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Nuclear localization of ISWI ATPase Smarca5 (Snf2h) in mouse

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1. ABSTRACT

Nucleosome movement is, at least in part, facilitated by ISWI ATPase Smarca5 (Snf2h). *Smarca5* gene inactivation in mouse demonstrated its requirement at blastocyst stage; however its role at later stages is not completely understood. We herein determined nuclear distribution of Smarca5 and histone marks associated with actively transcribed and repressed chromatin structure in embryonic and adult murine tissues and in tumor cells. Confocal microscopy images demonstrate that Smarca5 is localized mainly in euchromatin and to lesser extent also in heterochromatin and nucleoli. *Smarca5* heterozygous mice for a null allele display decreased levels of histone H3 modifications and defects in heterochromatin foci supporting role of Smarca5 as a key regulator of global chromatin structure.

2. INTRODUCTION

DNA is in mammals organized into a complex structure consisting of small nucleoprotein units recognized as nucleosomes. Each nucleus spatially handles nucleosomes to form dynamic structure that each cell division can reorganize itself and is referred to as chromatin. There exist ATP dependent mechanisms that move DNA along histone octamer structure or can displace nucleosomes from particular DNA sequence. SWI/SNF (mating type switch/sucrose non-fermenting) superfamily encodes four classes of chromatin remodeling proteins that coordinate by distinct mechanisms (1) chromatin structure changes upon cell fate decisions. Both major SWI/SNF ATPases, Smarca5 and Brg1, were shown using gene inactivation mouse models to be critically important for

early embryonic development (2, 3) and after birth (4, 5) as well as in tumor biology (6, 7 and references there). However, the mechanisms of how these ATPases regulate chromatin structure are still incompletely understood. Smarca5 is thought to be directly involved both in transcriptional regulation (8) as well as in chromatin assembly in *Drosophila* (9) with possible role of extranucleosomal histone proteins including histone 1 (10). We herein determined nuclear localization of Smarca5 and Brg1 and also a panel of histone modifications to distinguish major nuclear compartments in murine embryonic and adult tissues. Our localization data indicate that Smarca5 occurs in euchromatin and to lesser extent also within nucleoli and heterochromatin. Our data using *Smarca5* knockout mice also indicate that Smarca5 may regulate chromatin structure of these nuclear compartments.

3. MATERIALS AND METHODS

3.1. Preparation of *ex vivo* isolated adult murine tissues

Mouse tissues were *ex vivo* isolated from wild type C57Bl6 mice or those carrying a null allele of *Smarca5* (2) and handled according to Institutional board for animal handling. *Ex vivo* isolated murine tissues were fixed in 4% paraformaldehyde in PBS at 4˚C overnight, washed in PBS (for 1 hour at room temperature) and incubated in 7.5% sucrose and 15% sucrose (both for 2-3 hours at room temperature) respectively. The tissues were then sectioned using CM3050 cryostat (Leica), transferred to SuperFrost Plus microscope slides (Menzel-Glaser), permeabilized and blocked with 0.5% Triton X-100, 3% BSA in PBS (1 hour at room temperature), washed in PBS and incubated with the primary antibodies (see bellow).

3.2. Mouse embryos and cell lines

To isolate blastocysts the C57Bl6 mice were superovulated with an injection of 5 IU pregnant mare serum gonadotropin followed by the injection of 5 IU human chorionic gonadotropin 48 hours later. Injected females were mated with C57Bl6 males. Plugged females were sacrificed by cervical dislocation (3.5 days after mating) and blastocysts were obtained by flushing the uterus with embryonic stem (ES) cell culture media (2, 3). We also used ES cells cultured in DMEM media (Gibco) supplemented with leukemia inhibitory factor (LIF, Scintila) and 10% fetal bovine serum (Hyclone); all supplements were tested for ES cell culture. HeLa cells were cultured in DMEM media supplemented with 10% fetal bovine serum. The cells and blastocysts were fixed on slide in 3.5% paraformaldehyde in PBS at room temperature for 15-20 minutes. After fixation and brief wash in PBS, the cell lines were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at room temperature and blocked using 3% non-fat milk in PBS for 10 minutes at room temperature. The blastocysts were permeabilized and blocked in 0.5% Triton X-100, 3% BSA in PBS for 20 minutes at room temperature. After blocking, the cells and blastocysts were repeatedly rinsed with PBS and incubated with primary antibodies (see bellow) (11).

3.3. Immunostaining and imaging

Primary antibodies: anti-Smarca5 (cat.# ab3749, Abcam, dilution 1:100 in 1% BSA-PBS and cat.# MAB3644, Chemicon, dilution 1:500 in 1% BSA-PBS), anti-Brg1 (cat.# sc-10768, Santa Cruz Biotechnology, dilution 1:100 in 1% BSA-PBS), anti-H3K9 acetylation (cat.# 07-352, Upstate, dilution 1:1000 in 1% BSA-PBS), anti-H3K9 trimethylation (cat.# 07-442, Upstate, dilution 1:750 in 1% BSA-PBS), and anti-H3K4 dimethylation (cat.# ab7766, Abcam, dilution 1:500 in 1% BSA-PBS). The primary antibody was detected using secondary antibodies coupled either with Alexa Fluor 488 (cat.# A-11070, Invitrogen, dilution 1:300 in 2% BSA-PBS,) or Alexa Fluor 594 (cat.# A-11020, Invitrogen, dilution 1:300 in 2% BSA-PBS, incubation for 1 hour at room temperature). Following each of the antibody incubation, cells were repeatedly washed in 0.1% Tween 20-PBS. The fixed and immunostained samples were mounted onto microscope slides with Vectashield Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI). Images were acquired using confocal laser scanning microscope Leica TCS SP2 with AOBS system (oil immersion 63x objective N.A. 1.4). Digital processing of confocal images data, including fluorescence intensity profiles acquisition and determination of heterochromatin content, was performed using ImageJ 1,38x software. Y-axis in intensity profile graphs represents fluorescence of respective fluorophor and X axis represents distance. For statistical analyses Student's two-sample t-test with equal variances was used.

4. RESULTS

4.1. Smarca5 localization in euchromatin and defects of euchromatin structure in *Smarca5* +/- mice

To determine nuclear localization of Smarca5 in mouse we have used the following tissues: mouse adult testes (to study primary spermatocytes) and spleen where predominant expression of Smarca5 was demonstrated previously (7, 12). We have also used mouse embryos at blastocyst stage (Day E3.5), since this stage has been shown to become severely affected in mice deleted of *Smarca5* gene by homologous recombination (2). In addition, we have also used *ex vivo* isolated murine cell lines: pluripotent embryonic stem (ES) cells and primary skin fibroblast (PSF) cells. We also used the abovementioned tissues and cell lines isolated from mice heterozygous for null allele of Smarca5 (+/-), because these tissues contain significantly lower levels of Smarca5 (2). Firstly, we have characterized nuclear staining of the chromatin compartments using antibodies recognizing acetylated histone H3K9 (H3K9Ac) and methylated histone H3K4 (not shown) residues that distinguish actively transcribed chromatin from regions that are densely packed and stained positively by antibodies recognizing trimethylated histone H3K9 (H3K9Me3) and by DAPI (reviewed in 13). We have also used antibodies recognizing dimethylation of histone H3K79 representing modification that is enriched in euchromatin and also in heterochromatin. Secondly, we have determined nuclear localization of Smarca5 in all analyzed murine tissues and cell lines using two independent antibodies and found that Smarca5 is largely enriched in euchromatin (Figure 1 and Figure 2B). Thirdly, we overlaid the immunostaining of Smarca5 with histone modifications within euchromatin

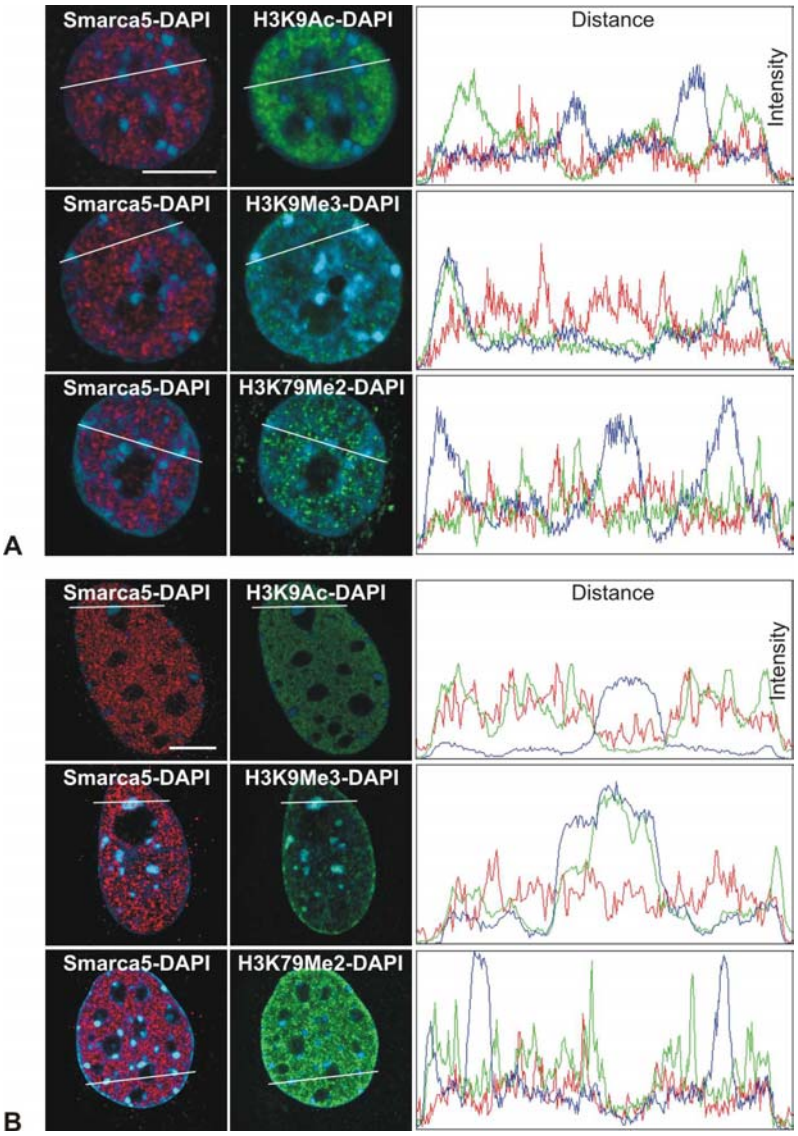

Figure 1. Smarca5 nuclear localization in euchromatin. Confocal laser scanning microscopy (nuclei: overlaid immunostaining, graphs: intensity profiles of immunostaining above background across the nucleus (indicated by white line)) of ES cells (N=30) (A) and PSF cells (N=15) (B), using antibodies to Smarca5 (red), to histone modifications (green): H3K9Ac, H3K9Me3, H3K79Me2 and DAPI (blue) (see M&M section). Scale bar represents 5 micrometers.

and found that Smarca5 imperfectly overlays H3K9Ac and H3K79Me2 (Pearson's coefficient = 0,03 and 0,05 respectively; random mutual localization). These data suggests that some but not all loci enriched with either H3K9Ac or H3K79Me2 may be enriched with Smarca5. Specificity of Smarca5 immunostaining was obtained using negative control antibody immunostaining and also using cells with manipulated Smarca5 levels e.g. Smarca5 +/- ES and +/- PSF cell lines. Partial depletion of Smarca5 in the mouse tissues and cell lines heterozygous for null allele (+/-) compared to wild type controls was observed in euchromatin of all studied cell types (Figure 2A). Unexpectedly, we observed in *Smarca5* heterozygous nuclei that the immunostaining of H3K9Ac and H3K79Me2 were significantly decreased compared to wild type nuclei indicating that these (H3K9Ac and H3K79Me2) modifications of chromatin may be partly dependent on Smarca5 levels (Figure 3A). The mechanisms of how Smarca5 influence histone H3K79 dimethylation and H3K9 acetylation are currently under our scientific investigation. We have also analyzed nuclear localization of Smarca5 in tumor cells including human cervical cancer (HeLa) and murine erythroleukemia (MEL) cells. The localization data indicate that Smarca5 is enriched in these neoplastic highly proliferating cells within euchromatin compartment and that tumorous cell features such as high proliferation and block of differentiation did not affect localization pattern of Smarca5 (Figure 3B,C). Close homologue of Smarca5, Brg1, that shares some of the structural/functional properties (nucleosome sliding) and phenotypic outcome

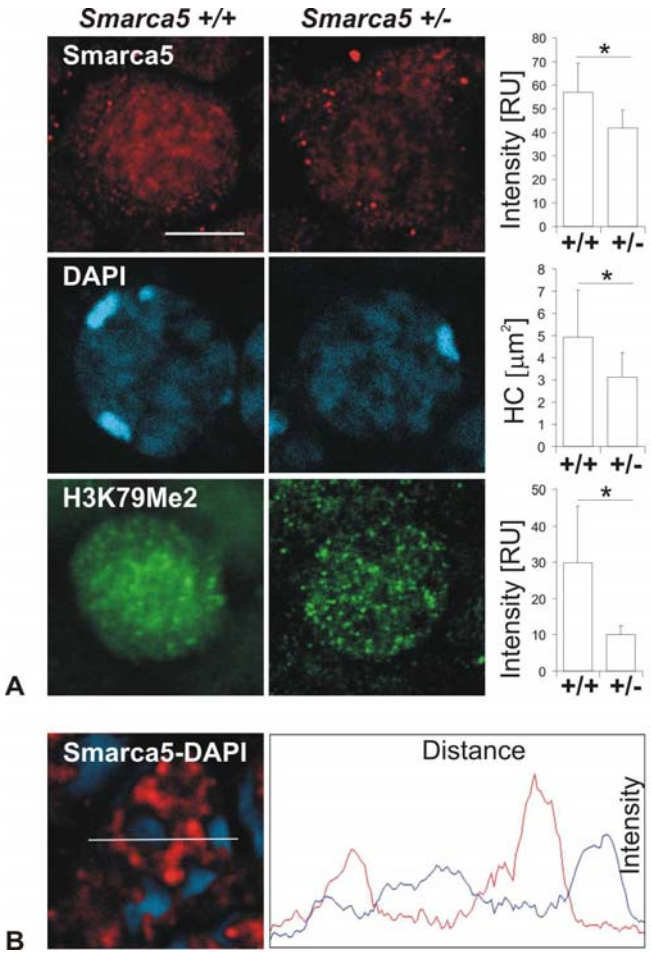

Figure 2. Decreased Smarca5 levels coincide with decreased global H3K79 dimethylation and heterochromatin content (HC). Confocal laser scanning microscopy of *ex vivo* isolated testes (primary spermatocytes) either wild type or heterozygous for *Smarca5* null allele (A). The panel display level of Smarca5 (upper, N=60) and H3K79Me2 (lower, N=30) immunostaining intensities and the middle panel display heterochromatin content (HC) (N=30). Smarca5 nuclear localization in euchromatin of spleen cell (B). Graph represents intensity profile of Smarca5 (red) and DAPI (blue). Scale bar represents 5 micrometers.

(requirement at blastocyst stage in mouse) was also tested in murine tissues and cell lines and its localization was also consistently observed in euchromatin (not shown). However, levels of Brg1 in *Smarca5* heterozygous ES cells remain unchanged compared to wild type controls (Figure 3A). Localization of Smarca5 and Brg1 in euchromatin supports their role in transcription regulation of actively transcribed genes in euchromatin (2, 7, 14, 15).

4.2. Low levels of Smarca5 in heterochromatin and nucleoli in mouse. Defects of heterochromatin structure in *Smarca5* +/- mice

Confocal laser scanning microscopy of H3K9Me3 and DAPI staining of the murine nuclei allowed us to determine protein localization within heterochromatin (16). As negative control we have used control isotypic antibodies and also antibody recognizing H3K9Ac and H3K4Me. Localization of H3K79Me2 was associated partly with heterochromatin; this contention is supported by studies indicating that this modification is also associated with repressed transcription (17). Intensity profiles of Smarca5 across the heterochromatin foci indicated that low levels of Smarca5 are enriched in heterochromatin albeit lower than in euchromatin (Figure 4A,B). Interestingly, homologue of Smarca5, Brg1 was also detectable in heterochromatin at very low levels. In addition, depletion of Smarca5 in mouse tissues heterozygous for null allele correlated with significantly decreased levels of Smarca5 in heterochromatin (Figure 4C). Unexpectedly we have also observed that *Smarca5* heterozygous nuclei displayed also decreased DAPI staining that suggests depletion of heterochromatin content (Figure 2A and 4C). To test whether the heterochromatin content measured by integration of DAPI can be further decreased in *Smarca5* -/- cells we have studied blastocysts derived from *Smarca5* +/- females mated with +/- males. The blastocysts were subject to Smarca5 staining and DAPI staining and as shown in Figure 4D, in one half of the embryos we have detected decreased nuclear expression of Smarca5 at the level similar to heterozygotes. In addition, in one quarter of the offspring Smarca5 levels were further decreased indicating that these embryos are -/-, and in these embryos

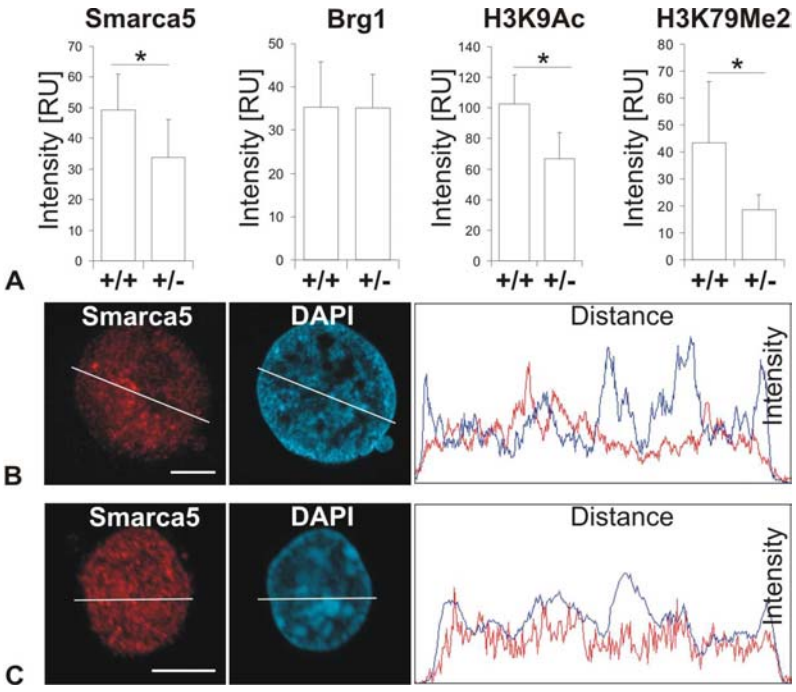

Figure 3. Levels of Smarca5 (N=30), H3K9Ac (N=20) and H3K79Me2 (N=20) are significantly (*) decreased ($p<0.05$) in *Smarca5* heterozygous ES cells whereas Brg1 level remains unchanged (A). Confocal laser scanning microscopy. B, C: Smarca5 nuclear localization in euchromatin of cancer cells. Confocal laser scanning microscopy (nuclei: immunostaining, graph: intensity profiles of immunostaining and DAPI staining above background across the nucleus (indicated by white line)) of HeLa cells (N=20) (B) and MEL cells (N=10) (C), using antibodies to Smarca5 (red) and DAPI (blue). Scale bar represents 5 micrometers.

the DAPI staining displayed significant decrease of staining compared to both heterozygous and wild type nuclei (Figure 4D). These data therefore indicate that Smarca5 levels may be important for determining heterochromatin size. Localization of Smarca5 in heterochromatin and role of its levels in heterochromatin content supports its role in nucleosome assembly in mouse (9).

Smarca5 has also been identified as important coregulator of rRNA expression (reviewed in 18) and therefore we have determined its localization in nucleoli of the murine tissues. As negative control staining we have used either isotypic control or H3K79Me2 antibodies. As positive control we have used human HeLa cells that were repeatedly shown to contain high levels of nucleolar Smarca5 (not shown) (19). Analysis of Smarca5 revealed its discrete nucleolar localization in all studied tissues (Figure 4E,F) supporting previously reported regulatory role of Smarca5 in rRNA expression in mouse (19).

5. DISCUSSION

Gene inactivation studies of Smarca5 indicated that it is required for early embryonic development in mouse around embryo implantation (2) and that pluripotent stem cells lacking Smarca5 are not able to develop properly. We have provided another line of evidence that chromatin remodelling protein Smarca5 is highly expressed in murine and human leukemia cells and its expression is important during early differentiation decisions of progenitor cells (7). We have therefore decided to study nuclear localization of Smarca5 in mouse and compare it to distribution of known chromatin marks (13) that would enable us to understand processes that Smarca5 regulates. We used comprehensive approach using also mouse tissues derived from Smarca5 +/- animals with significantly depleted Smarca5 levels. It is of note that Smarca5 +/- mice display unique phenotypic features: are smaller than wild type littermates and decreased levels of Smarca5 in primary spermatocytes appear to regulate expression in offspring differently as compared to the offspring derived from wild type spermatocytes (12). Our data collectively demonstrate that Smarca5 is mainly localized in euchromatin and traces of its expression are detectable in both heterochromatin and nucleoli. Furthermore, Smarca5 +/- mice display defects in histone modifications in euchromatin (H3K9Ac, H3K79Me2) (Figure 2A, 3A) and also defects in heterochromatin structure (Figure 2A, 4C,D). These data are supported by reports indicating that Smarca5 is involved in gene expression regulation (14, 18 and references there) and also in regulating nucleosome assembly rather than its disruption (9). In addition, our study indicated that homologue of Smarca5, ATPase Brg1, that shares some but not all of chromatin remodelling properties (20, 21) and that is also required for proper chromatin development in mouse (3) is also localized mainly in euchromatin. Euchromatin distribution of Smarca5 and Brg1 in cancer and leukemia cells (Figure 3B,C) support several lines of evidence that these chromatin remodelers may regulate genes required for tumor development (reviewed in 6).

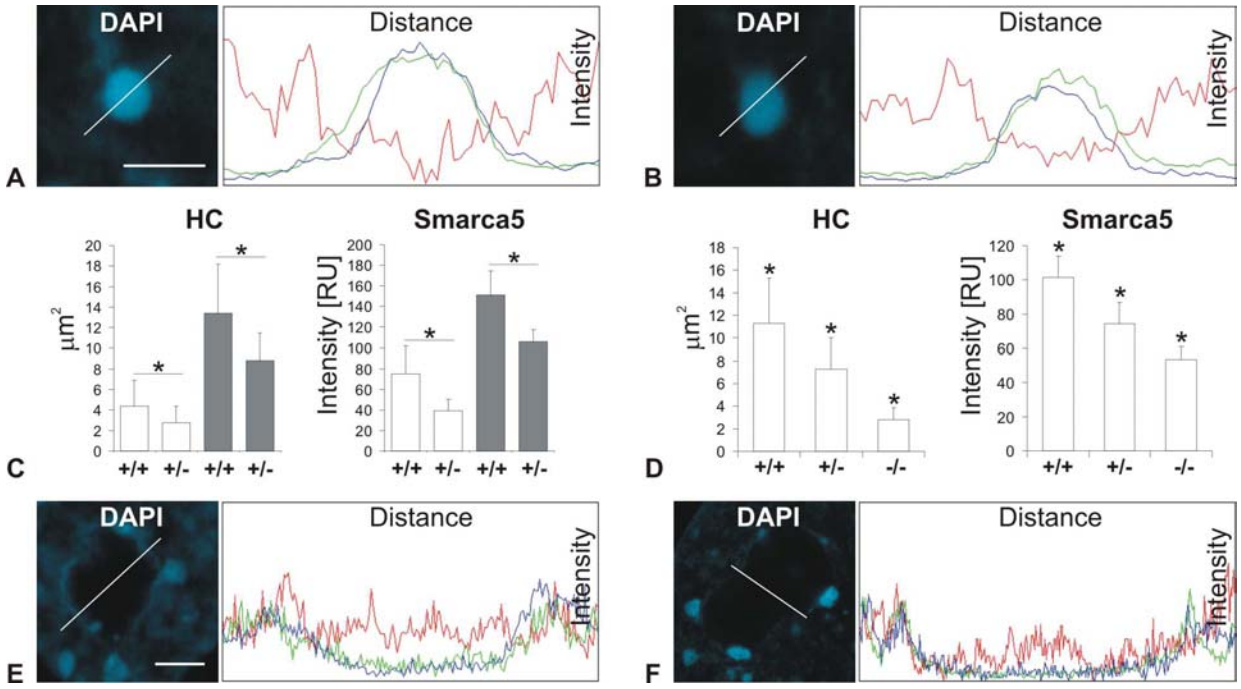

Figure 4. Smarca5 localization in heterochromatin and nucleoli. Confocal laser scanning microscopy of ES cells (N=15) (A) and PSF cells (N=15) (B) using antibodies to Smarca5 (red), H3K9Me3 (green) and DAPI (blue) (intensity profiles of immunostaining above background across the heterochromatin focus (indicated by white line). Smarca5 levels (N=30) and heterochromatin content (HC) (N=20) are significantly (*) decreased ($p<0.05$) in *Smarca5* heterozygous ES (white bars) and PSF (grey bars) cells (C) and as well as in *Smarca5* heterozygous (+/-) and null (-/-) mutant blastocysts (N=20) (D). Confocal laser scanning microscopy of ES cells (N=15) (E) and PSF cells (N=15) (F) using antibodies to Smarca5 (red), H3K79Me2 (green) and DAPI (blue) (intensity profiles of immunostaining above background across the nucleoli (indicated by white line)). Scale bar represents 2 micrometers.

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