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Trends in Genetics

Review

The evolution of gene regulation on sex chromosomes

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Sex chromosomes have evolved repeatedly across the tree of life. Most work has focused on the loss of coding regions from sex-limited chromosomes through the accumulation of deleterious mutations. By comparison, less is known about how the regulatory landscape evolves. We review theories of how regulatory landscapes evolve on sex chromosomes and the overall impact they have on gametolog expression. We integrate empirical studies on sex chromosomes with theoretical work to synthesize how regulatory evolution could occur on sex chromosomes. Recent findings have revealed that downregulation of ancestral alleles is probably widespread on Y chromosomes and that regulatory evolution plays a key role in the evolution of sex chromosomes.

Highlights

Sex chromosomes rapidly accumulate deleterious mutations. Coding-sequence degeneration is well documented, but regulatory mutation has not been explored empirically. Regulatory mutation may have a primary role in the early evolution of the expression of Y-linked gametologs.

The evolution of the expression of genes on sex chromosomes likely does not follow a simple trajectory of pseudogenization and dosage compensation. Gametologs can also be maintained on the Y chromosome through purifying selection. Gametologs important for male function can also evolve novel regulatory elements, leading to the gain of new expression domains.

The completion of additional Y-chromosome reference assemblies will greatly add to our understanding of the early evolution of regulatory elements. However, a major rate-limiting step will be the functional annotation of these regions.

Evolution of heteromorphic sex chromosomes

Sex chromosomes evolve from homologous autosome pairs. The classic model of sex chromosome evolution predicts that linkage between **sexually antagonistic** (see Glossary) mutations and the master sex-determination locus selects for suppression of recombination between the X and Y chromosomes [1,2]. Since the classic model of sex chromosome evolution was proposed, empirical support for the role of sexually antagonistic mutations has been scarce [3–5]. This has led to the synthesis of several other models. Alternative explanations for recombination suppression have included meiotic drive [6], heterozygote advantage [7], and genetic drift (reviewed in [8]). Recently, a new model (degeneration by regulatory evolution, DRE) has been developed in which inversions can be favored to be retained on sex chromosomes after rapid divergence of regulatory regions between the X and Y chromosomes [9]. This model has been supported through simulations and does not require the accumulation of sexually antagonistic mutations to suppress recombination.

For all models, once recombination is suppressed, the Y chromosome rapidly undergoes **sequence degeneration** through the accumulation of deleterious mutations. This process can occur through **selective interference** [10–12]. Sequence degeneration can also occur in the absence of selective interference. In this scenario, regulatory and coding-sequence substitutions accumulate on the Y chromosome in a gene-by-gene manner, leading to silencing of Y-linked **gametologs** [9,13]. In many species, recombination suppression has continued in a stepwise manner, extending the non-crossover region into multiple, independent **evolutionary strata** [14–17]. Although extensive sequence degeneration does occur, it is not the inevitable outcome in all species. Many species possess small sex-determination regions and the majority of the remaining sex chromosomes freely recombine [18,19].

At the sequence level, the accumulation of deleterious mutations has been extensively demonstrated within coding regions of genes that are ancestrally shared between the X and Y chromosomes (**gametologs**). Over time, the accumulation of missense, nonsense, and frameshift mutations renders most coding regions nonfunctional on the Y chromosome. On some of the

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most highly degenerated Y chromosomes, only a handful of ancestral Y-linked alleles remain [17,20]. Degeneration of regulatory regions has been indirectly shown by identifying gametolog-specific expression changes. This has been most thoroughly studied using RNA-seq technologies to interrogate the entire sex-linked transcriptome [21–25]. However, the overall lack of high-quality Y-chromosome assemblies, combined with limited annotations of sex-linked regulatory elements, has prevented direct quantification of the number and location of mutations important for regulatory evolution on sex chromosomes. To date, only a handful of chromosome-level scaffolds have been produced, and these are restricted to mammals [17,20,26–28], fish [16,29,30], birds [21,27], and *Drosophila* [31,32]. With new sequencing technologies, the completion of additional sex-chromosome assemblies (Box 1) will provide a foundation to characterize factors that influence gene regulation such as *cis*-regulatory evolution, **DNA methylation**, and chromatin changes. We review here the recent theoretical and empirical advances that have expanded our understanding of regulatory evolution on heteromorphic sex chromosomes. Although we focus on Y chromosomes, these concepts also apply to W chromosomes that evolve in a similar manner (Box 2).

Sex-linked gametologs rapidly evolve expression differences

As the X and Y chromosomes differentiate, gametolog expression can diverge substantially, and the Y-linked allele follows one of several different evolutionary fates. Owing to the widespread accumulation of deleterious alleles, theory predicts that expression should be lost from most gametologs across the Y chromosome either through silencing of deleterious coding substitutions [12,33] or through degeneration by regulatory evolution [9,13]. Transcriptome sequencing has confirmed that lowered Y expression is widespread across Y chromosomes of animals [22,34–36] and plants [23–25,37–39]. Studies of young sex chromosomes have revealed that gametologs can rapidly evolve sex-biased expression before the accumulation of deleterious mutations within coding regions [23,24,40], and this may be due to the accumulation of mutations within ***cis*-regulatory elements** (discussed in the section 'Models of regulatory evolution'). In many species, **dosage compensation** can occur through upregulation of the X-linked allele to restore the expression of genes lost from the Y chromosome [41]. This can be essential to

Box 1. Improved technology has vastly accelerated the assembly of sex chromosomes

The repetitive nature of Y chromosomes makes their sequencing and assembly particularly challenging. To date, most reference chromosome assemblies have avoided the heterogametic sex entirely. To overcome the highly repetitive nature of sex-limited chromosomes, long-read sequencing approaches are necessary. The earliest assemblies of Y chromosomes were completed for mammalian genomes using single-haplotype iterative mapping and sequencing (SHIMS) of bacterial artificial chromosome inserts [20,21,26,55,57,62,94]. These inserts contained ~150 kb fragments which were able to span the large repeat units of the Y chromosome. The SHIMS method has been used to create higher-quality assemblies than those assembled using enrichment-based [95], reference-guided approaches [96], and *de novo* short-read assemblies [97].

In addition to the SHIMS method, long-read technologies (PacBio sequencing and Oxford Nanopore Technology) have created an increasingly efficient way to obtain highly accurate chromosome-scale assemblies. These techniques can sequence long single-molecule reads up to 100 kb [98], enabling the assembly of highly repetitive regions. These assemblies can be further error-corrected using short-read technologies to increase the accuracy of base pair calling [99]. Chromosome conformation capture and deep sequencing (Hi-C) techniques can also be used to link contigs into full chromosome-scale scaffolds. The first two nonmammalian high-quality Y chromosomes were sequenced using a combination of PacBio sequencing, Illumina short-read sequencing, and Hi-C scaffolding in *Drosophila miranda* [31] and threespine stickleback fish [16]. Since then, multiple sex chromosomes have been assembled using similar approaches in insects [100], fish [29,30], birds [27], and mammals [27,28,101]. These approaches are even robust enough to assemble dense heterochromatic Y regions [86]. In some situations, the SHIMS method may be superior to long-read sequencing approaches. A *de novo* assembly of the human Y chromosome was found to be less contiguous than the SHIMS-assembled Y chromosome [102]. However, the speed of the long-read approach may be an important tradeoff in rapidly increasing the number of representative Y-chromosome assemblies across the tree of life.

Glossary

Adaptive evolution: evolutionary changes that increase the fitness of an organism.
Ampliconic genes: large arrays of duplicated gene families that can maintain high sequence similarity through gene conversion.
***Cis*-regulatory elements:** noncoding DNA elements used to modify expression (promoters, enhancers, insulators).
DNA methylation: a chemical modification to the DNA sequence that has context-dependent effects on expression.
Dosage compensation: the balancing of gene expression resulting in equal expression levels between the homogametic and heterogametic sex.
Evolutionary strata: the stepwise suppression of recombination across sex chromosomes that leads to the formation of distinct domains of sequence divergence.
Gametologs: sex-linked genes that are homologous through sharing a common evolutionary origin on the ancestral autosome.
Heterochromatin: the transcriptionally repressive form of chromatin structure that is associated with repetitive sequences, DNA methylation, and repressive histone modifications.
Neo-sex chromosomes (neo-Y, etc.): the formation of a new sex chromosome either via fusion of an autosome to an existing sex chromosome or the *de novo* evolution of a sex-determining locus on a new autosome.
Purifying selection: the removal of deleterious mutations from a population.
Selective interference: decreased efficiency of selection when advantageous and disadvantageous mutations are in linkage disequilibrium with each other. Examples of this include Muller's ratchet, genetic hitchhiking, and background selection.
Sequence degeneration: the gradual erosion of sex-limited gametolog function through the accumulation of deleterious mutations.
Sexually antagonistic: the direction of selection acting on a trait differs depending on the sex.

Box 2. Similarities and differences between Y- and W-chromosome evolution

Heteromorphic sex chromosomes can occur in males (X/Y) and females (Z/W), both of which have evolved independently across taxa [103]. The formation of W and Y chromosomes is predicted to occur through similar processes driven by the suppression of recombination [104]. Following recombination suppression, sequence degeneration, loss of gene expression from the sex-limited chromosome, and retention of dosage-sensitive genes through purifying selection have all been documented on ancient W chromosomes [21,105]. Despite the similarities, there are differences in sex-specific gene content on the Y and W chromosomes. Interestingly, the chicken W chromosome has not accumulated genes with functions restricted to female tissues [21]. This is in stark contrast to the over-representation of testis-biased genes on Y chromosomes [20,52]. In addition, although some multicopy gene families have been found on avian W chromosomes [106–108], gene-family amplification appears in higher copy numbers on Y chromosomes [20,26,32]. It remains unclear why fewer female-specific genes have been acquired and amplified on W chromosomes. The difference may depend on differences in gametogenesis. For example, spermatogenesis has a longer developmental window that is prone to meiotic conflict. During oogenesis, competition between gametes must be resolved before the end of meiosis I, before gametes are split into polar bodies. During spermatogenesis, transcripts are shared through cytoplasmic bridges [109], resulting in potential conflicts throughout meiosis and in developing spermatids. In addition, testis tissue has been found to be highly transcribed compared with other tissues including ovaries [110]. Elevated rates of transcription in testis may provoke the formation of new genes [111].

maintain early embryonic viability of the heterogametic sex [17,42]. In mammals, there is a second stage, where one X chromosome is inactivated to restore balanced gene expression in females [43]. This process evolved gene-by-gene and does not apply universally across gametologs [44].

A second class of gametologs retain expression from the Y chromosome. These genes have signatures of strong **purifying selection** to maintain function on the Y chromosome [17,21,22,42]. Genes with essential cellular functions, that are broadly expressed across multiple tissues, have been independently retained across multiple lineages of mammalian Y chromosomes [17]. Similar patterns of retention have also been documented more broadly across the independently evolved Y and W chromosomes of amniotes [42], as well as in fish [16], and *Drosophila* [45]. These retained genes are often enriched for haploinsufficiency phenotypes [16,17,21,42], suggesting that they may be dosage-sensitive. Gene-editing approaches that knock out the Y-specific gametolog will be essential to test whether many of these retained genes are actually dosage-sensitive. Retention of essential genes has also been demonstrated on plant sex-limited chromosomes. Genes that are important for the haploid phase of the plant life cycle are retained on Y chromosomes, despite widespread sequence degeneration [46,47].

Although there are clear signs of purifying selection within the coding regions of broadly expressed gametologs, comparative analyses of the regulatory regions are lacking. An outstanding question is whether ancestral regulatory elements are maintained through purifying selection (see Outstanding questions). Alternatively, regulatory elements could evolve rapidly but still maintain optimal expression levels by gaining new transcription factor-binding sites that compensate for the repeated loss of ancestral regulatory elements during Y-chromosome degeneration. Although it remains to be demonstrated whether this process has a prevalent role in maintaining the expression of broadly expressed Y gametologs, the evolution of compensatory transcription factor-binding sites has been documented elsewhere in the genomes of *Drosophila* [48] and mammals [49,50] for genes that are functionally critical.

Y-linked gametologs can also gain novel expression patterns relative to gametologs on the X chromosome [22,51]. Many gametologs on the Y chromosome are enriched for male-specific functions in spermatogenesis. This pattern can be caused by the biased retention of genes important for spermatogenesis on the Y chromosome followed by the loss of spermatogenesis function from the X-linked gametolog through regulatory evolution [52]. This could also occur through the evolution of spermatogenesis-specific regulation of the Y-linked gametolog after the X and Y chromosomes diverged [53]. Most Y-linked gametologs have been associated

with spermatogenesis, but recent evidence shows that some Y-linked gametologs can also exhibit elevated expression in non-reproductive tissues in humans [51], suggesting male-specific functions that extend beyond the testes.

In addition to evolution of gametologs on the Y chromosome, many novel genes are acquired on the Y chromosome via translocation from autosomes. These genes are often further duplicated within the Y chromosome and evolve testis-specific functions. **Ampliconic gene** families evolve rapidly following the suppression of recombination [16,54] and can grow into massive arrays of duplicated genes on highly degenerated Y chromosomes [20,26,32,55–57]. Although gene duplication occurs genome-wide [58,59], it appears that the Y chromosomes are especially susceptible, which may be driven by intrachromosomal conflicts between the sex chromosomes [60]. Comparisons between sex chromosomes have shown that gene amplification may be accelerated on X, Y, and Z chromosomes, which all pass through the male germline, indicating that selection pressure on male functions may drive the expansion [20,61,62] (Box 2). Manipulating copy number through gene editing will provide insight into whether amplicon expansion is essential for male fertility, as observed for the *Slx* and *Slx1* amplicons in house mice [63]. In addition, Y-chromosome gene duplication may be an alternative way to maintain the expression of genes important for spermatogenesis. Recombination between duplicated genes through gene conversion could aid in purging deleterious mutations that accumulate in coding or regulatory regions [64].

Multiple mechanisms contribute to altered expression patterns of gametologs

Although gain of expression occurs on Y chromosomes, the mechanism that has garnered most empirical and theoretical attention is the loss of expression of Y-linked gametologs. Loss of gene expression can be caused by many factors, including *cis*- and *trans*- regulatory changes [65,66]. To date, *trans*-acting factors that broadly act to downregulate Y-linked gametologs have not been identified. The gene-by-gene loss of expression independently observed across taxa is most likely due to *cis*-regulatory evolution acting locally, including changes in **heterochromatin**, the accumulation of transposable elements, differential DNA methylation, and *cis*-regulatory DNA changes at promoters and enhancers (Figure 1).

Mutations within *cis*-regulatory regions (promoters and enhancers) that alter transcription factor-binding sites or disrupt chromatin accessibility can lead to downregulation if the binding of transcriptional activators is inhibited [66]. For example, deletions in the regulatory region of the mammalian Y-linked sex determination gene, *Sry*, prevents the expression of a reporter gene in the primordial gonad [67], indicating the presence of an essential regulatory element. Mutations within regulatory regions can also lead to deleterious upregulation of genes if sites of repressors are lost or novel activators are gained [66].

The accumulation of repetitive transposable elements may contribute to allele-specific gene expression across sex chromosomes (Figure 1). After suppression of crossing over, transposable elements accumulate on the sex-limited chromosome. It is well established that repetitive transposable elements can be silenced through the accumulation of heterochromatin [68], and the spreading of heterochromatin can affect expression of adjacent coding regions [69–71]. Accumulation can occur rapidly, as apparent by the high density of transposable elements on younger sex-limited chromosomes [16,31]. On the **neo-Y chromosomes** of *Drosophila miranda*, the accumulation of transposable elements has been associated with the onset of constitutive heterochromatin formation on the neo-Y [72], visualized cytogenetically as well as through ChIP-seq targeting two repressive histone modifications (H3K9me3/H3K9me2). The accumulation of transposable elements and the spreading of heterochromatin are associated with the silencing of gametologs throughout the neo-Y chromosome, raising the possibility that transposable

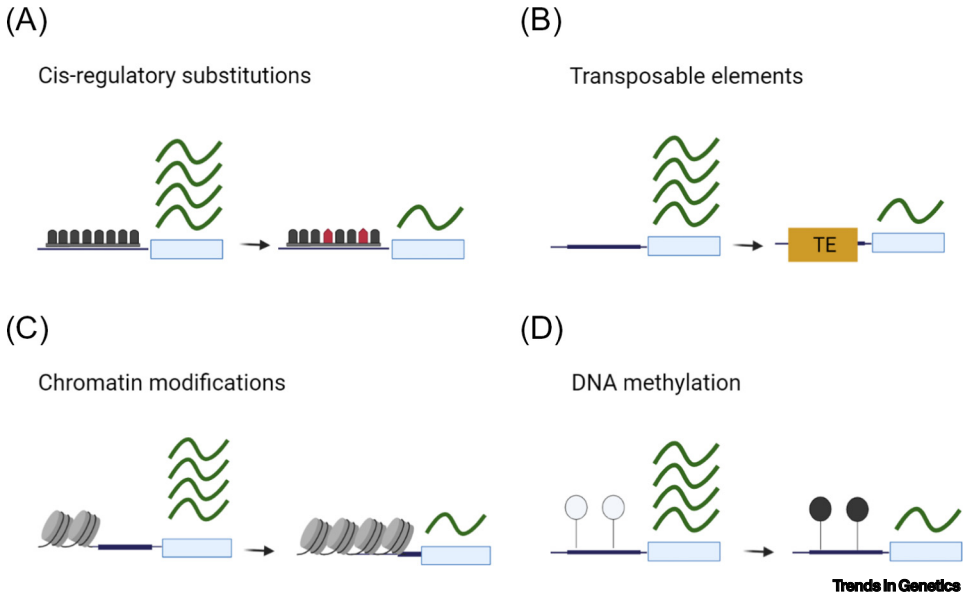

Figure 1. Gene expression differences on sex-limited chromosomes evolve through multiple mechanisms. (A) *Cis*-regulatory substitutions, (B) transposable elements, (C) nucleosome occupancy due to chromatin changes, and (D) DNA methylation all likely contribute to evolution of the expression of gametologs on sex chromosomes. Each of these mechanisms evolves rapidly and can lead to both the gain and the loss of gene expression. Wavy green lines represent relative changes in gene expression. Figure created with Biorender.com. Abbreviation: TE, transposable element.

element accumulation may directly lead to silencing of genes. However, it remains difficult to determine the temporal order of events. It is possible that transcriptionally repressed genes may instead be an opportunistic location for transposable element insertion, leading to heterochromatinization after Y-gametolog silencing.

The accumulation of transposable elements does not always lead to the downregulation of gametologs. Transposable elements can also rewire transcriptional networks by the introduction of *cis*-regulatory elements [73]. In *D. miranda*, some transposable elements contain binding sites for *MSL* (male-specific lethal). The accumulation of these elements on the X chromosome cause the upregulation of gametologs, resulting in dosage compensation in males [74,75]. Transposable elements are also responsible for the evolution of genetic sex determination in multiple species of fish [76–78]. In each case, novel *cis*-regulatory elements introduced by the elements allowed neofunctionalization of existing genes to initiate male development. Given the considerable number of transposable elements that accumulate on the Y chromosome, the cooption of novel binding sites from these insertions may be a mechanism to transcriptionally rewire genes throughout Y-chromosome evolution. Additional analyses of regulatory evolution on the Y chromosome will reveal the pervasiveness of this mechanism.

DNA methylation is another gene-regulator modifier likely playing a role in sex chromosome evolution (Figure 1). Changes in DNA methylation can have context-dependent effects on gene expression but are often associated with repression of transcription, especially when found at repetitive regions and CpG islands in mammals [79]. Similar mechanisms may affect expression of gametologs on sex-limited chromosomes. An analysis of a set of XY gametologs in *Silene latifolia* revealed that many Y-linked gametologs within the oldest evolutionary strata had high levels of methylation in the promoter relative to their X-chromosome counterparts, and many of these genes were in close proximity to transposable elements [38]. Additional work that

compares X- and Y-specific methylomes will clarify the relationship between DNA methylation and gene expression on sex chromosomes.

Models of regulatory evolution

Mutations that randomly occur within regulatory elements can accumulate through selective interference [37] or through **adaptive evolution**. In the case of adaptive evolution, mutations that downregulate gene expression would be beneficial to prevent the expression of coding regions that have accumulated deleterious mutations, rendering the protein sub- or nonfunctional. Selection could also favor downregulation of gametologs that produce toxic proteins, although this pattern has not been observed empirically, as these types of mutations may be rare and quickly lost from populations. Recent models suggest that regulatory evolution could occur before the degeneration of coding regions [9,13]. In the degeneration by regulatory evolution (DRE) model, *cis*-regulatory mutations evolve first on the Y chromosome, lowering expression. Deleterious mutations that accumulate within the coding regions of gametologs with lowered expression are more recessive, which selects for the accumulation of additional *cis*-regulatory mutations to further reduce expression. Importantly, this model does not require selective interference for deleterious mutations to initially accumulate. As silencing continues, stabilizing selection to maintain dosage balance leads to upregulation of the X-linked allele through the evolution of *trans*-acting transcription factors [13].

The DRE model aligns with many patterns observed across sex chromosomes. First, Y-chromosome gametologs can be silenced before amino acid substitutions have accumulated, as observed across two species of *Rumex* [37] and *D. miranda* [80]. This is consistent with mutations first accumulating in *cis*-regulatory elements in the DRE model. Second, if there is an absence of stabilizing selection for dosage compensation, Y-gametolog silencing and degeneration still occurs [13]. This helps to explain the widespread loss of Y-chromosome alleles in species that do not exhibit dosage compensation for every gene [22,81,82]. In these species, many Y gametologs are lost without coordinated upregulation of the X gametolog to compensate for dosage. Third, if stabilizing selection to maintain expression from both sex chromosomes is strong, but *trans* factors do not exist to permit gene-by-gene dosage compensation, Y-chromosome degeneration does not occur [13]. This finding may help to explain the retention of similar genes across multiple sex chromosomes [16,17,21,22,42]. Many gametologs that are retained on Y chromosomes through strong purifying selection are enriched for expression across multiple tissues [17]. A single *trans* factor may not easily evolve to universally upregulate the X gametolog to compensate for dosage across multiple developmental stages and tissues.

The later stages of adaptive silencing through the DRE model have not yet been clearly observed on sex chromosomes. As amino acid substitutions accumulate, selection should favor substitutions within *cis*-regulatory elements on the sex-limited chromosome to further downregulate the gametolog. Adaptive silencing would be evident if there was a negative correlation between amino acid substitutions and Y-gametolog expression level. However, loss of Y-linked expression was not associated with high levels of divergence in coding regions on the recently evolved sex chromosome in *Rumex rothschildianus* [37], and thus failed to provide support for widespread adaptive silencing. In addition, this pattern has not been observed across the young neo-Y chromosomes of *Drosophila*. Expression of Y-linked gametologs was not correlated with amino acid divergence on the young neo-Y of *Drosophila albomicans* [36,83], which evolved only ~120 000 years ago, or on the slightly older neo-Y of *D. miranda* (~1 million years ago) [80,84]. A correlation may only be observable during a narrow window of degeneration after amino acid substitutions begin to accumulate, but before widespread degeneration and complete silencing has occurred. Surveys of additional sex chromosomes at different states of degeneration will help to test this hypothesis.

The DRE model provides a theoretical framework for how degeneration occurs first in the regulatory region, followed by the accumulation of deleterious mutations within the coding region. To date, empirical studies have not broadly surveyed the molecular evolution of regulatory regions relative to coding sequences on sex chromosomes (Figure 2). Some signatures of adaptive evolution have been noted within noncoding sequences on the *D. melanogaster* X chromosome [85], but there has not been a widespread focus on the Y chromosome. Future work will also need to focus on how regulatory evolution proceeds when genes with multiple types of functional constraints evolve on the same linked Y chromosome (Figure 3, Key figure). One clear finding from comparative analyses of Y-chromosome assemblies is that not all gametologs have the same fate of pseudogenization and dosage compensation. In addition, the DRE model does not integrate the evolution of beneficial mutations on the Y chromosome, where Y-chromosome gametologs evolve neo- or subfunctionalization through the gain of novel regulatory elements. Signatures of positive selection are prevalent across Y gametologs [32,35,46], and expansion of Y-gametolog expression domains may be more common than was previously assumed [51].

A greater challenge will be to assess the overall importance of the different types of mechanisms that alter gametolog expression. Current models use a single mutation rate that affects all regulatory and coding regions equally. These mutations generally have small deleterious effects and act in an additive fashion to progressively erode Y-gametolog function. In actuality, regulatory mutations will have a mixture of effect sizes and will occur at different rates. Transposable element

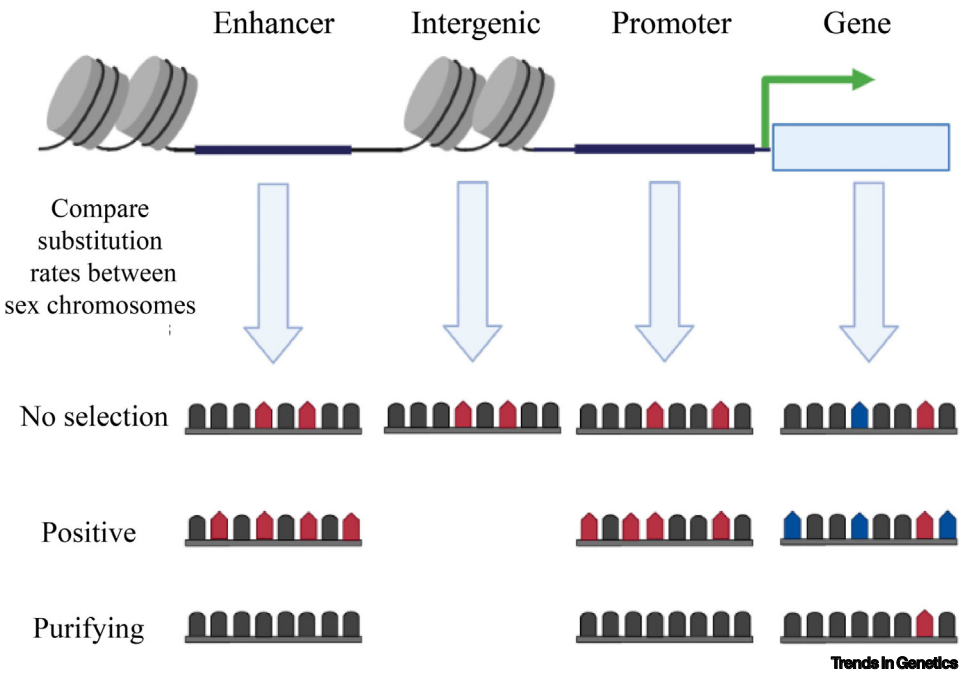

Figure 2. Testing for models of selection on sex-linked regulatory regions. Positive and purifying selection in functional regions throughout the genome can be identified by comparisons with neutrally evolving intergenic regions. If loss of gene expression is not driven by selection, regulatory regions would be predicted to accumulate mutations (red) at a similar rate as intergenic regions. A higher substitution rate (red mutations) within enhancers or promoters compared with intergenic regions would indicate positive selection. A lower substitution rate (red mutations) within enhancers or promoters compared with intergenic regions would indicate negative selection. Within coding regions, positive selection is indicated by more non-synonymous mutations (blue) relative to synonymous mutations (red), whereas purifying selection is indicated by fewer non-synonymous mutations relative to synonymous mutations. Positive selection on coding regions is not necessarily associated with regulatory elements under positive selection. Figure created with Biorender.com.

Key figure

Evolution of regulatory elements on sex chromosomes

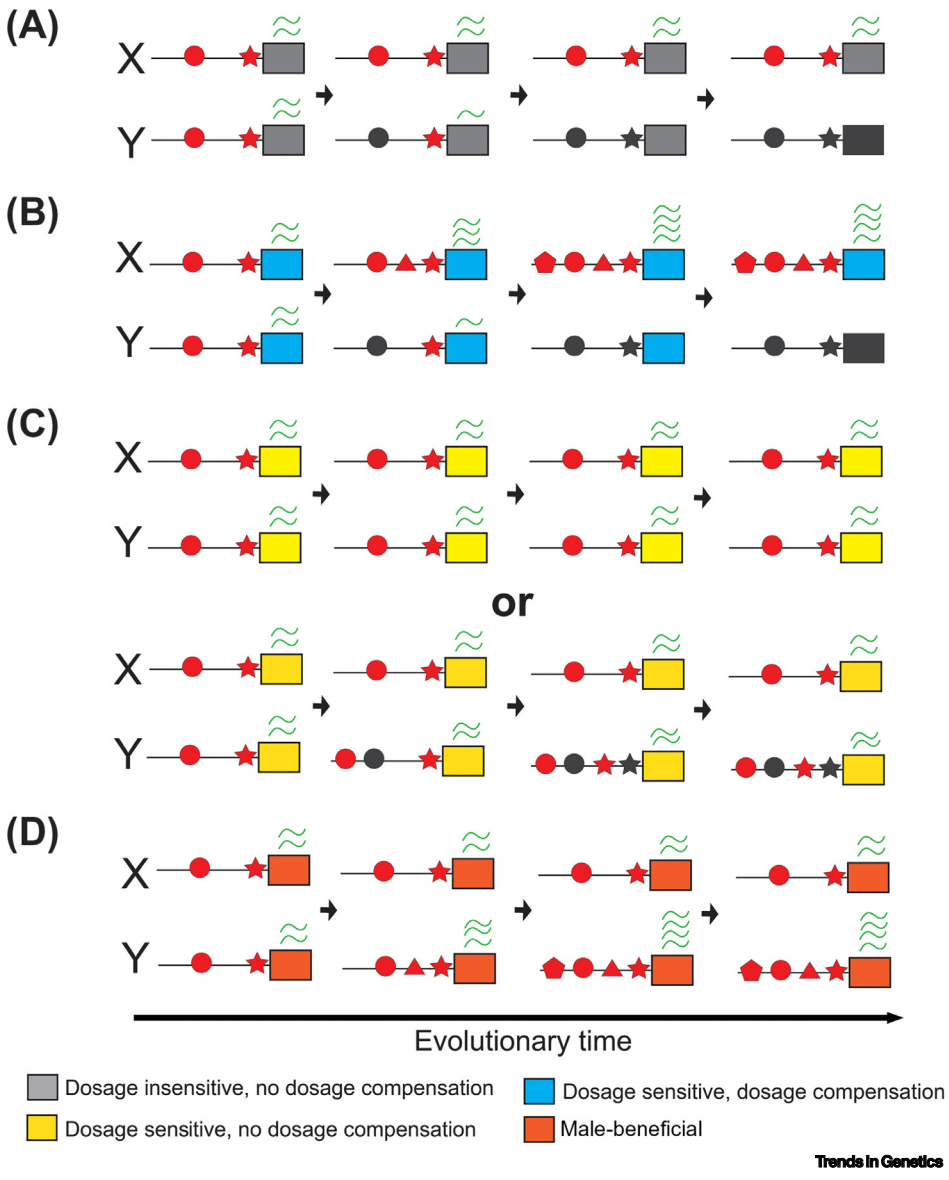

Figure 3. (A) Gametologs that are not dosage sensitive can accumulate mutations within *cis*-regulatory elements, downregulating Y-linked genes. These gametologs may degenerate without X-chromosome dosage compensation. Mutation accumulation can also lead to the upregulation of Y-linked genes (not shown). (B) Stabilizing selection selects for the loss of Y-chromosome expression and gain of X expression for gametologs that are dosage-sensitive [degeneration by regulatory evolution (DRE) model]. (C) Gametologs that are dosage-sensitive may maintain regulatory function on the Y chromosome through purifying selection, circumventing the need for dosage compensation. These genes could also evolve new *cis*-regulatory regions to compensate for the loss of ancestral regulatory elements and maintain expression levels. (D) Gametologs can evolve new functions important for male fitness. These genes will accumulate regulatory mutations that lead to novel expression patterns (e.g., testis-specific expression). Coding regions are represented by rectangles. *Cis*-regulatory elements are represented by the shapes upstream of the coding region. Regions that have degenerated are shown in black. Wavy green lines represent relative changes in gene expression.

insertions, for instance, may immediately trigger complete Y-gametolog repression through the spreading of repressive chromatin or DNA methylation. Understanding which types of regulatory change are most prevalent early in the evolution of Y chromosomes will be aided by producing additional high-quality reference sequences that focus on annotating functional regulatory regions for comparative analyses.

Concluding remarks

One of the major obstacles limiting our understanding of regulatory evolution on sex chromosomes is the lack of high-quality assemblies. The high repeat content of Y chromosomes makes assemblies and mapping reads a challenge. However, recent sequencing approaches that combine long-read technologies with chromatin-interaction maps or optical mapping have made the production of reference chromosomes feasible in many cases [16,31,86] (Box 1).

As high-quality assemblies are completed for the sex chromosomes across diverse taxa, detailed transcriptome and epigenome annotations (Box 3) will be necessary to empirically validate theoretical models of regulatory evolution. By utilizing approaches targeted towards regions of chromatin accessibility, DNA methylation, and histone modifications, we can gain an understanding of how gene regulatory modifications are evolving on young and old sex chromosomes. An ongoing challenge will be the identification of long-distance enhancer elements important for gene regulation on the Y chromosome (Box 3). An additional challenge will be to discover regulatory mechanisms that are unique to the sex-limited chromosome. For example, some genes on the Y chromosome in *D. melanogaster* contain gigantic introns composed of simple satellite repeats [87]. These introns

Outstanding questions

Does DNA methylation and/or the accumulation of heterochromatin play a role in the early downregulation of Y-linked gametologs? Does the accumulation of SNP variants within regulatory elements have a larger impact?

Are regulatory elements broadly under selection to downregulate Y-linked alleles?

Which patterns of regulatory evolution on sex chromosomes are generalizable across species?

Can purifying selection maintain regulatory regions on sex chromosomes?

Are transposable elements a common mechanism to introduce novel transcription factor-binding sites on Y chromosomes?

Does pleiotropy constrain the evolution of some regulatory elements on the Y chromosome?

Box 3. Challenges remain in annotating regulatory regions across the Y chromosome

Our ability to understand how regulatory regions evolve on sex chromosomes is dependent on broadly annotating enhancer and promoter regions. Highly contiguous assemblies enable the identification of previously unannotated regions of the genome, such as repeat-dense heterochromatic regions [86,100,112] including centromeres and pericentromeric regions [16,100,113–115]. Annotation of these regions will require the use of ChIP-seq in many different species to identify repressive histone modifications and variants (e.g., H3K9me3, HeK9me2, CENPA).

Regulatory regions are challenging to annotate throughout the genome and on sex chromosomes. Regulatory regions can be located far from the genes they regulate, may interact with multiple genes, and do not always have distinct sequence signatures. Putative enhancer regions can be identified using next-generation sequencing targeted towards accessible chromatin regions (assay for transposase-accessible chromatin, ATAC-seq). In addition, ChIP-seq can be used to locate functional regulatory regions. H3K4me3 modifications are associated with promoters, and H3K4me1 and H3K27ac (acetylation) modifications are associated with enhancers [114]. Hi-C contact maps can be used to find chromatin interactions between enhancer elements and promoter regions of genes [116,117]. It is important to note that genomic approaches for regulatory annotation utilize short-read sequencing, which may be challenging to accurately align to highly repetitive, degenerate sex chromosomes. In addition, sequence identity between recently evolved sex chromosomes may be high, limiting the number of variants available to map X- and Y-specific reads.

To empirically test models of regulatory evolution on Y chromosomes, it will be necessary to detect signatures of selection in annotated regions. Detection of recent positive selection may be challenging on nonrecombining sex chromosomes because the entire linked region will uniformly have reduced genetic variation within populations [118–120]. Scans of selection will need to be coupled with estimates of divergence between the X and Y chromosomes over longer evolutionary timescales to understand whether variants are accumulating at a higher rate within *cis*-regulatory elements compared with neutrally evolving regions.

Once candidate regulatory elements are identified, a growing number of approaches can be used to test function. Reporter constructs such as luciferase expression assays can be used to validate whether an element of DNA has regulatory function *in vitro* [121,122]. CRISPR/Cas9 editing has also proved to be a promising approach to test regulatory function throughout the genome by inducing mutations within promoter regions [123] or by targeting regulatory regions based on chromatin accessibility [124,125]. To date, gene editing has only been used to target limited sites on the ancient Y chromosomes of *D. melanogaster* [126] and the house mouse [127]. Gene editing may have a higher efficiency on younger Y chromosomes because these are generally composed of fewer repeats and less heterochromatin.

are transcribed and recruit specialized RNA-binding proteins that are required for successful transcription and processing.

An important challenge remaining is to untangle the timing and importance of different types of regulatory evolution over the course of Y-chromosome evolution (Figure 3). Y-chromosome degeneration occurs rapidly after the suppression of recombination [1,13]. One empirical approach is to study overall rates of regulatory evolution in species with more recently evolved sex chromosomes. Fish [29,88,89], plants [37,90,91], and insects [92,93] have proved to be useful taxa in which to study the convergent evolution of sex chromosomes over relatively short timescales.

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Declaration of interests

The authors declare no conflicts of interest.

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