Genetic Control of Gonadal Sex Determination and Development

Stevant, Isabelle; Nef, Serge

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Genetic control of sex determination and gonad development

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Corresponding Author:	Serge Nef geneva, SWITZERLAND
First Author:	Isabelle Stévant
Order of Authors:	Isabelle Stévant
	Serge Nef
Abstract:	Sex determination is the process by which the bipotential gonads develop as either testes or ovaries. With two distinct potential outomes, the gonadal primordium offers a unique model for the study of cell fate specification and how distinct cell populations diverge from multipotent progenitors. This review focuses on recent advances in our understanding of the genetic programs and epigenetic mechanisms that regulate gonadal sex determination and regulation of cell fate commitment in the bipotential gonads. We rely primarily on mouse data to illuminate the complex and dynamic genetics programs controlling cell fate decision and sex-specific cell differentiation during gonadal formation and gonadal sex determination.

Highlights (900 characters with space)**:**

New technologies in molecular biology, such as single cell RNA sequencing and epigenetic techniques, are emerging and being refined at a fast pace. They provide an opportunity to gain new insights into mechanisms driving the fate of progenitor cells and the transcriptional epigenetic dynamics underlying the process of sex determination.

A single multipotent progenitor cell population undergoes fate restriction and differentiates into either the supporting or interstitial/stromal progenitors of the gonad. The supporting cells differentiate from these early progenitors in two sequential steps with first a commitment toward the supporting cell lineage followed by a sex-specific differentiation toward pre-granulosa and Sertoli cells.

Accumulating evidence suggest that epigenetic mechanisms contribute to establish the male fate. The expression of the male determining gene *Sry* is regulated by histone modification and DNA methylation.

Genetic control of gonadal sex determination and development

Authors: Isabelle Stévant[1,2,3], Serge Nef[1,2,*].

Affiliations:

[1]Department of Genetic Medicine and Development, University of Geneva, 1211 Geneva, Switzerland;

[2]iGE3, Institute of Genetics and Genomics of Geneva, University of Geneva, 1211 Geneva, Switzerland;

[3]SIB, Swiss Institute of Bioinformatics, University of Geneva, 1211 Geneva, Switzerland;

Contact information:

[*]Corresponding Author: Serge.Nef@unige.ch

Summary (max 120 words)**:**

Sex determination is the process by which the bipotential gonads develop as either testes or ovaries. With two distinct potential outomes, the gonadal primordium offers a unique model for the study of cell fate specification and how distinct cell populations diverge from multipotent progenitors. This review focuses on recent advances in our understanding of the genetic programs and epigenetic mechanisms that regulate gonadal sex determination and regulation of cell fate commitment in the bipotential gonads. We rely primarily on mouse data to illuminate the complex and dynamic genetics programs controlling cell fate decision and sex-specific cell differentiation during gonadal formation and gonadal sex determination.

Keywords:

transcriptomics, gonadal sex determination, ovary, testis, granulosa cells, Sertoli cells, supporting cells, progenitors, differentiation, lineage specification, cell fate decision, gene expression, epigenetic regulation

A fundamental goal of developmental biology is to shed light on the mechanisms behind cell fate determination and differentiation of progenitor cells into distinct cell types. In mammals, the testes and ovaries initially develop from bipotential primordia composed of progenitor cells that can differentiate into either testicular or ovarian cells. This process is called **gonadal sex determination** (see Glossary). This unique ability of the gonadal progenitor cells provides a valuable model to explore how gene regulation and chromatin landscape regulate cell fate determination and sex-specific differentiation.

The decision to adopt an ovarian or testicular fate relies on robust genetic networks, as well as a delicate balance of expression levels for several pro-testis and pro-ovary factors. In particular, initiation of the male pathway in XY gonad is driven by activation of the testis pathway and simultaneous repression of the ovarian pathway, while commitment of the XX gonads towards the female fate mostly depends on the continuous activation of female-promoting genes [1]. Accumulating evidence suggests that **epigenetic** regulation also contributes to the bipotential state of the developing gonad and is a key element fine-tuning the expression levels of sex-determining genes.

In this review, we will lay out our current understanding of the complex genetic programs mediating gonadal sex determination in mice, as well as their epigenetic control. We first focus on how the bipotential gonad and its progenitors are established prior to sex determination. We then review the dynamics of gene expression at play during sex fate choice and the establishment of sexual dimorphisms. Finally, we highlight how the process of gonadal sex determination is controlled by epigenetic mechanisms regulating gene expression during gonadal development.

Establishment of the bipotential gonad prior to sex determination

In mammals, **the genital ridges**, or bipotential gonads, constitute the primordia from which ovaries and testes differentiate. In the mouse, they develop at around embryonic day E9.5 in a precise region of the ventral surface of the mesonephros (Figure 1A and B) and form paired narrow bands of proliferating cells on either side of the dorsal mesentery (Figure 1C). The growth of the genital ridges occurs via proliferation of the **coelomic epithelium** (CE), and subsequently via fragmentation of the underlying basement membrane, allowing the delamination of the proliferating CE into the inner mesenchymal region of the mesonephros (Figure 1C) [2]. It is not clear whether the fragmentation is an active or a passive mechanism that is initiated by an increase of the number of cells. Thickening of the genital ridges start at E10.3 following an anteroposterior axis (Figure 1B). Concurrently with the thickening, the genital ridges are colonized by the migrating primordial germ cells (Figure 1C) [3–7].

The precise molecular mechanisms that define the location of the gonads on the surface of the mesonephroi remain to be elucidated. One interesting lead might reside in a recent study in chicken embryos which suggests that Sonic Hedgehog signaling mediated by cytokine BMP4 (bone morphogenetic protein 4) initiates gonadogenesis by establishing the dorsoventral patterning of the mesoderm and by inducing ingression of the coelomic cells [8]. Activation of the Hedgehog signaling in the mesonephric capsule progenitor cells (epithelial cells in the flanked regions of the genital ridges) induces the ectopic formation of gonads. However, significant differences between mouse and chick gonadal morphogenesis [9] call for a similar study in mammals in order to establish whether this process is conserved among vertebrates.

Full development of the genital ridges requires the correct ingression and the proliferation of coelomic epithelial cells to establish the pool of gonadal somatic progenitor cells prior to gonadal sex determination. Many important factors involved in both ingression and cell proliferation have been identified by studying mutant mice (see Table 1). To our knowledge, GATA4 is the earliest transcription factor expressed specifically in the genital ridges. GATA4 is present in the CE cells of the rostral part of the genital ridges as early as E10.0, and its expression extends along the rostrocaudal axis until E10.4 [2]. It is involved in the initiation of the formation of the genital ridges by controlling the basement membrane fragmentation and the CE cell proliferation. It also controls the expression of *Nr5a1* (also called *Sf1*) and *Lhx9*. Whether it does so in a direct or indirect manner is still unclear. These two critical genes are also expressed specifically in the gonadal progenitor cells [10–12] (Table 1) and their expression pattern follows *Gata4* expression with a slight delay. It is worth noting that no expression of these two genes is observed when the *Gata4* gene is deleted [2]. The origin and the establishment of the gonadal somatic cell lineages are not yet fully resolved. Experiments tracing cell lineage *in vitro* prior to gonadal sex determination have revealed that, in XX and XY gonads, the ingressing CE cells constitute the most important source of gonadal somatic cells in both sexes and contribute to the supporting cell and the steroidogenic cell lineages [13,14]. These results are corroborated by transcriptomic reconstruction of the gonadal CE cell differentiation trajectories that shows that both supporting and steroidogenic precursor cells derive from a common progenitor cell population [15,16]. This suggests that CE cells are multipotent and that their fate is controlled either by asymmetrical cell division driven by molecular determinants, or by external signals from other cells within the gonads [13,17–19].

Recently, NUMB – an inhibitor of the Notch signaling – has been found to be involved in the polarity of the CE cells. NUMB is asymmetrically distributed in the daughter cells during cell division. Its disruption in XY bipotential gonads leads to the accumulation of undifferentiated cells as well as a reduction of Sertoli and Leydig cells [20]. While it is clear that the supporting cells derive from the CE, the steroidogenic cell lineage derives from multiple sources. Studies demonstrated that, while the vast majority of the steroidogenic cell precursor reside within the gonads and might derive from the CE, a significant portion (~30%) of them originate from cells migrating from the mesonephros after gonadal sex determination [13,21–27]. This phenomenon has been observed in both sexes, suggesting they probably derive from a common mesonephric steroidogenic precursor niche.

Despite significant efforts, details about the formation of the genital ridges and the characteristics of the somatic cells composing the bipotential gonads remain poorly understood. This is mostly due to the lack of specific marker genes and reporters, the small size of the tissue, and the difficulty to access it. New arising technologies, such as single-cell RNA-sequencing and cyclic single-molecule fluorescence *in situ* hybridization [28] hold great potential for studying cells from the whole mesonephric region during genital ridge formation, including the mesonephros, the mesonephric duct and the genital ridges.

Transcriptional events underlying gonadal sex determination and sex-specific cell differentiation

Because gonadal sex determination is mostly a cell-autonomous process driven by antagonistic genetic programs, intensive efforts have been deployed to uncover the dynamics of gene expression at play during sex fate choice and the establishment of sexual

dimorphisms. Time-series transcriptomic studies of XX and XY mouse gonads during sex determination were first conducted using microarrays technologies [1,29–34], and more recently using high throughput technics such as RNA-seq [35] and single-cell RNA-seq [15,16] (**Figure 2**). Although evaluating the dynamics of gene expression during gonadal sex determination is pitted with technical difficulties (see Box 1), these complementary studies greatly contributed to the understanding of the genetic programs driving gonadal somatic cell sex determination.

Supporting cell commitment and differentiation

As the bipotential gonads form at E10.5, the gonadal cells do not display any sexual dimorphism at the transcriptomic level, with the exception of few genes located on the sex chromosomes [30,35]. The CE derived somatic progenitor cells (expressing *Gata4* and *Sf1*) express epithelial-like stem cell related genes as well as proliferation genes and do not yet show any transcriptomic signature of a commitment toward the supporting or the steroidogenic fate [15,16]. Supporting cell commitment and sex-specific differentiation occurs sequentially with an initial decision whereby XY and XX multipotent progenitors adopt a supporting cell fate and share a similar transcriptomic identity followed then by sex-specific differentiation into Sertoli and pre-granulosa cells [15,16]. More precisely, around E11.0-E11.5, the commitment of the supporting cell lineage from the multipotent somatic progenitor cells is mediated by a common genetic program consisting in the rapid up-regulation of hundreds of genes in both XX and XY [15,16]. This program poises the expression of pro-Sertoli (*Fgf9*, *Dmrt1*) and pre-granulosa (*Wnt4*, *Runx1*, and *Dax1*) genes at the onset of supporting cell commitment [1,16,34]. Moreover, this up-regulation of genes also coincides with the activation of the *Sry* gene and its direct target *Sox9* in XY supporting

cells [15,16]. It is likely that the genetic program engaging the cells into the supporting cell lineage also confers them their bipotential properties. This is supported by the fact that transgenic expression of *Sry* in XX supporting cells in the exact same time window as the XY supporting cells is sufficient to drive Sertoli cell development [36,37]. By E12.5, the supporting cell progenitors have adopted their sex-respective identity by engaging their differentiation as Sertoli cells in XY, and pre-granulosa cells in XX. The differentiation of the supporting cells into Sertoli or pre-granulosa cells follow different courses. In XY, a massive wave of activation of male-specific genes, such as *Amh* and *Dhh*, is observed between E11.5 and E12.5, soon after *Sry* expression and up-regulation of *Sox9*. In parallel, a large proportion of the genes that are up-regulated at the time of the supporting cell commitment is down-regulated at this stage [1,15,16,34,35]. In XX supporting cell progenitors, female-specific genes, such as *Foxl2*, are up-regulated from E12.5 onward, and less gene repression is observed compared to XY [16,34,35]. Further analysis determined that XX and XY bipotential supporting progenitors at E11.5 present female-biased priming, suggesting that the natural progression of these progenitors is to adopt the female identity, unless SRY activation induces the repression of the female program and promotes Sertoli cell differentiation [34]. Transcriptomic analysis of granulosa cells at a later embryonic stage (E13.5 and E16.5) and at an early post-natal day (P6) revealed that the differentiation of granulosa cells takes place over several days. This is in contrast with Sertoli cells, which differentiate within a 24 hours period. Between E12.5 and E16.5, pre-granulosa cells gradually up-regulate female-specific genes but also progressively down-regulate the same set of genes that is rapidly down-regulated in pre-Sertoli cells between E11.5 and E12.5 [16]. After birth, the granulosa cells complete their differentiation as the

process of folliculogenesis begins. In summary, the temporal differences in gene expression observed between XX and XY supporting cell progenitors is likely due to a delay of cell differentiation in the pre-granulosa cells compared to the Sertoli cells.

Interstitial/stromal cell specification as steroidogenic progenitors

In parallel of the supporting cell differentiation, the remaining CE-derived progenitor cells also undergo transcriptomic changes during gonadal development [15,16,34]. In early testis development, these progenitor cells are confined within the interstitial compartment, while the Sertoli cells enclose germ cells to form the testis cords. In the early developing ovary, no particular structures are yet observed, and the progenitor cells appear intermingled with the pre-granulosa cells and the germ cells, but are later found in the stromal compartment of the ovaries during folliculogenesis. The interstitial and the stromal compartments are both composed of heterogeneous cell populations. They are thought to contain the major source of steroidogenic progenitor cells that differentiate as fetal Leydig cells from E12.5 in testes, and theca cells during the first week *post-partum* in ovaries. They are also the siege of the vasculature development from migrating mesonephric endothelial cells [18,38–40].

The study of the interstitial and the stromal cells is challenging due to their heterogeneity and the lack of specific markers (see Box 1). A first transcriptomic study attempted to isolate these cells with Mafb-eGFP transgene [34]. They found the interstitial and the stromal cells display gradual sexual dimorphism from E11.5 to E13.5, partly driven by the differentiation of the fetal Leydig cells comprised in the Mafb-eGFP positive cells [34]. More refined studies using single-cell RNA-sequencing on Nr5a1 expressing cells [15,16]

showed that the CE derived interstitial/stromal progenitor cells gradually up-regulate genes known as markers of steroidogenic precursor cells such as *Pdgfra, Arx, Ptch1* [41–44]. XX stromal cells progression appears slightly delayed compared to that of the XY interstitial cells. Gradual transcriptomic sexual dimorphism is observed from E12.5, concomitantly with the differentiation of the supporting cells. However, the observed sexual dimorphism is driven by differences in level of expression of genes rather than expression of genes specific to one or the other sex. Unfortunately, the low number of foetal Leydig cells, together with the absence of theca cells in the dataset, prevented from studying the steroidogenic cell differentiation program. Whether the steroidogenic precursor cells migrating from the mesonephroi display a similar transcriptome progression is still unknown.

Alternative splicing and sex determination

Alternative splicing (AS) is a ubiquitous regulatory mechanism involving the selection of specific exons/introns to produce different transcripts from a single gene, thus expanding the complexity of the proteome [45,46]. While it is well established that AS plays a major role in the development of various organs and contributes to cell differentiation and lineage determination [47], the extent of AS occurring during the process of gonadal sex determination and its functional relevance remains unclear. Recently, a RNA-Seq study performed in mouse fetal gonads during the process of sex determination detected widespread stage- and sex-specific regulation of transcript isoform usage during gonadal development [35]. Although it is still difficult to predict the exact molecular consequences of these differential splicing events, AS has been reported in genes known to play important function in gonadal development. It includes the Wilms' tumor suppressor gene *Wt1* [48],

the FGF9 receptor *Fgfr2* (fibroblast growth factor receptor 2) [49] as well as *Lef1*, a key mediator of the canonical WNT signalling pathway [50–55]. Overall, these findings suggest an important regulatory role of AS in sex determination and early gonadal development.

Evaluation of the dynamics of gene expression during gonadal sex determination allow us to better understand how sexual dimorphisms are established upstream from the gonadal morphological changes, and revealed the complexity of the transcriptomic program at play during testicular and ovarian development. However, efforts still need to be deployed to elucidate the gene networks responsible for sex fate decision, and in particular to understand the mechanisms of activation and repression of gene expression.

Mechanisms of gene expression regulation during gonadal development

The transcriptome is a direct read-out of the genetic program responsible for cell fate decision and differentiation. But it is also the result of many upstream events occurring at the DNA level to promote or prevent transcription. Transcription factors play a key role in the control of gene expression by binding on enhancer and promoter regions. The accessibility of the enhancer regions is an important mechanism of gene expression regulation. It involves 3D DNA conformation, chromatin modifications, and DNA demethylation. There is increasing evidence for the role of epigenetics and DNA regulatory elements in gonadal sex determination, starting with the activation of the testis determining factor *Sry* itself [56].

Regulation of Sry expression

The mechanisms controlling the precise spatiotemporal expression of *Sry* is not yet fully resolved [57]. Bisulfite sequencing highlighted two loci upstream of *Sry* that present dynamic methylation status in the time period that coincide with *Sry* expression [58–60] (Figure 4A). The first region overlaps with the transcription start site (TSS) of the untranslated circular *Sry* RNA (Region I), while the second overlap with the TSS of the translated *Sry* transcript that is expressed during gonadal sex determination (Region II). These two regions present CpG dinucleotide hypermethylation at E8.5, when *Sry* is not expressed. Around E11.5, both regions are hypomethylated in the gonad, while the hypermethylated status is maintained in other tissues. By E15.5, region I is still hypomethylated while region II is hypermethylated again [58,60]. These results suggest that DNA methylation might be responsible for the tissue-specificity and the temporal regulation of *Sry* by protecting the cis-regulatory regions.

In addition to DNA demethylation, *Sry* expression requires the demethylation of H3K9me2 (dimethylated Lys 9 on histone 3, a histone mark associated with transcriptional repression) by the histone demethylase JMJD1A (Figure 4A). Disruption of *Jmjd1a* results in frequent sex-reversal due to an accumulation of H3K9me2 around the *Sry* promoter, leading to a lower expression [61]. Conjointly with the H3K9 demethylation, *Sry* promoter display enrichment of permissive histone marks, including H3 lysine four trimethylation (H3K4me3) and H3 acetylation (H3ac) [60].

Together, DNA methylation and histone modifications are actively involved in the spatiotemporal expression of *Sry* by making the enhancers and the promoter accessible for the binding of multiple transcription factors (reviewed in [62]).

Sox9 cis-regulatory regions

During male gonadal sex determination, SRY up-regulates *Sox9* by targeting the testis specific enhancer of *Sox9* expression (TES), which includes the core element TESCO [63]. Deletion of TES and TESCO in XY mice reduces *Sox9* expression by ~50% without provoking sex reversal [64]. These results confirmed the important role of TES/TESCO to control *Sox9* expression levels in the testis, and suggest the presence of additional enhancers that remained to be identified. A more recent study combined **ATAC-seq** (Assay for Transposase-Accessible Chromatin using sequencing) on XY and XX gonads at E10.5 and E13.5 together with ChIP-seq for H3K27ac to screen for new putative *Sox9* enhancers. 16 candidate enhancer regions were screened using transgenic mice carrying the enhancer regions upstream to a *LacZ* reporter. Two out of the 16 candidates showed testis-specific activity (Enh13 and Enh14). Deletion of the Enh14 did not result in alteration of *Sox9* expression. However, the homozygous deletion of Enh13, a 557 base pair element located 565 kb upstream from *Sox9* TSS, resulted in a complete XY male-to-female sex reversal (Figure 4B). The study also demonstrated that SRY preferentially binds to the Enh13 rather than on TESCO, indicating that Enh13 is critical for the up-regulation of *Sox9*, whereas TESCO is responsible for the stabilisation of *Sox9* up-regulation [65].

Stabilisation of male and female fate

Sertoli cells and granulosa cells come from a common progenitor cell population and maintain the ability to trans-differentiate into their opposite sex counterpart even after birth [66–70]. Although many transcription factors controlling the differentiation of the Sertoli cells have been identified, the way they act to control cell fate decision remain poorly understood.

The epigenetic regulator CBX2 (chromobox protein homologue 2) is part of the polycomb repressive complex 1 (PRC1) which binds to H3K27me3 (trimethylated Lys 27 on histone 3) to maintain chromatin compaction and repress gene expression [71]. Disruption of *Cbx2* in XY mice embryos results in ovary development [72,73]. It was originally proposed that *Cbx2* acts as an activator of the male fate through indirect positive regulation of *Sry* [56,73]. However, recent findings suggest instead that *Cbx2* is required to stabilize the male fate by blocking the upregulation of bivalent female determining genes [74]. By directly binding to *Lef1*, a Wnt downstream target in XY gonads, CBX2 inhibits Wnt signaling and promotes the stabilization of the male fate and the differentiation of the Sertoli cells. In XX E13.5 gonads, or in XY gonads that lack *Cbx2*, *Lef1* promotes the up-regulation of the female pathway that antagonizes the male fate, allowing the differentiation of the pre-granulosa cell (**Figure 4C**).

Two complementary studies recently monitored open chromatin regions (using **DNAseI-seq** and **ATAC-seq**) and histone modification indicative of active enhancers and promoters (**ChIP-seq** for H3K27ac) in purified XX and XY gonadal supporting cells before (E10.5) and after (E13.5 and E15.5) gonadal sex determination in mice [75,76]. At E10.5, the XX and XY progenitor cells have similar chromatin accessibility landscapes, consistent with their transcriptional state [1,30]. Differentiation of the supporting lineage into Sertoli or granulosa cells is accompanied by an increase in open chromatin regions that neighbor Sertoli- or granulosa-promoting genes, respectively. These open chromatin regions are enriched for transcription factors binding motifs that are known to promote supporting cell development. Interestingly, in Sertoli cells, granulosa-promoting genes display accessible but inactive regulatory regions (i.e. depleted in H3K27ac histone modification) that are

enriched in binding motifs for DMRT1 and SOX9. This suggest that the pro-granulosa genes are repressed by Sertoli cell transcription factors right after gonadal sex determination to restrict the cell fate to Sertoli type. These findings are consistent with expression data indicating that commitment to the male fate requires both upregulation of Sertoli-promoting genes and simultaneous repression of granulosa-promoting genes [1].

Until recently, most of our knowledge about the antagonistic genetic programs underlying gonadal sex determination was based on the observation of sex reversals induced by targeted knock-out genes. The integration of genome-wide screening of chromatin state and transcription factor binding sites starts to uncover the regulatory networks that control the fine-tuned timing of gene expression observed during gonadal sex determination.

Concluding remarks

Although we have limited the scope of this review to embryonic sex determination it is now evident that the initial decision for gonads to differentiate into either testes or ovaries is not permanent and has to be actively maintained throughout life [68,70]. Disruption of the delicate balance of the genetic programs required to maintain cell identity in adult gonads results in a change of fate of somatic gonadal cell types, with Sertoli cells transdifferentiating into granulosa cells and vice versa. Unfortunately, there is only scarce information about the epigenetic and transcriptional changes driving adult transdifferentiation and how similar/divergent they are when compared to the process of embryonic sex determination. With the emergence of single-cell RNA sequencing technology (scRNA-seq) as well as other genome-wide techniques describing the chromatin landscape at the single cell resolution (e.g. single cell ATAC-seq) it is now

possible to reconstruct the transcriptomic programs and the dynamics of chromatin regulatory landscapes driving transdifferentiation of adult testis and ovarian cells.

It is likely that future progress in our understanding of the transcriptomic and epigenetic mechanisms regulating embryonic sex determination and adult testis and ovarian cell transdifferentiation will improve our capacity to identify causative variants in **disorders of sex development** (**DSD**) patients. Currently, the majority of DSD cases related to defects in gonadal development and differentiation do not receive a genetic diagnosis [77]. It is hypothesized that a significant proportion of pathogenic variants or in/dels may be localized in critical regulatory intergenic regions that are not covered by classical exome sequencing. We expect that a better understanding of the regulatory regions controlling the fine balance of expression of key sex-determining genes, in the mouse as well as in human, will improve the success rate of identifying causal variants in patients with DSD.

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Figure legends:

Figure 1:
(A) Schematic representation of a urogenital ridge showing the pronephros, mesonephros, and metanephros. The gonads (in green) develop from the thickening of the coelomic epithelium on the ventromedial surface of the mesonephros (adapted from [78]).
(B) Whole Tg(Nr5a1-GFP) mouse embryo at E10.5 (merge of bright-field and UV light). GFP fluorescence is localized in the somatic cells of the developing gonads.

(C) Schematic representation of a transverse section of the genital ridge during gonadal formation. The growth of the genital ridges occurs by the proliferation of the coelomic epithelium (shown in green) and subsequently by the fragmentation of the underlying basement membrane (pink dashed line), allowing the delamination of the proliferating CE into the inner mesenchymal region of the mesonephros. Concurrently with the thickening, the genital ridges are colonized by the migrating primordial germ cells (shown in purple). (adapted from [79]).

Figure 2:
Description of the existing large-scale transcriptomic studies of mouse gonadal sex determination. The blue and pink dots indicate the embryonic time points covered by the studies (in XY and XX gonads respectively), while the pale coloured rectangles inform about the method employed (microarrays, RNA-seq or single-cell (sc) RNA-seq). The input cells of each studies is indicated on the right of the graph.

Figure 3:
Comparison of the information obtained from transcriptomic studies using bulk of whole gonadal cells (left), bulk of sorted cell populations (center), or isolated single cells (right).

Figure 4:
(A) Dynamic of the accessibility of the cis-regulatory regions controlling *Sry* expression. Prior to sex determination (E8.5), the cis-regulatory regions upstream to *Sry* gene present high DNA CpG methylation and di-methylated state of lysine residue 9 on histone H3, a histone modification associated with transcriptional repression. These marks prevent the accessibility of the region for the transcription factors and thus inhibit *Sry* expression. Around E11.5, the same regulatory regions appear demethylase and histones H3 are demethylated with the action of JMJD1A. Thereby the transcription factors have access to the cis-regulatory regions and trigger the expression of *Sry* [61].
(B) Over-expression of *Sox9* in XY is controlled by the TESCO enhancer regions but also by the recently identified Enh13 regions, which is preferentially bound by SRY. Deletion of the Enh13 is sufficient to cause male-to-female sex-reversal in XY mice [65].
(C) In XY mice, CBX2 prevents the activation of the *Lef1* gene via the Wnt pathway by acting as a protector of the DNA compaction. This allows the stabilisation of the male pathway and assure a normal testicular development. In XX and in XY mice lacking *Cbx2*, the Wnt signalling activates *Lef1* gene expression, causing the up-regulation of the female pathway and the development of ovaries [74].

Tables:

Table 1: Critical factors involved in the genital ridges and early gonadal development.

Genes	Full names	Phenotypes	References
Gata4	GATA-binding protein 4	Conditional knockout of *Gata4* induced at E8.75 impairs epithelial proliferation and basement membrane fragmentation. Gata4 controls the expression of *Nr5a1* and *Lhx9*.	[2]
***Nr5a1* (*Ad4BP*, *Sf1*)**	Nuclear receptor subfamily 5, group A, member 1	Constitutive knockout of *Nr5a1* causes the degeneration of the gonadal ridge by apoptosis and complete absence of both the adrenal glands and the gonads.	[10,11]
Wt1	Wilms' tumor 1	Constitutive induced mutation of *Wt1* leads to a disruption of the urogenital development, in particular to the absence of gonads, caused by causes increased cell death.	[48,80]
Lhx9	LIM homeobox 9	Constitutive *Lhx9* knockout mice show absence of gonads due to coelomic epithelial cell proliferation failure at E10.5.	[12]
Emx2	Empty spiracles homeobox 2	Constitutive *Emx2* knockout mice display absence of gonads caused by impaired epithelial cells migration through the basement membrane, but the adrenal gland development is not affected.	[81]
Six1* and *Six4	SIX Homeobox 1 and 4	*Six1/Six4* double knockout results in smaller gonads with a male-to-female sex-reversal due to an impaired *Sry* expression. *Six1/Six4* regulates *Nr5a1* and *Zfpm2*, a direct regulator of *Sry*.	[82]
Insr* and *Igf1r	Insulin receptor and IGF receptor 1	*Insr* and *Igf1r* double knockout affects the expression of *Nr5a1* and reduces the proliferation rates of the somatic progenitor cells in both XX and XY prior gonadal sex determination. Mice present a male-to-female sex reversal and complete absence of adrenal glands.	[83,84]
Numb	NUMB endocytic adaptor protein	Conditional knockout of *Numb* and *Numbl* at E8.25 using a tamoxifen-inducible ROSA-CreER leads to disrupted cell polarity in the CE, a reduce number of supporting and steroidogenic cells, and a accumulation of undifferentiated cells in the developing gonad.	[20]
Nrg1	Neuregulin 1	Conditional *Nrg1* knockout using a WT1-$Cre^{Tg/+}$ results in a decrease of the coelomic epithelial cells, and a reduced number and delayed Sertoli cell differentiation.	[85]

Sry	Sex determining region of the chromosome Y	Sry initiates a dramatic increase in somatic cell proliferation at the coelomic epithelium of XY gonads starting at E11.25 into two distinct stages. Initially proliferation was observed largely in NR5A1-positive cells and contributed to the Sertoli cell population and later in NR5A1-negative cells below the coelomic epithelium that did not give rise to Sertoli cells.	[13,14,86]
Rspo1* and *Wnt4	R-spondin 1 and Wingless-type MMTV integration site family, member 4	Constitutive knockout of *Wnt4* and *Rspo1* results in impaired proliferation of the cells of the CE in XY gonads leading to a reduced number of Sertoli cells and the formation of a hypoplastic testis.	[87]

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Outstanding questions (3-4 questions)**:**

Ovaries and testes are two complex – and very different – organs that arise from a common primordium. A comprehensive atlas of all the cell lineages and cell types originating or migrating in these two organs both during embryo and child development, and later in adult life, will be required in order to obtain a basic framework to understand the full gonadal development and function.

The exact epigenetic modifications and transcriptional events mediating cell lineage specification and sex-specific differentiation during the process of primary sex determination are still unidentified or unclear. What are molecular mechanisms underlying the early stages of the gonadal primordium establishment? How the supporting and the interstitial/stromal cell lineages are defined at the onset of sex determination? Is sex fate decision maintained by constant and active repression by sexual antagonistic factors or by epigenetics modifications?

To what extent genetic variation in intergenic regions such as promoters, enhancers and silencers can impact the fine-tuning of key sex-determining genes and explain undiagnosed DSD cases.

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Comments reviewer

We thank the Reviewers for their constructive comments of the manuscript. We have taken into account their suggestions and changed the text accordingly. We remove some parts of the text, rewrote other parts and included new results and citations. In particular, we remove from the main text some of the technical details about single cell RNA sequencing. This technical part is now included in a new text box 1. In parallel, both sections dealing with *transcriptional events underlying gonadal sex determination* and *epigenetic regulation during gonadal development* have been significantly improved and completed with new text and references.
Responses to specific points are given below.

Reviewer #1:

1) The main problem is that basic concepts are often not introduced, thereby probably making it very hard / impossible for a non-expert reader to follow.
Response: as pointed out by the Editor, we believe that Reviewer 1 may have missed our glossary as many of the points he/she asks for is addressed in there.

Additional comments:
2) As there is only one instance discussion data that does not refer to mice, it might be better to delete that and solely focussing on what is known from work in mice.
Response: Although we focused our review on mouse data, in some instance data are available only in other species, such as the chicken in the case of hedgehog signaling and early gonadal formation (lines 60-73). We believe it is important and wishes to keep it unchanged in the final version of the review. We have rephrased the introduction of this instance to highlight it might be an interesting hypothesis to test in mammals.

3) As it is not commonly accepted that 'sex determination' is equivalent with the decision of the genital ridges to develop into either testis or ovary, it is recommended to use 'gonadal sex determination' for this process throughout the review, including line 22 and 33, replace 'primary sex determination' with 'gonadal sex determination', add to title and in lines 15, 42, 111, 113, 121, 123, 196, 257, 260, and 291 (and maybe some more) 'gonadal'.
Response: As suggested we replaced “sex determination” by “gonadal sex determination” throughout the manuscript. Few exceptions were made when it was obvious or introduced a repetition in the sentence or paragraph.

4) Replace in line 32 'testis' and 'ovary' with "testicular' and 'ovarian'.
Response: replaced

5) In lines 50/51 be consistent, i.e. either use singular ('genital ridge, or bipotential gonad'…'ovary and testis') or plural ('genital ridges, or bipotential gonads'…'ovaries and testes').
Response: We now consistently use the plural.

6) In Figure legend 1C it should read 'transverse' and not 'sagittal', as a transverse section is shown in the schematic.
Response: we modified the legend as suggested.

7) Line 73, GAT4 is not precociously expressed (that would implicate that it is normally expressed later, but I guess, the authors are talking about the normal, wild-type situation here) - it is one of the earliest expressed. This should be re-worded.

Response: This is a valid point, we reworded the sentence: “To our knowledge, GATA4 is the earliest transcription factor expressed specifically in genital ridges.”

8) Lines 94/95 reads '…by asymmetrical cell division of molecular determinants…', which does not make sense. Maybe better write '…by asymmetrical cell division driven by molecular determinants…', or something like this. Similarly, '…or by external signals from inside the gonads' sounds a bit funny. Maybe replace with something like '…or by external signals from other cells within the gonads'.
Response: We modified the sentence based on the Reviewer’s suggestions.

9) Line 173, it is not clear what 'Sf1-GFP' cells mean. This needs to be explained.
Response: Correct. For more clarity, we modified the sentence, removed “Sf1-GFP” and now mention “individual NR5A1+ somatic cells”.
Excerpt: “We recently purified and sequenced the mRNA of individual NR5A1+ somatic cells from XY and XX mouse gonads at E10.5, E11.5, E12.5, E13.5, E16.5 and P6 (last point in XX only) [28,29]”

10) Line 198, it was known before the single cell RNA-Seq experiments that ovarian development is not passive. So this statement here is misleading and should be removed.
Response: This is a relevant point. We removed this statement and replace it by “…revealed the complexity of the transcriptomic program at play during ovarian development “.

11) Line 232, as per official nomenclature (see http://www.informatics.jax.org/mgihome/nomen/), proteins are written in all capital, i.e. it should be 'JMJD1A'.
Response: We use now Jmjd1a instead of Kdm3a and wrote in capital letters when it is the protein or small italic letters when it is the mouse gene.

12) Line 238, 'Granulosa' should read 'granulosa' (it is not based on a name, hence not in capital like for example Sertoli).
Response: modified.

13) Line 274 should read 'beta-galactosidase' not 'beta-Gal'.
Response: corrected

14) The authors need to make sure that all citations mentioned in Figure 2 are included in the references and/or in the Figure legend (which is currently not the case).
Response: we checked again and we can confirm that all the articles mentioned in Figure 2 are included in the references.

Reviewer #2:

15) However, in some instances the authors could have better documented their statements through citations. In 2018, single cell RNA sequencing analyses from the Nef group and data on the mesonephric origin of a proportion of somatic cells in the testis (Kumar and De Falco, Nat Coms 2018 and previous articles) were major achievements. Given that the contribution of mesonephric cells accounts for around 30% of the fetal Leydig cells and other cell populations of the testis, this should be discussed in the present review.
Response: Following the Reviewer’s suggestion, we are now discussing the paper of Kumar et al 2018 (PMID: 30375389) about the contribution of mesonephric cells in the origin of fetal Leydig cells (line 101-106).

Minor Revisions
16) 42 'mediating sex determination' change to 'mediating sex determination in mice'

Response: the change has been made

17) 54 The growth of the genital ridges occurs via proliferation of the coelomic epithelium (CE), and subsequently via fragmentation of the underlying basement membrane, allowing the delamination of the proliferating CE into the inner mesenchymal region of the mesonephros (Figure 1C) [1,2]. The cited articles (Hatano 1996, Ikeda 1994) do not show what is described in the sentence, but rather relate the common origin of the AGP with the expression of Nr5a1. Instead, the article (Hu 2013) shows a correlation between proliferation of the CE and fragmentation of the basement membrane and should be cited.
Response: we now cite the article of Hu et al Plos Genet 2013 (PMID: 23874227) as a reference.

18) 69 Schmahl et al (2000)
Response: We included the paper of Jennifer Schmahl et al Development 2000 (PMID: 10654601) about the effect of SRY on somatic cell proliferation in Table 1 together with a small description of its effect on somatic cell proliferation at the coelomic epithelium of XY gonads at 11.25 dpc.

19) 74 please cite ref 13 earlier in the text
Response: done

20) 76 by controlling the basement membrane fragmentation.
Response: It is not clear to us what the Reviewer meant.

21) 86 the ingressing CE cells are the major source of somatic cells
It is not clear whether the fragmentation is an active or a passive mechanism that is initiated by an increase of the number of cells.
Response: This sentence has been included in line 59-60.

22) 87 though endothelial cell migration from the adjacent mesonephros occurs later in XY gonads during their development [14-17]. This sentence would be a good place to comment on the contribution of the mesonephric cells.
Response: We agree. The mesonephric source of Leydig cells is now discussed in the chapter about gonad formation (see lines 101-107).

23) line 91 Single CE labeled cells. Please insert a reference
Response: correct, the reference has now been included

24) 93 This suggests that CE cells are multipotent and their fate is controlled, either by asymmetrical cell division of molecular determinants, or by external signals from inside the gonads [14]. The idea of a common progenitor stems from many other investigators such as McLaren, Burgoyne, Albrecht, and Eicher. Their work should be given credit in this review.
Response: We provide now additional references including:
S.J. Palmer, P.S. Burgoyne Development, 1991. And M. Buehr, S. Gu, A. McLaren Mesonephric contribution to testis differentiation in the fetal mouse Development, 117 (1993)

25)134 The opposite phenomenon was observed with germ cells being primed with a male bias. The role of Sry in the sex dimorphism of the somatic fate is described. Please discuss the factors promoting differentiation of germ cells into oogonia.
Response: We wish not to discuss the factors promoting differentiation of PGCs into oogonia. We believe it will disrupt the natural flow of the text focusing mostly on the somatic compartment and distract the attention of the reader. In addition, if we discuss germ cell differentiation into oogonia, the same should be done for spermatogonia differentiation.

26) 158 Specific reporter genes or markers can be used to purify cell types prior to transcriptomic analysis. The authors discuss the different methodologies and cell populations that were analyzed. What's about the mesonephros? The recent data from Kumar and De Falco (Nat Coms, 2018) show that a proportion of fetal Leydig cells, peritubular myoid cells, endothelial cells, pericytes and some interstitial progenitor cells are derived from the mesonephros reaching the gonads from E11.5 onwards. Until recently, it was thought that the mesonephros only/mostly contributes endothelial cells, thus explaining why it was neglected in most of previous analyses. In the future, this new findings will have to be taken into account and should be discussed in a review on cell populations.
Response: Correct, this point has been included in the revised version.

27) 173 We recently purified and sequenced the mRNA of individual somatic cells (Sf1-GFP cells) from XY and XX mouse gonads at E10.5, E11.5, E12.5, E13.5, E16.5 and P6 (last point in XX only) [28,29]. See previous comment. Part of the cells constituting the gonads comes from the mesonephros. The authors used the Nr5a1-GFP reporter to purify the somatic cells of the gonads and, by doing this, excluded the cells coming from the mesonephros that do not express Nr5a1. This should be clarified.
Response: We wish not to mention a third time that some of the cell come from the mesonephros. As for point 25), we believe repeating this point will disrupt the natural flow of the text, distract the attention of the reader and ultimately not add much more to the text.

There is increasing evidence for the role of epigenetics and DNA regulatory elements in sex determination, starting by the activation of the testis determining factor.
28) 212 SRY itself. Typo error: Sry
Response: Corrected

29) 232 Jmjd1a
Response: Corrected

30) 237 KDM3A levels.
Protein or transcripts? Check the nomenclature
Response: Corrected

31) 240 It seems to me more relevant to follow the kinetics of gonadal development with firstly, sex determination with the control of the expression of Sry (Gonen et al) and then a potential mechanism promoting the restriction of the supporting cell fate (Maatouk et al).
Response: The structure of the text has been significantly modified so to follow the suggestion of the reviewer.

32) 291 Although we have limited the scope of this review to embryonic sex determination
Add 'in mice'.
Response: done

33) In human, sex reversal often describe/ed the differentiation of the genitalia. In many cases, the patients exhibit pure gonadal dysgenesis with streak gonads, which is not the case in mice. The mouse is a useful model but differences between both species exist and this should be stated.
Response: This is correct. However, our conclusion is broader and encompass human sex determination and their corresponding pathologies. We argue that a better understanding of the fine balance of expression of key sex-determining genes will improve the success rate of identifying causal variants in patients with DSD.
Taking into account the Reviewer's comment that differences exists between both species, we now state: "We expect that a better understanding of the regulatory regions controlling the fine balance of expression of key sex-determining genes, <u>in mice as well as in human</u>, will improve the success rate of identifying causal variants in patients with DSD."

Figure legends
34) 1 A- Whole Tg(Nr5a1-GFP) mouse embryo at E10.5 (merge of bright-field and UV light). For the general readership, it might be not so obvious that GFP is expressed in the UGR. Please provide a more detailed figure legend.
It might be clearer to show first the drawing highlighting the gonad within the urogenital ridge and then its position in the embryo at E10.5.
Response: As suggested, we swapped the drawing highlighting the gonad within the urogenital ridge (now Fig 1A) and then its position in the embryo at E10.5 (now Fig 1B). The legend of Fig 1B has also been completed.

35) 1C- A recent analysis showed that a proportion of gonadal progenitors originates from the mesonephros (De Falco et al, 2011, Kumar and De Falco, 2018). These cells give rise to more than 30% of fetal Leydig cells, as well as additional cell populations in testes and likely some theca cells in ovaries. I believe these data should be included in the schematic drawing to provide an up to date model for somatic cell development of the testis.
Response: The figure depicts the formation of the gonad prior to the mesonephric cell migration contributing to the steroidogenic cell population. To not exclude the possibility that mesonephric cells might also contribute to the initial gonadal cell population, we added mesonephric cells within the gonadal compartment in the schematic.

Table 1
36) WNT signaling contributes to the proliferation of the coelomic epithelium leading to hypoplastic testis in knock out models (Chassot et al Dev 2012).
Response: Correct, Table 1 has been completed by adding the factors Wnt4/Rspo1.

Reviewer #3:

37) Globally the manuscript is well written and pleasant to read. However, some parts are too long, and some references and aspects of the field are missing.
For example, authors should shorten along the manuscript the technical aspects and advantages of different transcriptomic approaches. It is well established for scientific audience that global RNAseq and single cell analysis cannot bring the same information especially for complex tissues.
Authors analyze in too much detail some paper that they want to highlight. As example should be shortened:
-the part on paper from Jameson et al (lane 125-138),
-some gain of space could be obtained on the description of single cell analysis recently published by the authors;
-authors made a description on methylation study of Sry promoter and did not developed the role of MAPK pathway involving Gadd45g and GATA4 (a couple of Dev Cell papers…even if one is cited)
-description of DNAseI-seq paper is long as the one of Science paper on Sox9's Enh13. Again, technical points are too much developed.

With the gain of space all along the manuscript, authors could add some missing points in the text:
-The work about the critical time window of Sry action has been omitted (for ex Hiramatsu et al Development 2009). As also suggested by multiple studies on Mus Poschiavinus Y chromosome in the C57BL/6J background, precise kinetic of expression of sex determining factors is crucial, this should at least be addressed;

Response: As suggested, we remove some part of the text, rewrote other parts and included some new results. In particular, we remove from the main text some of the technical details

about single cell RNA sequencing. This technical part as been now included in a new text box 1. In parallel, we included the description of two new publications concerning epigenetic regulation of sex determining genes (Garcia Moreno et al Dev biol *in press,* Garcia Moreno et al BioRxiv, doi: https://doi.org/10.1101/496984).
We did not follow all the suggestions made by Reviewer #3. The review focuses on the genetic aspects of sex determination, not on signaling pathways, that's why we did not develop MAPK/Gadd45g. The suggestion about time window of *Sry* expression was also omitted since the scope of TiG reviews is to describe studies published during the last 5 years.
Selected articles are described in more details

38) -on lanes 148-154 a manuscript by Zhao and colleagues in 2018 (about splicing) is cited but this concept has been previously enunciated by Rahmoun et al in a NAR paper from 2017, identifying Sox9 as a potential regulator of sex-specific splicing. This point reference should be cited.
Response: we included a new paragraph about alternative splicing and its potential role in regulating the process of sex determination (lines 200-215)

39) -only few things are explained on transcription factors known to be master regulators of genetic expression: At least two studies on ChIP-seq approaches during mouse sex determining period have been published during the past two years: for Sox9 (Rahmoun et al, NAR 2017) and FoxL2 (Nicol et al, HMG 2018); this should be reported and discussed;
Response: unfortunately, we did not discuss these two papers

40)-again, about transcription factors, the authors should cite the only paper of reprogramming experiment of embryonic Sertoli cells (Buganim et al 2012);
Response: unfortunately, we did not discuss these two papers

41)-on lane 226, authors cite Cbx2 Ko paper and say that forced expression of Sry or Sox9 rescued Cbx2 ko, but they forget to notify that it's happened only in XY embryo, not in XX.
Response: done

Reviewer #4:

42) Page3, line 33 - you might want to consider calling this "gonadal sex determination". Calling this "primary sex determination" is becoming more and more controversial with more discoveries of early effects of sex chromosomes outside the gonad. Maybe can say gonadal sex determination has been considered primary sex determination since it is the driver of the majority of male/female phenotypic characteristics, but might be more accurately called "gonadal
Response: a similar suggestion has been made by Reviewer #1. As suggested, we modified the manuscript and use now the term "gonadal sex determination"

43) Page 4, line 62 - "Sonic Hedgehog (SHH) signaling mediated by BMP4 initiates gonadogenesis by establishing the dorsoventral patterning of the mesoderm and by undergoing ingression of the coelomic cells". Do you mean "inducing ingression"
Response: that's correct. The text has been modified accordingly.

44) Page 6, line 99 - It might be worth mentioning that the loss of NUMB leads to the accumulation of undifferentiated cells in the gonad (as well as loss of supporting and Leydig lineages.
Response: a similar suggestion has been made by Reviewer #1. As suggested, we modified the sentenced to include that a loss of NUMB leads to the accumulation of undifferentiated cells.

45) Page 6, line 113 - "gonadal sex determination"?

Response: done

46) Page 7, line 121 - "around sex determination, and were performed…". Add the "and"
Response: done

47) Line 133 - "unless SRY represses the female program and promotes Sertoli cell…". Add an "s" on promote
Response: done

48) Page 8, line 146 - "that the supporting cell lineage is the first to operate sex fate…"
Do you mean "adopt sex fate"
Response: instead of “adopt sex fate” we propose to use the term “initiate sex fate”

49) Page 10, line 190 - "Their transcriptomes progress from E11.5 onward"
Response: done

50) line 192 - "present very few sexual dimorphisms"
Response: done

51) line 196 - "gene expression during sex determination allowed to better"
I think you mean, "allowed us to better understand"
Response: correct, we modified the text accordingly.

52) Page 13, line 266 - "…and suggest the presence of additional enhancers that remained to be identified"
Response: done

53) In the last "Outstanding Question":
"To which extend genetic variation in intergenic regions…."
Change to : "To what extent genetic…."
Response: done

Figure 1

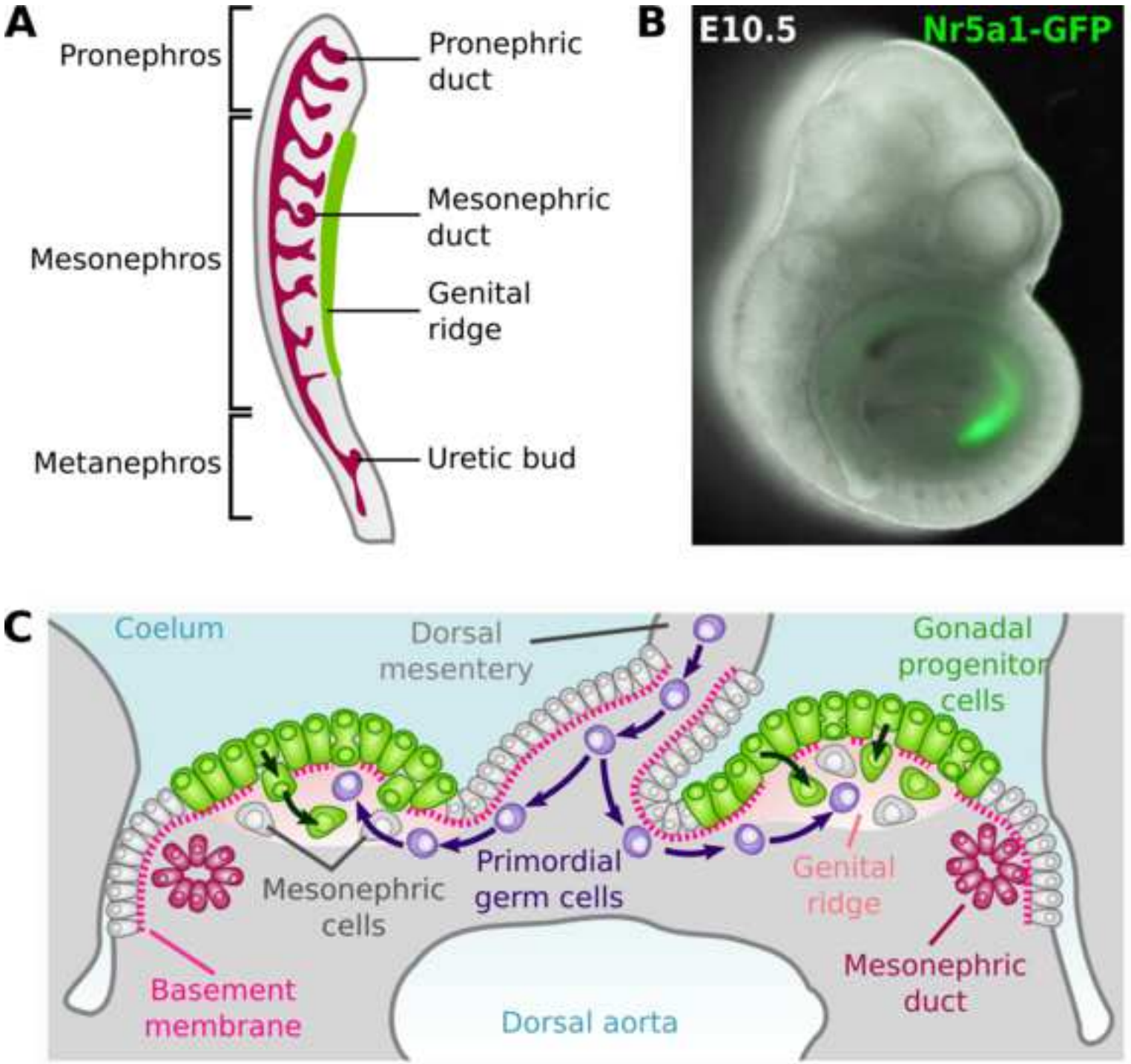


Figure 2

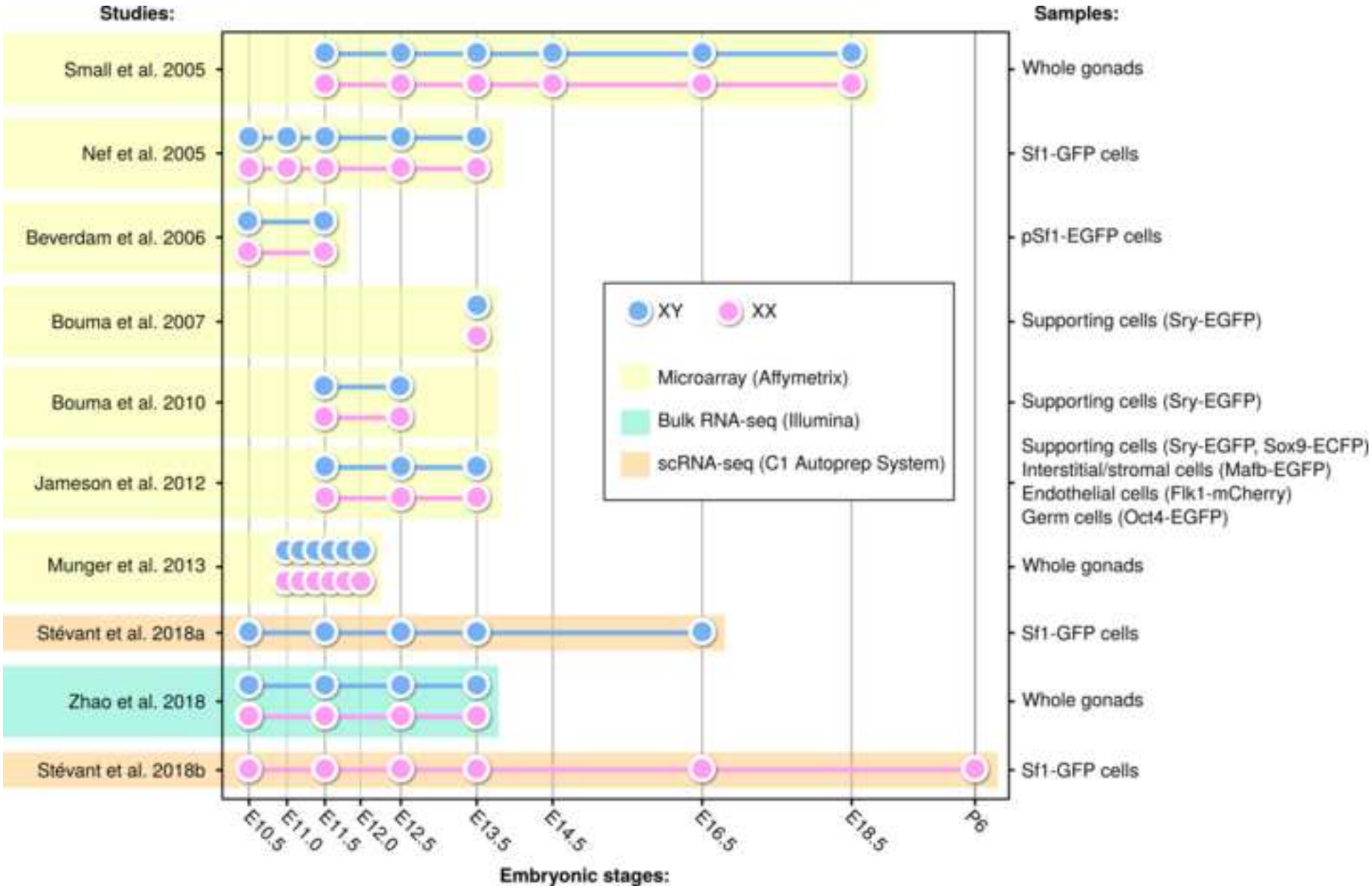


Figure 3

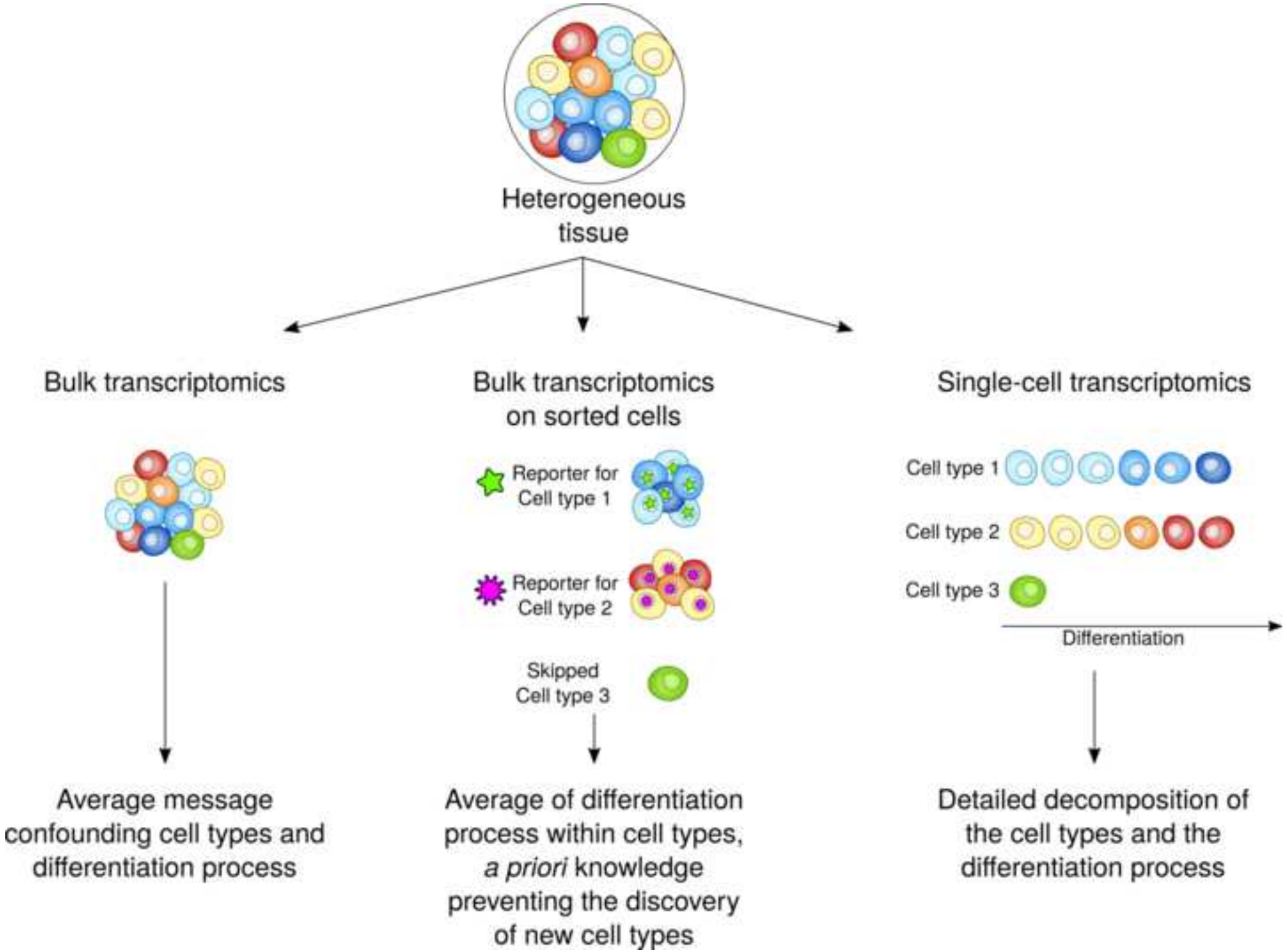
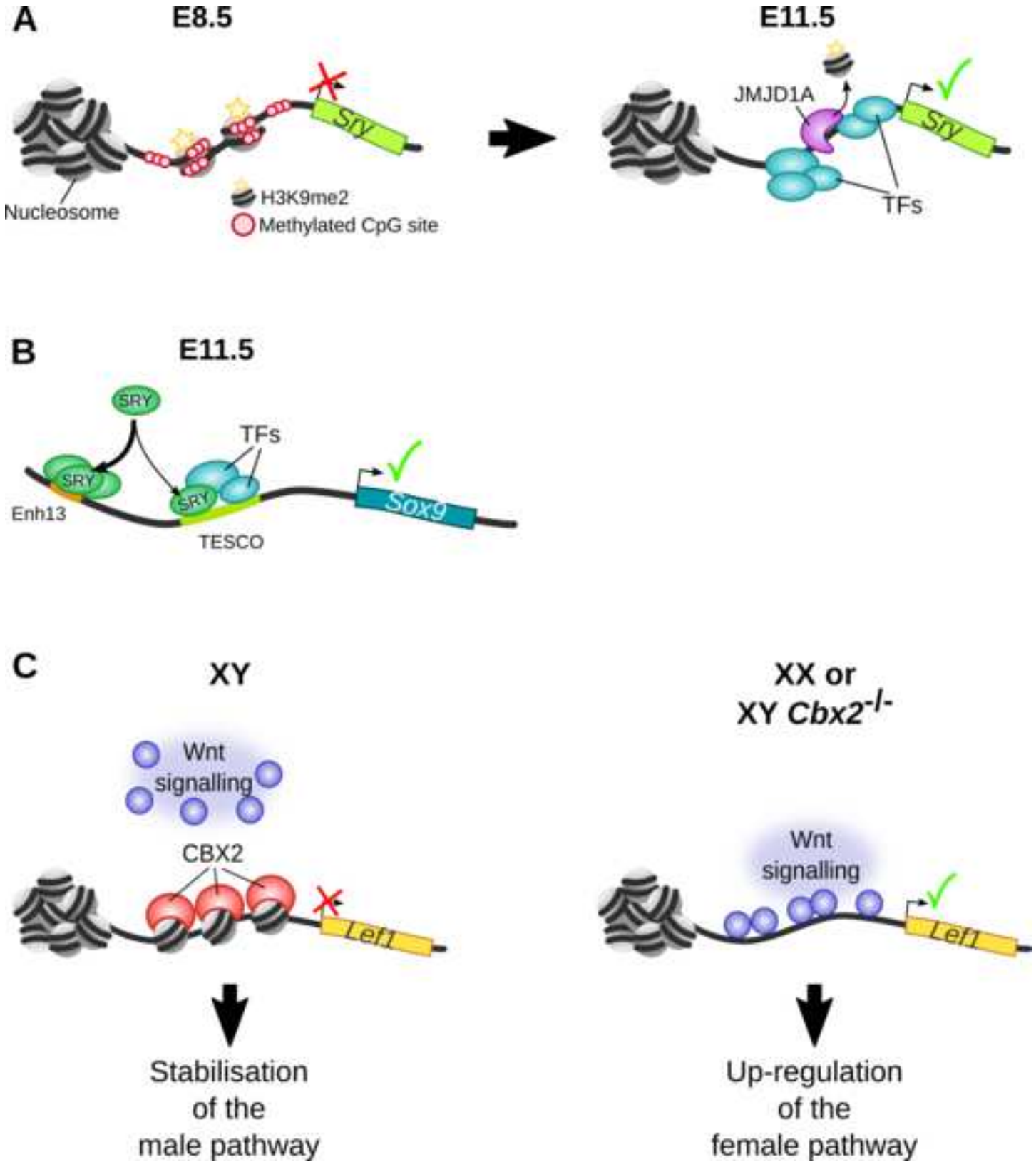


Figure 4

Click here to access/download;Figure;Figure 4 190214_gene_regulation.png



Text Box1

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Supplementary Material

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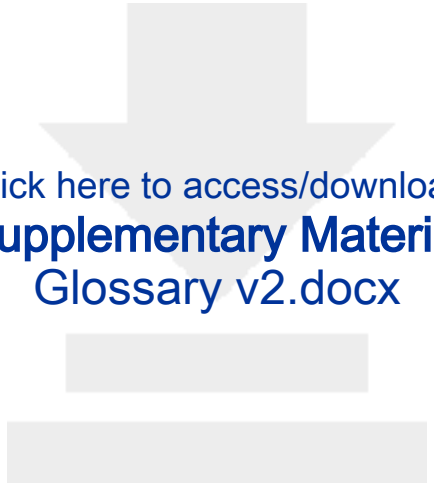

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manuscript with modifications highlighted