociation activity to chromatin, in addition to the previously described histone methyl transferases ACFI or SUVAR39 (38) or 2) by modulating the protein interaction network at pericentric HC. As such, both Parps could regulate various HP1-dependent processes, including 1) transcriptional silencing of HC (39, 40); 2) its participation in kinetochore formation and maintenance during chromosome segregation (41); or 3) its role in HC dynamics during DNA replication (5).

Distinct functions of Parp-2 and Parp-1 in endodermal differentiation and TIF1β-dependent transcriptional activity

Despite the established shared functions of Parp-1 and Parp-2 in cellular response to DNA damage and a similar contribution in the maintenance of HC integrity, both enzymes have distinct DNA and/or protein targets, which suggests that they might also play unique functions that have only started to be clarified. In the present study, we provide direct evidence that Parp-2 and Parp-1 display key specific functions throughout endodermal differentiation even though both proteins poly(ADP-ribose)ylate HP1α.

The dynamic accumulation of Parp-2 to centromeric regions in PrE cells, where it colocalizes and interacts with TIF1β, combined with the observation that the depletion of Parp-2 or the inhibition of its activity significantly impairs the relocation of TIF1β to nuclear foci, provide compelling evidence that Parp-2 and the associated poly(ADP-ribose) synthesis participate in the targeting of TIF1β to pericentric HC. One major mechanism by which TIF1β is targeted to centromeric regions is through HP1 interaction (30). This association is required for terminal differentiation of F9 to PE
and VE cells (19). The finding that TIF1β-HP1 association is not significantly disrupted in Parp-2-depleted cells argues for the existence of an additional Parp-2-regulated process involved in TIF1β compartmentalization during cellular differentiation. Accordingly, we show here that compromised TIF1β heterochromatic targeting is associated with impaired differentiation of shParp-2 into PrE cells, whereas terminal differentiation of the remaining cells is not affected. These observations indicate key functions of Parp-2 in initiating differentiation. It is conceivable that through TIF1β HC selective targeting, Parp-2 controls the expression of TIF1β-dependent yet-to-be-identified genes involved in early endodermal differentiation. In this respect, it is noteworthy that Parp-2 has recently been shown to associate with and to act as a transcriptional cofactor for PPARγ during adipocyte differentiation (27) and for TTF-1 during lung development (42). In addition, we found a severe down-regulation of the endoderm-specific gene HNF4 in Parp-2-depleted and Parp-1-Parp-2-depleted cells similar to that previously observed in TIF1β-HP1box−/− cells but not in Parp-1-depleted cells. Therefore, Parp-2 participates in the regulation of TIF1β functions, including HC targeting and expression of endoderm-specific genes through a process that might be independent of TIF1β-HP1 interaction.

In contrast to Parp-2, we show that Parp-1 and the associated polymerizing activity are crucial for terminal differentiation of F9 cells into PE or VE cells even though Parp-1 does not interact with HP1α and only weakly binds to TIF1β. Furthermore, Parp-1 is at least partly required for stable HP1α-TIF1β interaction. Given the essential role of TIF1β-HP1 interaction in terminal differentiation (19), it seems likely that the abrogated differentiation of shParp-1 F9 cells to PE or VE cells could be a consequence of the unstable TIF1β-HP1 association observed in these cells. However, alternative roles of Parp-1 dependent-poly(ADP-ribosylation) of HP1α that might govern differentiation appear to be involved as a significant level of TIF1β-HP1 interaction remained reproducibly detected in shParp-1 F9 that is sufficient for the wild-type-like expression of HNF4.

Together, this work highlights key distinct functions of Parp-1 and Parp-2 in endodermal differentiation, although redundant activities cannot be excluded, as indicated by the similar up-regulation of the mesoderm-specific Mest gene in the absence of either Parp-1, Parp-2, or both proteins.

**Toward a role of Parp-2 and Parp-1 in the histone subcode**

It is well established that the dynamic regulation of chromatin structure and function is accomplished by a tuned combination of histone modifications, defined as the “histone code” (5). In recent studies, poly(ADP-ribosylation) is turning out to be another of the many global epigenetic modulators involved in chromatin dynamics during physiological processes (28).

More recently, a histone subcode hypothesis has been reported, which predicts that the posttranslational modification of HP1 provides a second regulatory layer of the chromatin code involved in transcriptional repression (9). HP1 proteins can be extensively modified by phosphorylation, acetylation, methylation, sumoylation, and ubiquitination, similar to histones.

Here we identified a selective poly(ADP-ribosylation) of HP1α by both Parp-1 and Parp-2, thus describing PAR as an emerging new modification of subcode proteins. In addition, this property points out Parp-1 and Parp-2 as new regulators of the histone subcode. The next challenge will be to identify the site-specific residue poly(ADP-ribosyl)ated in HP1α, with the aim of further deciphering the tight regulatory network that regulates these highly similar proteins.

In conclusion, the work described here identifies another step toward the role of TIF1β-HP1 association in endodermal differentiation, defined by distinct physical interactions of Parp-1 and Parp-2 with TIF1β and HP1 isoforms and a selective poly(ADP-ribosylation) of HP1α. A model describing Parp-1 and Parp-2 as essential players in the histone subcode can be proposed in which the selective poly(ADP-ribosylation) of HP1α is a major regulatory event required for TIF1β relocation on heterochromatic foci and TIF1β-HP1α-mediated transcriptional activity throughout differentiation (Fig. 8). Identifying the mechanism by which Parp activity is spontaneously induced during this process, as shown in Supplemental Fig. 3, remains an exciting challenge. In addition, these findings reinforce previous evidence that PAR plays fundamental roles in pericentric HC structure and integrity and open the way toward forthcoming fasci-
nating issues aimed at understanding its contribution in HP1-mediated centromere function and cell division.

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