Noncoding Genes on Sex Chromosomes and Their Function in Sex Determination, Dosage Compensation, Male Traits, and Diseases

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Abstract

The mammalian Y chromosome has evolved in many species into a specialized chromosome that contributes to sex development among other male phenotypes. This function is well studied in terms of protein-coding genes. Less is known about the noncoding genome on the Y chromosome and its contribution to both sex development and other traits. Once considered junk genetic material, noncoding RNAs are now known to contribute to the regulation of gene expression and to play an important role in refining cellular functions. The prime examples are noncoding genes on the X chromosome, which mitigate the differential dosage of genes on sex chromosomes. Here, we discuss the evolution of noncoding RNAs on the Y chromosome and the emerging evidence of how micro, long, and circular noncoding RNAs transcribed

from the Y chromosome contribute to sex differentiation. We briefly touch on emerging evidence that these noncoding RNAs also contribute to some other important clinical phenotypes in humans.

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Mammalian Sex Chromosome Evolution

The organization and function of mammalian sex chromosomes owes as much to their peculiar evolutionary trajectory as to their fitness for purpose.

Sex chromosomes have arisen independently many times during vertebrate evolution when a new sex-determining gene is acquired by a chromosome pair. Linkage with sex instantly changes the dynamics of this chromosome such that other gene variants which confer an advantage to one or the other sex accumulate near the sex-determining locus, and recombination between the sex-specific chromosome and its erstwhile homologue is suppressed to keep the package of sex-specific variants together [Ellegren, 2011; Saunders et al., 2019]. Loss of recombination leads to accumulation of mutations and

deletions in the sex-specific chromosome, leading to its rapid degradation and eventual disappearance [Graves, 2006; Bachtrog, 2013]. Such cycles of acquisition, differentiation, and loss have been observed in many vertebrate and invertebrate lineages [Ortega et al., 2019].

Generation of novel sex determining genes has resulted in the huge variety among vertebrates such that it may appear that almost any gene can take on this function [Graves, 2013; Bachtrog et al., 2014]. However, there are patterns and limitations. Formally, sex determining genes may either promote or inhibit development of either male or female [Arnold, 2017]. Acquisition of a male-dominant gene defines a Y chromosome that is male-specific, such as the sex-determining region Y (*SRY*) gene in mammals, and sets up an XX female:XY male system [Charlesworth, 1996]. Acquisition of a male-inhibitor allele, on the other hand, defines a W chromosome that is female specific, such as *Dmw* in *Xenopus laevis*, and sets up a ZZ male:ZW female system [Yoshimoto et al., 2010; Yoshimoto and Ito, 2011]. In birds as well as many reptiles and fish, it is the dosage of a sex-determining gene that sends development down a male- or female-determining pathway [Alam et al., 2018]. For instance, the Double-sex and Mab-3 Related Transcription Factor 1 (*Dmrt1*) in birds is present on the Z but not the W chromosome [Jeng et al., 2019]. Two copies are required to be male, so males are ZZ and females ZW [Yoshimoto and Ito, 2011]. Dosage of a female-determining gene is also theoretically possible and sets up an XX female:XY male system as in *Drosophila* [Cline, 1984].

The origin of sex determining genes is usually one or other of the 60+ genes in the complex vertebrate sex-determining pathway [Bachtrog, 2013]. For instance, *Dmrt1*, which is sex determining in several reptiles and fish as well as birds, has a conserved role stabilizing testis development in mammals, and copies of this gene may be repurposed as a male-dominant gene (e.g., *Dmy* in medaka fish) or an inhibitor (e.g., *Dmw* in *X. laevis*) [Trukhina et al., 2013].

Dmrt1 is reported to be the key factor in sex determination in birds. Ioannidis et al. [2021] utilised a CRISPR/Cas9-based monoallelic targeting approach resulting in a single functional copy of *Dmrt1*. This led to chickens developing ovaries in place of testes, therefore demonstrating that the sex-determining system in avian species is dependent on *Dmrt1* dosage. *Dmrt1* is reported to be the key factor in sex determination in birds.

The mammalian *SRY* evolved from an ancient transcription factor, *SRY*-line HMG Box Transcription Factor 3 (*SOX3*) simply by a rearrangement that sent its ex-

pression into the developing gonad: homologues of this gene are sex determining in a frog and fish species [Graves, 1998]. Anti-müllerian hormone (*Amh*), which is essential for the male-specific inhibition of female reproductive ducts in mammals, is sex determining in some fish and perhaps the platypus [Veyrunes et al., 2008; Cortez et al., 2014]. The acquisition of a sex determining function by this variety of genes means that sex chromosomes in different lineages have quite different origins and bear different sets of genes [Graves, 1998].

Turnover of the sex-determining gene can be extremely frequent in reptiles, amphibians, and fish and may helped along by the influence of environmental cues like temperature on sexual development [Janzen and Paukstis, 1991; Holleley et al., 2015]. Turnover is rare in mammals and birds, perhaps because homeothermy makes temperature sex determination impossible. However, the cycle of Y chromosome attrition promotes turnover, even in mammals [Janzen and Paukstis, 1991].

The mammalian (including human) XY chromosome pair illustrates this cycle of birth, differentiation, degradation, and loss of a male-specific Y chromosome. The human Y chromosome is much smaller than the X, contains few protein-coding genes, and is largely composed of highly repetitive sequence (Fig. 1) [Quintana-Murci and Fellous, 2001]. Only the very tip of the Y retains homology with the X over a pseudoautosomal region (PAR) that contains about 25 genes. The PAR pairs at meiosis and is critical for sex chromosome segregation and spermatogenesis [Quintana-Murci and Fellous, 2001].

The mammalian X chromosome contains more than 1,000 protein-coding genes and is almost completely conserved among mammals, perhaps protected by the chromosome-wide X chromosome inactivation mechanism that ensures dosage compensation between the sexes [Prothero et al., 2009]. It has homology to a chromosome or chromosome regions in all vertebrates and shares *SOX3*, the gene from which *SRY* evolved [Graves and Peichel, 2010].

In contrast, the male-specific portion of the human Y (*MSY*) chromosome bears only 27 protein-coding genes, most of which have homology to conserved genes on the X, from which they obviously diverged [Skaletsky et al., 2003]. However, a few genes arose from the insertion of gene copies transposed or retrotransposed from autosomes (e.g., *DAZ1*) or the X (e.g., *PCDH11Y*) [Ghenu et al., 2016]. Several Y genes, including those that evolved from X genes (such as *RBM1Y*) have been greatly amplified and are present in tandem arrays on enormous loops, whose arms bear complementary (palindromic) sequenc-

Fig. 1. Structure of the human Y chromosome. Each terminal end of the Y chromosome harbours the pseudoautosomal regions (PAR1 and PAR2). The heterochromatin (grey section) is genetically inactive and the region within each of the PAR regions is the male specific region on Y (MSY). The block of black text illustrates Y-linked noncoding genes that play a role in male disease, while *circSRY* contributes to male sex determination.

es [Ghenu et al., 2016]. These sequences can undergo gene conversion, which could correct mutations that arise in the multiple arrays or may hasten their pseudogenization (presumably the reason for the high proportion of inactive copies). The reasons why these few genes have survived the inexorable degradation of the Y have been debated for decades. Some have obviously taken on male-specific functions (for instance *RBMV*, essential for spermatogenesis, evolved from *RBMX*, essential for brain development) [Tsend-Ayush et al., 2005] whereas others seem to be dose sensitive so their loss from the Y would present severe dosage imbalance (e.g., *KDM5C* and *ZFY*) [Bellott et al., 2014]. The degradation of the Y chromosome has proceeded to different extents in different lineages [Bachtrog, 2013]. The primate (especially human) Y seems to have been reasonably stable over the last few million years [Hughes et al., 2012], whereas the mouse Y retains few essential genes [Morgan and Pardo-Manuel De Villena, 2017] and some rodent species have lost the Y (and *Sry*) entirely [Mulugeta et al., 2016; Ortega et al., 2019]. While most attention has been given to protein-coding genes on the human Y chromosome, the transcription from at least a few noncoding RNAs (ncRNAs)

on the Y chromosome have been recognised relatively recently [Molina et al., 2017; Lai et al., 2019; Xiao et al., 2019].

Noncoding RNA Derivation and Function

ncRNAs do not encode proteins, but they may exhibit regulatory function [Farazi et al., 2008]. Regulatory ncRNAs can be divided into small RNAs with fewer than 200 nucleotides and long ncRNAs (lncRNAs) with a length greater than 200 nucleotides [Kim et al., 2009].

Small ncRNAs can be further subdivided into endogenous small interfering RNAs (endo-siRNAs), PIWI-associated RNAs (piRNAs), and microRNAs (miRNAs) [Farazi et al., 2008; Kim et al., 2009]. Endo-siRNA are derived from complementary double-stranded RNAs formed through sense-antisense transcript pairs and transposon transcripts [Golden et al., 2008]. These endo-siRNAs have been found to contribute to the repression of transposons [Song et al., 2011]. Small RNAs, piRNAs, on the other hand, are formed from single-stranded piRNA precursor transcripts [Vagin et al., 2006] and have

been reported in transposon silencing via heterochromatin formation and RNA destabilization [Klattenhoff and Theurkauf, 2008]. One of the most investigated ncRNA classes has been miRNAs. miRNAs are transcribed by RNA polymerase II and further processed in the cytoplasm by the enzyme Dicer, producing a 21–23-nt long miRNA duplex. One strand of this duplex can mediate sequence-specific binding to target messenger RNAs (mRNAs), which are subsequently silenced or degraded [Farazi et al., 2008; Kim et al., 2009].

Many long ncRNAs have also been identified [Okazaki et al., 2002; Carninci et al., 2005], varying in length from 200 bp to several kilobases. lncRNAs are transcribed by RNA polymerase II from different regions of the genome. Several derive from intergenic regions [Guttman et al., 2011; Ulitsky and David, 2013], while others derive from the sense or antisense strand of protein-coding genes [Wu et al., 2014]. Many lncRNAs are considered to be general by-products of transcription, RNA processing, and splicing, but they may also exhibit specificity of expression and function [Fatica and Bozzoni, 2014]. lncRNA functions involve regulating chromatin remodelling, controlling transcription rate of genes, and influencing post-transcriptional processes through inhibition or induction of translation [Mercer et al., 2009; Fatica and Bozzoni, 2014].

Circular RNAs (circRNAs) are emerging as an important class of regulatory ncRNAs. CircRNAs are a covalent closed loop and lack the polyadenylated tail seen in messenger RNA [Qu et al., 2015]. They originate from protein-coding genes [Pamudurti et al., 2017] and have been suggested to act as sponges that regulate miRNAs [Legnini et al., 2017; Pamudurti et al., 2017]. CircRNA expression also seems to be highly abundant in comparison with their linear isomers [Jeck et al., 2013]. Due to their closed structure, circRNAs are very stable and have exonuclease resistance properties [Holdt et al., 2018]. CircRNAs have been found to be highly tissue specific and expressed in peripheral blood of humans [Memczak et al., 2015] with potential use as biomarkers.

Role of Noncoding RNA from Sex Chromosomes in Sex Determination

Various ncRNAs are involved in the sex determination and dosage compensation in invertebrates. In *Drosophila melanogaster*, specification of sex is controlled by alternative splicing of the gene *Sex-lethal* (*Sxl*) which is triggered by the dosage of X chromosome “numerator genes”.

Noncoding transcripts from 2 regions upstream of the *Sxl* promoter affect the chromatin state around the promoter. The antisense transcript from one, in partnership with proteins of the 5 “numerator genes”, changes the chromatin conformation around the *Sxl* promoter and permits transcription of the full-length gene that is required for female development [Mulvey et al., 2014]. Expression of lncRNAs in transgenic lines demonstrate that they impact not only sex determination but alter levels of other lncRNA [Mulvey et al., 2014].

In invertebrate sex determination, a cell-autonomous process is usually occurring throughout the embryo [Girard et al., 2006; Lau et al., 2006; Watanabe et al., 2006]. Other invertebrate sex-determining systems involve small ncRNAs. *Bombyx mori* (silkworm) has an ZW female:ZZ male system. The female-specific W chromosome has a dominant female-determining action. However, it contains no coding genes and is practically all composed of transposable elements. The only transcripts from the sex-determining region of the W chromosome are noncoding piRNAs transcribed from a sequence *Fem*, which is present in tandem arrays [Kawaoka et al., 2011; Kiuchi et al., 2014]. *Fem* is expressed in embryos and silences a masculinizing gene on the Z chromosome, which in turn regulates splicing of the gene *Doublesex* (*dsx*) which determines sex in many insects [Arbeitman et al., 2016]. The *Dsx* transcript is spliced in a sexual dimorphic manner that leads to 2 proteins with female and male functions and coordinates female versus male development in not just the gonads but all the body. The *dsx* vertebrate homologue *Dmrt1* is a highly conserved transcription factor with an important role in morphogenesis of the testis [Gamble and Zarkower, 2012; Matson and Zarkower, 2012]. While differential splicing of *Dmrt1* has been shown in vertebrates [Huang et al., 2017], no evidence currently exists to indicate that vertebrate *Dmrt1* genes are regulated by piRNAs. However, ncRNAs may be involved in other vertebrate systems, and there is a growing list of ncRNAs that are transcribed differentially during sex determination in various species [Zhang et al., 2018; Yan et al., 2021].

Although it is established that the linear SRY gene is responsible for sex determination, there is also a testis-specific circular SRY (*circSRY*) that may play a role in regulation of sex determination in mammals [Capel et al., 1993]. This *circSRY* has been found in higher abundance than linear SRY in human testis. There are 16 *miR-138* binding sites on this *circSRY*, and it has previously been shown to interact with *miR-138* in human embryonic kidney cells (HEK293), and therefore could function as a *miR-138* sponge [Hansen et al., 2013].

Sex Chromosome lncRNA and Dosage Compensation

Tightly coupled with sex determination are mechanisms to correct for dosage differences in sex chromosomes. Species with highly differentiated sex chromosomes must contend with differential dosage of genes on the X (in XX female/XY male systems) or Z (in ZZ male/ZW female systems). In *Drosophila*, genes on the single X in XY males are hypertranscribed by binding with the male-specific lethal-dosage compensation complex (MSL-DCC) that acetylates histones. MSL-DCC contains 5 proteins and 2 lncRNAs, *roX1* and *roX2* (RNA on X), which function in binding to targets [Meller and Rattner, 2002] and provide binding specificity [Li et al., 2008].

In mammals, XX females have 2 copies of each of the 1,000 or so genes on the X, whereas XY males have only 1. The solution to gene dosage differences in mammals is to inactivate 1 of the X chromosomes. Again, lncRNAs also seem to regulate this process. In eutherian (placental) mammals, this is accomplished through a lncRNA called *Xist* (X inactive specific transcript) [Disteche, 2012]. The 19-kb transcript is transcribed only from the *Xist* locus on the inactive X chromosome and represses the expression of hundreds of genes [Lu et al., 2017]. In both mouse and human, *XIST* lncRNA coats the X chromosome to be inactivated, and its interaction with many protein and RNA factors (some of which are involved in *Drosophila* dosage compensation) assembles a silencing domain. Another lncRNA (*Firre*) attaches it to the nuclear membrane, where several proteins conspire to block transcription [Khosraviani et al., 2019]. There are several other lncRNAs involved in X chromosome inactivation in humans and mouse [Gendrel and Heard, 2014], including X-borne *XACT* on the human X [Vallot et al., 2013] and an antisense lncRNA *Tsix* in mouse [Maclary et al., 2013].

Marsupials too have an X chromosome inactivation system, albeit partial and incomplete and paternally imprinted. Searches for an *XIST* homologue over a decade were unsuccessful, and it was discovered that the region that flanks this gene in eutherians is disrupted in marsupials and monotremes [Hore et al., 2007]. Remarkably, however, a lncRNA was discovered in marsupials with no homology to *XIST* but with similar properties. This *RSX* (RNA on the silent X) is expressed from the paternal X and coats it, recruiting other silencing factors in much the same way as the unrelated *XIST* in eutherians [Grant et al., 2012]. The presence of these unrelated lncRNAs that do a similar job in related mammal lineages suggests that the genome of a common ancestor may have included several – or many – lncRNAs with the propensity to si-

lence large regions of chromatin, and different ones took over the sex chromosome silencing function in eutherians and marsupials [Graves, 2016].

In other systems, such as birds, which possess the ZZ male:ZW female system, there is no such chromosome-wide inactivation of one sex chromosome [Disteche, 2012; Livernois et al., 2012]. However, lncRNAs may be involved in dosage compensation in chickens. The gene *Dmrt1* has been found to play an important role in avian sex determination through directing testis development in ZZ embryos [Ioannidis et al., 2021]. *Dmrt1* overexpression induces the male-specific genes *Hemgn*, *Sox9*, and *Amh* [Lambeth et al., 2014]. However, *Dmrt1* knockdown results in feminization of the gonads, leading to an increase of female marker genes *Foxl2* and *Aromatase* [Smith et al., 2009].

Located on the Z chromosome of chicken (but not other bird lineages) is the male hypermethylation region (*Mhm*), a 2.2-kb sequence that is hypermethylated in males and transcribed only in female embryos [Yang et al., 2016]. *Mhm* produces a 9-kb lncRNA that accumulates in the nucleus adjacent to the *Dmrt1* locus [Teranishi et al., 2001]. A second differentially methylated region has been discovered on the chicken Z chromosome [Sun et al., 2019]. Although it has no sequence homology with *Mhm1*, it shares several characteristics: highly repetitive structure, higher chromatin accessibility, and enrichment with active histone marks in females. The transcription of lncRNA in a female-biased way, and reduced male:female expression ratios of nearby genes confer a degree of dosage compensation [Bisoni et al., 2005], ultimately affecting development of gonads and other tissues [Roeszler et al., 2012]. It has been suggested that *Mhm* is involved in gonadal sex differentiation in chickens, contribution to the reduced expression of *Dmrt1* in females [Huang et al., 2005]. This process is thought to occur by *Mhm* lncRNA altering the chromatin conformation thereby disrupting transcription of *Dmrt1*. This is supported by ZZ chicken embryos possessing altered *Dmrt1* expression [Roeszler et al., 2012], while adult chicken testis that contain an insertion of *Mhm* plasmids dampen the expression of *Dmrt1*. Collectively, evidence of these ncRNAs in sexual determination demonstrate the pivotal regulatory roles across species.

Y-Borne ncRNAs and Male Traits and Diseases

ncRNAs have been identified across all human chromosomes, including the Y and X chromosomes [Uszczyńska-Ratajczak et al., 2018]. As well as 27 protein-cod-

ing genes, the male-specific region of the human Y harbours a number of lncRNAs (including circRNAs) and miRNAs [Skaletsky et al., 2003]. Very few studies have determined whether these linear Y-linked ncRNAs play a role in testis determination or other male traits in humans. The haploid nature of the Y chromosome means that conventional methods of chromosomal analysis such as genome-wide association studies cannot be utilised.

Although it was initially thought that the ncRNAs present on the Y chromosome are only involved in male-exclusive phenotypes such as spermatogenesis and male gonad development [Skaletsky et al., 2003], there is recent emerging evidence to suggest that they play a role in regulating male disease [Molina et al., 2017; Huang et al., 2019; Lai et al., 2019; Lin et al., 2019; Xiao et al., 2019; Brownmiller et al., 2020; Hao and Chen, 2021]. Figure 1 shows Y-borne noncoding genes not only involved in sex determination but other phenotypes in males.

For instance, Y-linked lncRNAs have been described that play a part in early-stage central nervous system (CNS) development in human males [Johansson et al., 2019]. The noncoding transcripts of Y-linked lnc-*KDM5D*, *TTY14*, and *TTY15* were found to be expressed in early CNS development and played functional roles in newborn chimpanzees [Johansson et al., 2019]. These Y-linked lncRNAs all have characteristics that suggest a potential functional role such as tissue specificity, polyadenylation, and conservation across primate evolution [Johansson et al., 2019].

There are also emerging concepts that Y chromosome borne ncRNAs play important roles in regulating male disease. Associations between the Y chromosome and cardiovascular disease, lung cancer, and prostate cancer have been demonstrated [Molina et al., 2017; Xiao et al., 2019; Brownmiller et al., 2020].

Molina et al. [2017] demonstrated the first lncRNA on the Y chromosome that could be involved in a disease process. The study investigated the role of lncRNA Lysine Demethylase 5D-4 (*lnc-KDM5D-4*) in atherosclerosis and coronary artery disease. The authors discovered an intergenic lncRNA transcribed from the Y chromosome and demonstrated that knocking *lnc-KDM5D-4* down changed the expression of several genes putatively involved in lipid metabolism. This demonstrates a function of *lnc-KDM5D-4* in lipid metabolism and suggested that its disruption could lead to formation of increased lipid droplets in hepatocytes. This increase is a known factor that contributes to the chronic inflammatory process underpinning coronary artery disease.

The noncoding testis-specific transcript, Y-linked 15 (*TTY15*) on the human Y has been implicated in several male diseases [Huang et al., 2019; Lai et al., 2019; Xiao et al., 2019; Hao and Chen, 2021]. Xiao et al. [2019] found that *TTY15* was upregulated in prostate cancer tissues with the ability to promote prostate cancer progression. Forkhead box protein A1 (*FOXA1*) plays an important role in prostate cancer development and is also a regulator of *TTY15*. *TTY15* “sponges” the *let-7* family to alter the expression of Fibronectin 1 (*FN1*), which is a relevant mediator of cancer metastasis [Xiao et al., 2019]. In another study Lai et al. [2019] found that *TTY15* inhibits non-small cell lung cancer (NSCLC) metastasis and proliferation. These authors found that lower expression levels of *TTY15* had an association with tumor-node-metastasis and concluded that low expression correlates with worse prognosis among patients with NSCLC [Lai et al., 2019].

Additionally, a group of Y-linked long intergenic non-coding RNAs (lincRNAs) have been implicated in male NSCLC radiation sensitivity [Brownmiller et al., 2020]. Expression of 3 Y-linked lncRNAs, *linc-SPRY3-2*, *linc-SPRY3-3*, and *linc-SPRY3-4*, were detected in radiosensitive male NSCLC cell lines following radiation and not in radioresistant male NSCLC cell lines [Brownmiller et al., 2020]. The radioresistant male NSCLC cell lines demonstrated a loss of the Y chromosome (LOY) [Brownmiller et al., 2020]. Microarray and RNA sequencing analysis illustrated a negative correlation amongst the LOY, *linc-SPRY3-2/3/4* expression, and overall survival. This study therefore suggests that *linc-SPRY3-2/3/4* expression and LOY could be a useful biomarker of successful radiation therapy in NSCLC patients [Brownmiller et al., 2020].

There is increasing evidence that circRNAs are involved in development and progression of male disease. To understand the role of circRNAs on the Y chromosome in the context of coronary artery disease, Lin et al. [2019] measured circRNAs in peripheral blood of coronary artery disease patients. They found several significantly upregulated Y-linked circRNAs. These included circRNAs associated with the MSY genes *UTY*, *KDM5D*, and *USP9Y* [Lai et al., 2019]. However, these studies are yet to define the mechanistic role of these circRNAs.

Concluding Remarks

The last few decades of sex chromosome research have come far in elucidating how the Y chromosome has evolved and how it contributes to sex determination. This

is true in terms of the protein-coding genes. The noncoding content of the Y and the X are still underexplored. There is an emerging appreciation that Y-linked ncRNAs may play a bigger role developmentally. Few examples through evolution are the *Fem* piRNA in the silkworm and the lncRNA *Sxl* in *Drosophila*. In humans, further evidence is also emerging that these ncRNAs contribute to development and health. Y-linked lncRNAs such as *lnc-KDM5D*, *TTY14*, and *TTY15* are increasingly found to be associated with disease. Since these functional ncRNAs play a time-critical role, it is important and more difficult to study the role of these molecules without genome editing. Nonetheless, further studies exploring the function of these Y-linked ncRNAs and how they interact with other chromosomes, including the X, are warranted.

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