Components of a Pathway Maintaining Histone Modification and Heterochromatin Protein 1 Binding at the Pericentric Heterochromatin in Mammalian Cells*

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Huawei Xin‡§, Ho-Guen Yoon§, Prim B. Singh‡, Jiemin Wong§, and Jun Qin§‡

From the ‡Verna and Marrs McLean Department of Biochemistry and Molecular Biology and §Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030 and ¶Nuclear Reprogramming Laboratory, Division of Gene Expression and Development, The Roslin Institute (Edinburgh), Midlothian, EH25 9PS, United Kingdom

Heterochromatin is a higher order chromatin structure that is important for transcriptional silencing, chromosome segregation, and genome stability. The establishment and maintenance of heterochromatin is regulated not only by genetic elements but also by epigenetic elements that include histone tail modification (e.g. acetylation and methylation) and DNA methylation. Here we show that the p33ING1-Sin3-HDAC complex as well as DNA methyltransferase 1 (DNMT1) and DMAP1-associated protein 1 (DMAP1) are components of a pathway required for maintaining proper histone modification and heterochromatin protein 1 binding at the pericentric heterochromatin. p33ING1 and DMAP1 interact physically and co-localize to heterochromatin in the late S phase, and both are required for heterochromatin protein 1 binding to heterochromatin. Although the p33ING1-Sin3-HDAC and DMAP1-DNMT1 complexes are recruited independently to pericentric heterochromatin regions, they are both required for deacetylation of histones and methylation of histone H3 at lysine 9. These data support a cooperative model for histone deacetylation, methylation, and DNA methylation in maintaining pericentric heterochromatin structure throughout cell divisions.

Heterochromatin is that portion of the genome that generally remains condensed throughout the cell cycle and replicates late in the S phase because of its unique, higher order chromatin structure. Heterochromatic DNA is predominantly present in the centromeric and telomeric regions of the chromosome that are composed of repetitive DNA sequence elements. In general, heterochromatic DNA sequences are heavily methylated on cytosine of the CpG dinucleotides. It is suggested that the chief heterochromatin duplication in the late S phase. This discrepancy raises the possibility that other factors may be involved in targeting HDAC activity specifically to heterochromatin during heterochromatin duplication in the late S phase.

In this paper we report the components of a pathway that maintain histone modification for HP1 binding. We find that the candidate tumor suppressor p33 inhibitor of growth family 1 (ING1) complex, which includes the core Sin3-HDAC1/2 complex, physically and functionally interacts with the DNMT1-DMAPI complex to maintain histone hypoacetylation and methylation of histone H3 of K9 at pericentric heterochromatin during cell division in human cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, Plasmids, and Transfections—293T and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The stable HeLa cell line expressing FLAG-DMAPI was cultured in medium supplemented with 1 μg/ml puromycin.

The M2 anti-FLAG antibody was from Sigma. Anti-acetyl-histone H3; H4, and anti-dimethyl-histone H3K9 antibodies were from Upstate Cell Signaling Solutions. Anti-trimethyl-histone H3K9 antibody was described previously (9). The DNMT1 monoclonal antibody was from
Imgenex. Anti-Sin3 and proliferation cell nuclear antigen antibodies were from Santa Cruz Biotechnology. Anti-HP1α and anti-HP1β antibodies were from Chemicon International. The M31 anti-HP1α rat antibody was from Serotec. Rabbit ING1 and DMAP1 antibodies were raised against bacterially produced His-ING1 and GST-DMAP1 (Bethyl Laboratories) and affinity-purified.

ING1 and DMAP1 were cloned into pET or pGEX4T-1 vectors and were expressed as His6 or GST fusion proteins in E. coli BL21 (DE3). For transient transfection in 293T cells, ING1 was cloned into a pEGFP-C2 vector (Clontech Laboratories), and FLAG-DMAP1 was cloned into a pcDNA3 vector (Invitrogen).

Transient transfection of GFP-ING1 and FLAG-DMAP1 in 293T cells was carried out with LipofectAMINE (Invitrogen). Cells were harvested 48 h after transfection. To establish the FLAG-DMAP1-stable cell line, FLAG-DMAP1 was cloned into a pBabe vector and then transfected into 293T cells for a retrovirus particle package. The virus-containing medium was used for HeLa cell transduction.

In Vitro Pull-down Assay, Immunoprecipitation, Mass Spectrometry, and Cell Cycle Synchronization—An in vitro pull-down assay was performed using Sepharose-immobilized His6-ING1 or GST-ING1 to pull down interacting proteins from HeLa nuclear extract, or DMAP1 transfected cells, respectively, and co-immunoprecipitated DNMT1 and ING1 were detected by Western blotting. PCNA, proliferating cell nuclear antigen; IP, immunoprecipitate.

RESULTS AND DISCUSSION

p33ING1 Interacts and Co-localizes with DMAP1 in the Late S Phase at Heterochromatin—Recent studies have shown that the human candidate tumor suppressor p33ING1 resides in an HDAC complex that includes Sin3-HDAC1/2 (12, 13). In a search for p33ING1-binding proteins, we carried out a His6-p33ING1 pull-down assay in HeLa nuclear extract followed by mass spectrometric identification. We found DMAP1 as a...
The specific binding of DMAP1 to ING1 (both p33 and p24ING1 isoforms, Fig. 1A) in HeLa nuclear extract was verified by a GST pull-down assay and by Western blotting (Fig. 1B), whereas proliferation cell nuclear antigen and HP1/H925 as controls cannot bind p33ING1 or p24ING1. In vitro−translated DMAP1 also binds to ING1 (Fig. 1C), and the conserved plant homeodomain is not required for binding (data not shown). In addition, DMAP1 can interact with ING1 within cells. When GFP-p33ING1 or p24ING1 and FLAG-DMAP1 are co-transfected into 293T cells, immunoprecipitation of DMAP1 by the FLAG antibody (M2) co-precipitates GFP-p33ING1 and GFP-p24ING1 but to a lesser extent (Fig. 1D). Furthermore, a small percentage of endogenous p33ING1 can also be co-immunoprecipitated with FLAG-DMAP1 from a cycling stable HeLa cell line that expresses FLAG-DMAP1 at a level similar to endogenous DMAP1. Importantly, the amount of co-immunoprecipitated p33ING1 is diminished when cells are blocked at the G1/S boundary using hydroxyurea. Similarly, DNMT1 is co-immunoprecipitated with DMAP1 from cycling cells but not from hydroxyurea-treated cells (Fig. 1E), in agreement with the observation that DNMT1 and DMAP1 interact throughout the S phase. These results show that DMAP1 and p33ING1 physically interact, and their interaction may be cell cycle-regulated (see below).

Because DNMT1 associates with DMAP1 at replication foci throughout the S phase but associates only with HDAC2 in the late S phase (8), our finding that DMAP1 physically interacts with p33ING1 raises the possibility that DMAP1 may bring p33ING1-Sin3-HDAC1/2 to DNMT1 in the late S phase. We examined p33ING1 and DMAP1 localization during the cell cycle by indirect immunostaining. To facilitate this analysis, we used the stable HeLa cell line that expresses FLAG-DMAP1 at a level similar to the endogenous DMAP1 (see Fig. 1B). We synchronized HeLa cells by a double block procedure in which nocodazole-arrested mitotic cells enriched by mitotic shake-off were blocked in mimosine and then released into drug-free medium. Cells were fixed and immunostained at different

**Fig. 2. Cell cycle-regulated localization of ING1 and DMAP1 to heterochromatin.** A, co-localization of ING1 and DMAP1 was regulated by the cell cycle. HeLa cells that stably express FLAG-DMAP1 were synchronized by a double block procedure. At different times after release, cells were fixed with cold methanol/acetone (90:10 in volume) and double immunostained with a mouse M2 antibody (red) and a rabbit anti-ING1 N terminus antibody (green) as described previously (10). DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI) (blue). B, ING1 and DMAP1 co-localize with HP1β in the late S phase. Co-immunostaining is shown of ING1 or DMAP1 with HP1β in the late S phase cells 6.5 h after release from the double block procedure.
times after release from mimosine using a mouse anti-FLAG antibody and a rabbit anti-p33ING1 N terminus antibody. Cell cycle progression was monitored by flow cytometry (data not shown). As shown in Fig. 2A, both FLAG-DMAP1 and p33ING1 were stained in a speckle pattern during early S phase (2.5 h after mimosine release), but they do not co-localize. Starting in mid S phase (4.5–5.5 h), the two proteins appear to begin forming bright foci and partially co-localize (data not shown). When cells enter late S phase (6.5 h), they form large foci and co-localize to the highest extent. The large DMAP1 and ING1 foci disappear when cells exit the S phase (7.5 h) and resume the speckled pattern in the G2 phase. These results indicate that p33ING1 and DMAP1 interaction is regulated and confined to the late S phase.

Because heterochromatin is replicated in the late S phase, we examined whether DMAP1 or p33ING1 co-localizes with a heterochromatin maker, HP1α, in the late S phase cells. HP1β shows distinct, large foci and co-localizes with DMAP1 or p33ING1 foci in the late S phase, i.e. 6.5 h after release (Fig. 2B). This observation supports the idea that p33ING1 and DMAP1 are recruited to the heterochromatin region during the late S phase and suggests that the two proteins and possibly their interaction may be involved in heterochromatin duplication.

**Fig. 3.** The requirement of the p33ING1-Sin3 complex and the DNMT1-DMAP1 complex for binding of HP1 and trimethyl-H3K9 to heterochromatin. A, HP1α and trimethyl-H3K9 were immunostained in cells transfected with siVimentin, siING1, or siSin3. B, HP1α and trimethyl-H3K9 were immunostained in cells transfected with siDMAP1 or siDNMT1. C, HP1α and β were immunostained in cells transfected with siVimentin, siING1, or siDMAP1. D, p33ING1, DMAP1, Sin3, or DNMT1 proteins were depleted by RNAi. DAPI, 4’,6’-diamidino-2-phenylindole.

HP1α and trimethyl-K9 form distinct, large and small foci and co-localize in siVimentin-transfected control cells (Fig. 3A). These large foci may correspond to the pericentric heterochromatin region. In contrast, these large foci are significantly disrupted, and HP1α and trimethyl-K9 are uniformly distributed throughout the nucleus with some small foci remaining in
siING1- and siSin3-transfected cells, a pattern similar to that seen in trichostatin A-treated cells (7). These results demonstrate that the p33ING1-Sin3 complex is required for the localization of HP1α and for the concentration of trimethyl-H3K9 to the heterochromatin region. This is consistent with a recent study showing that an mSin3-associated protein, mSds3, is essential for pericentric heterochromatin formation in a mouse knock-out model (16). The human SDS3 protein is an integral component of the p33ING1-Sin3 complex.2

The interaction and co-localization of DMAP1 and p33ING1 to heterochromatin during the late S phase (Figs. 1 and 2) suggest that they may function in a pathway maintaining the heterochromatin structure. Indeed, transfection of HeLa cells with siDMAP1 leads to delocalization of HP1α and trimethyl-H3K9 foci (Fig. 3B). Therefore, DMAP1 is also required for HP1α and trimethyl-H3K9 association with heterochromatin. Given the previous observation that recruitment of DMAP1 to replication foci throughout the S phase requires DNMT1 (8), we next tested the role of DNMT1 in localizing HP1α and trimethyl-H3K9 to heterochromatin. A similar effect was observed when DNMT1 was down-regulated (Fig. 3B). Therefore, the DNMT1-DMAP1 complex also functions in the pathway of maintaining heterochromatin structure.

To corroborate the above results, we examined HP1β, another heterochromatin marker. To co-stain HP1α and -β, we used mouse anti-HP1α and rat anti-HP1β (M31) antibodies for immunostaining. As shown in Fig. 3C, HP1α and -β form similar foci, and more importantly, they co-localize to a large degree in siVimentin-transfected control cells. Similarly to HP1α, the large HP1β foci are also significantly disrupted, and HP1β stains uniformly throughout the nucleus with some small foci remaining when cells are transfected with siING1 or siDMAP1. As shown in Fig. 3D, the protein levels of ING1, DMAP1, Sin3, and DNMT1 are significantly reduced by siRNA transfection. Collectively, these results demonstrate that the loss of these proteins in the cell has a dramatically adverse effect on the localization of heterochromatin markers to heterochromatin.

Both p33ING1-Sin3 and DMAP1-DNMT1 Complexes Are Required to Maintain Histone Deacetylation and H3K9 Methylation at the Pericentric Heterochromatin—We first investigated the global changes in histone acetylation and methylation using total extracted histones from siING1- and siDMAP1-transfected cells. As shown in Fig. 4A, neither acetylation nor H3K9 methylation of bulk histones is significantly affected by the loss of ING1 or DMAP1 (Fig. 3D), suggesting that they may have only a restricted effect on histone modification of specific chromosome regions, such as the pericentric heterochromatin. Chromatin-associated HP1β is somewhat decreased in the absence of ING1 or DMAP1, but the total HP1β level in whole cell

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lysate does not change. This is consistent with the dynamic nature of HP1 binding to heterochromatin (9, 17, 18). The loss of ING1 or DMAP1 impinges on the integrity of heterochromatin, which in turn is likely to change the dynamics of HP1 binding.

Next we made use of chromatin immunoprecipitation assays to directly examine the role of p33ING1-Sin3 and DMAP1-DNMT1 in regulating histone acetylation and methylation in pericentric heterochromatin (11). For this purpose, specific pairs of PCR primers were designed to allow the amplification of the chromosome 4 centromeric regions and X chromosome-linked \( \alpha \)-satellite repetitive DNA (19). These two types of centromeric DNA are found in the pericentric heterochromatin territories of chromosome 4 and X, respectively. The \( MT\alpha 2 \) and \( D3 \) genes were chosen as control euchromatin loci. As shown in Fig. 4B, a knockdown of ING1 or DMAP1 results in elevated levels of acetylated H3 and H4, diminished dimethylated and trimethylated H3K9, and a concomitant increase in acetylated H3K9 in the chromosome 4 centromeric region. Similar results were obtained when the chromosome 10 centromeric region was analyzed (data not shown). In contrast, the knockdown of ING1 and DMAP1 has no significant effect on the acetylation or methylation patterns over the euchromatic \( MT\alpha 2 \) and \( D3 \) gene promoter regions. On the other hand, the effect on the X

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**Fig. 4. Regulation of histone acetylation and methylation by and recruitment of the p33ING1-Sin3 and DNMT1-DMAP1 complexes at pericentric heterochromatin.** A, the knockdown of ING1 or DMAP1 does not grossly change histone acetylation and methylation. Total histones and chromatin-associated proteins from mock-, siING1-, and siDMAP1-transfected cells were resolved on SDS-PAGE, and the status of histone acetylation and methylation was measured with acetylation- and methylation-specific antibodies by Western blotting. Note the decreased association of HP1/\( H9252 \) on chromatin in siING1- and siDMAP1-transfected cells, but the HP1/\( H9252 \) total protein level does not change. B, increased histone acetylation and diminished histone methylation at pericentric heterochromatin in siING1- and siDMAP1-transfected cells are shown. DNA immunoprecipitated by various antibodies (specific for all acetylated histone H3, all acetylated H4, acetylated H3K9, dimethylated H3K9, and trimethylated H3K9) in the chromatin immunoprecipitation experiments was amplified by specific primers for the chromosome 4 centromere region, a promoter of the \( MTA2 \) gene, the \( \alpha \)-satellite of the X chromosome, and a promoter of the \( D3 \) gene. C, independent recruitment is shown of the p33ING1-Sin3 complex and the DNMT1-DMAP1 complex to the pericentric heterochromatin. Chromatin immunoprecipitation analysis shows associations of Sin3, ING1, DMAP1, and DNMT1 with pericentric heterochromatin in mock, and various siRNA transfected cells. WCL, whole cell lysate; IgG, immunoglobulin G; Chro-4, chromosome 4; X-Chro, X chromosome.
chromosome-linked α-satellite region is less dramatic with little effect on both acetylation and methylation, which is consistent with the observation that facultative heterochromatinization of the X chromosome may affect the histone modifications differently at the X centromere when compared with the other chromosomes (4). We also found that a knockdown of Sin3 or Dnmt1 led to a similar change in histone modifications (data not shown). These results establish an essential role of the p33ING1-Sin3 and DMAP1-DNMT1 complexes for histone deacetylation in pericentric heterochromatin and demonstrate that the p33ING1-Sin3-HDAC and DMAP1-DNMT1 complexes are required for maintaining histone H3K9 methylation in pericentric heterochromatin. In addition, it demonstrates that DNA methyltransferase is also important for maintaining histone hypoacetylation and methylation in pericentric heterochromatin. These results and published work indicate that histone deacetylation and histone and DNA methylation may be interdependent at the pericentric heterochromatin region in maintaining a heterochromatin structure that is conducive for HP1 binding.

Independent Recruitment of p33ING1 and DMAP1 to Pericentric Heterochromatin—Because our data demonstrate a physical interaction between p33ING1 and DMAP1, we used a chromatin immunoprecipitation assay to investigate their recruitment to specific pericentric heterochromatin loci. As shown, Sin3, p33ING1, DMAP1, and DNMT1 all associate with the centromeric region of chromosome 4 (Fig. 4C, top row). Interestingly, although p33ING1 and Sin3 show interdependence for binding to the chromosome 4 centromeric region, a clear difference can be seen when examining their binding to the X chromosome α-satellite sequence. The binding of Sin3 to the X chromosome α-satellite is independent of p33ING1. In contrast, DNMT1 recruits DMAP1 to heterochromatin, consistent with the previous finding that DNMT1 recruits DMAP1 to DNA replication foci (8). Despite the fact that ING1 and DMAP1 physically interact and co-localize to heterochromatin in the late S phase (Figs. 1 and 2), they independently associate with heterochromatin at the chromosome 4 centromeric and X chromosome-linked α-satellite regions.

The three epigenetic elements that characterized heterochromatin are hypoacetylation and hypermethylation of histones, and hypermethylation of DNA (3). The relationships among them are beginning to be understood. Previous studies have shown that a histone H3-specific HDAC, Clr3, is required for H3K9 methylation in fission yeast (20), an H3K9 methyltransferase can direct DNA methylation in fungi and plants (21, 22), and a DNA methyltransferase and a SWI/SNF-like protein that regulates DNA methylation are required for deacetylation of histone H4 in plants (23, 24). Therefore, these three characteristics may be interdependent, and all may be required for maintenance of heterochromatin structure.

In this study, we found that the loss of the p33ING1-Sin3-HDAC complex and the DMAP1-DNMT1 proteins also leads to hyperacetylation and hypomethylation of histones at pericentric heterochromatin. Thus, in addition to the H3K9-specific methyltransferase Suv39h, our data demonstrate that a histone deacetylase complex as well as a DNA methyltransferase complex are required for maintaining histone modifications at the pericentric heterochromatin in human cells. The mechanism of maintaining heterochromatin seems to be evolutionarily conserved. The loss of any one of these three enzymes that are important to maintain the characteristics of pericentric heterochromatin can lead to destabilization of the higher order structure that is necessary for binding of HP1 proteins. Although it is clear that both DMAP1-DNMT1 and p33ING1-Sin3 are required for H3K9 methylation at pericentric heterochromatin, it remains to be established whether they are required for DNA methylation of the cytosome residue at pericentric heterochromatin.

Because p33ING1 and DMAP1 are recruited independently to heterochromatin, DMAP1 does not appear to be necessary for localizing the p33ING1-Sin3-HDAC complex to heterochromatin. Thus, the functional significance of the interaction between p33ING1 and DMAP1 is not yet clear. One possibility is that DMAP1 activates the HDAC activity of the p33ING1 complex localized at the pericentric heterochromatin by interacting with the p33ING1 subunit. This model is suggested by the requirements of both p33ING1-Sin3 and DMAP1 for deacetylation of histones at the pericentric heterochromatin. Alternatively, DNMT1-DMAP1 could recruit HDAC2 independently of their interaction with the p33ING1-Sin3 complex. In this scenario, DMAP1 may stimulate the DNMT1-associated HDAC2 activity or stabilize their interaction and/or heterochromatin binding because the binding of DNMT1 to heterochromatin is independent of DMAP1 but is required for deacetylation of histones. Nevertheless, the activities of a histone deacetylase and a DNA methyltransferase need to cooperate at pericentric heterochromatin regions to bring about histone methylation for HP1 binding.

Data presented here reveal components of a pathway for maintaining histone modification at the pericentric heterochromatin during cell division in HeLa cells. Our findings may also provide a molecular mechanism for the links between DNA hypomethylation, genomic instability, and cancer (25, 26). Mice with a hypomorphic allele of Dnmt1 that retains 10% of wild type DNA methyltransferase activity develop cancer because of genomic instability (25). This instability may be caused by a failure to maintain histone modification at pericentric heterochromatin when DNMT1 activity is low. Similarly, the requirement of the p33ING1-Sin3-HDAC and DMAP1-DNMT1 complexes for this process suggests that other components in these complexes may also be important for preventing cancer development.

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