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Heterochromatin and ND10 are cell-cycle regulated and phosphorylation-dependent alternate nuclear sites of the transcription repressor Daxx and SWI/SNF protein ATRX

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Summary

Placing regulatory proteins into different multiprotein complexes should modify key cellular processes. Here, we show that the transcription repressor Daxx and the SWI/SNF protein ATRX are both associated with two intranuclear domains: ND10/PML bodies and heterochromatin. The accumulation of ATRX at nuclear domain 10 (ND10) was mediated by its interaction with the N-terminus of Daxx. Binding of this complex to ND10 was facilitated by the interaction of the Daxx C-terminus with SUMOylated promyelocytic leukemia protein (PML). Although ATRX was present at heterochromatin during the entire cell cycle, Daxx was actively recruited to this domain at the end of S-phase. The FACT-complex member structure-specific recognition protein 1 (SSRP1) accumulated at heterochromatin simultaneously with Daxx and accumulation of both proteins depended on ATRX phosphorylation. Both Daxx and SSRP1 were released from heterochromatin early in G_2 phase and Daxx was recruited back to ND10, indicating that both proteins localize to heterochromatin during a very short temporal window of the cell cycle. ATRX seems to assemble a repression multiprotein complex including Daxx and SSRP1 at heterochromatin during a specific stage of the cell cycle, whereas Daxx functions as an adapter for ATRX accumulation at ND10. A potential functional consequence of Daxx accumulation at heterochromatin was found in the S- to G_2-phase transition. In Daxx$^{-/-}$ cells, S-phase was accelerated and the propensity to form double nuclei was increased, functional changes that could be rescued by Daxx reconstitution and that might be the basis for the developmental problems observed in Daxx knockout animals.

Key words: Nuclear structure, ND10, Heterochromatin, Daxx, ATRX, SSRP1

Introduction

The nucleus is a highly structured environment principally divided into the chromatin component and the interchromatin space. Chromatin-based specializations of nuclear space are best recognized microscopically when genomic processes are highly repetitive, for example transcription and assembly of ribosomal subunits. Large protein complexes are assembled at many individual genomic sites and therefore cannot be visualized, but are currently characterized by affinity-column chromatography. One set of multicomponent complexes with a changing cast of characters is the chromatin remodeling SWI/SNF family (for reviews, see Flaus and Owen-Hughes, 2001; Klochendler-Yeivin et al., 2002). The basic chromatin remodeling complexes altered by specialized modifiers might be active at different promoters, in different specialized cells or in different parts of the cell cycle. The large multidomain proteins might therefore function as a matrix or scaffold for the assembly of different complexes, a scenario that would allow a high diversity of specific interactions.

Specific protein accumulation at large intranuclear domains does not necessarily represent the site of this protein's activity. For instance, several components of the splicing machinery are accumulated in domains known as speckles or SC35 domains, but the proteins seem to be at least partly inactive in these locations (for review, see Dirks et al., 1999). At the same time, genes activated during the differentiation process are relocating to the periphery of speckles, suggesting a dual role for these domains that seem to participate in the formation of local euchromatic 'neighborhoods' (Moen et al., 2004; Shopland et al., 2003). In-vivo time-lapse image sequences have shown that proteins can move to different sites concomitant with changes in transcriptional or splicing activity of such proteins (Tsukamoto et al., 2000). Other interchromosomal sites known as nuclear domain 10 (ND10) or promyelocytic leukemia protein (PML) bodies respond to stress by releasing Daxx and Sp100, leading to the different inhibition of heat-shock-protein synthesis (Nefkens et al., 2003). Even though evidence is accumulating that ND10-associated proteins function directly within this structure (for review, see Borden, 2002), most of these proteins seem to act outside of ND10. For instance,

the proteins released from ND10 must bind to different intranuclear sites to induce repression of heat-shock genes. The examples of speckles and ND10 suggest that proteins are not always active at the site of highest concentration and have led to the depot hypothesis for the PML-based accumulation of proteins at ND10 (Negorev and Maul, 2001).

The highest concentration of Daxx, originally identified as a proapoptotic Fas-interacting protein (Kawai et al., 2003; Yang et al., 1997) and also demonstrated to have anti-apoptotic activity (Michaelson et al., 1999; Michaelson and Leder, 2003), is found at ND10 (Everett et al., 1999a; Ishov et al., 1999; Torii et al., 1999). Daxx recruitment to ND10 is facilitated by interaction of the C-terminus of the protein with PML bound to the small ubiquitin-related modifier (SUMO; SUMOylated PML) (Ishov et al., 1999). In addition to PML, the C-terminus domain of Daxx can also interact with other proteins, including several repressors (for a review, see Michaelson, 2000; Michaelson and Leder, 2003), and, in those cases, Daxx cannot be accumulated at ND10. Another protein-binding domain is located at the N-terminus of Daxx and facilitates binding with a human cytomegalovirus tegument transactivator pp71, sequestering pp71 to ND10 (Hofmann et al., 2002; Ishov et al., 2002). Besides the major concentration at ND10, Daxx is also accumulating at the condensed heterochromatic areas in subpopulation of cells and is therefore probably present at these sites during a limited part of the cell cycle (Ishov et al., 1999). Condensed chromatin marks pericentromeric heterochromatin, particularly in mouse cells, and contains much of the repressed genome. The repressed genome is replicated during the latter part of S-phase and requires the reestablishment of epigenetic markers such as deacetylated histones and methylated DNA to remain repressed after successive cell divisions. Proteins that accumulate at these sites during and after replication are therefore potentially involved in such processes. Because characterization of these proteins by column-based protein isolation depends on large quantities of cells, this approach is impractical for analysis of complexes that span a short window of the cell cycle. Immunohistochemical techniques are, however, exquisitely sensitive in resolving phases of the cell cycle as various stages of S-phase can be distinguished by the pattern generated after bromodeoxyuridine (BrdU) incorporation (Nakayasu and Berezney, 1989) and the G_2/M-phase by the increasing amounts of phosphorylated histone 3 (H3) (Hendzel et al., 1997; Zeitlin et al., 2001).

We investigated the change of Daxx location during the cell cycle relative to the potential of interacting with several heterochromatin-associated proteins and the mechanisms that result in such temporal changes in nuclear domain association. Heterochromatin protein 1α (HP1α) is associated with heterochromatin and ND10 (Lehming et al., 1998; Orphanides et al., 1999; Seeler et al., 1998). ATRX, a member of the Swi2/Snf2 family of chromatin remodeling proteins (Picketts et al., 1996), has been described at heterochromatin (Sutherland et al., 2001; Yarnell et al., 2001) and at several non-chromatin-associated domains (McDowell et al., 1999; Xue et al., 2003). A murine homologue of human protein SSRP1 [a component of the facilitates chromatin transcription (FACT) complex] has a demonstrated cell-cycle association with heterochromatin (Hertel et al., 1999) and is involved in cell-cycle control (Orphanides et al., 1999; Yarnell et al., 2001). Here, we describe the cell-cycle dependent and phosphorylation-induced changes in Daxx, ATRX and SSRP1 localization and the consequences of Daxx loss for S-phase progression.

Materials and Methods

Cells and growth conditions

HEp2 cells, mouse primary embryonic fibroblasts (MPEFs) and PML$^{-/-}$ mouse fibroblasts have been described (Ishov et al., 1999). Daxx$^{-/-}$ mouse fibroblasts were produced as described below. The following kinase inhibitors (all inhibitors from Calbiochem, La Jolla, CA) were added to cells 1 hour before fixation: A3 (200 μM), HSP25 (100 μM), PD98059 (200 μM), SB202190 (5 μM), SB203580 (5 μM), staurosporine (0.2 μM) and roscovitine (200 μM).

Antibodies

Antibodies against PML have been described (Ishov et al., 1999). Sources for antibodies used were: ATRX rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HP1α monoclonal antibody (mAb) and anti-SSRP1 antiserum were provided by F. Rauscher and S. Lippard respectively; anti-tubulin mAb and anti-FLAG M2 mAb (Sigma, St Louis, MO); rabbit antiserum against a hemagglutinin (HA) epitope (Clontech, Palo Alto, CA); rabbit anti-phospho-H3 antiserum (Upstate, Lake Placid, NY); anti-BrdU mAb (Roche Diagnostics, Indianapolis, IN); sheep anti-BrdU antiserum (Kamiya Biomedical, Seattle, WA); rabbit anti-HDAC2 (ZYMED Labs, San Francisco, CA). To prove the specificity of immunostaining by Daxx rabbit antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) that visualized an unspecific band in Daxx$^{-/-}$ cells (see Fig. 4c), key immunostaining experiments were repeated using rabbit antibody raised against amino acids 678-690 of mDaxx.

Immunolocalization of proteins

At different times after plating, cells were fixed and immunostained as described (Ishov and Maul, 1996). Cells were analysed using a Leica TCS SP2 spectral confocal microscope. LCS Leica confocal software was used in balancing signal strength. For cell-cycle analysis, 400 cells were evaluated in randomly selected fields for BrdU and phospho-H3 labeling. To determine the proportion of cells with double nuclei, 1000 cells were evaluated for directly abutting nuclei with no space visible in the DAPI staining.

BrdU labeling

Cells were labeled with 20 μM BrdU (Roche Diagnostics) for 30 minutes at 37°C. For pulse-chase experiments, cells were washed after labeling by PBS and incubated for an additional 3 hours in the normal growth medium. After fixation, cells were incubated with various primary antibodies for 1 hour and labeled with the corresponding secondary antibodies for 30 minutes before refixation in 2% paraformaldehyde. Cells were incubated in 2 N HCl/H_2O for 30 minutes at room temperature, washed and labeled with anti-BrdU antibodies.

Daxx knockout (KO) production and characterization

The mouse *Daxx* gene was cloned from a 129 SV λ phage genomic library. The 5′ flanking segment used for the targeting construct was a 1.0 kb PCR product containing exon 2, intron 2 and part of exon3. It was amplified using primers MSA1 (5′-GGCGAAGCTTAGGAAG-TGCTACAAGTTG-3′) located in exon 2 and MSA2 (5′-GC-CGTTCGAAACGAGGAGTCTGGGTCATC-3′) located in exon 3, and cloned into the *Hin*dIII site of pSP72 (Promega, Madison, WI).

The 3′ flanking segment, a 6.7 kb fragment from the *Eco*RI site in intron 7 to the end of the genomic clone (*Not*I site), was cloned in the same vector. The targeting construct (10 μg) was linearized using *Not*I and transfected by electroporation into 129 SV embryonic stem (ES) cells. This strategy replaces part of exon 3 and all of exons 4-7 with an intact *neo* gene. After selection with G418, surviving colonies were analysed using the polymerase chain reaction (PCR) for homologous recombination at the *Daxx* locus.

Correctly targeted clones were cultivated with FVB×FVB blastocysts and transferred into pseudopregnant FVB foster mothers. Germ-line transmission of the targeted *Daxx* allele was predicted by black eye color and agouti coat color in F1 mice and confirmed by PCR analysis. Genotyping of F2 mice was performed using primers GenDaxx227Up and mDaxxGen4Down or GenDaxx227Up and NeoI. Of 99 mice tested, 38 were $Daxx^{+/+}$ and 61 were $Daxx^{+/-}$, suggesting that $Daxx^{-/-}$ is lethal. Results of embryo analysis indicated lethality at embryonic day 10-10.5 (E10-E10.5). Fibroblasts were collected from $Daxx^{-/-}$, $Daxx^{+/+}$ and $Daxx^{+/-}$ embryos.

Daxx reconstitution in a retroviral system

The pBABE-puro plasmid (Morgenstern and Land, 1990) encoding the puromycin resistance gene was digested with *Sna*BI/*Bam*HI for cloning of Daxx wild-type and truncation mutants that maintain either the first 625 amino acids (DaxxΔC) or the last 112 amino acids (DaxxC). Corresponding fragments were tagged with the HA epitope at the 5′ end and obtained from plasmids pEBBmDaxx, pEBBmDaxxΔC and pEBBmDaxxC. To generate the FLAG epitope, primers 5′-GATCCATGGACTACAAGACGATGACGACAAGC-3′ and 5′-GATCGCTTGTCGTCATCGTCTTTGTAGTCCATG-3′ were annealed and cloned into the *Bam*HI site of pBABEmDaxx wild-type, pBABEmDaxxΔC and pBABEmDaxxC. For preparation of retroviral stocks, Phoenix-ampho packaging cells (Grignani et al., 1999) were transfected with corresponding vector DNA. Viral supernatants were collected 36-48 hours after transfection and either used immediately or stored at –80°C. Diluted viral stocks were added three times to $Daxx^{-/-}$ cells for at least 12 hours. Cells were selected for 1 week with puromycin and the clones expanded and immunostained with anti-HA antibody to confirm the proportion of cells reconstituted by the corresponding construct. Because expression appears to be a rather unstable proportion of positives, cells were monitored after every third passage.

Isolation of chromatin-associated proteins and phosphatase assay

Chromatin-associated proteins were isolated as described by Mendez and Stillman (Mendez and Stillman, 2000). Cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid and 2% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitors [1 mM EDTA, 100 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin]. For phosphatase treatment, 2×10^5 cells were washed in RIPA buffer. Half of this fraction was used for treatment with 400 U of λ protein phosphatase (New England Biolabs, Mississauga, Ontario, Canada) for 30 minutes at 30°C before SDS polyacrylamide-gel electrophoresis (SDS-PAGE) analysis.

Co-immunoprecipitation assays

Cells were collected in PBS and incubated for 20 minutes in NLB (10 mM NaCl, 10 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100) containing 250 mM NaCl supplemented with protease inhibitors (1 mM EDTA, 100 μg ml^{-1} PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin). Nuclei were collected, washed again with NLB containing 250 mM NaCl and incubated in NLB containing 450 mM NaCl for 30 minutes at 4°C with rotation. After removal of nuclear debris by centrifugation, the nuclear extracts were adjusted to 220 mM NaCl with NLB containing 50 mM NaCl and incubated overnight at 4°C with anti-FLAG-M2-conjugated beads (ProBond Resin; Sigma). Eluted proteins were separated by 4-12% gradient SDS-PAGE and analysed by western blotting.

Results

Daxx is a chromatin-associated protein

In mouse cells, Daxx is located at high concentration in two different nuclear compartments. Co-staining with anti-PML antibodies indicated that Daxx is part of ND10 in most cells (Fig. 1a), whereas co-staining with antibodies to the chromatin-associated protein HP1α showed that the second domain of Daxx aggregation observed in a subpopulation of cells constitutes heterochromatic areas (Fig. 1b), which localized with condensed chromatin, as demonstrated by Hoechst

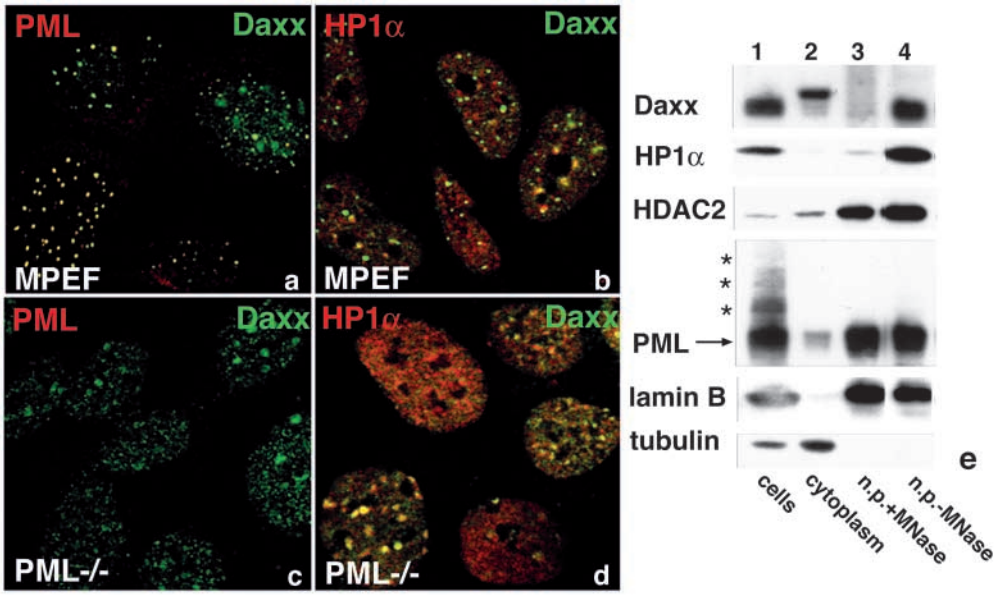


Fig. 1. Daxx is a chromatin-associated protein. (a) MPEF double labeled for PML (red) and Daxx (green). Both proteins localize at ND10, and Daxx also accumulates in a different shaped domain (upper right nucleus). (b) MPEF double labeled for HP1α and Daxx. Daxx localizes with HP1α at heterochromatin (middle right). (c) In $PML^{-/-}$ cells, Daxx accumulates in heterochromatin-like domains that localize with HP1α (d). (e) Western blot of intact MPEF cells (lane 1), cytoplasm (lane 2), nuclear insoluble fractions (n.p.) digested by micrococcal nuclease (MNase) (lane 3) and control (lane 4). Treatment with MNase resulted in the complete solubilization of Daxx and HP1α (lane 3). Loads in lanes 1 and 2 correspond to 10% of cells used in lanes 3 and 4. The asterisk indicates SUMOylated PML.

staining (not shown). Use of the same staining sequence with PML$^{-/-}$ cells revealed no ND10-like staining of Daxx (Fig. 1c) and the only Daxx-positive domains remained associated with HP1α (Fig. 1d). This confirmed the importance of PML for ND10 formation and indicated that Daxx association with heterochromatin is PML independent. In addition to the most condensed accumulations at ND10 and heterochromatin, Daxx was also located throughout the nucleoplasm.

Daxx is associated with ND10, which is part of the nuclear matrix (Ascoli and Maul, 1991), and with chromatin at heterochromatic areas. To confirm the chromatin association of Daxx, cells were fractionated into a cytoplasmic and a nuclear fraction, with further digestion of the latter with micrococcal nuclease (MNase) to separate chromatin-bound proteins from the matrix fraction. Anti-tubulin and anti-lamin-B antibodies were used to monitor the separation of cytoplasmic and nucleoplasmic fractions. Daxx in the cytoplasmic fraction (Fig. 1e, lane 2) migrated more slowly than Daxx in the complete cell extract, potentially reflecting some modification of the protein by cytoplasmic enzymes. During the fractionation procedure most of the PML remained in the insoluble nuclear fraction in both MNase-treated and untreated samples, confirming the nuclear matrix association of PML. Upon cell fractionation, PML became deSUMOylated (Fig. 1e, asterisks in the unfractionated cells show the SUMOylated forms, compare lanes 1, 3, 4), probably owing to activation of the SUMO-isopeptidases SENP1/2 during the fractionation procedure. Because Daxx interacts with SUMOylated PML at ND10 (Ishov et al., 1999), the matrix fraction of Daxx (associated with the SUMOylated PML at ND10) should be released from the insoluble fraction under these conditions. Therefore, the Daxx fraction associated with the nuclear insoluble fraction probably represents the chromatin-associated protein, including the heterochromatic fraction (Fig. 1e, lane 4). Treatment of the nuclear fraction with MNase resulted in the complete solubilization of Daxx (Fig. 1e, lane 3), confirming the association of Daxx with chromatin. The chromatin-associated protein HP1α was similarly released from chromatin after MNase treatment, but this treatment led to only partial depletion of histone deacetylase 2 (HDAC2) from the insoluble fraction, potentially reflecting some matrix association of this protein. The bulk of bound Daxx fractionated most convincingly with HP1α and not with the nuclear-matrix-associated deSUMOylated PML, indicating that a subfraction of Daxx is associated with chromatin. To test these findings in situ, cells were treated with MNase and immunostained with antibodies against Daxx, PML and HP1α. As expected, Daxx and HP1α completely disappeared from heterochromatic areas, whereas ND10-associated Daxx remained localized with PML (data not shown), confirming a chromatin association of part of Daxx and the nuclear matrix nature of ND10.

Cell-cycle-dependent distribution of Daxx

Daxx interacts with SUMOylated PML, resulting in the deposition of Daxx at ND10 in most interphase nuclei (Ishov et al., 1999), and is present in the heterochromatic regions in a few cells, potentially reflecting a cell-cycle dependence of accumulation at heterochromatin. To test for this possibility, we used a marker of G_2/M cells, phosphorylated histone H3. Mitotic (M) and premitotic cells (G_2/M phase, potentially prophase in Fig. 2a,b) showed the strongest H3 label but Daxx was not localized to specific domains in these cells (Fig. 2a,c). The G_2-phase cell with substantial phospho-histone-H3 labeling (Fig. 2a, top right) and the two G_1-phase cells with no red signal showed specific Daxx accumulation in precise ND10-like domains. However, in the two cells with low phospho-histone-H3 labeling (indicated by question marks), Daxx was localized to more diffuse areas, confirmed to be condensed chromatin by Hoechst staining (not shown). Thus, Daxx is not present in heterochromatin during mitosis, G_2 phase or G_1 phase.

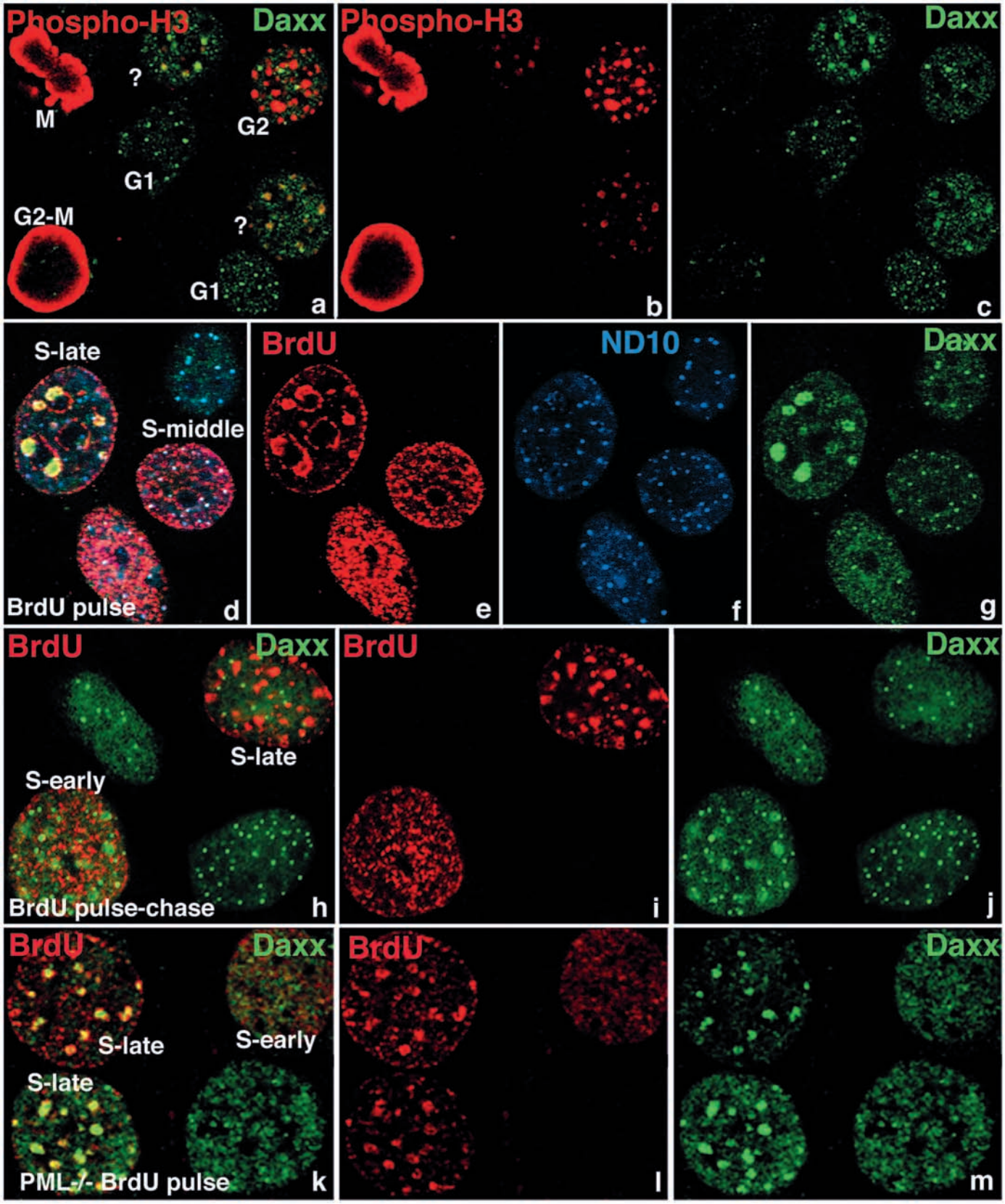
Fig. 2. Cell-cycle-dependent distribution of Daxx. (a-c) MPEF labeled for phospho-histone H3 and Daxx show that no Daxx accumulates at heterochromatin during G_1, G_2 or M phase. (d-g) MPEF pulsed with BrdU for 30 minutes and triple-labeled for BrdU, ND10 and Daxx show Daxx located at heterochromatin during late S-phase but not during middle S-phase. (h-j) MPEF pulsed with BrdU for 30 minutes, chased for 3 hours and double-labeled for BrdU and Daxx show Daxx in ND10-like domains but not in heterochromatin of the cell that was at late S-phase during the pulse, and on heterochromatin in cell that was at early S-phase during the pulse. (k-m) *PML*$^{-/-}$ cells pulsed with BrdU for 30 minutes and labeled for BrdU and Daxx show Daxx concentrated at heterochromatin only during late S-phase despite the absence of ND10.

BrdU labeling patterns allow discrimination between cells in early S phase (low brightness, punctative), middle S phase (bright throughout the nucleus) and late S phase (perinuclear and condensed heterochromatin) (Nakayasu and Berezney, 1989). In Fig. 2d, the cell without incorporated BrdU has Daxx aggregated in ND10. BrdU labeling in early and middle S-phase cells contain Daxx in ND10. In late S-phase, indicated by heterochromatin replication (Fig. 2d, left), Daxx location coincided with replicating heterochromatin. Significantly, there was almost no labeling of Daxx in ND10 (blue staining) at that stage. Therefore, Daxx was mostly dissociated from ND10, becoming associated with heterochromatin at late S phase.

To analyse the period of Daxx accumulation at condensed chromatin, cells were labeled for 30 minutes with BrdU and then chased for 3 hours. Cells that were at late S phase during BrdU pulse reconstituted Daxx in precise ND10-like locations at the moment of fixation (Fig. 2g, top right) and cells that were at early S phase at the time of the BrdU pulse had, by the time of fixation, accumulated Daxx in irregular heterochromatin areas, characteristic of late S-phase (Fig. 2g, bottom left). Comparison of Daxx accumulation in BrdU-pulse and pulse-chase experiments indicated that Daxx accumulated in heterochromatin during late S-phase, concomitant with reduction at ND10 in most cells. During G_2 phase, Daxx left heterochromatin and reappeared at ND10. In PML$^{-/-}$ cells, which do not contain ND10, Daxx was also recruited to heterochromatin only at late S phase (Fig. 2k-m), suggesting that Daxx accumulation at heterochromatin is an active late-S-phase-associated process. It appears that Daxx leaves one compartment of the nucleus (ND10) and is actively recruited into another (heterochromatin) during a precise time of the cell cycle (late S phase) when this part of the genome is replicated, and Daxx returns to ND10 soon after the completion of DNA replication.

ATRX localizes with Daxx at heterochromatin and at ND10

To identify proteins that might mediate the recruitment of Daxx



to heterochromatin, we focused on ATRX reported to be localized to heterochromatin and at a few non-chromatin sites (McDowell et al., 1999; Berube et al., 2000) and in a complex with Daxx (Xue et al., 2003). In almost all cells, ATRX had two patterns of accumulation: heterochromatin, where it localized with HP1α (Fig. 3a-d) and ND10 (Fig. 3e-h). Daxx localized

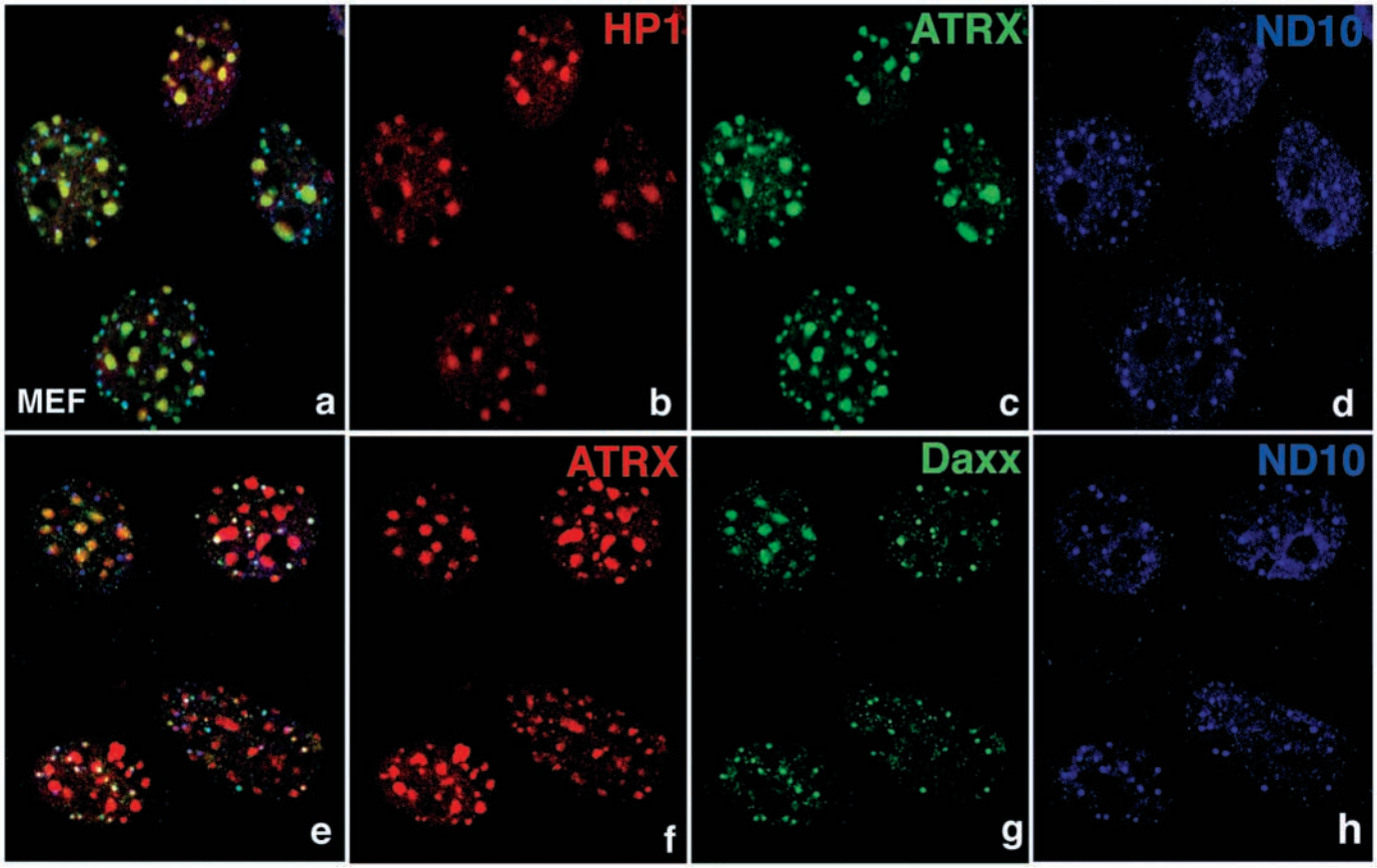


Fig. 3. ATRX localizes with Daxx at heterochromatin and at ND10. (a) MPEF cells triple-labeled for HP1α, ATRX and ND10, and the respective color separations (b-d) show that ATRX localizes with heterochromatin and ND10. (e) MPEF cells triple-labeled for ATRX, Daxx and ND10 and the respective individual colors (f-h) shows that Daxx localizes with ATRX to ND10 in most cells and, in some cells, with heterochromatin-like domains (upper left).

with ATRX at ND10 in most cells (Fig. 3e-h), whereas Daxx was observed at heterochromatic areas with ATRX only in a subpopulation of cells (Fig. 3e-h, top left). Thus, ATRX is a constitutive component of ND10 and heterochromatin.

Daxx is required for ATRX recruitment to ND10

To examine the potential role of Daxx in ATRX accumulation at ND10 and heterochromatin, *Daxx*$^{-/-}$ cell lines were derived from *Daxx*$^{-/-}$ embryos. Partial deletion of *Daxx* in both alleles was previously shown to be lethal at E8.5-E9 (Michaelson et al., 1999), even though the residual *Daxx* mRNA, encoding the C-terminal 479 amino acids of the Daxx protein, was still expressed. We used a different strategy to construct of *Daxx*$^{-/-}$ animals, eliminating the last 512 amino acids of the Daxx protein. The remaining part of *Daxx* mRNA, encoding the first 227 amino acids of the protein, appeared to be unstable and was not detectable by either northern or western blot analysis (not shown). *Daxx*$^{-/-}$ embryos were developmentally retarded by E8 and disintegrated completely by E11.5-E12.5, whereas *Daxx*$^{+/-}$ mice did not differ developmentally from *Daxx*$^{+/+}$ mice during the prenatal and postnatal periods (Fig. 4a).

Next, we collected cells of E9.5 embryos and characterized the derived *Daxx*$^{+/+}$, *Daxx*$^{+/-}$ and *Daxx*$^{-/-}$ cell lines by PCR analysis (Fig. 4b), western blot (Fig. 4c) and immunofluorescence (Fig. 4d-f). *Daxx*$^{-/-}$ cells did not produce Daxx protein, even though these cells still had intact ND10 (Fig. 4f, anti-PML staining), demonstrating that Daxx is not required for ND10 integrity. Immunostaining for ATRX in *Daxx*$^{-/-}$ cells did not reveal this protein at ND10, although the heterochromatin-associated pattern of ATRX remained the same as in *Daxx*$^{+/+}$ cells (Fig. 3, Fig. 4g). These results suggest that Daxx is required for ATRX localization to ND10.

Daxx has separate ND10- and heterochromatin-targeting domains

The potential requirement for Daxx in ATRX accumulation at ND10 was further tested in *Daxx*$^{-/-}$ cells reconstituted with wild-type and truncation Daxx mutants that maintain either the first 625 amino acids (DaxxΔC) or the last 112 amino acids (DaxxC) tagged with FLAG and HA epitopes using a retrovirus-based expression system (Fig. 5, top). After puromycin selection, cells were screened for clones that produced quantities of reconstituted proteins that were, by western blot analysis, similar to the level of Daxx production in *Daxx*$^{+/+}$ cells. Clones were characterized for the distribution of Daxx and corresponding mutants using anti-HA antibody. Fig. 5a,d show a representative distribution of wild-type Daxx

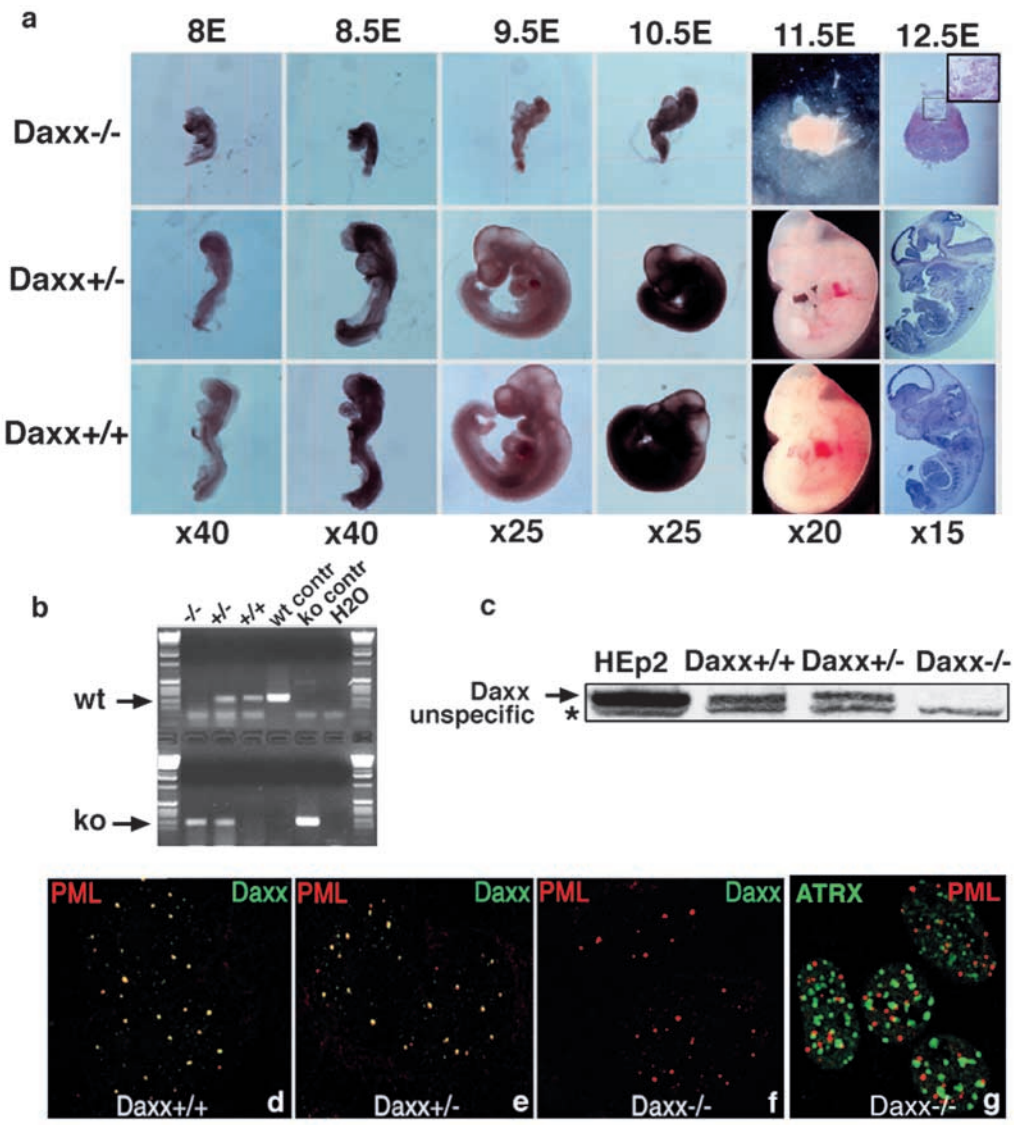


Fig. 4. Characterization of Daxx knockout (KO) embryos and cell line. (a) Embryos were collected at the indicated days after mating (E), genotyped for Daxx wild type (wt) and KO and photographed. At E8, *Daxx*$^{-/-}$ embryos were developmentally retarded and were prominently so by E10.5. At E11.5, *Daxx*$^{-/-}$ embryos started to dissolve and were completely disintegrated by E12.5 (inset in *Daxx*$^{-/-}$ E12.5 shows 40× magnification). *Daxx*$^{+/-}$ embryos did not differ developmentally from *Daxx*$^{+/+}$ embryos. (b) Genotyping of *Daxx*$^{-/-}$, *Daxx*$^{+/-}$ and *Daxx*$^{+/+}$ cell lines by PCR indicating the presence of corresponding alleles, whereas controls (wt control, KO control, H_2O) demonstrated the specificity of the signals. (c) Western-blot analysis of *Daxx*$^{-/-}$, *Daxx*$^{+/-}$ and *Daxx*$^{+/+}$ cell lines. Daxx-specific signal (arrow) was present in HEp2, *Daxx*$^{+/+}$ and *Daxx*$^{+/-}$, but not in *Daxx*$^{-/-}$ cells. Unspecific signal is marked by the asterisk. (d-g) Cells labeled for Daxx and PML demonstrate colocalization of Daxx and PML to ND10 in *Daxx*$^{+/+}$ (d) and *Daxx*$^{+/-}$ (e), but not in *Daxx*$^{-/-}$ (f), cells. (g) *Daxx*$^{-/-}$ cells double-labeled for ATRX and PML show ATRX accumulated in heterochromatin but not at ND10.

upon reconstitution. The Daxx wild type localizes to heterochromatin in some cells (Fig. 5a, top and bottom cells) and in ND10 (Fig. 5d, all cells except bottom left, in which Daxx is visible only in heterochromatin-like aggregates), and so resembles endogenous Daxx distribution. Upon reconstitution by DaxxΔC, anti-HA antibody also labeled heterochromatic areas in a subpopulation of cells that localized with HP1α (Fig. 5b, top and middle left cells) but no colocalization was observed with PML at ND10 (Fig. 5e), indicating that the amino-acid sequence of DaxxΔC contained the heterochromatin-targeting but not the ND10-targeting signal. However, DaxxC did not localize with HP1α (Fig. 5c) but was exclusively accumulating at ND10 with PML (Fig. 5f). We demonstrated previously that the homologous region of human Daxx is sufficient for interaction with SUMOylated human PML and for subsequent targeting of Daxx to ND10 in human cells. Accumulation of DaxxC in murine cells at ND10 seemed to follow a similar scenario. Moreover, murine Daxx interacts with murine PML in the context of yeast cells and this interaction is mediated by the C-terminus of Daxx (A.M.I. and G.G.M., unpublished).

We questioned whether the reconstructed cells maintain the cell-cycle-dependent redistribution of Daxx. Reconstituted Daxx wild type localized to heterochromatic areas only at late S-phase and in a ND10-like pattern for the rest of interphase, like the endogenous Daxx (shown by double labeling of incorporated BrdU) (Fig. 5g). In DaxxΔC reconstituted cells, HA-antibody-labeled heterochromatin only at late S-phase (Fig. 5h, bottom right cell), whereas DaxxΔC exhibited a homogeneous and micropunctative nucleoplasmic distribution for the rest of interphase. These findings, together with similar accumulation of Daxx in the absence of ND10 (Fig. 2k-m), indicate that the cell-cycle-dependent recruitment of Daxx to condensed chromatin is an active S-phase-associated process. Cell-cycle analysis of DaxxC reconstituted cell lines revealed retention of this mutant at ND10 even during late S-phase (Fig. 5i). Therefore, the active recruitment of Daxx to condensed chromatin at the end of S-phase might lead to the reduction of the soluble nucleoplasmic Daxx and, as a consequence, its passive release from ND10. Analysis of reconstituted cell lines demonstrates in situ that the Daxx protein has two separate

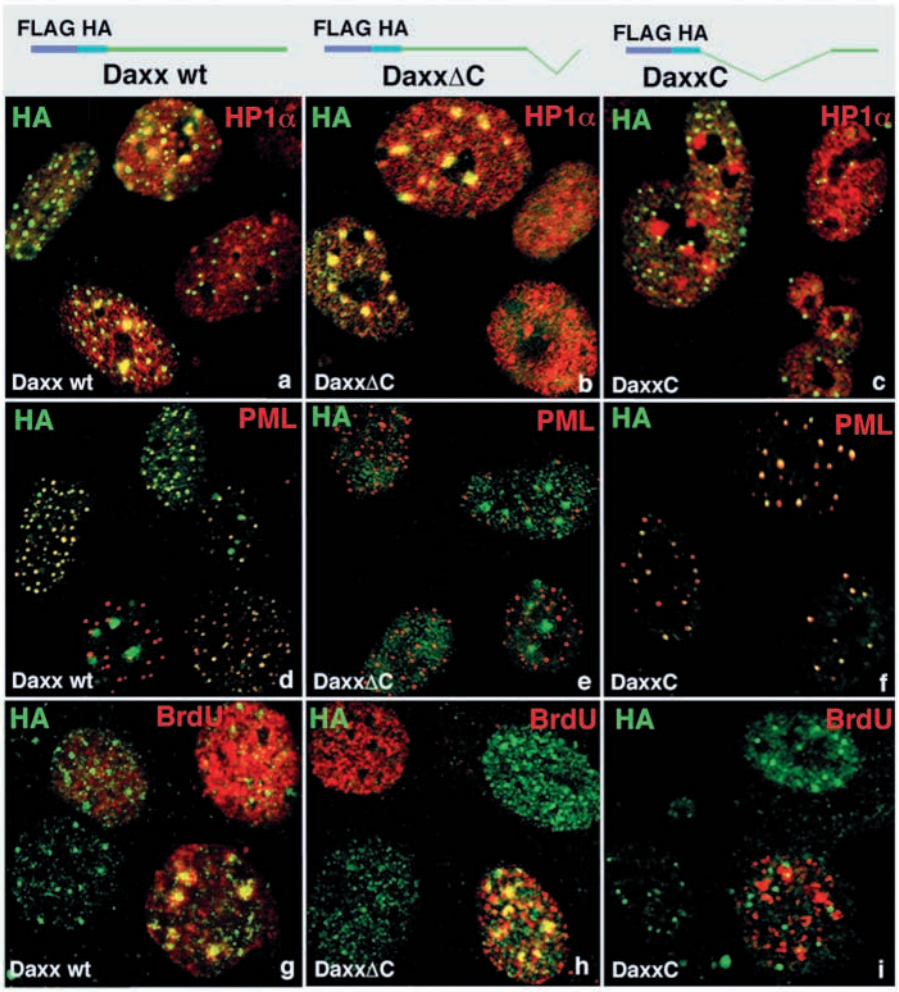


Fig. 5. Production and cell-cycle analysis of cells reconstituted by Daxx wild-type (wt) and truncation mutants. Schematic of constructs is given at the top. Co-localization of reconstituted Daxx wt with HP1α at condensed chromatin (a, upper and lower nuclei) and with PML at ND10 (d) resemble those of endogenous Daxx. (g) BrdU incorporation followed by labeling of BrdU and Daxx by HA antibody; reconstituted Daxx wt accumulated at heterochromatin only at late S-phase (lower right nucleus). (b) DaxxΔC localized with HP1α at heterochromatin (upper and left nuclei) and is homogeneously dispersed in other cells. (e) DaxxΔC does not accumulate at ND10. (h) DaxxΔC accumulation at late BrdU incorporation sites (lower right). (c) DaxxC does not accumulate with HP1α at heterochromatin but maintains exclusive PML/ND10 associated pattern (f) throughout the cell cycle, including cells with a late S-phase BrdU incorporation pattern (i, lower right).

intranuclear targeting domains: the C-terminus, responsible for ND10 targeting and a domain in the remaining part of the protein, responsible for the active recruitment to heterochromatin at late S-phase.

ATRX accumulates at ND10 as a consequence of in vivo interaction with DaxxΔC

To confirm that Daxx is required for ATRX accumulation at ND10 (Fig. 3, Fig. 4g), we evaluated ATRX distribution in the Daxx reconstituted cells. Double staining for PML and ATRX in Daxx wild-type reconstituted cells (Fig. 6a) revealed ATRX accumulations in ND10 localizing with Daxx (Daxx labeled by HA antibody, Fig. 6d). Despite DaxxC accumulation at ND10 upon reconstitution (Fig. 5f, Fig. 6e), it did not recruit ATRX to this nuclear compartment (Fig. 6b,e). Reconstitution by DaxxΔC also did not change the intranuclear distribution of ATRX, which was located in heterochromatin but not at ND10 (Fig. 6c,f).

Daxx recruited ATRX to ND10, suggesting that the two proteins might be components of a multiprotein complex. Upon co-immunoprecipitation using anti-FLAG antibody, ATRX-derived signal was observed in samples reconstituted by Daxx wild-type and DaxxΔC, but not in the extracts of cells reconstituted with empty plasmid or DaxxC (Fig. 6g), suggesting that Daxx and ATRX interact with each other directly or indirectly, and that DaxxΔC contains a domain sufficient for the interaction. In combination with the immunohistochemical localization of ATRX after Daxx reconstitution, these data suggest that the ΔC domain of Daxx is sufficient for interaction with ATRX, whereas C-terminal domain of Daxx binds to SUMOylated PML, resulting in accumulation of ATRX at ND10.

SSRP1 accumulates at heterochromatin in late S-phase

To determine whether the specific redistribution of Daxx to heterochromatin at late S-phase was unique to this protein, we analysed the cell-cycle-dependent distribution of the high-mobility-group protein SSRP1 that was recently purified within the ATRX complex (W. Wang, personal communication). Immunofluorescence analysis of SSRP1 revealed its accumulation in heterochromatin-like domains in a subpopulation of cells, but no association with PML at ND10 (Fig. 7a). Double staining with anti-HP1α antibodies confirmed the heterochromatic localization of SSRP1 in a subpopulation of cells (Fig. 7b, bottom left two cells). Moreover, SSRP1 localized with Daxx at heterochromatic areas (Fig. 7c, middle cell), indicating that both proteins accumulate there at late S-phase. The BrdU incorporation analysis (Fig. 7d) and

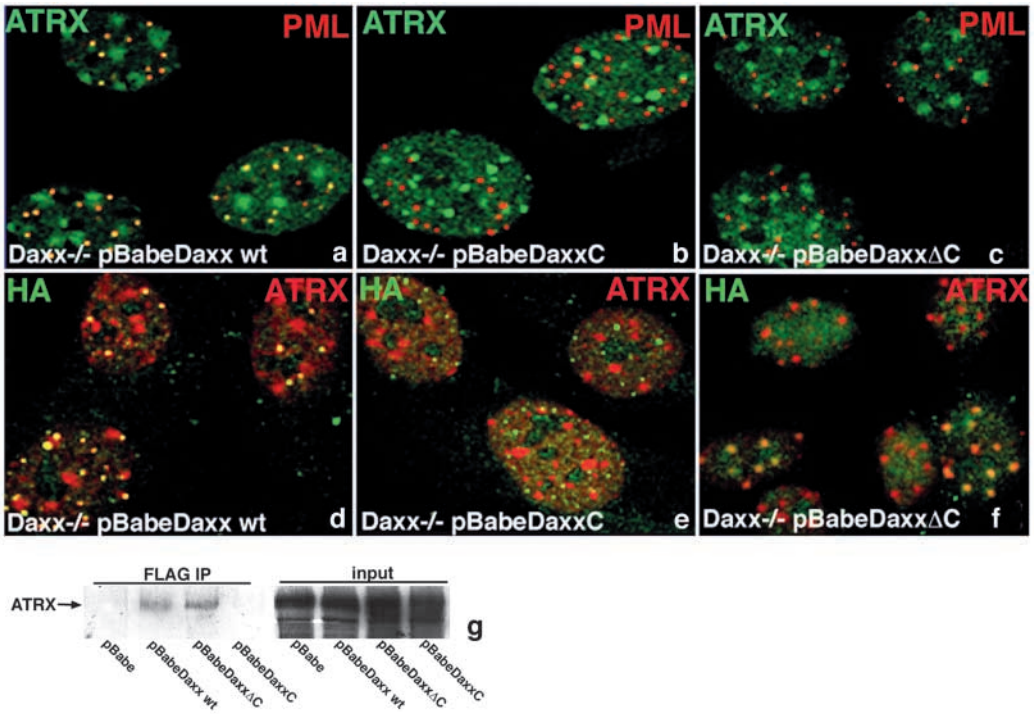


Fig. 6. Intranuclear distribution of ATRX in Daxx reconstituted cells. (a) Daxx wild-type (wt) reconstituted cells labeled for ATRX and PML show colocalization of the proteins at ND10 (yellow), indicating a Daxx requirement in ATRX recruitment to ND10. (d) The same cells stained with ATRX and HA show colocalization of ATRX with Daxx at ND10-like domains. (e) DaxxC accumulation at ND10-like domains but no recruitment of ATRX to ND10 (b). In cells reconstituted with DaxxΔC and double stained for ATRX and PML (c) or HA and ATRX (f), ATRX does not accumulate at ND10 but has exclusive accumulation in irregular domains (c) while localizing with HA-DaxxΔC in a subpopulation of cells at irregular heterochromatic domains (f, lower left and right cells). (g) Western-blot analysis of interaction between ATRX and Daxx. Nuclear extracts produced from *Daxx*$^{-/-}$ cell lines reconstituted with empty vector (lane pBabe), Daxx wt (lane pBabe wt), DaxxΔC (lane pBabeDaxxΔC) and DaxxC (lane pBabeDaxxC) were incubated with anti-FLAG antibody to immunoprecipitate Daxx (FLAG IP) and probed for the presence of ATRX. No ATRX-derived signal was detected in extracts from the empty vector and DaxxC-reconstituted cells, whereas the signal was present in immunoprecipitates of Daxx wt and DaxxΔC cell extracts. Corresponding input (20%) for the ATRX load is shown (right).

immunostaining with anti-phospho-H3 antibodies (Fig. 7e,f) confirmed that SSRP1 accumulates at condensed chromatin at late S-phase and is homogeneously distributed throughout the nucleoplasm in the remaining stages of interphase.

Heterochromatic accumulation of Daxx and SSRP1 depends on cellular kinase activity

To identify the cell-cycle-specific signal that attracts Daxx and SSRP1 to heterochromatin during a narrow window of the cell cycle, the intranuclear distribution of proteins was analysed after a 1 hour exposure to the phosphorylation inhibitors A3, HSP25, PD98059, SB202190, SB203580, staurosporine and roscovitine, and the phosphatase inhibitor okadaic acid. Daxx and SSRP1 did not accumulate at heterochromatin only upon roscovitine treatment (Fig. 8a,c,d,f) nor was ATRX released from ND10 (Fig. 8b,e), and Daxx remained with PML (Fig. 8d) at these conditions. ND10 integrity was apparently not affected by the roscovitine treatment.

Daxx, PML, HP1α and ATRX are phosphoproteins (Berube et al., 2000; Ecsedy et al., 2003; Everett et al., 1999b; Zhao et al., 2001). Treatment by kinase inhibitor roscovitine might affect the phosphorylation status of these proteins and result in gel mobility changes of the affected protein (Fig. 8g). Roscovitine treatment did not affect the quantities or mobility of HP1α, PML or the double band of Daxx that might represent unmodified and phosphorylated forms of the protein. PML SUMOylation was also unaffected. The only noticeable change in mobility was observed for the ATRX protein. After 1 hour of roscovitine treatment, a faster migrating, presumably dephosphorylated, ATRX band appeared. The mobility shift observed resembles the mobility shift of ATRX after phosphatase treatment (Fig. 8g). Potentially, the cell-cycle-dependent specific phosphorylation of ATRX might be a mechanism leading to the cell-cycle-specific accumulation of Daxx and SSRP1 at condensed chromatin. The incomplete shift of ATRX after roscovitine treatment is similar to that obtained after protein phosphatase treatment and might reflect a site-specific dephosphorylation of ATRX, suggesting that there is a roscovitine-sensitive kinase(s) involved in ATRX phosphorylation. More extensive experiments, including mapping the ATRX roscovitine-sensitive phosphorylation positions, should be performed to reveal details of the Daxx-ATRX interaction.

Daxx-dependent changes in cell-cycle progression

The very narrow window of Daxx accumulation at condensed

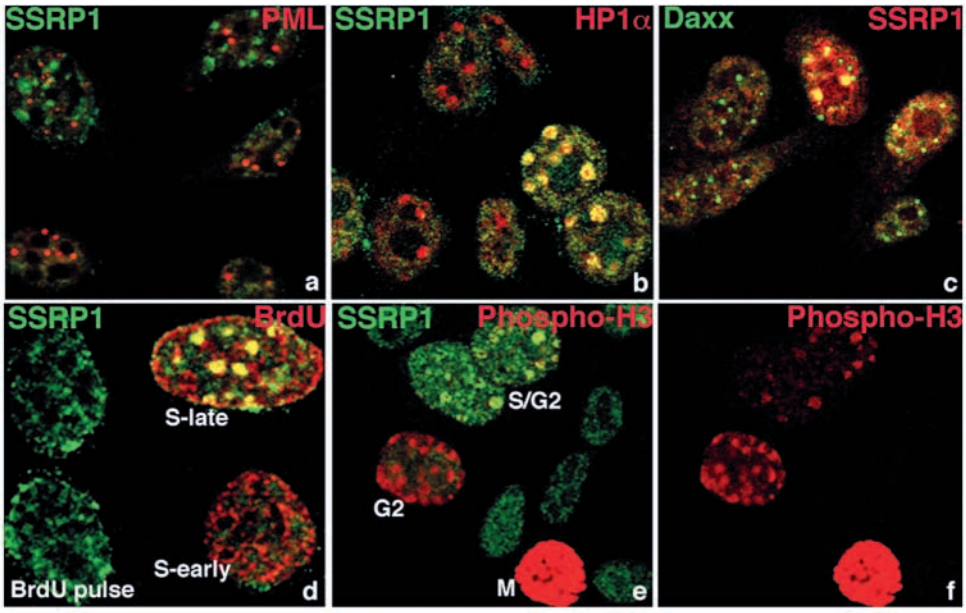


Fig. 7. Cell-cycle-dependent intranuclear distribution of SSRP1. (a) Cells double-labeled for SSRP1 and PML show that SSRP1 does not accumulate at ND10 but is present at irregular domains in some cells (upper left and right cells). (b) Double-labeling for SSRP1 and HP1α reveals SSRP1 at heterochromatin in some cells (two cells at lower right). (c) Double-labeling for Daxx and SSRP1 demonstrates co-localization of the proteins in heterochromatic domains (upper middle cell). (d) Cells pulsed with BrdU and labeled for SSRP1 and BrdU show homogeneous nuclear distribution of SSRP1 in most cells but localization with the late BrdU incorporation pattern (S-late). (e) Cells labeled for SSRP1 and phosphorylated histone H3 shows accumulation of SSRP1 at heterochromatin at the beginning of histone H3 phosphorylation corresponding to late S-phase and early G_2 phase (S/G2) but not late G_2 phase (G2). (f) The phosphorylated histone H3 signal shown separately.

chromatin suggests that Daxx is involved in the cell-cycle transition. We therefore analysed the cell-cycle progression of *Daxx*$^{-/-}$ cells reconstituted with empty plasmid (vector), wild-type Daxx and Daxx deletion mutants combining BrdU and phospho-H3 labeling (Table 1). S-phase cells incorporated BrdU and G_2/M-phase cells were labeled with the anti-phospho-H3 antibody; the G_1/G_0-phase cells were negative for both markers. We find little difference between the control (empty vector) and the DaxxC reconstituted cells. DaxxC therefore does not have an effect on the cell-cycle progression. However, the Daxx wild-type and DaxxΔC reconstituted cells had a substantially higher proportion of cells in S-phase, suggesting that Daxx has an effect on S-phase prolongation, possibly on the degree of S-G_2 phase transition. Unexpectedly, the DaxxΔC cells had a more than twice the proportion of cells in G_2/M phase as did the Daxx wild-type cells although they had a similar proportion of S-phase cells. Expansion of the G_2 phase by the presence of DaxxΔC might reflect an inhibition of processes that are facilitated by the ΔC domain of Daxx, but cannot be finished owing to the absence of the C-terminal part of the protein.

Table 1. Determination of cell-cycle stages in Daxx reconstituted cell lines

Cell line	% G_1/G_0	% S	% G_2/M	% Double nuclei
Daxx$^{-/-}$ + vector	64.0	29.6	6.4	10.7
Daxx wild type	56.6	37.2	6.2	3.3
DaxxΔC	48.7	35.5	15.8	4.6
DaxxC	65.5	28.4	6.1	14.1

Over 400 cells, double-labeled with BrdU and anti-phospho-H3 antibodies, were analysed in randomly selected fields and categorized for stages of cell cycle. Over 1000 cells per cell line were evaluated for the double-nucleus formation.

Many cells were observed in the *Daxx*$^{-/-}$ strain that contained double nuclei (Table 1). Daxx wild-type and DaxxΔC, but not DaxxC reconstitution, rescued the double-nucleus phenotype of the *Daxx*$^{-/-}$ cells. Double-nucleus formation suggests a problem in the coordination of cell-cycle control after DNA replication and incomplete mitosis, which might contribute to the developmental problems observed in *Daxx*$^{-/-}$ embryos.

Discussion

Daxx binds with its C-terminus to SUMOylated PML and is thus accumulated at ND10 (Ishov et al., 1999). An increasing number of repressors such as Pax 3 (Hollenbach et al., 1999; Lehembre et al., 2001) and HDAC2 (Li et al., 2000; Tang and Maul, 2003) can bind to the same C-terminus of Daxx. Daxx might function as a repressor not at ND10 but elsewhere in the nucleus, presumably at condensed chromatin. Besides the C-terminus, Daxx has an additional protein interaction domain located in the ΔC region, which was found to bind the cytomegalovirus tegument transactivator pp71, leading to the sequestration of pp71 at ND10 (Hofmann et al., 2002; Ishov et al., 2002). The reconstitution of *Daxx*$^{-/-}$ cells with wild-type Daxx and the respective mutants demonstrated that the same domain also binds the SWI/SNF chromatin-associated protein ATRX, resulting in deposition of ATRX at ND10 and, reciprocally, accumulation of Daxx at condensed chromatin.

We analysed the dual accumulation of Daxx at condensed

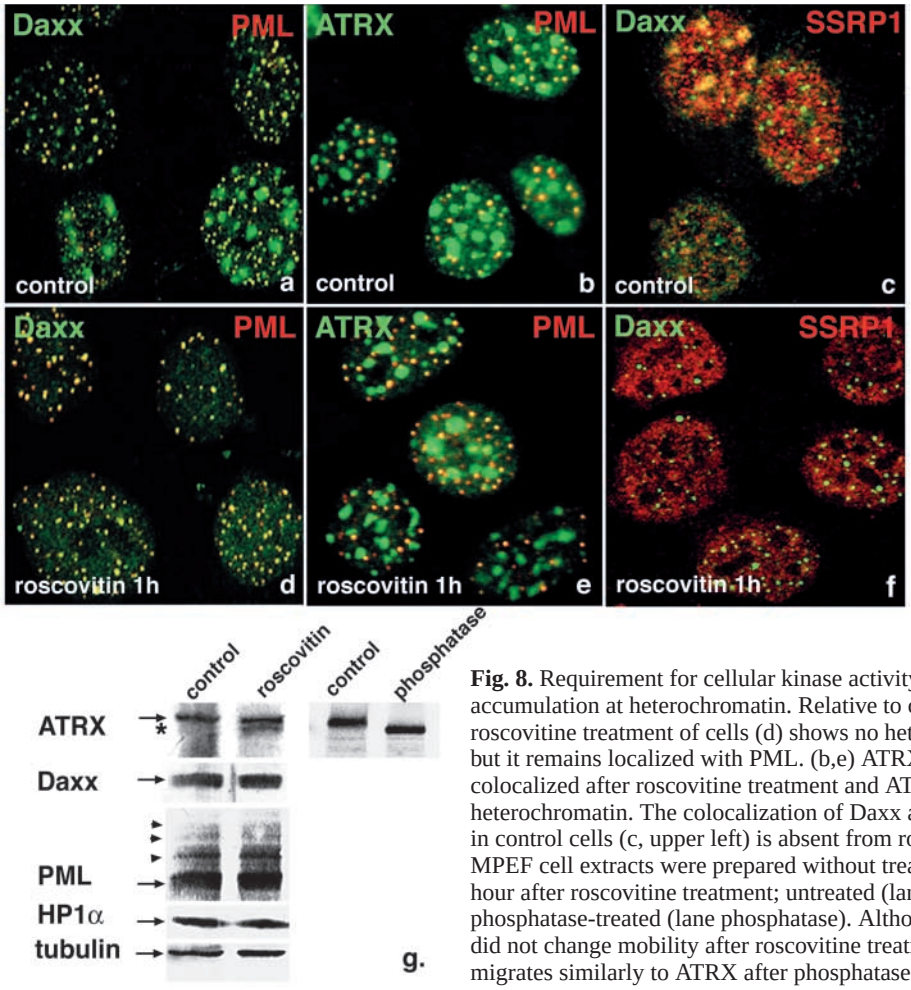


Fig. 8. Requirement for cellular kinase activity in Daxx and SSRP1 accumulation at heterochromatin. Relative to control (a), Daxx after roscovitine treatment of cells (d) shows no heterochromatic accumulation but it remains localized with PML. (b,e) ATRX and PML remain colocalized after roscovitine treatment and ATRX still accumulates at heterochromatin. The colocalization of Daxx and SSRP1 in heterochromatin in control cells (c, upper left) is absent from roscovitine-treated cells (e). (g) MPEF cell extracts were prepared without treatment (control lane), and 1 hour after roscovitine treatment; untreated (lane control 1) or protein-phosphatase-treated (lane phosphatase). Although Daxx, PML and HP1α did not change mobility after roscovitine treatment, the new ATRX band (*) migrates similarly to ATRX after phosphatase treatment.

chromatin and ND10 in light of the hypothesized nuclear depot function of ND10 (Negorev and Maul, 2001). This hypothesis suggests that Daxx might not act at ND10 but instead at heterochromatin, where it might achieve its reported transcription repressive activities by interacting with chromatin-associated proteins such as HDAC2, Dek and acetylated histone H4 (Hollenbach et al., 2002). Extraction experiments confirmed that Daxx is a chromatin-associated protein. By combining several markers of the cell cycle, we demonstrated that heterochromatic association of Daxx occurs in late S-phase, concomitant with a decrease in Daxx content at ND10. The change in localization of Daxx between two intranuclear domains implies the involvement of some cell-cycle-dependent signaling. Analysis of cell-cycle-dependent

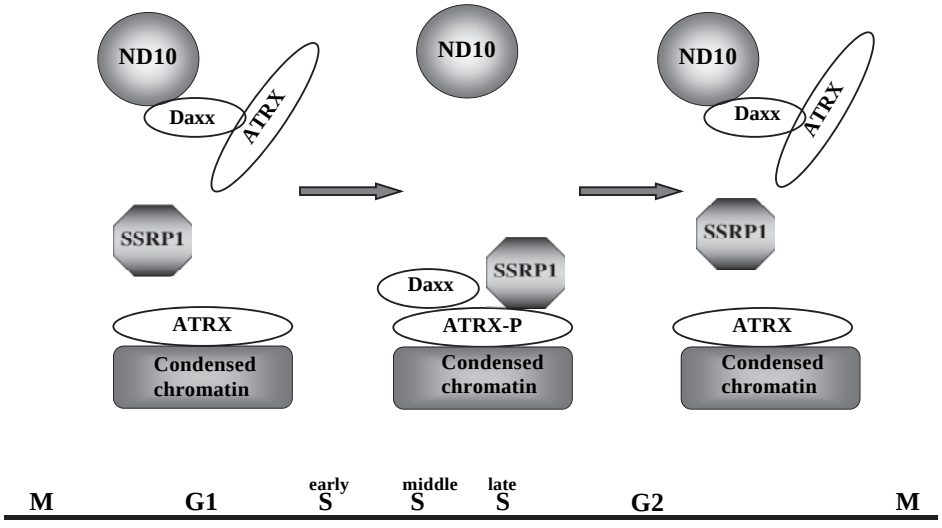


Fig. 9. Timeline of the reconfiguration of a multiprotein complex at condensed chromatin. Proteins from different nuclear domains accumulate together at heterochromatin in an apparent ATRX-based complex during late S-phase. Signaling for this change is due to a shift in phosphorylation of the heterochromatin-based ATRX. This complex is resolved during G_2 phase, when ATRX recruitment to ND10 is mediated by Daxx.

accumulation of Daxx in the absence of ND10 (in *PML*$^{-/-}$ cells) and in Daxx-reconstituted *Daxx*$^{-/-}$ cells indicates that S-phase-specific heterochromatic accumulation of Daxx is an active process. The PML-bound C-terminal domain of Daxx (DaxxC reconstitution, Fig. 5i), however, is not released from ND10 during S-phase. The release of Daxx from ND10 is therefore more likely to be a passive consequence of Daxx accumulation at chromatin that changes the dynamic balance between ND10-associated Daxx and the soluble nucleoplasmic Daxx. Daxx shuttles between two nuclear domains, being actively recruited to heterochromatin during or after replication of its DNA and released after completion of this stage of the cell cycle (and, presumably, after the reestablishment of epigenetic markers of transcription repression at these regions). The very narrow window of Daxx association with heterochromatin, together with the apparent active deposition of protein at this nuclear structure, suggests that Daxx participates in the post-replication chromatin modification.

Because the domain of ATRX interaction and heterochromatin targeting overlaps within the Daxx molecule, Daxx accumulation at chromatin probably occurs through direct or indirect interaction with ATRX. Column chromatography (Xue et al., 2003) and co-immunoprecipitation analysis indicate that Daxx is a member of ATRX complex. However, it is still unclear why Daxx and ATRX remain together at ND10 during almost all of interphase, whereas Daxx accumulates at heterochromatin, the main constitutive ATRX intranuclear domain, only at late S-phase. Roscovitine treatment experiments revealed that specific phosphorylation of ATRX (potentially, by the cyclin-B kinase, that is mostly inactivated by roscovitine) is the most likely mechanism of Daxx accumulation at heterochromatin. Another, unknown, modification of ATRX underlies the interaction with Daxx leading to the accumulation of ATRX at ND10.

SSRP1, a member of the FACT complex (Orphanides et al., 1999), has been isolated as a member of the ATRX complex (W. Wang, personal communication). This protein also accumulates at heterochromatin during late S-phase and is released from these sites by roscovitine treatment. Association of SSRP1 with heterochromatin is Daxx independent, because the association occurs in *Daxx*$^{-/-}$ cells (not shown). Presumably, SSRP1 accumulates at heterochromatin owing to the same signaling as Daxx but involving a different interaction domain of ATRX or a different intermediate protein. Alternatively, SSRP1 might itself be phosphorylated (Krohn et al., 2003) to interact with ATRX-containing complex at heterochromatin. These associations, observable in vivo, suggested the formation of a multiprotein complex at the pericentromeric regions during or immediately after DNA replication. The constitutive heterochromatin protein ATRX might be a scaffold protein for this complex formation induced by specific cell cycle signaling altering the composition of complex components. Deposition of ATRX to ND10 through interaction with Daxx might balance the availability of active ATRX at heterochromatin. The combinatory scheme of the ATRX-based cell-cycle-dependent complex assembly at condensed chromatin is presented in Fig. 9.

Functionally, Daxx (and, potentially, the Daxx-ATRX interaction) seems to have two cell-cycle effects that correlate with the observed accumulation of Daxx to heterochromatin. In the absence of Daxx, S-phase seems to proceed more quickly. This defect can be rescued by Daxx wild-type and DaxxΔC reconstitution, although DaxxΔC reconstitution prolongs G_2 phase. The lack of the C-terminus of Daxx, which is involved in many protein interactions, might slow down the completion of specific processes that depend on these interactions.

The second effect is the propensity of *Daxx*$^{-/-}$ cells to form double nuclei, which appears to be related to the faster S-phase and can also be rescued by reconstituting Daxx or the C-terminal deletion mutant. Overexpression of ATRX in transgenic animals leads to abnormal embryo growth, mostly affecting neural development (Berube et al., 2002). Changes in the pattern of DNA methylation caused by ATRX mutations (Gibbons et al., 2000) correlate with its DNA methyltransferase 3 homology and might be partly responsible for abnormalities during development. In light of the recently proposed interplay between methylation of DNA and histones (Fuks et al., 2003), one can anticipate even more global involvement of ATRX in epigenetic control. Daxx is not essential for the DNA replication, but S-phase progression is shortened in *Daxx*$^{-/-}$ cells. Depletion of other chromatin remodeling proteins, such as ACF1 and SNF2H, also interferes with the cell cycle but results in delay of methylated pericentromeric DNA replication in late S-phase and extension of S-phase (Collins et al., 2002). Apparently, Daxx depletion leads to the opposite effect, namely the number of S-phase cells in *Daxx*$^{-/-}$ is lower than upon Daxx reconstitution. Daxx might, as a member of the ATRX-containing complex, participate in post-replication chromatin modification(s), potentially reestablishing the repressive chromatin state at the pericentromeric DNA. Presumably, in the absence of Daxx, cells proceed more quickly through certain post-replication checkpoints and are unable to recover some epigenetic markers that, although not essential for the survival of cells in culture, might lead to the embryonic lethality found in *Daxx*$^{-/-}$ animals. Remarkably, ATRX knockout embryos are lethal at almost the same stage of development (Gibbons et al., 2003), suggesting a similarity between the two proteins functions and pointing to the potential importance of the Daxx-ATRX complex during the developmental process. The known interactions of the C-terminus of Daxx with an increasing number of transcription repressors are at the center of hypothetical mechanisms relating to the formation of a chromatin modifying complex with specific relevance to maintaining or reestablishing the epigenetic state of silent heterochromatin after replication.

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