REVIEW ARTICLE

Divergent actions of long noncoding RNAs on X-chromosome remodelling in mammals and *Drosophila* achieve the same end result: dosage compensation

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Abstract

Organisms with heterochromatic sex chromosomes need to compensate for differences in dosages of the sex chromosome-linked genes that have somatic functions. In-depth cytological and subsequent biochemical and molecular studies on dosage compensation started with Mary F. Lyon's proposal in early 1960s that the Barr body in female mammalian somatic cells represented one of the randomly inactivated and heterochromatinized X chromosomes. In contrast, *Drosophila* was soon shown to achieve dosage compensation through hypertranscription of single X in male whose chromatin remains more open. Identification of proteins that remodel chromatin either to cause one of the two X chromosomes in somatic cells of very early female mammalian embryos to become condensed and inactive or to remodel the single X in male *Drosophila* embryos to a more open state for hypertranscription provided important insights into the underlying cellular epigenetic processes. However, the most striking and unexpected discoveries were the identification of long noncoding RNAs (lncRNAs), X-inactive specific transcript (Xist) in mammals and roX1/2 in *Drosophila*, which were essential for achieving the contrasting chromatin organizations but leading to similar end result in terms of dosage compensation of X-linked genes in females and males. An overview of the processes of X inactivation or hyperactivation in mammals and *Drosophila*, respectively, and the roles played by Xist, roX1/2 and other lncRNAs in these events is presented.

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Introduction

It is a great privilege to write this review in memory of Mary F. Lyon whose extraordinary X-inactivation hypothesis proposed in 1961 (Lyon 1961) has had a lasting impact on mammalian genetics. This hypothesis was also influential in selection of my own research when I initiated Ph.D. research at Calcutta University in 1967. I also had the privilege to visit her at the MRC Radiobiology unit, Harwell in 1973. The attention and affection that I, as a less than 28 year old researcher, received from her during that visit remains refreshing even today.

The concept of dosage compensation, first indicated by C. Stern in 1929, and later elaborated by H. J. Muller (Muller 1932, 1950), was proposed to explain equalized expression of X-linked genes in male and female *Drosophila* in spite of them having one and two dosages of X-chromosome genes, respectively. In the 1950s and 1960s, while Muller and others believed the dosage compensation in *Drosophila* to involve regulation of each X-linked gene by specific modifiers to achieve the dosage compensation, R. B. Goldschmidt (1954) believed that dosage compensation was a direct consequence of different physiologies of male and female flies (see Smith and Lucchesi 1969).

Lyon first proposed the hypothesis of X inactivation in 1961 to explain the nature of Barr body seen in mouse somatic cells. In subsequent and more detailed, she (Lyon 1962) elaborated the X-inactivation hypothesis to make it applicable to mammals in general and to link it to dosage compensation (also see Gartler 2014). The X-inactivation hypothesis was quickly confirmed through genetic, cytological as well as functional approaches, which established that of the two X chromosomes in female somatic cells, one was inactivated early in development, that the inactivation in eutherians was random and irreversible, and the inactive X chromosome or the Barr body was transcriptionally silent

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and replicated during the late S phase (Takagi and Makino 1966; Lyon 1972), qualifying it to be classified as facultative heterochromatin (Brown 1966). Another important discovery was that the inactivation process 'spread' along the X chromosome from a single locus (Russell 1963; Cattanach and Issacson 1967), which was named as the X-inactivation centre (XIC/Xic). The spreading of X chromosome inactivation from an inactivation centre found support in the observations that an autosomal piece inserted in the X chromosome could also get genetically inactive and late replicating when the rearranged X chromosome was inactivated and likewise, a segment of X chromosome when inserted in an autosome was found to escape inactivation (Russell 1963).

On the basis of a wider and pale stained appearance of the single X chromosome in polytene nuclei of male *Drosophila*, Dobzhansky (1957) suggested 'this single X works apparently twice as hard as does each of the two X's in the female'. This forerunner of the 'hyperactive male X' model for achieving dosage compensation in *Drosophila* was formally supported by the transcription autoradiographic studies on polytene chromosomes of *Drosophila* by Mukherjee and Beermann (1965). A few years later, DNA replication studies suggested that unlike the inactive X in female mammalian cells, the single X chromosome in male polytene nuclei completed replication faster than autosomes or the Xs in female cells (Berendes 1966). Thus contrary to Lyon's X-inactivation hypothesis for achieving dosage compensation in mammals, that in flies appeared to be achieved by hyperactivation of the single X chromosome in somatic cells of male flies. These early results also indicated that like the inactive X chromosome in mammalian females, the dosage compensation in *Drosophila* was also operative at chromosome level, although Muller (see Muller and Kaplan 1966) did not fully believe in the hyperactive X model. When I joined Dr A. S. Mukherjee's lab at the Zoology department of Calcutta University in 1967 for my doctoral research, I chose to work on dosage compensation in *Drosophila* and, because of this, I followed Lyon's and others' early works on the mammalian X inactivation to design my own study on dosage compensation in *Drosophila* (Lakhotia and Mukherjee 1969, 1970; Lakhotia 1970).

The phenomenon of dosage compensation became a very good and popular model system to study the aspects of gene regulation and chromatin organization. Genetic, cell biological and molecular studies during the last five decades, following the initial chromosomal studies in mammals as well as *Drosophila*, have unraveled the complex network of proteins and RNA that help organize chromatin in the X chromosome in a sex-specific manner and thus achieve dosage compensation. In the context of strong belief in notions of 'junk' and 'selfish' DNA (Ohno 1972; Orgel *et al.* 1980), the discoveries that long noncoding RNAs like X-inactive specific transcript (Xist) in mammals (Brown *et al.* 1991; Brockdorff *et al.* 1991) and roX1 and roX2 in *Drosophila* (Amrein and Axel 1997; Meller *et al.* 1997) were essential to achieve the chromosomal level modifications were pathbreaking and helped establish the importance of long lncRNAs against the dogma (Lakhotia 1996, 1999). In recent years, the lncRNAs have become very attractive candidates for understanding the complex regulatory circuits in eukaryotes, and ever increasing numbers are being identified to be responsible for or associated with a variety of human disorders (Lakhotia 2012; Bergmann and Spector 2014; Cech and Steitz 2014; Legeai and Derrien 2014; Chujo *et al.* 2015; Iyer *et al.* 2015).

Roles of the lncRNAs like Xist in mammals and roX1 and roX2 in *Drosophila* in establishment and maintenance of the inactive X and hyperactive X in mammals and *Drosophila*, respectively, have been extensively reviewed in recent years (Georgiev *et al.* 2011; Koya and Meller 2011; Horabin 2012; Mank 2013; Vallot and Rougeulle 2013; Briggs and Reijo Pera 2014; Chery and Larschan 2014; Ferrari *et al.* 2014; Gartler 2014; Nakagawa and Kageyama 2014; Marchese and Huarte 2014; Peeters *et al.* 2014; Keller and Akhtar 2015; Valencia and Wutz 2015). An overview of dosage compensation in eutherians and *Drosophila*, and the lncRNAs associated therewith is presented here.

X inactivation and lncRNAs in eutherians

Soon after Lyon's hypothesis was proposed (Lyon 1961, 1962), studies on different X-autosome translocations suggested a single site on the X chromosome from which inactivation spreads in *cis* (Russell 1963; Cattanach *et al.* 1969). This site, variously named as X-inactivation centre (XIC) or X-controlling element (Xce), but now commonly called XIC/Xic (Spusta and Goldman 1999), is a *cis*-regulatory element from which inactivation spreads along the X chromosome to bring about chromosome-wide alterations in chromatin organization of the inactive X. The random inactivation of one of the two X chromosomes is a multistep process beginning with the choice of inactive X chromosome followed by initiation and spreading of inactivation and subsequent maintenance of the inactive X in heterochromatinized or silenced state (Plath *et al.* 2002).

Noncoding transcripts from the XIC bring about the initiation, spreading and maintenance of inactivation along one of the X chromosomes in female mammals

Identification of the *XIST* gene mapping to XIC in human and transcribed exclusively from the inactive X (Brown *et al.* 1991) was soon followed by characterization of the mouse homolog *Xist* (Brockdorff *et al.* 1991). The mouse Xist RNA is 15 kb with six exons while the human XIST RNA is 17-kb long with eight exons. There is significant divergence in their sequence but five repetitive sequence motifs in exons 1–6 are relatively better conserved (Spusta and Goldman 1999). The conserved repeat sequences at 5′ end of Xist mediate silencing while its association with X chromosome occurs through functionally redundant but unconserved sequences dispersed through its length (Wutz *et al.* 2002). A number

of other lncRNAs produced from the Xic region have been identified in recent years to have significant roles in the X-inactivation process (see below).

Most studies on X inactivation have used the mouse or human system and revealed remarkable similarities as well as specific differences in the roles of Xist in achieving X inactivation. The imprinted paternal X chromosome is first inactivated in four cell preimplantation mouse embryo and extra-embryonic tissues followed, during blastocyst formation, by reactivation of the paternal X in inner cell mass; it is around day 6.5 that the Xist-mediated random X inactivation in developing epiblast cells is initiated and then stably inherited (Briggs *et al.* 2014; Valencia and Wutz 2015). The imprinted paternal X inactivation is not observed in humans and may actually be uncommon in eutherians. XIST RNA is detectable in one to eight cell stage human embryos by PCR analysis and/or fluorescence *in situ* hybridization; however, it is not clear if all cells of early human embryos express XIST during these early stages (Briggs *et al.* 2014).

Silencing of X-chromosome by Xist RNA

The Xist is exclusively transcribed from the Xic of the inactive X and the transcript spreads along the length of X chromosome in *cis*. Xist initially associates with X-chromosome at a few focal loci from where these transcripts spread along its length to promote chromosome-wide heterochromatinization. One of the earliest chromosome-wide changes following Xist RNA coating is the depletion of the basic transcriptional machinery and loss of euchromatic histone modifications like H3K4me2/me3 and H3/H4 acetylation. Xist in combination with PRC2, a Polycomb group repressive protein complex comprising of Suz12, Eed, Rbbp4/7 and the methyltransferase Ezh2 (or Ezh1), directs H3K27me2/3 of the inactive X (Marchese and Huarte 2014). Jarid2, a PRC2 cofactor, is an important intermediate between PRC2 and Xist RNA for the initial targeting of the PRC2 complex to the X chromosome during onset of X inactivation (da Rocha *et al.* 2014; Valencia and Wutz 2015). The spreading of X inactivation is believed to be facilitated by a hierarchy of defined Polycomb stations that spread H3K27 methylation in *cis* (Pinter *et al.* 2012). Potential RNA-binding proteins like YY1 or ATRX have been implicated in X-chromosome inactivation (Valencia and Wutz 2015). X chromosome coated with Xist RNA seems to be organized into a compact structure through interaction with SATB1, which enables efficient formation of the silencing complex (Brockdorff 2009). The nuclear matrix-associated SAF-A/hnRNP U protein is believed to act as a platform to immobilize Xist RNA along the X chromosome (Fackelmayer 2005; Hasegawa *et al.* 2010). Nakagawa and Prasanth (2011) suggested that S/MAR sequences might function as booster elements in the X chromosome, providing entry sites for SATB1 and SAF-A/hnRNP U to facilitate formation of the inactivation core complex and the subsequent chromosome-wide silencing. Extraordinarily high frequency of the long interspersed nuclear element 1 (LINE1) in the X chromosome has been suggested to be responsible for the efficient spreading of the inactivation signal (Lyon 2003) since LINE1 contains experimentally confirmed S/MAR sequences that interact with SATB1 *in vivo*. However, because of the repetitive nature of LINEs, it has been difficult to assign locus-specific function (Pinter *et al.* 2012). In spite of the correlative evidence in favour of LINES (Bailey *et al.* 2000; Wang *et al.* 2006; Chow *et al.* 2010; Jachowicz and Torres-Padilla 2015), many species without active LINEs also show X inactivation (Cantrell *et al.* 2009), indicating role of other X-enriched repeats like the low-complexity simple SINE repeats (Chow *et al.* 2005; Pinter *et al.* 2012).

Recently, McHugh *et al.* (2015) identified proteins that specifically associate with Xist and may initiate cascade of the inactivation process. Upon initiation of its expression, Xist localizes to sites on the X chromosome by binding to the chromatin-interacting SAF-A protein. Direct interaction of Xist with SHARP at these sites is suggested to recruit SMRT. The Xist – SHARP – SMRT complex may recruit HDAC3 directly to the X chromosome or may induce the histone deacetylation activity of the already present HDAC3 at active genes across the X chromosome. Xist directed removal of the activating histone acetylation triggers compaction of chromatin and transcriptional silencing. After initiating the silenced state, Xist recruits PRC2 across the X chromosome in an HDAC3-dependent manner, either through a direct interaction between PRC2 and HDAC3 or indirectly through HDAC3-induced transcriptional silencing or chromatin compaction. Thus, Xist and its interacting proteins achieve initiation of the inactive state by recruiting transcriptional silencers (HDAC3) and maintaining the inactive state by recruiting stable epigenetic silencer-like PRC2 (McHugh *et al.* 2015). A three-dimensional structured illumination microscopic analysis (Cerase *et al.* 2014) revealed that the Xist RNA and PRC2 are in common zone but are not physically close and this has raised doubts about a direct interaction between Xist RNA and PRC2 as has been strongly suggested by earlier biochemical studies. It is possible that PRC2 recruitment on the inactive X is an indirect consequence of changes in underlying chromatin configuration following the Xist-mediated chromatin silencing (Brockdorff 2013; Cerase *et al.* 2014). On the other hand, a more recent super resolution microscopy (STORM) study (Sunwoo *et al.* 2015) reported that only about 50 hubs of Xist and PRC2 exist in a statistically significant spatial association on the inactive X in mouse; since this number is much smaller than expected on the basis of microscopic and biochemical studies, Sunwoo *et al.* (2015) suggest that Xist and PRC2 complexes methylate nucleosomes in a hit and run mode.

Initiation of Xist transcription: panoply of lncRNAs

Random inactivation of one of the two X chromosomes in early embryonic stage involves a decision as to which of the two X chromosomes would express Xist. Recent studies

reveal that besides the Xist, this region produces several other lncRNAs, some of which promote and some suppress Xist expression (Vallot and Rougeulle 2013; Briggs and Reijo Pera 2014; Marchese and Huarte 2014). Balance of these transcripts seems to determine Xist activity and therefore, choice of the inactive X. Among the other lncRNAs produced by Xic are Jpx and Ftx, which are potential activators of the X inactivation process in mouse (Vallot and Rougeulle 2013). Another noncoding transcript, RepeatA (RepA) is encoded within the first exon of Xist and binds independently with the inactivating PRC2 *in vitro* (Zhao *et al*. 2008; Marchese and Huarte 2014). Tsix is yet another lncRNA transcribed from the Xic on the active X chromosome in antisense direction from Xist (Lee *et al.* 1999). It inhibits the accumulation of Xist transcripts on the future active X through negative regulation of expression of Xist in *cis*, and of Jpx lncRNA. The Jpx activates *Xist* by removing the repressive RNA-binding protein CTCF from the *Xist* promoter (Sun *et al.* 2013b). Both RepA and Tsix have been shown to bind PRC2 and thus, while RepA and Tsix may function as 'decoys' for PRC2, Xist transcripts act as 'guides' to localize PRC2 and other members to correct regions on X chromosome for silencing (Marchese and Huarte 2014). Sun *et al.* (2006) suggested that Tsix does not destabilize Xist RNA on the chromosome that produces Tsix, rather Xist transcription on the future inactive-X occurs due to upregulation of its promoter and that Tsix-directed DNA methylation of the Xist promoter on active X in female mice is invoked only as a secondary mechanism after the X inactivation is underway.

Sustenance of Tsix expression requires another lncRNA, Xite, which influences Tsix promoter activity (Ogawa and Lee 2003). However, since truncated Xite RNAs can also function as effectively as the full-length Xite lncRNA in regulating Tsix expression, it is not clear if the genetic control of *Tsix* locus by *Xite* depends only on its RNA (Marchese and Huarte 2014). Linx is another lncRNA which may possibly participate in the control of Tsix expression in mouse (Nora *et al.* 2012). However, it may not be conserved in human, especially since Tsix-like function is missing in humans.

A novel 251.8-kb long and mostly unspliced lncRNA in humans is XACT, which is expressed from a region about 40 Mb from the human XIC (Vallot *et al.* 2013). The XACT transcripts surround the active X chromosome(s) in human pluripotent and early differentiating cells, when XCI is still highly dynamic. In the absence of Xist, XACT can coat both the X chromosomes. Monoallelic repression of XACT appears to correlate with the establishment of stable, irreversible X inactivation. Based on its expression, it is suggested that XACT could protect the active X during the early steps of X inactivation (Vallot and Rougeulle 2013; Briggs and Reijo Pera 2014).

The *Xist* gene has to be kept inactive in mammalian males. It is suggested that the imprinting of maternal X and methylation of the Xist promoter prevents Xist transcription in early mouse male embryos (Panning and Jaenisch 1996; Barr *et al.* 2007). Tsix transcripts may also keep the Xist gene on single X in male mouse inactive. Ray *et al.* (1997) reported expression of XIST in human 5–10 cell stage preimplantation male as well as female embryos. The XACT transcripts coat the active X in male as well as female human embryonic stem cells (Vallot and Rougeulle 2013) and thus may keep the XIST inactive in male cells.

The XIC/Xic is conserved in eutherians, especially between the most studied mouse and human X chromosomes. Human orthologues of many of the lncRNAs from the Xic in mouse are known but their functions are yet to be fully understood. Several significant differences between events at and products of the Xic in mouse and man are, however, known. Thus, unlike in mouse where Xist expression is tightly associated with embryonic stem cell differentiation, its expression in human embryonic stem cells is variable (Makhlouf and Rougeulle 2011). Further, in human preimplantation embryos, X chromosomes are coated by XIST but are active suggesting some level of disconnect between XIST expression and X-chromosome inactivity in human (Okamoto *et al.* 2011). The Tsix transcripts in mouse are antisense to Xist, they inhibit accumulation of Xist transcripts on the future active X and are involved in transition from imprinted to random X inactivation. Interestingly, however, the XIST antisense transcript in humans bear very little similarity to the mouse Tsix and its other properties also do not favour its role in XIST repression, especially because TSIX and XIST can be concomitantly expressed from the same X chromosome in human cells (Vallot and Rougeulle 2013). In view of such differences between mouse and human XIC/Xic, it would be greatly interesting to examine the Xic regions in other eutherians. They may uncover more variability, especially since the lncRNAs generally show less sequence conservation as their functions are dependent more upon structure than sequence (Lakhotia 1996, 2012).

roX1 and roX2 lncRNAs and the dosage compensation complex (DCC) in *Drosophila*

Like the mammalian Xist, the *Drosophila* roX1 and roX2 lncRNAs are essential for achieving dosage compensation in flies but they do so by bringing about changes in chromatin organization that are opposite to those effected by Xist. These RNAs are essential to modify the chromatin organization of the X chromosome in somatic cells of male *Drosophila* so that its genes can transcribe at higher rates to produce nearly as much products as the two Xs together in corresponding female cells (Lucchesi 1998; Kelley *et al*. 1999; Kelley and Kuroda 2000; Lucchesi *et al*. 2005; Georgiev *et al*. 2011; Maenner *et al*. 2012; Mank 2013; Straub *et al*. 2013; Chery and Larschan 2014; Ferrari *et al*. 2014; Keller and Akhtar 2015).

The DCC

Unlike in mammals, the genetic regulation of sex determination and dosage compensation is intimately connected in

Drosophila. The initial cascade of gene activity, triggered after the ratio of autosomes and X chromosomes is determined by the X-linked *sisterless* and *runt* genes, not only drives the embryo to female or male path of development but also sets the stage for dosage compensation (Lucchesi 1998; Lucchesi *et al.* 2005). The *sxl* gene is the master regulator in both pathways, acting through alternative splicing of its transcripts in males and females. Its splicing in females produces the functional RNA-binding and DNA-binding Sxl protein while a truncated and apparently nonfunctional protein is produced in males. The Sxl protein in female embryos controls the female specific alternative splicing of the transformer (tra) transcripts, which in turn initiates sexual dimorphism. The Sxl protein maintains the female fate through autoregulation of the *sxl* gene activity (Bell *et al.* 1991; Lucchesi 1998; Lucchesi *et al.* 2005; Salz and Erickson 2010; Chery and Larschan 2014). In the absence of functional Sxl protein, males produce the male-specific lethal-2 (Msl-2) protein, which is a core component of the male-specific lethal (MSL) or DCC that is essential for the nearly two-fold upregulation of transcriptional activity of the single X chromosome in males (Kelley *et al.* 1997). In females, the Msl-2 synthesis is kept suppressed by Sxl.

The MSL complex or DCC comprises of at least five proteins, namely, male-specific lethal-1 (Msl-1, scaffolding protein), Msl-2 (RING finger protein), Msl-3 (chromodomain protein), males-absent-on-the-first (Mof, histone acetyl transferase) and maleless (Mle, DNA/RNA helicase), and two lncRNAs, roX1 and roX2 (Kelley *et al*. 1995; Lucchesi 1998; Lucchesi *et al*. 2005; Gelbart and Kuroda 2009; Georgiev *et al*. 2011). The DCC paints the male X chromosome along its length and keeps the histone H4 hyperacetylated at lysine 16. Each of these five proteins is essential for modifying the male X-chromatin organization for hyperactivity while the roX1 and roX2 lncRNAs are necessary for the orderly distribution of the DCC along the male X-chromosome. These two transcripts are redundant since any one of them can suffice although absence of both of them disrupts dosage compensation and results in male lethality (Lucchesi 1998; Lucchesi *et al.* 2005; Chery and Larschan 2014).

roX transcripts and spreading of DCC along the male X chromosome

Two polyadenylated but noncoding RNAs on X (roX1 and roX2, respectively) were initially identified as more abundant male-specific brain transcripts, which were found by RNA:RNA *in situ* hybridization, to exclusively 'paint' the X chromosome in male polytene nuclei which gives them the names roX1 and roX2 (Amrein and Axel 1997; Meller *et al.* 1997; Kelley 2004; Lucchesi *et al.* 2005). The *roX1* and *roX2* genes are located on X chromosome and produce transcripts that are dissimilar in size and sequence. The major roX1 transcripts are ~4-kb long while nearly 21 different alternatively spliced forms of roX2 are produced, with the most abundant form being only ~0.5-kb long (Park *et al.* 2005). Male-specific transcription of both the *roX* genes appears to be regulated by the Msl-2 through gene internal enhancers (Bai *et al.* 2004; Rattner and Meller 2004). Preexisting roX RNA is suggested to positively autoregulate *roX1* expression and presence of newly assembled MSL complex around the *roX* gene seems to sustain its transcription and X-chromosome specific spreading in males (Lim and Kelly 2013).

The functional Sxl protein in females destabilizes the msl-2 pre-mRNA and also silences the msl-2 mRNA through interaction with its 5′ and 3′ UTRs (Zhou *et al.* 1995; Bashaw and Baker 1997; Beckmann *et al.* 2005). The consequent absence of Msl-2, thus keeps the *roX1/2* genes inactive in females. Further, Msl-1 and to a lesser extent Msl-3 and roX1/2 RNAs also become destabilized and partially degraded in the absence of Msl-2 so that DCC does not assemble in females (Georgiev *et al.* 2011).

It is believed that X chromosomal chromatin entry sites (CES) or high affinity sites (HAS) facilitate binding of Msl-1 and Msl-2 with male X chromosome which promotes association of roX transcripts and other members of DCC (Lyman *et al.* 1997; Lucchesi 1998; Lucchesi *et al.* 2005; Maenner *et al.* 2012; Straub *et al.* 2013; Chery and Larschan 2014; Keller and Akhtar 2015). The X chromosome is 2-fold enriched in GA-rich Msl recognition element (MRE), which are also present in most of the CES/HAS (Maenner *et al.* 2012; Chery and Larschan 2014). An enrichment of $(dC-dA)_n$.$(dG-dT)_n$ sequences on the X chromosome was reported earlier (Pardue *et al.* 1987) while recent whole genome sequencing revealed the C/A_n and G/T_n repeats to be a sequence signature for the X chromosome; these seem to contribute to the enrichment of MRE and HAS motifs on this chromosome (Georgiev *et al.* 2011). Like the LINES or SINES which may provide entry sites for Xist RNA on inactive X in mammals (see above), the MREs also appear to have evolved from transposon insertions followed by expansion of GA-rich sequences (Chery and Larschan 2014).

Location of the *roX1* and *roX2* genes on X chromosome facilitates the spreading of these transcripts in *cis* when activated in male cells, although unlike the Xist, these transcripts can also associate with X chromosome in male cells when ectopically expressed from an autosomal site (Maenner *et al.* 2012; Straub *et al.* 2013; Chery and Larschan 2014; Keller and Akhtar 2015). Both the *roX* genes carry strong HAS sites, which facilitate cotranscriptional assembly of DCC *in situ* as the nucleation point for its spreading in *cis* to neighbouring HAS (Park *et al.* 2003; Maenner *et al.* 2012). The Msl-1, Msl-2 and Msl-3 proteins function as scaffolds that facilitate proper association of the DCC with the X chromosome. Msl-2 binds to a dimer of Msl-1 to form the core complex that can identify and associate with the HAS. Msl-3 functions as an adaptor molecule through its N-terminal chromodomain (CD) and C-terminal Morf-related gene (MRG) domain. The CD possibly recognizes the H3K36me3, a mark preferentially seen on the 3′ ends of actively transcribed

genes and whose reduction causes X-specific depletion of H4K16ac (Keller and Akhtar 2015). Thus it is possible that the MSL complex spreads on X-linked genes through interaction of the Msl-3 CD with H3K36me3. The MRG domain of Msl-3, on the other hand, interacts with Msl-1 and also stimulates Mof's histone acetyltransferase activity (Morales *et al.* 2005; Keller and Akhtar 2015). Mle is a RNA/DNA helicase which helps incorporation of roX1/2 into the DCC through transient RNA-mediated interactions. Mle and Msl-2 bind distinct stem-loop structures in roX1/2; Mle remodels the roX1/2 stem-loop structures and hands over the roX1/2 RNAs to Msl-2 for their integration in the DCC (Keller and Akhtar 2015). The transient association of Mle with the DCC as well as the roX1/2 RNAs requires cofactor like upstream of N-Ras (UNR) protein which has a binding site on roX RNAs close to the known roX remodelling site of the Mle helicase and which inhibits MSL–DCC assembly in females by repressing Msl-2 synthesis (Abaza *et al.* 2006; Mittili *et al.* 2014). The Mof acetylates the lysine 16 of H4 (H4K16ac) to unfold the chromatin and increase its transcription potential (Maenner *et al.* 2012; Chery and Larschan 2014; Keller and Akhtar 2015).

In spite of the dramatic differences in size and sequence, the roX1 and roX2 RNAs are redundant for all known functions (Kelley 2004; Lucchesi *et al.* 2005). Phylogenetic analysis revealed conserved multiple GUUNUACG (roX box) sequences on both roX transcripts which function redundantly but deletion of all the roX boxes affects assembly of the DCC and H4K16 acetylation on the male X chromosome (Park *et al.* 2008; Maenner *et al.* 2012). *In silico* analysis suggested that the conserved roX box at the 3′ end of roX RNAs can form a stable stem-loop structure of about 80 nucleotides (Maenner *et al.* 2012). Like the A and C regions of Xist being involved in distinct functions, removal of the 3′ stem–loop region affects male viability without much effect on localization of the DCC on X chromosome, while removal of large part of 5′ end of roX1 affects its exclusive localization on the X chromosome (Maenner *et al.* 2012). Figueiredo *et al.* (2014) found that in the absence of both the roX transcripts, the DCC binds with heterochromatic chromocentre and some sites on chromosome 4; they have, therefore, suggested that one of the functions of roX1/2 transcripts is to prevent binding of DCC to heterochromatic regions, which may be an ancestral property of DCC.

Some studies have suggested that at the level of resolution offered by polytene chromosomes, an autosomal segment inserted in the male X chromosome does not show hyperactivity (Lakhotia 1970) or recruitment of DCC (Fagegaltier and Baker 2004; Georgiev *et al.* 2011). However, a ChIP study (Gorchakov *et al.* 2009) showed that active genes on a 65-kb autosomal segment inserted on the male X chromosome show MSL binding. It is possible that length of the inserted autosomal DNA may influence spreading of DCC.

Additional proteins that are essential facilitators of the DCC have also been identified. JIL-1 kinase, which can phosphorylate serine 10 of H3 is 2-fold more enriched on the male X chromosome with its chromosomal location correlating with presence of the H3K36me3 and H4K16ac marks of actively transcribed chromatin (Maenner *et al.* 2012). Wang *et al.* (2013) have identified CG4747, a putative H3K36me3-binding protein, which, together with Set2, facilitates targeting of the DCC to active genes. The CG1832 or chromatin-linked adaptor for MSL protein (CLAMP) recruits DCC to the MRE/HAS sites on the X chromosome (Larschan *et al.* 2012; Wang *et al.* 2013). Since the MREs are not X-specific sequences, CLAMP may increase specificity for Msl 1/2 binding on the X chromosome and thus, recruit the DCC to the MRE/HAS sites (Larschan *et al.* 2012) and, like the mammalian YY1, keep it tethered to the target sites. MSL complex also recruits Topo II to the hyperactive X chromosome through association with an unidentified RNA (Cugusi *et al.* 2013).

In a recent study using GAL4-Mof or GAL4-Msl-2 fusion proteins, Sun *et al.* (2013a) suggested that unlike the popular belief, Msl-2 does not mediate the 2-fold hyperactivation of the male X, but its presence in the DCC overrides the high level of Mof-mediated histone acetylation and counteracts the potential overexpression of X-linked genes to achieve the proper two-fold upregulation in males. It remains to be seen if these somewhat divergent results are due to the GAL4 fusion proteins or would apply in general.

As expected from the altered chromatin organization of the X chromosome in male cells, reflected in its pale staining and increased width in larval salivary gland polytene nuclei (Dobzhansky 1957; Mukherjee and Beermann 1965; Lakhotia and Mukherjee 1969), chromatin remodelers also interact with the DCC. Null mutations for Nurf301 or ISWI cause severe distortion in the X chromosome in male polytene nuclei (Deuring *et al.* 2000; Corona *et al.* 2002; Bai *et al.* 2007). Other epigenetic regulators of chromatin organization like HP1 (Spierer *et al.* 2005), DNA supercoiling factor, SCF (Furuhashi *et al.* 2006) and the nuclear pore and/or matrix components like NUP153 and Megator (Mendjan *et al.* 2006; Vaquerizas *et al.* 2010) have also been found to affect the association of DCC members and organization of the X chromosome in male *Drosophila*. Recent studies in our laboratory (Chaturvedi D. and Lakhotia S. C., unpublished data) have revealed that the hsrω-n lncRNAs, which organize the nucleoplasmic omega speckles and thus, regulate intranuclear dynamics of hnRNPs and some other RNA-binding proteins (Lakhotia 2011, 2012; Singh and Lakhotia 2015) also interact with the DCC since misexpression of these transcripts affects the altered male X chromosome organization and association of DCC following absence or downregulation of some members of the DCC or chromatin remodelers. The hsrω-n transcripts are known to interact with ISWI (Onorati *et al.* 2011; Singh and Lakhotia 2015), Megator (Zimowska and Paddy 2002; Singh and Lakhotia 2015), HP1 (Lakhotia *et al.* 2012) while other recent observations in our laboratory (Chaturvedi D. and Lakhotia S. C., unpublished data) reveal genetic

interaction of hsrω transcripts with Nurf 301, Nurf 38 and GCN5 chromatin remodelers, which in turn affect the organization of the *Drosophila* male X chromosome.

The MOF and MLE also have functions beyond dosage compensation through transcriptional and splicing regulation of autosomal genes (Lucchesi 1998; Lucchesi *et al.* 2005; Gelbart and Kuroda 2009; Georgiev *et al.* 2011; Maenner *et al.* 2012; Cugusi *et al.* 2015; Keller and Akhtar 2015). Therefore, these proteins have also been reported to be members of another complex, the nonspecific lethal (NSL) complex (Georgiev *et al.* 2011; Keller and Akhtar 2015). The NSL complex and its interaction with MOF have been reported in mammals too. The mammalian MSL-associated MOF acetylates nucleosomal histone H4 almost exclusively on lysine 16, while the NSL-associated MOF exhibits a relaxed specificity and also acetylates nucleosomal histone H4 on lysines 5 and 8 (Georgiev *et al.* 2011; Keller and Akhtar 2015). It remains to be seen if the NSL complex in flies and/or mammals requires any lncRNA for its functions.

Concluding remarks

The phenomenon of dosage compensation is primarily a requirement only in species which have sex chromosomes that vary in their copy number between the two sexes. Intriguingly, however, the extent of dosage compensation for the sex chromosome-linked genes ranges from absence to partial to complete in the limited number of species that have been studied in-depth (Mank 2013). The mechanism of dosage compensation also varies since it is only in mammals and dipteran insects like *Drosophila*, that a whole chromosome level inactivation or hyperactivation is known. *Caenorhabditis* achieves complete dosage compensation through partial repression of gene activity in both the X chromosomes in females (Lucchesi *et al.* 2005; Mank 2013). It is significant that the two groups, mammals and diptera like *Drosophila*, that show chromosome-wide inactivation or hyperactivation to achieve dosage compensation, employ lncRNAs to epigenetically modify the chromatin in opposing manner. Such opposing effects of chromosome-wide 'painting' with lncRNAs reflect the versatility of RNA molecules. It is interesting to note that in spite of the apparent similarity in spreading of Xist or roX transcripts on the inactive or the hyperactive X chromosome, respectively, the spatial gradient effect seen in the spreading of inactivation of female mammalian X is not seen in *Drosophila*, so that in spite of the whole chromosomal effect, individual genes remain under 'piecemeal' (Muller 1950) regulation in *Drosophila*. A question that awaits resolution is how some genes on the X chromosome, located within the broad zone of Xist 'painting', escape inactivation (Lopes *et al.* 2011; Peeters *et al.* 2014). It also remains to be understood why some alleles of an X-linked gene in *Drosophila* show dosage compensation while another allele of the same gene is not compensated (Muller 1950). It is significant that the human and mouse Xist RNAs show substantial sequence divergence but still achieve the same end result. In this context, the divergence between the functionally redundant roX1 and roX2 lncRNAs is more remarkable. This also raises the question that if roX1 and roX2 are indeed fully redundant, why have both genes survived selection in spite of great sequence divergence? It is possible that there may be subtle but significant differences in their actions which may not have yet been looked into or may not be apparent under the constant laboratory environment under which all these studies are undertaken. Another vexing question that needs resolution is if the single active X in male and female mammals is transcriptionally hyperactive when compared with autosomes (Mank 2013)?

Discovery of the phenomena of X inactivation in mammalian females by Lyon and X hyperactivation in *Drosophila* males by Mukherjee and Beermann in early 1960s catalysed active research that rapidly established a general correlation between the cytological appearance of chromatin, transcriptional inactivity/activity and late/early replication. These, in turn, stimulated extensive genetic, biochemical and molecular approaches to understand the factors that impinge upon chromatin organization. Studies on dosage compensation in mammals and *Drosophila* have contributed significantly to our current appreciation of biological relevance of lncRNAs and the rapidly expanding field of epigenetic modifications of chromatin. It is hoped that the increasingly powerful genetic, cell and molecular biological methods, combined with more specific questions, will not only resolve some of the uncertainties but would also bring out unanticipated variables in diverse groups to achieve dosage compensation as the end result. Variability is so characteristic of living systems since anything that works can survive the natural selection.

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