The Trithorax group protein Ash2l and Saf-A are recruited to the inactive X chromosome at the onset of stable X inactivation

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SUMMARY
Mammals compensate X chromosome gene dosage between the sexes by silencing of one of the two female X chromosomes. X inactivation is initiated in the early embryo and requires the non-coding Xist RNA, which encompasses the inactive X chromosome (Xi) and triggers its silencing. In differentiated cells, several factors including the histone variant macroH2A and the scaffold attachment factor SAF-A are recruited to the Xi and maintain its repression. Consequently, in female somatic cells the Xi remains stably silenced independently of Xist. Here, we identify the Trithorax group protein Ash2l as a novel component of the Xi. Ash2l is recruited by Xist concomitantly with Saf-A and macroH2A at the transition to Xi maintenance. Recruitment of these factors characterizes a developmental transition point for the chromatin composition of the Xi. Surprisingly, expression of a mutant Xist RNA that does not cause gene repression can trigger recruitment of Ash2l, Saf-A and macroH2A to the X chromosome, and can cause chromosome-wide histone H4 hypoacetylation. This suggests that a chromatin configuration is established on non-genic chromatin on the Xi by Xist to provide a repressive compartment that could be used for maintaining gene silencing. Gene silencing is mechanistically separable from the formation of this repressive compartment and, thus, requires additional pathways. This observation highlights a crucial role for spatial organization of chromatin changes in the maintenance of X inactivation.

KEY WORDS: X inactivation, Xist RNA, SAF-A, Nuclear matrix, Dosage compensation

INTRODUCTION
In mammals X inactivation establishes dosage compensation between males and females. Early in embryogenesis one of the two female X chromosomes is transcriptionally silenced by expression of Xist (Heard and Disteche, 2006). The nuclear non-coding Xist RNA is required for the initiation of X inactivation (Marahrens et al., 1997; Penny et al., 1996). Xist is transcribed from and associates with the inactive X chromosome (Xi) (Clemson et al., 1996) and triggers gene silencing. However, in differentiated cells Xist is dispensable for the maintenance of gene repression on the Xi (Brown and Willard, 1994; Csankovszki et al., 2001; Csankovszki et al., 1999).

X inactivation is recapitulated during the differentiation of mouse embryonic stem (ES) cells. Female ES cells possess two active X chromosomes, one of which becomes inactivated upon differentiation. Using this system the sequence of events leading to the formation of a stably silenced Xi has been dissected (Lucchesi et al., 2005). Xist localization initially leads to the formation of a nuclear compartment from which RNA polymerase II and transcription factors are excluded (Chaumeil et al., 2006; Okamoto et al., 2004). This repressive compartment can be formed when a mutant Xist RNA is expressed that lacks the 5′ repeat A motif and cannot initiate gene repression (Chaumeil et al., 2006). At the onset of X inactivation genes are located on the outside of the Xi chromosome territory and relocate into the repressive compartment upon silencing in a repeat A-dependent manner (Chaumeil et al., 2006).

Xist expression causes the recruitment of Polycomb group (PcG) proteins (de Napoles et al., 2004; Plath et al., 2004; Silva et al., 2003). Polycomb repressive complex 2 (PRC2) contains the PcG proteins Eed and Suz12, and the histone methylase Ezh2, which catalyzes trimethylation of lysine 27 of histone H3 (H3K27me3) along the Xi (Plath et al., 2003). Likewise, PRC1 complexes containing several PcG proteins, including Ring1B (Rnf2 – Mouse Genome Informatics), are implicated in mono-ubiquitylation of histone H2A lysine 119 (uH2A) on the Xi (de Napoles et al., 2004; Fang et al., 2004; Leeb and Wutz, 2007). The recruitment of PcG proteins to the Xi is regulated during differentiation (Plath et al., 2004). PcG proteins have been implicated in the maintenance of X inactivation in keeping with their well-established function in maintaining repression of developmental control genes (Wang et al., 2001). Factors that contribute to maintaining the silent state of the Xi further include the histone variant macroH2A (Costanzi and Pehrson, 1998; Mermod et al., 1999; Mietton et al., 2009; Rasmussen et al., 2004), histone H4 hypoacetylation (Keohane et al., 1996) and DNA methylation (Sado et al., 2000; Sado et al., 2004). Recently, it has been shown that the Smchd1 protein is required for the maintenance of DNA methylation patterns and gene repression on the Xi (Blewitt et al., 2008). In human cell lines, the nuclear scaffold protein SAF-A (also known as HNRNPU) has also been reported as being enriched on the Xi (Helbig and Fackelmayer, 2003), where it forms a stable structure, suggesting a role in stabilization of Xi silencing (Fackelmayer, 2005).

Here, we describe an approach aimed at further characterizing the chromatin composition of the Xi. We identify the mammalian homologue of the fruit fly Trithorax group (TrxG) protein absent, small or homeotic discs 2 (Ash2) as a novel factor on the Xi that is recruited at maintenance of X inactivation.

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MATERIALS AND METHODS
Screening of autoimmunity sera
Screening of autoimmunity sera was performed on HEK293 cells grown on Roboz slides. Briefly, slides were washed in phosphate-buffered saline (PBS), pre-extracted in CSK buffer [10 mM Pipes (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂] with 0.5% Triton X-100, washed in PBS and fixed in 4% paraformaldehyde (PFA). After blocking in PBS, 3% bovine serum albumin (BSA) and 0.1% Tween-20 for 1 hour, slides were incubated with human sera diluted 1:50 and 1:300 in blocking buffer and rabbit α-H3K27me3 antiserum (1:1000). Subsequently optimal dilutions were determined: serum #184, 1:500/1:500 [immunofluorescence (IF)/western analysis]; serum #516, 1:600/1:500.

Cell culture and generation of ES cell lines
ΔAS ES cells (Wutz et al., 2002), clone 36 ES cells (Wutz and Jaenisch, 2000), and Eed deficient (Schoof et al., 2006) and Ring1B deficient ES cells (Leeb and Wutz, 2007) were cultured as described previously. Xist was induced with 1 μg/ml doxycycline. Differentiation medium contained 100 nM all-trans retinoic acid and no leukaemia inhibitor factor (LIF). For SAF-A expression, human SAF-A transcript variant 2 (IAPRT970D051D6, RZPD, Germany) or mouse Saf-A transcript variant 2 (Fantom 2, RIKEN, Japan) were amplified introducing EcoRI and BamHI sites and cloned into a modified pcAGGS vector (Niwa et al., 1991). The hrGFP cDNA (Stratagene) was inserted into the BamHI site. SAF-A deletions were introduced by digesting pcAGGS-SAF-A with SacI and re-ligating the plasmid (del1-3). The fragment between the first and second SacI site in the SAF-A cDNA (del1-2) or an oligomer restoring the SAP domain was subsequently deleted (del1-3 + oligo). Primer sequences were GCGGAATTCGAATCGGGGCCCTCACCATG and GCGGATCCCTATTACAC for hrGFP; the sequence of the inserted double MCS was GCGGAATTCGAATCGGGGCCCTCACCATG and GCGGATCCCTATTACAC for mouse SAF-A; and GCGGATCCCTATTACAC for mouse hrGFP. After selection with hygromycin B (130 μg/ml), resistant clones were subcloned for stable Saf-A:hrGFP expression.

H4 acetylation staining and DNA FISH
H4 acetylation staining was performed on native metaphases as described (Chaumeil et al., 2006). Briefly, ES cells were arrested with colcemid (0.1 μg/ml; Sigma) for 1 hour, trypsinized and incubated in hypotonic medium diluted with sterile water (1:1) for 10 minutes. Cells were cytospun [1800 rpm (380 g), 8 minutes] on poly-l-lysine coated slides and incubated for 30 minutes in KCM buffer [120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl (pH 8), 0.5 mM EDTA and 0.1% Triton X-100] containing 3% BSA. After staining with α-hyperacetylated H4 (penta) antibody (Upstate) diluted 1:500 in KCM, 10% normal goat serum, slides were washed three times with KCM for 5 minutes and incubated with goat anti-rabbit IgG secondary antibody in KCM, 1% normal goat serum for 40 minutes. After three washes in KCM, slides were fixed in 3% PFA and counterstained with DAPI. After image acquisition, DNA FISH was performed as recommended by the supplier (Star Fish Mouse X, Cy3 labelled, Cambio).

RNA and protein analysis
Northern analysis was performed using 15 μg of RNA (Trizol; Invitrogen) as described (Wutz and Jaenisch, 2000). Quantitative expression analysis of X-linked genes was performed as described (Blewitt et al., 2008). Total proteins were extracted in 10 mM Tris-HCl (pH 7.6), 0.5 mM EDTA, 1 mM EGTA, 0.4 M NaCl at 95°C for 15 minutes. For polyacrylamide gel electrophoresis, samples were diluted 1:4. For immunoprecipitation, nuclear proteins were extracted in 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 0.1 mM EDTA, 25% glycerol, 0.4 M NaCl after the cytoplasmatic fraction had been separated. The following antibodies were used for IF/western analysis: α-H3K27me3 [1:1000/– (Leeb and Wutz, 2007)]; α-hrGFP (+/-/5000; Stratagene); α-LaminB (∼/1:5000; Abcam); α-Ash2L (1:600/1:1000; Abcam); α-BAZ2A (1:300/1:107A).

RESULTS
An antibody screen for components of the Xi facultative heterochromatin
In order to characterize the chromatin composition of the Xi, we examined a collection of antisera directed against chromatin proteins for specific staining of the Xi. A polyclonal rabbit antisera specific for Ash2L, the mammalian homologue of the Drosophila absent, small or homeotic discs (Ash2) protein, recognized the Xi in differentiated mouse cells (Fig. 1). We also screened 800 human autoimmune sera by immunofluorescence staining of female human embryonic kidney (HEK) 293 cells. These cells have two prominent inactive X chromosomes, on which enrichment for factors of the Xi has previously been observed. Four autoimmune sera showed an Xi active marking as described (Kohlmaier et al., 2004). Briefly, cells were pre-extracted with CSK for 2 minutes, fixed for 10 minutes in 4% PFA in PBS, permeabilized for 5 minutes at room temperature in 0.1% sodium citrate/0.5% Triton X-100, and blocked for 1 hour in PBS, 5% BSA, 0.1% Tween-20. Antisera were diluted in blocking solution and washed performed in PBS, 0.1% Tween-20. Specificity of the Ash2L antibody was confirmed by pre-incubation with 10 μg/ml of blocking peptide (Bethyl Laboratories, BP300-107) for 15 minutes. For RNA fluorescence in situ hybridization (FISH), cells were pre-extracted in CSK, fixed in 4% PFA in PBS for 10 minutes at 4°C, dehydrated, hybridized and washed as described (Wutz and Jaenisch, 2000). RNA FISH probes were generated by random priming (Stratagene, USA) using Cy3-dCTP (Amersham). Images were obtained using a fluorescence microscope (Zeiss Axioplan) equipped with a Coolsnap fx CCD camera (Photometrics) and the MetaMorph image analysis software (Universal Imaging, USA). Brightness and contrast were adjusted in Photoshop 7.0 (Adobe).

Analysis of Saf-A:hrGFP expressing cells, immunostaining and RNA FISH
For hrGFP analysis, cells were grown on Roboz slides (CellPoint Scientific, USA), washed twice in PBS and either directly visualized or fixed with 4% PFA for 10 minutes and counterstained with DAPI. Immunostaining was performed as described (Kohlmaier et al., 2004). Briefly, cells were pre-extracted with CSK for 2 minutes, fixed for 10 minutes in 4% PFA in PBS, permeabilized for 5 minutes at room temperature in 0.1% sodium citrate/0.5% Triton X-100, and blocked for 1 hour in PBS, 5% BSA, 0.1% Tween-20. Antisera were diluted in blocking solution and washed performed in PBS, 0.1% Tween-20. Specificity of the Ash2L antibody was confirmed by pre-incubation with 10 μg/ml of blocking peptide (Bethyl Laboratories, BP300-107) for 15 minutes. For RNA fluorescence in situ hybridization (FISH), cells were pre-extracted in CSK, fixed in 4% PFA in PBS for 10 minutes at 4°C, dehydrated, hybridized and washed as described (Wutz and Jaenisch, 2000). RNA FISH probes were generated by random priming (Stratagene, USA) using Cy3-dCTP (Amersham). Images were obtained using a fluorescence microscope (Zeiss Axioplan) equipped with a Coolsnap fx CCD camera (Photometrics) and the MetaMorph image analysis software (Universal Imaging, USA). Brightness and contrast were adjusted in Photoshop 7.0 (Adobe).
mass spectrometric analysis identified SAF-A as a likely target of
serum #184. This was confirmed by western analysis of cells
expressing a full-length and a Myc-tagged SAF-A protein (Fig. 2B).
Enrichment of SAF-A on the Xi has been reported previously
(Helbig and Fackelmayer, 2003). In contrast to previous antisera,
which recognized the Xi only in matrix preparations after chromatin
extraction, serum #184SAF-A recognized the Xi in over 80% of the
cells using standard immunofluorescence staining. Serum #184SAF-A
also recognized SAF-A enrichment on the Xi in mitotic cells (Fig.
2A), showing that SAF-A is maintained on the Xi throughout the
cell cycle. We conclude that an antisera screen is useful for
characterization of chromatin of the Xi and has led to the
identification of Ash2l as a novel component.

Ash2l and SAF-A recruitment on the Xi requires
Xist but not gene silencing
To investigate whether Xist was required for Ash2l recruitment, we
derived immortalized mouse 3T3 cell lines from female day 13.5
embryonic fibroblasts homozygous for a conditional
Xist allele

Fig. 1. Ash2l is a component of the Xi. (A) 3T3 cells were derived
from female mouse embryos homozygous for a conditional
endogenous Xist allele and heterozygous for the rtTA transactivator and
da doxycycline-inducible tetOP-Cre. Focal enrichment of Saf-A on the Xi
was observed before Cre induction (–Cre) and was lost after
doxycycline-induced deletion of Xist (+Cre). DNA is stained by DAPI
(blue). (B) Immunofluorescence staining (IF) of ∆SX ES cells after 6 days
of differentiation with Xist induction using serum #516 to detect
macroH2A (green) and Ash2l (red) on the X chromosome. Specificity of
the staining was confirmed by incubation with an Ash2l blocking
peptide (lower panel). A merged picture with DNA stained by DAPI
(blue) is shown. Scale bars: 5 μm. (C) Ash2l recruitment was analyzed in
cloned ES cells harboring an inducible Ash2l RNAi construct or control
clones on day 6 of differentiation in the presence of doxycycline. The
number of cells showing Ash2l foci and Xist clusters is given (left).
Ash2l RNAi clones 1 and 2 contain independent siRNA sequences.
Western analysis (right) of the clones after 6 days of differentiation shows
strong RNAi targeted downstream of the Col1a1 locus emphasizing
the presence of Ash2l RNAi. The number of cells showing Ash2l foci and
Xist clusters is shown. Western analysis (right) of clones 1 and 2
showing a target sequence downstream of the Col1a1 locus shows
that induction of RNAi with doxycycline (dox) reduces Ash2l protein.
Lamin B was used as a loading control.

Fig. 2. A human autoimmune serum recognizes SAF-A on the Xi.
(A) IF of HEK293 cells using serum #184 (green) and an antibody
specific for H3K27me3 (red). Enrichment of the target antigen is
observed in interphase (upper panels) and in mitosis (lower panels).
DNA is stained by DAPI. Scale bars: 5 μm. (B) Western analysis of total
protein extracted from 293 cells using serum #184 identifies a band of
120 kDa (left). Bands corresponding to transgenic SAF-A proteins are
identified in 293 cells transiently transfected with the SAF-A constructs
indicated (right). Lamin B was used as a loading control. (C) 3T3 cells
homozygous for a conditional Xist allele and carrying an inducible Cre
transgene were stably transfected with SAF-A:hrGFP. Focal enrichment
of Saf-A on the Xi was observed before Cre induction (–Cre) but was
lost after doxycycline-induced deletion of Xist (+Cre). DNA is stained by
DAPI (blue).
(Csankovszki et al., 1999), which also carried a tetracycline regulated Cre recombinase transgene (Schonig et al., 2002). In these 3T3 Xist<sup>2LOX</sup>/Xist<sup>2LOX-R26-ntTA</sup>L<sup>C</sup>L<sup>C</sup> cells, Xist can be deleted by the addition of doxycycline. Focal Ash2l staining was observed in 90% of these cells (Fig. 1A). When Xist deletion was induced by the addition of doxycycline, Ash2l foci were lost within 3 days (Fig. 1A). We also observed Ash2l recruitment on day 6 of differentiation in ΔSX ES cells that expressed a mutated Xist that lacked the repeat A and, thus, did not cause gene silencing (Fig. 1B). This indicated that recruitment of Ash2l by Xist did not require gene silencing. We confirmed the specificity of the Ash2l antiserum using a blocking peptide that eliminated Ash2l but not macroH2A staining (Fig. 1B).

To unambiguously establish the identity of Ash2l on the Xi, we used RNAi. We constructed ES cell lines in which siRNAs could be produced from a microRNA precursor construct (Paddison et al., 2004). In undifferentiated clone 36 ES cells, Xist can be induced from a cDNA transgene integrated on chromosome 11 (Wutz and Jaenisch, 2000). In Xist-expressing clone 36 ES cells, focal Ash2l staining was observed in over 70% of the cells after day 6 of differentiation. Two independent inducible Ash2l RNAi cassettes were introduced downstream of the Colla1 gene by gene targeting (see Fig. S2 in the supplementary material). Induction of Ash2l RNAi decreased Ash2l protein (Fig. 1C) and led to a reduction in both the intensity and the frequency of focal Ash2l staining on day 6 of differentiation. This showed that the Ash2l antiserum was specific and confirmed Ash2l as a novel component of the Xi. We further examined gene silencing in cells after induction of Ash2l RNAi. Xist triggered the silencing of a puromycin resistance marker that is co-integrated with the Xist transgene with equal efficiency in parental and Ash2l RNAi cells. This indicates that under our conditions Ash2l is not required for gene silencing by Xist.

Serum #184SAF-A did not recognize the mouse Saf-A protein in western or immunofluorescence analysis, indicating exclusive specificity for the human protein (data not shown). To investigate Saf-A recruitment in X inactivation further, we introduced a Saf-A green fluorescence protein fusion (Saf-A:hrGFP) as a transgene into 3T3 Xist<sup>2LOX</sup>/Xist<sup>2LOX-R26-ntTA</sup>L<sup>C</sup>L<sup>C</sup> cells. We observed a focal Saf-A:hrGFP signal on the Xi in over 70% of these cells. When Xist deletion was induced with doxycycline, Saf-A staining was lost and Saf-A assumed a uniform nuclear distribution, showing that Saf-A recruitment to the Xi required Xist (Fig. 2C). To further test Saf-A recruitment by Xist, we introduced the Saf-A:hrGFP transgene into clone 36 ES cells, in which Xist can be induced from a transgene on chromosome 11, and into ΔSX ES cells, in which a mutant Xist that lacks repeat A and does not initiate gene silencing can be induced (Fig. 3A,B). Focal Saf-A:hrGFP signals overlapping H3K27me3 foci were observed in 60% of ΔSX Saf-A:hrGFP ES cells and 20% of 36<sup>SX</sup>Saf-A:hrGFP ES cells after 6 days of differentiation with Xist induction. The lower number of Saf-A foci in 36<sup>SX</sup>Saf-A:hrGFP ES cells compared with ΔSX Saf-A:hrGFP ES cells is in part explained by differences in transgene expression. Focal signals were not observed in cultures differentiated without Xist induction. This showed that, similar to Ash2l, Saf-A recruitment also required Xist expression but not gene silencing. Previously, a long residence time for SAF-A on the Xi was observed suggesting that SAF-A was part of a highly stable structure in the territory of the Xi (Fackelmayer, 2005). We further tested the kinetics of Saf-A loss after turning off Xist expression in ES cell differentiation. In differentiated ΔSX Saf-A:hrGFP ES cells, focal Saf-A:hrGFP signals decreased in parallel with Xist clusters (Fig. 3C). Saf-A:hrGFP loss was faster than that of H3K27me3, which disappeared with a delay from the X chromosome (Fig. 3C). Similarly, Saf-A:hrGFP staining was lost in differentiated 36<sup>SX</sup>Saf-A:hrGFP ES cells, where gene repression was maintained (Fig. 3D). Saf-A recruitment to the Xi was therefore strictly dependent on Xist, consistent with the observation that...
recruitment requires the RNA-binding domain of SAF-A (Helbig and Fackelmayer, 2003). We further investigated whether the SAP domain, which has been implicated in DNA binding, was also required. For this we examined the localization of SAF-A:hrGFP deletions in 293 cells. We observed that deletions within the SAP domain abrogated the localization of SAF-A to the Xi and led to a loss of association with mitotic chromosomes (see Fig. S3 in the supplementary material). This suggests that DNA binding of SAF-A is required together with its RNA-binding ability for the association with the Xi. We conclude that Ash2l and Saf-A are components of the Xi in differentiated cells that require Xist for recruitment to the Xi but not gene silencing.

Concurrent recruitment of Ash2l and Saf-A by Xist in ES cell differentiation

We next characterized the timing of Ash2l, Saf-A and macroH2A recruitment in ES cell differentiation (Fig. 4). In undifferentiated ΔSXist ES cells, focal Saf-A:hrGFP signals were not observed after induction of Xist expression. After 2 days of differentiation, only 5% of the cells showed a focal Saf-A:hrGFP signal. The percentage of cells showing Saf-A foci increased to 45% between days 3 and 4, and finally reached 66% on day 8, which corresponds to 80% of Xist-expressing cells (Fig. 4B). In parallel cultures, we detected Ash2l, macroH2A and H3K27me3 by immunofluorescence staining. Ash2l recruitment was first observed in few cells on day 2 of differentiation, with a sharp rise in the percentage of cells showing Ash2l foci between days 3 and 4 (Fig. 4C). Thus, recruitment of Ash2l largely followed the same kinetics as Saf-A. We followed the recruitment of macroH2A using serum #516macroH2A (Fig. 4D). Detection of macroH2A was less efficient and at the final time point we detected clear macroH2A foci in only 30% of the cells, compared with over 70% for Ash2l. This could be due to a less prominent enrichment of macroH2A on the Xi over the nuclear average (Mietton et al., 2009). The number of cells showing macroH2A enrichment increased with slower kinetics than that of Saf-A and Ash2l, and reached a maximum between days 4 and 8 (Fig. 4D). Previously, a later time point for macroH2A recruitment was also determined the recruitment of H3K27me3, which is an early marker of X inactivation. Focal H3K27me3 signals were observed in 12% of undifferentiated ΔSXist ES cells and in 75% of the cells after 3 days of differentiation (Fig. 4A), in agreement with our previous results (Kohlmaier et al., 2004). Therefore Saf-A, Ash2l and macroH2A were recruited about 2 days later in ES cell differentiation than H3K27me3. The timing of recruitment overlapped with the transition to Xi maintenance and suggested that Ash2l and Saf-A mark the shift to stable X inactivation.

Ash2l, Saf-A and macroH2A recruitment is regulated by a chromosomal memory

We have previously observed that the recruitment of PcG complexes to the Xi in differentiated cells is regulated by a chromosomal memory (Kohlmaier et al., 2004). In differentiated ES cells, Xist expression is not sufficient for efficient PcG complex recruitment but a memory is required that is established by expression of Xist in early differentiation. Establishment of this memory does not require gene silencing and occurs at the onset of Xi maintenance (Kohlmaier et al., 2004). Thus, recruitment of this memory and Ash2l, Saf-A and macroH2A are temporally correlated. To explore whether recruitment of these proteins was also regulated by a memory, we tested whether Ash2l, Saf-A and macroH2A recruitment required Xist expression during early differentiation. For this we induced Xist expression at progressively later time points in differentiating ΔSXist ES cells and analyzed recruitment after 12 days of differentiation (Fig. 5A). Focal Saf-A signals were observed in 64% and 71% of the cells when Xist was induced from the beginning of differentiation or one day thereafter. However, when Xist was induced after 3 or 6 days of differentiation, only 20% or 16% of the cells showed Saf-A foci, respectively (Fig. 5A). Similarly, efficient recruitment of macroH2A and Ash2l required that Xist was induced within one day after the onset of differentiation (Fig. 5A). Induction of Xist after 3 days of differentiation resulted in a markedly reduced efficiency of recruitment. This demonstrated that early Xist expression in ES cell differentiation regulated the ability of Xist to recruit Ash2l, Saf-A and macroH2A efficiently at later time points in differentiation.

**Fig. 4. Timing of Saf-A and Ash2l recruitment in ΔSXist ES cell differentiation.** An analysis of H3K27me3 (A), Saf-A (B), Ash2l (C) and macroH2A (D) recruitment by Xist in either undifferentiated ΔSXist ES cells or after 1, 2, 3, 4 and 8 days of differentiation in the presence of doxycycline is shown. The percentage of cells showing focal enrichment is given. The experiments were performed twice for macroH2A and Ash2l, and three times for Saf-A and H3K27me3. A representative experiment is shown (n=100).
We next investigated for how long \( Xist \) needed to be expressed in early differentiation. For this, we induced \( Xist \) in \( \Delta SX \) \( Saf-A:hrGFP \) ES cells for 1, 2, 3 and 4 days from the start of differentiation, then turned off \( Xist \) and re-induced expression at day 8 for 4 more days (Fig. 5B). We observed efficient recruitment of \( Saf-A \), \( Ash2l \) and \( H3K27me3 \) when \( Xist \) had been induced during the first 3 days of differentiation (Fig. 5B). When \( Xist \) was induced for less than three days at the beginning of differentiation, recruitment of all three proteins was significantly reduced. This indicated that \( Xist \) expression throughout the first 3 days of differentiation led to a change on the chromosome that enabled efficient recruitment at later stages, consistent with the establishment of a memory structure at the transition to stable X inactivation (Kohlamaier et al., 2004). Continuous \( Xist \) expression throughout differentiation was then not required for efficient \( Ash2l \), \( Saf-A \) and macroH2A recruitment, showing that memory maintenance did not require \( Xist \).

To investigate whether recruitment of \( Saf-A \) and \( Ash2l \) by \( Xist \) was dependent on Polycomb complex function, we analyzed ES cells with a disruption of \( Eed \) or \( Ring1B \), which lack PRC2 and PRC1 activity, respectively. Immunostaining showed that \( Ash2l \) was efficiently recruited by \( Xist \) RNA in clone 36 ES cells with a disruption of \( Eed \) or \( Ring1B \) (see Fig. S4A, B and Table S1 in the supplementary material). Furthermore, \( Saf-A \) was efficiently recruited by \( Xist \) in \( \Delta SX \) \( Saf-A:hrGFP \) ES cells with a disruption of \( Eed \) (see Fig. S4C in the supplementary material). Taken together, these data suggest that recruitment of \( Ash2l \) and \( Saf-A \) is independent of Polycomb complex function. Conversely, we observed that \( Xist \) triggered efficient \( H3K27me3 \) and \( ubH2A \) in clone 36 ES cells expressing an \( Ash2l \) microRNA precursor (see Fig. S4D and Table S2 in the supplementary material). This indicates that \( Ash2l \) is also not required for PRC1 or PRC2 recruitment by \( Xist \), which is consistent with our observation that recruitment of Polycomb complexes occurs before \( Ash2l \) and \( Saf-A \) recruitment in ES cell differentiation.

**Gene silencing is not required for global hypoacetylation of histone H4 on the Xi**

Our results showed that PcG complexes, \( Ash2l \), \( Saf-A \) and macroH2A were recruited by \( Xist \) lacking repeat A, suggesting that most chromatin marks of the Xi did not depend on gene silencing. We therefore investigated histone H4 hypoacetylation, which is a prominent mark of the Xi in differentiated cells (Keohane et al., 1996). Histone deacetylation contributes to the stability of gene silencing on the Xi (Csankovszki et al., 2001). It has been demonstrated that H4 hypoacetylation is maintained on the Xi when \( Xist \) is conditionally deleted in female mouse fibroblasts (Csankovszki et al., 1999). Stability of histone H4 hypoacetylation contrasts all other chromatin marks of the Xi, which depend on \( Xist \) expression for their recruitment. To investigate whether \( Xist \) repeat A and gene silencing were required for X chromosome-wide histone H4 hypoacetylation, we performed immunofluorescence staining on native chromosome spreads of \( \Delta SX \) ES cells that were differentiated for 1, 3 or 5 days. Hypoacetylated X chromosomes were detected in spreads of \( \Delta SX \) cells, which were differentiated for 3 or more days in the presence of doxycycline (Fig. 6A, B). Before day 3 of differentiation, hypoacetylated chromosomes were not observed, consistent with previous reports in differentiating female ES cells (Chaumeil et al., 2002; Keohane et al., 1996). Albeit the majority of the chromosomal sequences were deacetylated, we observed bands of acetylated histone H4 on the Xi (Fig. 6A and gene silencing were required for X chromosome-wide histone H4 hypoacetylation, which is a prominent mark of the Xi in differentiated cells (Keohane et al., 1996). Histone deacetylation contributes to the stability of gene silencing on the Xi (Csankovszki et al., 2001). It has been demonstrated that H4 hypoacetylation is maintained on the Xi when \( Xist \) is conditionally deleted in female mouse fibroblasts (Csankovszki et al., 1999). Stability of histone H4 hypoacetylation contrasts all other chromatin marks of the Xi, which depend on \( Xist \) expression for their recruitment. To investigate whether \( Xist \) repeat A and gene silencing were required for X chromosome-wide histone H4 hypoacetylation, we performed immunofluorescence staining on native chromosome spreads of \( \Delta SX \) ES cells that were differentiated for 1, 3 or 5 days. Hypoacetylated X chromosomes were detected in spreads of \( \Delta SX \) cells, which were differentiated for 3 or more days in the presence of doxycycline (Fig. 6A, B). Before day 3 of differentiation, hypoacetylated chromosomes were not observed, consistent with previous reports in differentiating female ES cells (Chaumeil et al., 2002; Keohane et al., 1996). Albeit the majority of the chromosomal sequences were deacetylated, we observed bands of acetylated histone H4 on the X chromosome in \( \Delta SX \) cells. This was in contrast to the fully deacetylated pattern known from the silent Xi (Keohane et al., 1996; Wutz and Jaenisch, 2000) and suggested that bands of acetylated histone H4 could indicate regions of active genes. This showed that expression of \( Xist \) lacking repeat A had led to the formation of an X chromosome with all Xi-associated chromatin features but on which genes were active. For ease of reference, we propose the term Xiag (Xi with active genes) for this novel chromosome configuration.

Notably, in male \( \Delta SX \) cells the Xiag was the only X chromosome and was apparently fully functional. To further test the effect of chromosome-wide modifications on the Xiag on the expression of...
X-linked genes, we performed quantitative RT-PCR in undifferentiated and differentiated ΔSX ES cells either expressing Xist or not. In undifferentiated ΔSX ES cells, the expression of 13 X-linked genes tested was not affected by the expression of Xist lacking repeat A (see Fig. S5A in the supplementary material). After 4 days of differentiation, three of the 13 X-linked genes were expressed at reduced levels in cells that expressed Xist compared with in cells differentiated without induction (see Fig. S5B in the supplementary material). This suggests that chromatin modifications on the Xiag had a modulatory effect on the expression of a minority of X-linked genes after differentiation.

To further investigate whether histone H4 hypoacetylation was maintained in the absence of gene silencing in ΔSX ES cells, we turned off Xist expression on day 4 of differentiation and analyzed chromosome spreads on day 9 of differentiation (Fig. 6C). We still detected hypoacetylated chromosomes at this time point showing that in differentiated ΔSX ES cells hypoacetylation of histone H4 could be maintained independently of Xist and gene repression. Delayed induction of Xist on day 4 of differentiation triggered chromosomal hypoacetylation to a lesser extent. In addition, we did not detect maintenance of hypoacetylation after turning off Xist, suggesting that Xist expression in early ES cell differentiation was required for stable deacetylation of the chromosome. To investigate whether other marks associated with active chromatin would behave in a similar way, we studied histone H3 lysine 4 di- and tri-methylation (see Fig. S6 in the supplementary material). We observed that Xist expression led to a reduction in methylation of lysine 4. However, we were unable to detect maintenance of either reduced di- or tri-methylation when Xist was turned off. Furthermore, when using commercial antisera specific for histone H3 lysine 9 acetylation, we did not detect a deacetylated X chromosome, despite a clearly reduced signal on the centromers and Y chromosome (see Fig. S6 in the supplementary material). Taken together, our results demonstrate that a stable chromatin configuration was established on the Xiag independent of gene silencing and specifically correlated with hypoacetylation of histone H4.

DISCUSSION
Xi maintenance highlights a developmental chromatin switch in ES cell differentiation

We observe the coordinated recruitment of Ash2l, Saf-A and macroH2A to the inactive X chromosome at a defined time point in ES cell differentiation. Recruitment of these proteins requires the expression of Xist and occurs at the transition to stable X inactivation. By immunofluorescence staining and protein tagging methods, recruitment of these proteins to the Xi was not observed in undifferentiated ES cells or at early time points in differentiation. This shows that, at least quantitatively, the recruitment of Ash2l, Saf-A and macroH2A to the Xi are dependent on the context of differentiated cells. Changes in the composition and the dynamics of chromatin have previously been reported during the differentiation of ES cells (Meshorer et al., 2006). This has led to the view that ES cell differentiation involves a fundamental change in chromatin structure and function. Our observations of coordinated changes highlight a specific stage in the differentiation process when a discrete transition of the composition of the facultative heterochromatin of the Xi occurs.

Recruitment of Ash2l, Saf-A, macroH2A and PcG complexes can be observed when a mutated Xist RNA is expressed that does not initiate gene silencing. We also show that chromosome-wide histone H4 hypoacetylation is triggered without gene repression. Albeit these components of the facultative heterochromatin of the Xi do not necessarily cause gene repression, we show that these chromatin modifications can lead to a modulation of the expression of three out of 13 X-linked genes examined after differentiation, but not in undifferentiated ES cells. At present we cannot entirely rule out that certain genes might become silenced by Xist lacking repeat A, but our data show that the majority of genes are minimally affected by chromatin modifications on the Xiag.

The observation of an X chromosome that carries all modifications known from the Xi and on which genes are transcribed is surprising. We suggest that this might be the result of a separation of chromatin of genes and non-genic sequences on the Xi. A key observation to explain this is that at the initiation of X
inactivation $Xist$ can be shown to accumulate in the repetitive core of the X chromosome where it generates a repressive compartment. At this stage, genes are positioned in the periphery of the chromosome territory in association with the transcription machinery (Chaumeil et al., 2006). Gene silencing is mediated in a separate step that requires repeat A of $Xist$ and involves cell type-specific pathways (Agrelo et al., 2009). Upon silencing, genes are recruited into the repressive compartment of the Xi (Chaumeil et al., 2006). $Xist$ lacking repeat A can establish a repressive compartment (Chaumeil et al., 2006). However, in the absence of repeat A silencing is not initiated and genes remain in the periphery of the chromosome territory; they are thus spatially separated from the $Xist$ covered centre of the chromosome territory (Chaumeil et al., 2006).

We interpret the changes in the composition of the facultative heterochromatin of the Xi during ES cell differentiation as a mark for the establishment of a stable chromatin structure for the maintenance of the repressive compartment. Spatial separation of genes from the repressive compartment in cells expressing $Xist$ lacking repeat A could prevent them from being subject to chromatin modifications and silencing. Thus, the separation of genes and non-germinal chromat in the Xi highlights a crucial role for the 3D organization of chromatin in X inactivation.

**The TrxG protein Ash2l is a novel component of the Xi**

Using antisera screening, we were able to establish Ash2l as a component of the Xi. Ash2l has previously been associated with TrxG complexes (Angulo et al., 2004; Steward et al., 2006; Wysocka et al., 2003; Yokoyama et al., 2004). TrxG proteins are linked to PcG regulation and have been implicated in gene activation rather than repression (Schuettengruber et al., 2007). Thus, the observation of Ash2l on the Xi is unexpected. Ash2l is a component of complexes containing HCF-1 or homologues of the MII and Set1 histone methyltransferases (Wysocka et al., 2003; Yokoyama et al., 2004). Human ASH2L is required for trimethylation of lysine 4 on histone H3 (Steward et al., 2006), which is associated with active promoters (Mikkelsen et al., 2007). In tumor lines, Ash2L is essential for cell proliferation and can also have oncogenic function when overexpressed (Luscher-Firzlaff et al., 2008). We were, thus far, unable to detect TrxG proteins other than Ash2l on the Xi. Although this could reflect limitations of the antibodies, staining procedure or analyzed cell types, it is likely that Ash2l is recruited to the Xi independently of other TrxG proteins. This would also be consistent with the observation that histone H3 lysine 4 trimethylation is not enriched but actually depleted along the Xi (Kohlmaier et al., 2004). We suggest that Ash2l could act as a regulatory component of PcG/TrxG-mediated memory on the Xi. A study in *Drosophila* imaginal wing discs has previously implicated Ash2 in the formation of a chromatin structure to assist the access of proteins that activate or repress gene expression (Angulo et al., 2004). Recruitment of Ash2l to the Xi could serve a similar structural role, which is independent of the known function of Ash2l for histone H3 lysine 4 trimethylation.

**Saf-A, Ash2l and macroH2A recruitment requires $Xist$ and is regulated by a memory**

$Xist$ is required for Saf-A, Ash2l and macroH2A recruitment to the X chromosome. Once $Xist$ expression is lost, these proteins are displaced from the chromosome showing that their recruitment is reversible and dependent on $Xist$. However, $Xist$ induction in differentiated cells is not sufficient for efficient recruitment. This suggests an additional regulator for Saf-A, Ash2l and macroH2A recruitment. We have previously reported that the recruitment of PcG complexes to the Xi in differentiated cells depends on $Xist$ expression during a time window in early ES cell differentiation (Kohlmaier et al., 2004; Schoeftner et al., 2006). To explain this, we have proposed a chromosomal memory that is required in combination with $Xist$ for efficient recruitment of PcG complexes in differentiated cells. Here, we find that the recruitment of Ash2l, Saf-A and macroH2A is similarly regulated by a chromosomal memory. $Xist$ expression during the first 3 days of ES cell differentiation is required for efficient recruitment of these proteins by $Xist$ induction in differentiated cells. The effect on recruitment efficiency is not dependent on continuous $Xist$ expression throughout differentiation or on gene silencing. The molecular basis of this chromosomal memory remains unclear thus far. We note that histone H4 hypoacetylation has properties that would be expected for such a memory. Histone H4 hypoacetylation is observed at the time of memory establishment, it can be established independently of gene silencing and it is maintained independently of $Xist$.

SAF-A has been previously reported to be enriched on the Xi (Helbig and Fackelmayer, 2003). This required the presence of the RNA-binding RGG domain of SAF-A. We now show that $Xist$ is required for Saf-A recruitment to the Xi. Yet, Saf-A recruitment by $Xist$ is not observed in undifferentiated ES cells. We find that the DNA-binding SAP domain is required for Saf-A recruitment by $Xist$ in addition to its RNA-binding RGG domain. Because the SAP domain is necessary for chromatin association, it is conceivable that it recognizes a specific chromatin state, which is established by $Xist$ during early differentiation. Such a signal could be the hypoacetylation of histone H4. SAF-A has binding affinity for AT-rich scaffold/matrix attachment region sequences (Kipp et al., 2000). Both $Xist$ RNA and SAF-A have been detected in the nuclear matrix fraction after removal of chromatin (Clemson et al., 1996; Helbig and Fackelmayer, 2003). SAF-A has been implicated in many processes, including transcription, RNA transport, DNA replication and circadian rhythm (Onishi et al., 2008). Saf-A is required for cell proliferation and a hypomorphic mutation in mice causes embryonic lethality (Roshon and Ruley, 2005). Although this precludes a direct genetic investigation of Saf-A function in X inactivation, our results suggest that Saf-A together with Ash2l could be components for either memory imposition or stabilization of the Xi.

The transition to stable X inactivation represents a unique opportunity for studying chromosome organization in a defined state during the differentiation of ES cells. Different proteins with apparently unlinked function and structure, such as Saf-A, Ash2 and macroH2A, act together in regulating the facultative heterochromatin of the Xi. This highlights the importance of defining the interface between transcription regulation and 3D chromatin organization in future studies.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.035956/-/DC1
References


Table S1. Recruitment of Ash2l by Xist in Eed and Ring1B deficient ES cells

<table>
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<th>Clone 36</th>
<th>Eed&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Ring1B&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
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<td>Ash2l foci</td>
<td>60±7</td>
<td>59±4</td>
<td>55±3</td>
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Table S2. Recruitment of Polycomb complex function by Xist in clone 36 Ash2l RNAi ES cells

<table>
<thead>
<tr>
<th></th>
<th>Clone 36</th>
<th>Clone 1</th>
<th>Clone 2</th>
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</thead>
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<tr>
<td>H3K27me3</td>
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<td>78±3</td>
<td>79±8</td>
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<td>ubH2A</td>
<td>77±1</td>
<td>80±6</td>
<td>74±6</td>
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