Role of ATRX in chromatin structure and function: implications for chromosome instability and human disease

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Abstract

Functional differentiation of chromatin structure is essential for the control of gene expression, nuclear architecture, and chromosome stability. Compelling evidence indicates that alterations in chromatin remodeling proteins play an important role in the pathogenesis of human disease. Among these, α -thalassemia mental retardation X-linked protein (ATRX) has recently emerged as a critical factor involved in heterochromatin formation at mammalian centromeres and telomeres as well as facultative heterochromatin on the murine inactive X chromosome. Mutations in human ATRX result in an X-linked neurodevelopmental condition with various degrees of gonadal dysgenesis (ATRX syndrome). Patients with ATRX syndrome may exhibit skewed X chromosome inactivation (XCI) patterns, and ATRX-deficient mice exhibit abnormal imprinted XCI in the trophoblast cell line. Non-random or skewed XCI can potentially affect both the onset and severity of X-linked disease. Notably, failure to establish epigenetic modifications associated with the inactive X chromosome (Xi) results in several conditions that exhibit genomic and chromosome instability such as fragile X syndrome as well as cancer development. Insight into the molecular mechanisms of ATRX function and its interacting partners in different tissues will no doubt contribute to our understanding of the pathogenesis of ATRX syndrome as well as the epigenetic origins of aneuploidy. In turn, this knowledge will be essential for the identification of novel drug targets and diagnostic tools for cancer progression as well as the therapeutic management of global epigenetic changes commonly associated with malignant neoplastic transformation.

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Emerging roles of α -thalassemia mental retardation X-linked protein in chromatin remodeling and control of gene expression

The α -thalassemia mental retardation X-linked protein (ATRX) is a member of the Switch 2, sucrose non-fermenting 2 (SWI2/SNF2) family of helicases/ATPases that exhibits chromatin remodeling activity (Picketts *et al.* 1996, Gibbons *et al.* 1997, Villard *et al.* 1997, Xue *et al.* 2003, Tang *et al.* 2004a). The ATRX gene maps to the long arm of the human X chromosome and is subject to X chromosome inactivation (XCI; Gibbons *et al.* 1995, 2008). It encodes a 280 kDa protein with an unusual N-terminal plant homeodomain (PHD) flanked by a coil-coil motif termed the ATRX-DNMT3-DNMT3L (ADD) domain due to its similarity to a protein region found in this group of DNA methyl transferases. The protein also contains several helicase domains at the carboxyl terminal region conferring ATPase activity and essential for interaction with several factors including the human methyl CpG binding protein MeCP2 (Picketts *et al.* 1998, Bérubé *et al.* 2000, Argentaro *et al.* 2007, Nan *et al.* 2007). ATRX also encodes a truncated isoform of ~200 kDa lacking the helicase domain (Garrick *et al.*

2004). Comparison of the nucleotide sequence revealed that the human and mouse genes exhibit 85% sequence identity and, therefore, a high level of evolutionary conservation (Picketts *et al.* 1998). In addition to the helicase domains, the protein contains a stretch of glutamic acid residues and a coil-coil domain. Hence, it has the potential to establish a direct interaction with DNA as well as with several functional partners, presumably assembling with different chromatin remodeling or transcriptional regulatory complexes according to the cell type, stage of the cell cycle, or stage of differentiation (Ishov *et al.* 2004, Tang *et al.* 2004a, Argentaro *et al.* 2007, Nan *et al.* 2007, Kernohan *et al.* 2010).

Spontaneous mutations in the human ATRX gene induce a complex neurological syndrome that includes mild α -thalassemia, mental retardation with facial anomalies, and in extreme cases gonadal dysgenesis (ATRX syndrome; Gibbons *et al.* 1995, 2003). Somatic mutations in ATRX were initially associated with a hematological phenotype consistently detected in patients with ATRX syndrome, exhibiting a mild form of α -thalassemia that is characterized by a reduction in α -globin gene expression and the accumulation of abnormal hemoglobin, detectable as intracellular

inclusions in peripheral blood cells (Gibbons *et al.* 2003). Therefore, ATRX was initially described as a critical transcriptional regulator of globin gene expression. However, the diverse clinical manifestations of ATRX syndrome remained unexplained (Gibbons *et al.* 1995, 2003). Since then, more than 120 mutations have been reported in human ATRX (Picketts *et al.* 1996, Gibbons *et al.* 2008). Interestingly, most female carriers are unaffected due to preferential inactivation of the X chromosome that harbors the mutation. These patterns of 'skewed' XCI are commonly observed in patients with X-linked mental retardation (XLMR) and other X-linked conditions (Migeon 2006, Raymond 2006, Muers *et al.* 2007). Thus, like most cases of XLMR, ATRX syndrome is predominantly observed in males (Gibbons & Higgs 2000, Raymond 2006).

Analysis of mice heterozygous for a null *Atrx* allele revealed that this pattern of skewed XCI is mainly due to a positive, tissue-specific, selection of cells expressing normal levels of ATRX (Muers *et al.* 2007). However, non-random XCI has recently been described in a 4-year-old girl with a totally skewed XCI pattern in which the X chromosome carrying a common ATRX mutation (R246C) remained active (Badens *et al.* 2006). Interestingly, she was conceived following IVF and showed abnormal methylation patterns at the FMR1 loci (Badens *et al.* 2006). Expansions in FMR1 are associated with mental retardation in cases of fragile X syndrome (Raymond 2006). Whether skewed XCI in this patient was due to some form of cell selection or whether the mutation affected the process of XCI remains to be determined.

ATRX: in sickness and in health

Various studies have shown that ATRX is present at pericentric heterochromatin domains in human and mouse somatic cells and is involved in regulating DNA methylation at ribosomal DNA as well as subtelomeric repetitive sequences of the human genome (Gibbons *et al.* 1997, 2000, Picketts *et al.* 1998, McDowell *et al.* 1999). Notably, ATRX has recently emerged as a major epigenetic regulator of chromatin structure and function during mitosis as well as meiosis (De La Fuente *et al.* 2004, Ritchie *et al.* 2008, Baumann *et al.* 2010) with important clinical implications for chromosome stability during development as well as malignant neoplastic transformation (Bérubé *et al.* 2002, 2005, Banaszynski *et al.* 2010, Kernohan *et al.* 2010, Elsasser *et al.* 2011, Jiao *et al.* 2011). For example, seminal studies have recently demonstrated that ATRX establishes a functional interaction with the cohesin proteins (SMC1/SMC3), the methyl cytosine binding protein MeCP2 as well as the chromatin insulator CTCF to regulate histone modifications as well as expression patterns at a subset of imprinted genes in the mouse brain (Kernohan *et al.* 2010). Mutations in human SMC1A and SMC3 result in

Cornelia de Lange syndrome, and abnormal MeCP2 function is associated with Rett syndrome. Both conditions exhibit a neurodevelopmental phenotype. Notably, studies suggest that ATRX might control the expression of select imprinted genes in the brain through a potential role in modulating chromatin loop formation and large-scale chromatin structure (Kernohan *et al.* 2010). This is consistent with its role in the neuropathogenesis of ATRX syndrome in which spontaneous mutations result in several degrees of mental retardation in humans and reduced cortical size in the brain of mice with a conditional deletion (Bérubé *et al.* 2002, 2005).

Surprisingly, identical ATRX mutations in different patients can result in variable hematological and neurological phenotypes (Gibbons *et al.* 2003, 2008). While the molecular mechanisms involved in this process remained obscure for some time, recent analysis of genome-wide binding sites in mouse and human cells using chromatin immunoprecipitation (ChIP) sequencing has provided intriguing evidence that ATRX is enriched at variable number tandem repeats (VNTRs). The size of VNTR elements varies among different individuals. Therefore, the different phenotypes observed in patients carrying the same mutation might be explained by variation in the size of the tandem repeats affected in different patients (Law *et al.* 2010).

In addition to its prominent role in the control of transcription at specific loci, ATRX regulates chromatin structure and function at both centromeric heterochromatin and telomeric domains. For example, ATRX binds pericentric heterochromatin through a direct interaction of its ADD domain with histone H3 methylated at lysine 9 (H3K9me3; Dhayalan *et al.* 2011). Notably, ATRX establishes a functional interaction with the transcriptional regulator DAXX at pericentric heterochromatin as well as at promyelocytic leukemia nuclear bodies (Tang *et al.* 2004a), where it is part of a protein complex with chromatin remodeling activity (Xue *et al.* 2003). The ATRX–DAXX complex plays a critical role in the replication-independent deposition of the histone variant H3.3, functioning as a *bona fide* histone chaperone at specific genomic regions including telomeric domains (Drane *et al.* 2010, Goldberg *et al.* 2010, Lewis *et al.* 2010). ATRX also interacts with canonical telomeric DNA sequences where, in addition to its role in deposition of H3.3, it is essential for the maintenance of transcriptional repression of telomeric RNA in mouse and human ES cells (Goldberg *et al.* 2010, Wong *et al.* 2010). Importantly, functional ablation of ATRX induced changes in chromatin composition and telomere instability in mouse ES cells (Wong *et al.* 2010).

Consistent with these observations, several lines of evidence indicate that ATRX plays a prominent role in the maintenance of chromosome stability in mammalian somatic and germ cells. For instance, RNAi knockdown in mouse oocytes induced abnormal chromosome–microtubule interactions and defects in meiotic spindle

formation at the metaphase II stage of meiosis (De La Fuente *et al.* 2004). In addition, lack of ATRX function in HeLa cells results in severe chromosome cohesion and congression defects (Ritchie *et al.* 2008). Furthermore, our recent studies indicate that lack of ATRX function in the female germ line interferes with the establishment of epigenetic modifications associated with chromosome condensation and centromere stability resulting in the transmission of aneuploidy during the transition to the first mitosis. Importantly, ATRX plays a direct role in recruiting the transcriptional regulator DAXX to pericentric heterochromatin domains in the oocyte genome (Baumann *et al.* 2010). The type of chromosome segregation defects found in ATRX-deficient oocytes, namely premature centromere separation and chromosome non-disjunction, are also the two most common causes of aneuploidy in oocytes from women of advanced reproductive age (Vialard *et al.* 2006). Hence, ATRX-deficient ova constitute an invaluable model to determine the epigenetic origins of aneuploidy in mammalian oocytes. Collectively, these results indicate that ATRX plays a critical role in the maintenance of chromosome stability during mitosis as well as meiosis (De La Fuente *et al.* 2004, Ritchie *et al.* 2008, Baumann *et al.* 2010).

Notably, almost 80% of patients with ATRX syndrome exhibit urogenital abnormalities ranging from undescended testes, testicular dysgenesis, and in extreme cases male to female sex reversal. The spectrum of abnormalities encountered in these patients is strongly suggestive of a potential role in sexual differentiation, although the mechanisms involved in this process remain to be elucidated (Tang *et al.* 2004b, Gibbons *et al.* 2008). ATRX associates with pericentric heterochromatin as well as with the Y chromosome on mouse neonatal spermatogonia (Baumann *et al.* 2008) and is present in both somatic and germ cells of adult rat and human testes (Tang *et al.* 2011), where it establishes a physical interaction with the androgen receptor and is required for Sertoli cell proliferation (Bagheri-Fam *et al.* 2011).

Most importantly, intriguing new evidence indicates that ATRX expression and function might be abnormally regulated in several types of cancers (Gibbons *et al.* 2003, Haas *et al.* 2009, Steensma *et al.* 2009, Elsasser *et al.* 2011, Jiao *et al.* 2011). Mutations in the PHD domain of this protein exacerbate the hematopoietic phenotype in premalignant conditions such as myelodysplastic syndrome (Gibbons *et al.* 2003). In addition, abnormal ATRX gene expression patterns have recently been observed in acute myeloid leukemia (AML; Serrano *et al.* 2006), where AML patients with reduced ATRX expression exhibit karyotypic abnormalities (Serrano *et al.* 2006). Moreover, genome-wide analysis of somatic mutations by exome sequencing in human tumors revealed that frameshifts and non-sense mutations in chromatin remodeling proteins, including ATRX, are frequently observed in pancreatic neuroendocrine

tumors (Elsasser *et al.* 2011, Jiao *et al.* 2011). This results in complete loss of DAXX or ATRX function in 43% of cancers characterized by high levels of chromosome instability (Jiao *et al.* 2011).

ATRX is an epigenetic marker of the murine inactive X chromosome

ATRX is the first member of the SWI2/SNF2 family of chromatin remodeling proteins to be detected in association with facultative heterochromatin on the inactive X chromosome (Baumann & De La Fuente 2009). ATRX remains stably associated with Xi during interphase as well as metaphase where it exhibits a banding pattern along the entire inactive X chromosome in mouse ovarian granulosa cells, embryonic fibroblasts as well as trophoblast stem (TS) cells, suggesting a potential involvement in XCI (Fig. 1). ChIP in mouse embryonic fibroblasts indicates that ATRX is preferentially enriched at the H3K9me hot spot similar to *bona fide* markers of the inactive X such as H3K9me2 and H3K27me3 (Baumann & De La Fuente 2009). Moreover, ATRX co-localizes with macroH2A on a late replicating X chromosome (Baumann & De La Fuente 2009). The specific role(s) of ATRX in dosage compensation is not clear at present. However, several lines of evidence indicate that ATRX might be involved in the maintenance rather than the onset of XCI. For example, although the association of ATRX to the H3K9me hot spot can be initially detected by ChIP assays on day 5 of embryonic stem (ES) cell differentiation (Baumann & De La Fuente 2009), a significant enrichment of this chromatin remodeling factor as well as its chromosome-wide distribution over the Xi is not detected until day 8 following spontaneous differentiation (Baumann & De La Fuente 2009). Notably, conditional deletion of ATRX in the mouse preimplantation embryo interferes with imprinted inactivation of the paternal X chromosome in extraembryonic tissues and severely disrupts trophoblast giant cell formation following implantation indicating a prominent role for ATRX in trophoblast development (Garrick *et al.* 2006).

The patterns of *Atrx* gene expression during preimplantation development in mice suggest the existence of diverse requirements for dosage compensation for X-linked genes in different tissues. For example, *Atrx* becomes inactivated in the epiblast by the blastocyst stage but subsequently escapes XCI in extraembryonic tissues (Garrick *et al.* 2006, Patrat *et al.* 2009). This indicates that escape from XCI for this factor occurs on a tissue-specific manner following implantation. Expression of *Atrx* in the trophoblast may, therefore, be required for the XCI process in placental derivatives during mammalian development (Garrick *et al.* 2006, Patrat *et al.* 2009). However, further studies are required to determine whether ATRX is strictly necessary for

Figure 1 ATRX accumulates in a non-linear banding pattern along the chromatids of the inactive X chromosome. (A) In addition to its prominent association with pericentric heterochromatin, ATRX (red) decorates the inactive X chromosome (Xi; arrow) in metaphase spreads from diploid trophoblast stem (TS) cells. Preferential association of BrdU (green) on the allocyclic replicating Xi is confirmed on the same metaphase spread. (B) Immuno-FISH analysis using an X chromosome painting probe confirmed that ATRX is preferentially enriched at one of the two X chromosomes in TS cells. Up to four ATRX bands are detectable on the Xi (inset), whereas Xa exhibits ATRX staining only at pericentric heterochromatin (inset). The ideogram represents a characteristic Giemsa staining pattern. The approximate localization of the murine *Xist* locus as well as additional X-linked genes including *Atrx* is indicated.

global transcriptional silencing of the inactive X chromosome in the epiblast, as well as to elucidate the specific roles of this chromatin remodeling protein in the maintenance of imprinted XCI.

Comparative aspects of XCI

In mammals, the process of dosage compensation takes place through the transcriptional inactivation of one of the two X chromosomes during early embryonic development (Lyon 1961). This process is thought to have evolved ~150 million years ago and is conserved among female mammals (Deakin *et al.* 2009). However, the molecular mechanisms controlling XCI as well as the type and number of genes subject to inactivation differ significantly between human and mouse (Okamoto & Heard 2009, Berletch *et al.* 2010, Okamoto *et al.* 2011).

Inactivation of one of the two X chromosomes in female mammals is an essential process for normal embryogenesis as functional uniparental disomy for the X chromosomes results in embryonic mortality (Takagi & Abe 1990, Migeon *et al.* 1993, Marahrens *et al.* 1997). A major breakthrough towards the molecular analysis of XCI came with the isolation and cloning of a non-coding

RNA expressed exclusively from the inactive X chromosome, the X inactive-specific (*XIST*) transcript. Its location and exclusive expression from the inactive X chromosome in both human and mouse somatic cells suggested a role in dosage compensation (Borsani *et al.* 1991, Brockdorff *et al.* 1991, Brown *et al.* 1991). Since its initial identification, the process of XCI has emerged as one of the prime examples of an epigenetic mechanism capable of regulating gene expression in response to critical developmental transitions. Importantly, accumulating evidence also indicates the existence of a link between XCI and the maintenance of chromosome stability in several human diseases including breast and ovarian cancer (Pageau *et al.* 2007, Agrelo & Wutz 2009, Hall *et al.* 2009).

The underlying molecular mechanisms involved in the regulation of XCI in mice have been the subject of intense investigation and are reviewed in detail elsewhere (Clerc & Avner 2003, Payer & Lee 2008, Barakat *et al.* 2010). However, comparative analysis of XCI reveals prominent differences in the molecular mechanisms and developmental regulation of dosage compensation between human and mouse (Okamoto & Heard 2009, Chang & Brown 2010, Okamoto *et al.* 2011).

Nucleotide sequence analysis revealed that the *XIST/Xist* transcript consists of a 17 kb nucleotide sequence in human and 15 kb in mouse, which exhibit no protein coding potential (Brockdorff *et al.* 1992, Brown *et al.* 1992). The number and orientation of exons varies between species; however, a highly conserved sequence has been detected at the most 5' region of exon 1 in several mammals suggesting an important functional role of this region for the onset of XCI (Hendrich *et al.* 1993). Elegant studies subsequently led to the identification of additional regulatory elements at the XCI center (XIC) in mice and revealed the presence of a complex multifunctional locus controlling the expression of several key regulatory and non-coding RNAs that are essential for i) counting the number of X chromosomes per diploid genome, ii) determining which X chromosome will become inactive (choice), and iii) regulating the initiation and spreading of the inactivation process throughout an entire X chromosome following a developmental transition (Lee & Lu 1999, Lee *et al.* 1999, Ogawa & Lee 2003, Stavropoulos *et al.* 2005, Bacher *et al.* 2006, Augui *et al.* 2007, Navarro *et al.* 2008, Lee 2009). However, while the critical 5' region of *Xist* has been evolutionarily conserved between human and mouse, many of the regulatory elements found downstream from the murine *Xist* sequence are not conserved in human XIC (Chang & Brown 2010). Moreover, XCI in marsupials takes place in the absence of *Xist* expression suggesting that either an alternative non-coding RNA or prominent epigenetic mechanisms may be set in place for the onset and maintenance of XCI in the marsupial embryo (Mahadevaiah *et al.* 2009,

Okamoto & Heard 2009, Rens *et al.* 2010, Okamoto *et al.* 2011).

The nuclear and chromosomal localization of the *Xist* transcript provides a remarkable example of the role of chromatin conformation in the control of gene expression. For instance, *Xist* RNA remains in close apposition with the inactive X chromosome, blocking the expression of most genes associated with this chromosome and contributing to the formation of a heterochromatin domain or Barr body (Brockdorff *et al.* 1992, Brown *et al.* 1992, Clemson *et al.* 1996). Spreading of inactivation to other genes involves chromatin changes such as global histone methylation and histone hypoacetylation (Jeppesen & Turner 1993, Clemson *et al.* 1996, Marks *et al.* 2009).

Epigenetic mechanisms of XCI

Ectopic *Xist* expression in autosomes leads to gene inactivation, late replication, and chromatin remodeling by decreasing acetylation of histone H4 (Lee & Jaenisch 1997, Hall *et al.* 2002). However, XCI is maintained after deletion of the XIC region indicating that the XIC is no longer required once dosage compensation is established (Brown & Willard 1994, Csankovszki *et al.* 2001). These studies suggest that epigenetic modifications might be critical for the maintenance of transcriptional silencing of the inactive X chromosome (Brockdorff 2002, Heard & Disteche 2006).

During the initiation phase, between 48 and 72 h following ES cell differentiation, *Xist* RNA coating is strictly required for XCI. In contrast, maintenance of the inactive state is independent of *Xist* RNA during the irreversible stage (>72 h) in ES cells as well as in somatic cells (Csankovszki *et al.* 2001, Chaumeil *et al.* 2006). Maintenance of XCI in somatic cells is, therefore, achieved through clonal inheritance of multiple epigenetic modifications and potentially reinforced by non-coding RNAs, DNA methylation, and late replication (Brown & Willard 1994, Bernardino *et al.* 2000, Brockdorff 2002, Heard & Disteche 2006). Non-coding RNAs recruit specific epigenetic marks to the active as well as the inactive X chromosome and thereby establish an asymmetric chromatin environment between the two X chromosomes in the female embryo (Bernstein *et al.* 2006, Pandey *et al.* 2008, Kanduri *et al.* 2009). In addition, *Xist* RNA establishes a transcriptionally silent nuclear domain devoid of RNA polymerase II and transcription factors (Chaumeil *et al.* 2006). Although no major differences were detected between the two X chromosomes at the level of the 30 nm chromatin fiber (Naughton *et al.* 2010), the complex interplay between histone modifications and chromatin remodeling factors induces dramatic changes in large-scale chromatin structure, which are also reflected by transcriptional repression as well as by transient interaction between the two X chromosomes (Xu *et al.* 2006, Naughton *et al.*

2010) and finally result in a close association of the Xi with heterochromatin domains around the nucleolus (Zhang *et al.* 2007).

The molecular composition of the large protein complexes modulating the epigenetic landscape of the human and mouse inactive X chromosomes are beginning to be unraveled (Table 1). Importantly, the kinetics of changes in histone modifications as well as the recruitment of specific chromatin remodeling factors and histone variants associated with the allocyclic, heterochromatic Xi has been characterized following induced differentiation in ES cells (O'Neill *et al.* 1999, Costanzi *et al.* 2000, Brockdorff 2002, Heard & Disteche 2006). These changes play distinct, albeit complementary, roles in the maintenance of transcriptional silencing (Fig. 2). Nevertheless, our understanding of the specific function of the myriad epigenetic modifications in heterochromatinization and/or maintenance of the inactive state is far from complete. For example, the mechanisms involved in recruitment of such diverse protein complexes to the inactive X chromosome are not fully understood, nor is it known how transcriptional repression and large-scale chromatin remodeling are coordinated to spread a heterochromatic state over an entire chromosome. Analysis of the kinetics of XCI after exposure of female murine ES cells to retinoic acid indicates that 'coating' of the inactive X chromosome by *Xist* RNA occurs within 24–48 h following induction of differentiation (Heard *et al.* 2001) with transcriptional silencing taking place within an additional 24 h (Ng *et al.* 2007). Trimethylation of histone H3 lysine 9 (H3K9me3) together with histone H4 trimethylated at lysine 20 (H4K20me3) is one of the earliest marks to be recruited to Xi almost immediately following the initial *Xist* RNA coating and before transcriptional repression is established during a precise developmental window (Heard *et al.* 2001, Boggs *et al.* 2002, Chadwick & Willard 2004, Chaumeil *et al.* 2006). Interestingly, these changes are initiated at a specific 5' region to the *Xist* promoter, the H3K9me 'hot-spot'. This region has been suggested to act as a putative nucleation center that might recruit additional epigenetic marks (Heard *et al.* 2001, Silva *et al.* 2003, Rougeulle *et al.* 2004).

Transcriptionally permissive histone modifications such as acetylation of histones H3/H4 are subsequently lost from the inactive X chromosome on day 2.5 of differentiation followed by deposition of silent chromatin marks such as polycomb complexes and a shift to late replication by day 4. Heterochromatinization and enrichment for histone variants such as macroH2A and chromatin remodeling proteins such as ATRX take place following day 5 of differentiation, and finally DNA methylation changes at specific promoters on day 9 of differentiation (Fig. 2; O'Neill *et al.* 1999, Brockdorff 2002, Heard & Disteche 2006, Baumann & De La Fuente 2009).

Binding of *Xist* RNA is thought to induce local heterochromatinization on the Xi by recruiting

Table 1 Epigenetic modifications associated with the inactive X chromosome.

Epigenetic modification	X _{inactive}	X _{active}	References
Non-coding RNAs			
<i>Xist</i>	+	—	Brown <i>et al.</i> (1992) and Clemson <i>et al.</i> (1996) ^a Lee <i>et al.</i> (1999)
<i>Tsix</i>	—	+	
Histone variants			
Histone H1	+	—	Chadwick & Willard (2004) ^a
MacroH2A	+	—	Costanzi <i>et al.</i> (2000)
Histone modifications			
ubH2A	+	—	Fang <i>et al.</i> (2004) and de Napoles <i>et al.</i> (2004)
H3K9me2	+	—	Heard <i>et al.</i> (2001) and Boggs <i>et al.</i> (2002) ^a
H3K9me3	+	—	Chadwick & Willard (2004) ^a
H3K4me2	—	+	Chadwick & Willard (2004) ^a
H3K27me3	+	—	Plath <i>et al.</i> (2003) ^a
H4 hypoacetylation	+	—	Jeppesen & Turner (1993) ^a and O'Neill <i>et al.</i> (1999)
Polycomb proteins			
RING1A/B	+	—	Fang <i>et al.</i> (2004) and de Napoles <i>et al.</i> (2004)
PHC1, PHC2	+	—	Plath <i>et al.</i> (2004) ^a
EED/EZH2	+	—	Mak <i>et al.</i> (2002), Plath <i>et al.</i> (2003) ^a and Silva <i>et al.</i> (2003)
CBX6, CBX7, CBX8	+	—	Bernstein <i>et al.</i> (2006)
CBX2	+	—	Plath <i>et al.</i> (2004) ^a and Bernstein <i>et al.</i> (2006)
BMI1	+	—	Plath <i>et al.</i> (2004) ^a
ASH2L	+	—	Pullirsch <i>et al.</i> (2010) ^a
Chromatin remodelling			
ATRX	+	—	Baumann & De La Fuente (2009)
Tumor suppressor			
BRCA1	+	—	Ganesan <i>et al.</i> (2002) ^a
Others			
PARP1	+	—	Nusinow <i>et al.</i> (2007) ^a
HP1	+	—	Chadwick & Willard (2004) ^a
SAF-A	+	—	Helbig & Fackelmayer (2003) and Pullirsch <i>et al.</i> (2010) ^a
SMCHD1	+	—	Blewitt <i>et al.</i> (2008)
DNA methylation			
Global (chromosome-wide)	—	+	Bernardino <i>et al.</i> (2000) ^a
Promoter regions	+	—	Mohandas <i>et al.</i> (1981) and Hellman & Chess (2007) ^a

+, enrichment; —, absence.

^aConserved in the human inactive X.

transcriptionally repressive protein complexes that spread in *cis* throughout the entire X chromosome. For example, a high resolution comprehensive map of local chromatin modifications at regulatory sequences within the XIC revealed that following the onset of *Xist* expression, a chromosome-wide increase in the levels of H3K27me3 is detected exclusively on the inactive X chromosome. Moreover, spreading of this epigenetic mark is associated with transcriptional silencing of most genes associated with the Xi (Marks *et al.* 2009). A 1.6 kb regulatory transcript (RepA) might also contribute to chromosome-wide silencing by facilitating the association of the inactive X chromosome with heterochromatin domains in the nucleus and by recruiting members of the polycomb repressive complex (PRC2) to the Xi (Chaumeil *et al.* 2006, Zhang *et al.* 2007, Pullirsch *et al.* 2010). Further studies are required to determine the precise mechanisms involved in translating a local epigenetic change at specific regulatory sequences of the XIC into chromosome-wide transcriptional silencing. However, facultative heterochromatin formation might be an essential mechanism in this process (Helbig & Fackelmayer 2003, Trojer & Reinberg 2007, Marks *et al.* 2009). Together with *Xist* RNA, long interspersed nuclear

elements (LINEs) contribute to the spreading of transcriptional silencing by creating a transcriptionally repressive chromatin environment rich in repetitive elements that in concert with the SATB1 protein function to nucleate heterochromatin formation at several regions throughout the inactive X chromosome (Agrelo *et al.* 2009, Chow *et al.* 2010).

Some epigenetic marks including ATRX are present in both pericentric heterochromatin of autosomes and heterochromatin at the inactive X chromosome. Interestingly, several ubiquitinated proteins such as ubiquitinated histone H2A (ubH2A) exhibit an exclusive association with the inactive X chromosome in somatic cells where it is present in a banding pattern that co-localizes with segments of *Xist* RNA on early mitotic chromosomes (Wang *et al.* 2001, Fang *et al.* 2004, de Napoles *et al.* 2004, Smith *et al.* 2004). Notably, recent studies have indicated that both ubiquitination and the enzymatic activity of Aurora B kinase are essential to recruit human *XIST* RNA to multiple binding sites in somatic cells (Hall *et al.* 2009). In turn, histone ubiquitination can induce additional post-translational modifications that might be of functional significance for the spreading of chromosome-wide transcriptional

Figure 2 Kinetics of epigenetic modifications imposed on the inactive X chromosome (Xi) following spontaneous differentiation of mouse embryonic stem (ES) cells. Time line of chromosome-wide association for different histone modifications, histone variants, and chromatin remodeling proteins known to contribute to the epigenetic landscape of Xi. Multiple epigenetic marks participate in the process of heterochromatinization and transcriptional silencing. Different shades of blue indicate the progressive transition from chromatin modifications involved in the onset versus the maintenance of the inactive state.

silencing (Smith *et al.* 2004). Although an attractive model, the transient nature of ubH2A staining during the early stages of XCI in TS cells (Fang *et al.* 2004) indicates that additional factors may be involved in maintaining the heterochromatic state following ES and TS cell differentiation (Hall *et al.* 2009). Interestingly, elegant studies in mouse ES cells have recently implicated the E3 ubiquitin ligase RNF12 as the first protein factor with a dose-dependent role in the initiation of XCI. However, the mechanisms involved in this process are yet to be determined (Jonkers *et al.* 2009).

Members of the PRC1 proteins such as BMI1, RNF2/RING1B, and CBX4 as well as the histone variant macroH2A1 are recruited to the inactive X chromosome in a cell cycle-dependent manner following ubiquitination by the CULLIN3/SPOP ubiquitin E3 ligase, where they play a role in the maintenance of XCI in somatic cells (Hernandez-Munoz *et al.* 2005). RING1B may also compact chromatin through a mechanism independent of histone ubiquitination (Eskeland *et al.* 2010).

MacroH2A interferes with transcription factor binding as determined by *in vitro*-reconstituted chromatin assays and inhibits the activity of chromatin remodeling proteins (Trojer & Reinberg 2007). Moreover, macroH2A may induce chromatin compaction by inhibiting the enzymatic activity of the poly (ADP-ribose) polymerase-1 (PARP1) protein, thus providing critical insight into the potential mechanisms of macroH2A as a transcriptionally repressive mark on the inactive X chromosome (Ouararhni *et al.* 2006, Nusinow *et al.* 2007, Trojer & Reinberg 2007, Timinszky *et al.* 2009).

PARP1 is a NAD^+ -dependent chromatin remodeling protein that becomes quickly activated in response to DNA damage by direct binding to double strand breaks (DSBs) through its zinc finger domains (Kim *et al.* 2004, 2005). Nevertheless, compelling evidence indicates that this protein might also be an important regulator of chromatin modifications under physiological conditions. For example, in both human and *Drosophila* cells, PARP1 activation resulting from nucleosome binding may induce the reversible modulation of chromatin structure during key developmental transitions in the absence of DNA damage (Tulin *et al.* 2002, Tulin & Spradling 2003, Kim *et al.* 2004). Notably, PARP1 has recently been found localized to the inactive X chromosome in a somatic cell line, in which macroH2A1.2-GFP is specifically over-expressed at Xi. In this model, PARP1 is required for the maintenance of transcriptional silencing of a reporter transgene providing the first evidence indicating a potential role for PARP1 in maintaining a transcriptionally repressive chromatin environment during XCI (Nusinow *et al.* 2007). Consistent with this hypothesis, our recent studies have indicated that PARP1 is a stable epigenetic mark on the inactive X chromosome during both interphase (Fig. 3A) and metaphase stages (Fig. 3B) in primary mouse embryonic fibroblasts.

Abnormalities of XCI: implications for chromosome instability

In principle, the XCI process can potentially be disrupted by environmental, toxicological, and/or disease conditions that affect i) chromatin structure and function, or ii) disrupt the establishment and/or maintenance of epigenetic modifications (Hall *et al.* 2009). In turn, abnormalities on X chromosome ploidy or epigenetic stability may lead to disease conditions arising from abnormal X-linked gene dosage. Specifically, recent studies have indicated that *XIST* function may be severely affected by defects in heterochromatin stability and large-scale chromatin structure in several types of human primary cell tumors (Pageau *et al.* 2007, Hall *et al.* 2009). Since the initial observations on the loss of the Barr body in breast and ovarian cancer cells five decades ago, studies have established that errors in chromosome segregation result in a high proportion of

Figure 3 Stable association of the poly (ADP-ribose) polymerase-1 (PARP1) protein with the inactive X chromosome Xi in mouse embryonic fibroblasts (MEFs) during interphase and metaphase. PARP1 (red) is co-localized with a condensed heterochromatic nuclear domain corresponding to the inactive X chromosome (Xi) or Barr body as detected by fluorescence *in situ* hybridization (green). PARP1 (red) decorates both chromatids on Xi (arrows) during mitosis. DNA is stained in blue (DAPI).

malignant cells lacking an inactive X chromosome. The most common mechanism involved in this process induces the formation of uniparental disomy for the active X chromosome as a consequence of non-disjunction resulting in the emergence of cells carrying two genetically identical X chromosomes (Pageau *et al.* 2007). Alternatively, abnormal epigenetic modifications commonly observed during malignant neoplastic transformation may lead to reactivation of the inactive X chromosome by disrupting the cascade of chromatin changes leading to heterochromatinization of large chromosomal segments or even an entire X chromosome (Pageau *et al.* 2007). Importantly, these mechanisms are not mutually exclusive and may occur in the same nucleus, leading to the clonal inheritance of malignant cells and subsequent tumor formation (Pageau *et al.* 2007). For example, functional ablation of the tumor suppressor BRCA1 might predispose cells to epigenetic instability and defects in DNA repair resulting in the formation of chromosomal rearrangements capable of interfering with XCI (Ganesan *et al.* 2002). Interestingly, some breast cancer cell lines as well as primary breast tumors exhibit improper localization of *Xist* RNA associated with abnormal heterochromatin formation (Pageau *et al.* 2007, Vincent-Salomon *et al.* 2007). In turn, abnormal heterochromatin formation might promote global changes in gene expression leading to chromosome instability and malignant neoplastic transformation (Pageau *et al.* 2007).

Notably, X chromosome instability was observed in female mice heterozygous for *Parp1* that also carry a

homozygous deletion of *Parp2* (*Parp1*^{+/-}/*Parp2*^{-/-}). In these mice, ~40% of female, but not male, embryonic fibroblast cells exhibit aneuploidy specific to the X chromosome (Ménissier de Murcia *et al.* 2003). Consistent with these studies, we have recently observed indicators of X chromosome instability in oocytes obtained from *Parp1* knockout female mice during the prophase I of meiosis (Yang *et al.* 2009). For example, analysis of homologous chromosome synapsis in fetal oocytes revealed a striking accumulation of RAD51 protein, a DNA recombination intermediate associated with DSBs formation (Moens *et al.* 2002, Burgoyne *et al.* 2007), at the meiotic chromosome cores of the fully synapsed X chromosome bivalent (Yang *et al.* 2009; Fig. 4). This indicates the presence of unresolved double strand DNA breaks in *Parp1* null oocytes at the pachytene stage, a time at which DSBs are fully repaired in control oocytes. Importantly, the X chromosome bivalent also exhibits abnormal chromatin modifications as detected by persistence of histone H2AX phosphorylation (Yang *et al.* 2009). The mechanisms involved in failure to repair DSBs in this model warrant further investigation. However, this might result from an increased susceptibility of the X chromosome bivalent to illegitimate recombination and, potentially, a contributing factor to the X chromosome instability reported in *Parp1*^{+/-}/*Parp2*^{-/-} female mice (Ménissier de Murcia *et al.* 2003).

Epigenetic instability on the inactive X chromosome has also been observed in human ES cells where a vast proportion of established cell lines exhibit an inactive X chromosome before induced cell differentiation with variable levels of *Xist* expression and loss of H3K27me3 (Hall *et al.* 2008, Silva *et al.* 2008). Epigenetic modifications are crucial to maintain cellular identity and XCI is closely linked to ES differentiation. Hence,

Figure 4 Persistence of RAD51 foci and genome instability at the X chromosome bivalent in *Parp1* null oocytes. (A) Pachytene stage oocyte with fully synapsed chromosomes labeled with the synaptonemal complex protein SYCP3 (green). Lack of PARP1 function during meiosis results in the persistence of double strand DNA breaks (RAD51 foci; red) in a single chromosome bivalent corresponding to the X chromosome (arrow) as determined by fluorescence *in situ* hybridization with an X chromosome probe (B; green).

a deeper understanding of the epigenetic mechanisms involved in XCI is needed to design optimal culture conditions that ensure the maintenance of the undifferentiated state, the timely onset of XCI upon cell differentiation as well as the potential therapeutic value of human ES cells (Lengner *et al.* 2010).

Abnormalities of XCI: implications for human disease

To date, more than 300 X-linked genes have been associated with human pathological conditions ranging from skin disease (Sun & Tsao 2008), renal dysfunction (Migeon 2008), and autoimmune conditions such as systemic lupus erythematosus (Pan & Sawalha 2009) in which the patterns of random and/or skewed XCI may have a profound influence on the clinical manifestation; for example, the early onset and/or severity of the disease (Migeon 2006). Moreover, abnormal XCI and lack of *Xist* transcription in patients with tiny ring X chromosomes result in a range of developmental and cognitive phenotypes including severe mental retardation (Migeon *et al.* 1993). Therefore, the initiation and maintenance of dosage compensation is a critical developmental transition with important clinical implications. Positive dosage compensation with transcriptional upregulation of a select group of genes on the active X chromosome is also a phenomenon of increasing importance due to the high levels of X-linked gene expression in both male and female brain tissue, in which up to 1300 loci exhibit parent of origin allelic expression patterns. Thus, skewing of XCI may have a significant impact on cognitive function (Nguyen & Disteche 2006, Hellman & Chess 2007, Gregg *et al.* 2010a, 2010b). Notably, α -thalassemia myelodysplastic syndrome (ATMDS) is a severe form of α -thalassemia resulting from acquired mutations in ATRX. This condition contrasts with the mild forms of α -thalassemia detected in patients with inherited mutations of the same amino acid sequence of ATRX and indicates that in addition to the initial mutation, an epigenetic component might play a critical role in mediating the severity of the hematological phenotype (Steensma *et al.* 2004, 2005). Although the majority of patients diagnosed with ATMDS are male, this condition may also occur sporadically in females (Haas *et al.* 2009). Preliminary analysis on the patterns of XCI in these patients revealed no overt alterations in this process. However, further studies are required to determine the behavior of the inactive X chromosome in female patients with myelodysplastic syndrome (Haas *et al.* 2009).

Concluding remarks

A growing body of evidence indicates that disruption of the epigenetic mechanisms involved in XCI may also have direct and wide-ranging implications in human

health. For example, loss of transcriptionally repressive heterochromatin marks on the Xi such as H3K27me3 has been observed in human fibroblasts obtained from patients with Hutchinson–Gilford progeria syndrome, a devastating condition inducing premature aging (Shumaker *et al.* 2006). Functional ablation of macroH2A1, another epigenetic marker of Xi, induces a metabolic disorder characterized by hepatic steatosis and upregulation of thyroxine-binding globulin, an X-linked enzyme in female mice (Boulard *et al.* 2010). Importantly, loss of the AT binding protein SATB1 in lymphoma cells interferes with the ability of *Xist* to induce transcriptional silencing and implicates SATB1 as an important factor in the XCI pathway (Agrelo *et al.* 2009). Expression of SATB1 and certain isoforms of macroH2A1 also associates with aggressive types of breast and lung cancer respectively, and hence might prove to be valuable biomarkers in clinical oncology (Agrelo & Wutz 2009, Agrelo *et al.* 2009, Sporn *et al.* 2009). These models provide direct evidence that the process of XCI has important implications for genome stability during cancer development as epigenetic regulatory pathways established in the developing conceptus may show reactivation and/or dysregulation during the complex process of malignant neoplastic transformation. Importantly, the presence of an unmethylated *XIST* promoter has been suggested as a potential marker for tumor progression (Kawakami *et al.* 2004). As we gain a better understanding of the epigenetic mechanisms regulating XCI and its impact on cellular differentiation, the clinical implications of this process will no doubt become increasingly appreciated.

Mutations in genes encoding for chromatin remodeling proteins containing a PHD result in a spectrum of human disorders ranging from neurodevelopmental syndromes, autoimmune conditions, myeloid leukemia, and other types of cancer (Baker *et al.* 2008). Among these, ATRX has recently emerged as a major epigenetic factor involved in transcriptional regulation, nuclear architecture, and chromosome stability in mammalian cells. Although ATRX was initially described as a critical transcriptional regulator of β -globin gene expression, major discoveries on the biological significance of ATRX have revealed important functions in the control of centromere and telomere stability, chromosome cohesion, and regulation of several imprinted genes in the postnatal mouse brain. Insight into the molecular mechanisms of ATRX function and its interacting partners in different tissues will no doubt contribute to our understanding of the pathogenesis of ATRX syndrome as well as the epigenetic origins of aneuploidy during development and differentiation. Altered chromatin states are a prominent hallmark of cancer cells (Davis & Brachmann 2003, Feinberg *et al.* 2006). Notably, loss of epigenetic marks in progeria can be rescued by epigenetic therapy (Columbaro *et al.* 2005). This provides encouraging evidence for the identification

of chromatin remodeling proteins as potential drug targets as well as the rational design of novel therapeutic management strategies of the abnormal epigenetic profiles observed during malignant neoplastic transformation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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