



Article
scientifique

Revue de la
littérature

2019

Accepted
version

Open
Access

This is an author manuscript post-peer-reviewing (accepted version) of the original publication. The layout of the published version may differ .

Genetic Control of Gonadal Sex Determination and Development

Stevant, Isabelle; Nef, Serge

How to cite

STEVANT, Isabelle, NEF, Serge. Genetic Control of Gonadal Sex Determination and Development. In: Trends in Genetics, 2019. doi: 10.1016/j.tig.2019.02.004

This publication URL: <https://archive-ouverte.unige.ch/unige:115790>

Publication DOI: [10.1016/j.tig.2019.02.004](https://doi.org/10.1016/j.tig.2019.02.004)

Trends in Genetics

Genetic control of sex determination and gonad development

--Manuscript Draft--

Manuscript Number:	TIGS-D-18-00173R1
Article Type:	Review
Keywords:	sex determination; ovary; testis; lineage specification; gene expression; epigenetic regulation
Corresponding Author:	Serge Nef geneva, SWITZERLAND
First Author:	Isabelle Stévant
Order of Authors:	Isabelle Stévant Serge Nef
Abstract:	<p>Sex determination is the process by which the bipotential gonads develop as either testes or ovaries. With two distinct potential outcomes, 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.</p>

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

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

6

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

12

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

1
2
3
4
5 1 **Genetic control of gonadal sex determination and development**
6
7
8 2

9
10 3 **Authors:** Isabelle Stévant^{1,2,3}, Serge Nef^{1,2,*}.
11
12
13

14 4 **Affiliations:**

15
16
17 5 ¹Department of Genetic Medicine and Development, University of Geneva, 1211 Geneva,
18
19 6 Switzerland;

20
21
22 7 ²iGE3, Institute of Genetics and Genomics of Geneva, University of Geneva, 1211 Geneva,
23
24 8 Switzerland;

25
26
27 9 ³SIB, Swiss Institute of Bioinformatics, University of Geneva, 1211 Geneva, Switzerland;
28
29

30 10
31
32
33 11 **Contact information:**

34
35 12 *Corresponding Author: Serge.Nef@unige.ch
36
37
38
39 13
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

14 **Summary** (max 120 words):

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

24
25
26
27
28
29
30
31 **Keywords:**

32 transcriptomics, gonadal sex determination, ovary, testis, granulosa cells, Sertoli cells,
33 supporting cells, progenitors, differentiation, lineage specification, cell fate decision, gene
34 expression, epigenetic regulation
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

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

1
2
3
4 51 **Establishment of the bipotential gonad prior to sex determination**

5
6
7 52 In mammals, **the genital ridges**, or bipotential gonads, constitute the primordia from which
8
9 53 ovaries and testes differentiate. In the mouse, they develop at around embryonic day E9.5
10
11 54 in a precise region of the ventral surface of the mesonephros (Figure 1A and B) and form
12
13
14 55 paired narrow bands of proliferating cells on either side of the dorsal mesentery (Figure
15
16 56 1C). The growth of the genital ridges occurs via proliferation of the **coelomic epithelium**
17
18 57 (CE), and subsequently via fragmentation of the underlying basement membrane, allowing
19
20
21 58 the delamination of the proliferating CE into the inner mesenchymal region of the
22
23
24 59 mesonephros (Figure 1C) [2]. It is not clear whether the fragmentation is an active or a
25
26 60 passive mechanism that is initiated by an increase of the number of cells. Thickening of
27
28
29 61 the genital ridges start at E10.3 following an anteroposterior axis (Figure 1B). Concurrently
30
31 62 with the thickening, the genital ridges are colonized by the migrating primordial germ cells
32
33
34 63 (Figure 1C) [3–7].

35
36 64 The precise molecular mechanisms that define the location of the gonads on the surface of
37
38 65 the mesonephroi remain to be elucidated. One interesting lead might reside in a recent
39
40
41 66 study in chicken embryos which suggests that Sonic Hedgehog signaling mediated by
42
43 67 cytokine BMP4 (bone morphogenetic protein 4) initiates gonadogenesis by establishing
44
45 68 the dorsoventral patterning of the mesoderm and by inducing ingression of the coelomic
46
47
48 69 cells [8]. Activation of the Hedgehog signaling in the mesonephric capsule progenitor cells
49
50
51 70 (epithelial cells in the flanked regions of the genital ridges) induces the ectopic formation
52
53 71 of gonads. However, significant differences between mouse and chick gonadal
54
55 72 morphogenesis [9] call for a similar study in mammals in order to establish whether this
56
57
58 73 process is conserved among vertebrates.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

115 **Transcriptional events underlying gonadal sex determination and sex-specific cell**
116 **differentiation**

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

1
2
3
4 120 dimorphisms. Time-series transcriptomic studies of XX and XY mouse gonads during sex
5
6 121 determination were first conducted using microarrays technologies [1,29–34], and more
7
8
9 122 recently using high throughput technics such as RNA-seq [35] and single-cell RNA-seq
10
11 123 [15,16] (**Figure 2**). Although evaluating the dynamics of gene expression during gonadal
12
13 124 sex determination is pitted with technical difficulties (see Box 1), these complementary
14
15 125 studies greatly contributed to the understanding of the genetic programs driving gonadal
16
17
18 126 somatic cell sex determination.

127 *Supporting cell commitment and differentiation*

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

1
2
3
4 166 process of folliculogenesis begins. In summary, the temporal differences in gene
5
6 167 expression observed between XX and XY supporting cell progenitors is likely due to a
7
8
9 168 delay of cell differentiation in the pre-granulosa cells compared to the Sertoli cells.

10
11
12 169 *Interstitial/stromal cell specification as steroidogenic progenitors*

13
14
15 170 In parallel of the supporting cell differentiation, the remaining CE-derived progenitor cells
16
17 171 also undergo transcriptomic changes during gonadal development [15,16,34]. In early
18
19 172 testis development, these progenitor cells are confined within the interstitial compartment,
20
21 173 while the Sertoli cells enclose germ cells to form the testis cords. In the early developing
22
23 174 ovary, no particular structures are yet observed, and the progenitor cells appear
24
25 175 intermingled with the pre-granulosa cells and the germ cells, but are later found in the
26
27 176 stromal compartment of the ovaries during folliculogenesis. The interstitial and the stromal
28
29 177 compartments are both composed of heterogeneous cell populations. They are thought to
30
31 178 contain the major source of steroidogenic progenitor cells that differentiate as fetal Leydig
32
33 179 cells from E12.5 in testes, and theca cells during the first week *post-partum* in ovaries.
34
35 180 They are also the site of the vasculature development from migrating mesonephric
36
37 181 endothelial cells [18,38–40].

38
39
40 182 The study of the interstitial and the stromal cells is challenging due to their heterogeneity
41
42 183 and the lack of specific markers (see Box 1). A first transcriptomic study attempted to
43
44 184 isolate these cells with *Mafb*-eGFP transgene [34]. They found the interstitial and the
45
46 185 stromal cells display gradual sexual dimorphism from E11.5 to E13.5, partly driven by the
47
48 186 differentiation of the fetal Leydig cells comprised in the *Mafb*-eGFP positive cells [34].
49
50 187 More refined studies using single-cell RNA-sequencing on *Nr5a1* expressing cells [15,16]

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

199 *Alternative splicing and sex determination*

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

221 **Mechanisms of gene expression regulation during gonadal development**

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

1
2
3
4 231 *Regulation of Sry expression*

5
6
7 232 The mechanisms controlling the precise spatiotemporal expression of *Sry* is not yet fully
8
9 233 resolved [57]. Bisulfite sequencing highlighted two loci upstream of *Sry* that present
10
11 234 dynamic methylation status in the time period that coincide with *Sry* expression [58–60]
12
13
14 235 (Figure 4A). The first region overlaps with the transcription start site (TSS) of the
15
16 236 untranslated circular *Sry* RNA (Region I), while the second overlap with the TSS of the
17
18 237 translated *Sry* transcript that is expressed during gonadal sex determination (Region II).
19
20
21 238 These two regions present CpG dinucleotide hypermethylation at E8.5, when *Sry* is not
22
23 239 expressed. Around E11.5, both regions are hypomethylated in the gonad, while the
24
25 240 hypermethylated status is maintained in other tissues. By E15.5, region I is still
26
27 241 hypomethylated while region II is hypermethylated again [58,60]. These results suggest
28
29 242 that DNA methylation might be responsible for the tissue-specificity and the temporal
30
31 243 regulation of *Sry* by protecting the cis-regulatory regions.
32
33

34
35
36 244 In addition to DNA demethylation, *Sry* expression requires the demethylation of H3K9me2
37
38 245 (dimethylated Lys 9 on histone 3, a histone mark associated with transcriptional repression)
39
40 246 by the histone demethylase JMJD1A (Figure 4A). Disruption of *Jmjd1a* results in frequent
41
42 247 sex-reversal due to an accumulation of H3K9me2 around the *Sry* promoter, leading to a
43
44 248 lower expression [61]. Conjointly with the H3K9 demethylation, *Sry* promoter display
45
46 249 enrichment of permissive histone marks, including H3 lysine four trimethylation
47
48 250 (H3K4me3) and H3 acetylation (H3ac) [60].
49
50

51
52
53 251 Together, DNA methylation and histone modifications are actively involved in the
54
55 252 spatiotemporal expression of *Sry* by making the enhancers and the promoter accessible for
56
57 253 the binding of multiple transcription factors (reviewed in [62]).
58
59
60

1
2
3
4 254 *Sox9 cis-regulatory regions*

5
6
7 255 During male gonadal sex determination, SRY up-regulates *Sox9* by targeting the testis
8
9 256 specific enhancer of *Sox9* expression (TES), which includes the core element TESCO [63].
10
11 257 Deletion of TES and TESCO in XY mice reduces *Sox9* expression by ~50% without
12
13
14 258 provoking sex reversal [64]. These results confirmed the important role of TES/TESCO to
15
16 259 control *Sox9* expression levels in the testis, and suggest the presence of additional
17
18 260 enhancers that remained to be identified. A more recent study combined **ATAC-seq** (Assay
19
20 261 for Transposase-Accessible Chromatin using sequencing) on XY and XX gonads at E10.5
21
22 262 and E13.5 together with ChIP-seq for H3K27ac to screen for new putative *Sox9* enhancers.
23
24 263 16 candidate enhancer regions were screened using transgenic mice carrying the enhancer
25
26 264 regions upstream to a *LacZ* reporter. Two out of the 16 candidates showed testis-specific
27
28 265 activity (Enh13 and Enh14). Deletion of the Enh14 did not result in alteration of *Sox9*
29
30 266 expression. However, the homozygous deletion of Enh13, a 557 base pair element located
31
32 267 565 kb upstream from *Sox9* TSS, resulted in a complete XY male-to-female sex reversal
33
34 268 (Figure 4B). The study also demonstrated that SRY preferentially binds to the Enh13 rather
35
36 269 than on TESCO, indicating that Enh13 is critical for the up-regulation of *Sox9*, whereas
37
38 270 TESCO is responsible for the stabilisation of *Sox9* up-regulation [65].
39
40
41
42
43
44
45
46

47 271 *Stabilisation of male and female fate*

48
49 272 Sertoli cells and granulosa cells come from a common progenitor cell population and
50
51 273 maintain the ability to trans-differentiate into their opposite sex counterpart even after birth
52
53 274 [66–70]. Although many transcription factors controlling the differentiation of the Sertoli
54
55 275 cells have been identified, the way they act to control cell fate decision remain poorly
56
57 276 understood.
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

300 enriched in binding motifs for DMRT1 and SOX9. This suggest that the pro-granulosa
301 genes are repressed by Sertoli cell transcription factors right after gonadal sex
302 determination to restrict the cell fate to Sertoli type. These findings are consistent with
303 expression data indicating that commitment to the male fate requires both upregulation of
304 Sertoli-promoting genes and simultaneous repression of granulosa-promoting genes [1].
305 Until recently, most of our knowledge about the antagonistic genetic programs underlying
306 gonadal sex determination was based on the observation of sex reversals induced by
307 targeted knock-out genes. The integration of genome-wide screening of chromatin state
308 and transcription factor binding sites starts to uncover the regulatory networks that control
309 the fine-tuned timing of gene expression observed during gonadal sex determination.

310 **Concluding remarks**

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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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

334 **Acknowledgments:**

335 The authors apologise to colleagues whose work has been omitted due to space constraints.
336 The authors are grateful to Dr. Luc Henry for proofreading and useful comments during
337 the manuscript preparation. Research in the Nef laboratory is funded by grants from the
338 Swiss National Science Foundation (grants 31003A_173070 and 51PHI0-141994) and by
339 the Département de l'Instruction Publique of the State of Geneva.

340

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

341 **References:**

342 1 Munger, S.C. *et al.* (2013) Fine time course expression analysis identifies cascades
343 of activation and repression and maps a putative regulator of mammalian sex
344 determination. *PLoS Genet.* 9, e1003630

345 2 Hu, Y.-C. *et al.* (2013) Gata4 is required for formation of the genital ridge in mice.
346 *PLoS Genet.* 9, e1003629

347 3 Richardson, B.E. and Lehmann, R. (2010) Mechanisms guiding primordial germ
348 cell migration: strategies from different organisms. *Nat. Rev. Mol. Cell Biol.* 11, 37–
349 49

350 4 Molyneaux, K. and Wylie, C. (2004) Primordial germ cell migration. *Int. J. Dev.*
351 *Biol.* 48, 537–543

352 5 Raz, E. (2004) Guidance of primordial germ cell migration. *Curr. Opin. Cell Biol.*
353 16, 169–173

354 6 Wylie, C.C. *et al.* (1986) Primordial Germ Cell Migration. In *The Cellular Basis of*
355 *Morphogenesis* pp. 433–448, Springer US

356 7 Wylie, C. (1999) Germ cells. *Cell* 96, 165–74

357 8 Yoshino, T. *et al.* (2016) Hedgehog–BMP signalling establishes dorsoventral
358 patterning in lateral plate mesoderm to trigger gonadogenesis in chicken embryos.
359 *Nat. Commun.* 7, 12561

360 9 Sekido, R. and Lovell-Badge, R. (2007) Mechanisms of gonadal morphogenesis are
361 not conserved between chick and mouse. *Dev. Biol.* 302, 132–142

362 10 Luo, X. *et al.* (1994) A cell-specific nuclear receptor is essential for adrenal and
363 gonadal development and sexual differentiation. *Cell* 77, 481–490

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

364 11 Sadovsky, Y. *et al.* (1995) Mice deficient in the orphan receptor steroidogenic factor
365 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in
366 the placenta and have normal embryonic serum levels of corticosteroids. *Proc. Natl.*
367 *Acad. Sci.* 92, 10939–10943

368 12 Birk, O.S. *et al.* (2000) The LIM homeobox gene Lhx9 is essential for mouse gonad
369 formation. *Nature* 403, 909–13

370 13 Karl, J. and Capel, B. (1998) Sertoli Cells of the Mouse Testis Originate from the
371 Coelomic Epithelium. *Dev. Biol.* 203, 323–333

372 14 Schmahl, J. *et al.* (2000) Sry induces cell proliferation in the mouse gonad.
373 *Development* 127, 65–73

374 15 Stévant, I. *et al.* (2018) Deciphering Cell Lineage Specification during Male Sex
375 Determination with Single-Cell RNA Sequencing. *Cell Rep.* 22, 1589–1599

376 16 Stévant, I. *et al.* (2018) Single-cell transcriptomics of the mouse gonadal soma
377 reveals the establishment of sexual dimorphism in distinct cell lineages. *bioRxiv*
378 DOI: 10.1101/410407

379 17 Palmer, S.J. and Burgoyne, P.S. (1991) In situ analysis of fetal, prepuberal and adult
380 XX---XY chimaeric mouse testes: Sertoli cells are predominantly, but not
381 exclusively, XY. *Development* 112, 265–8

382 18 Buehr, M. *et al.* (1993) Mesonephric contribution to testis differentiation in the fetal
383 mouse. *Development* 117, 273–81

384 19 Merchant-Larios, H. *et al.* (1993) The role of the mesonephros in cell differentiation
385 and morphogenesis of the mouse fetal testis. *Int. J. Dev. Biol.* 37, 407–415

386 20 Lin, Y.-T. *et al.* (2017) Numb regulates somatic cell lineage commitment during

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

387 early gonadogenesis in mice. *Development* 144, 1607–1618

388 21 Nishino, K. *et al.* (2001) Characterization of mesonephric cells that migrate into the
389 XY gonad during testis differentiation. *Exp. Cell Res.* 267, 225–232

390 22 Capel, B. *et al.* (1999) Migration of mesonephric cells into the mammalian gonad
391 depends on Sry. *Mech. Dev.* 84, 127–31

392 23 Smith, C.A. *et al.* (2005) Male-specific cell migration into the developing gonad is
393 a conserved process involving PDGF signalling. *Dev. Biol.* 284, 337–350

394 24 Liu, C. *et al.* (2015) Lineage specification of ovarian theca cells requires
395 multicellular interactions via oocyte and granulosa cells. *Nat. Commun.* 6, 6934

396 25 Kumar, D.L. and DeFalco, T. (2018) A perivascular niche for multipotent
397 progenitors in the fetal testis. *Nat. Commun.* 9, 4519

398 26 DeFalco, T. *et al.* (2011) Two distinct origins for Leydig cell progenitors in the fetal
399 testis. *Dev. Biol.* 352, 14–26

400 27 Liu, C. *et al.* (2016) Mapping lineage progression of somatic progenitor cells in the
401 mouse fetal testis. *Development* 143, dev.135756

402 28 Codeluppi, S. *et al.* (2018) Spatial organization of the somatosensory cortex
403 revealed by osmFISH. *Nat. Methods* 15, 932–935

404 29 Small, C.L. *et al.* (2005) Profiling Gene Expression During the Differentiation and
405 Development of the Murine Embryonic Gonad1. *Biol. Reprod.* 72, 492–501

406 30 Nef, S. *et al.* (2005) Gene expression during sex determination reveals a robust
407 female genetic program at the onset of ovarian development. *Dev. Biol.* 287, 361–
408 77

409 31 Beverdam, A. and Koopman, P. (2006) Expression profiling of purified mouse

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

410 gonadal somatic cells during the critical time window of sex determination reveals
411 novel candidate genes for human sexual dysgenesis syndromes. *Hum. Mol. Genet.*
412 15, 417–431

413 32 Bouma, G.J. *et al.* (2007) Transcriptional profile of mouse pre-granulosa and Sertoli
414 cells isolated from early-differentiated fetal gonads. *Gene Expr. Patterns* 7, 113–
415 123

416 33 Bouma, G.J. *et al.* (2010) New candidate genes identified for controlling mouse
417 gonadal sex determination and the early stages of granulosa and Sertoli cell
418 differentiation. *Biol. Reprod.* 82, 380–9

419 34 Jameson, S.A. *et al.* (2012) Temporal transcriptional profiling of somatic and germ
420 cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS*
421 *Genet.* 8, e1002575

422 35 Zhao, L. *et al.* (2018) Transcriptomic analysis of mRNA expression and alternative
423 splicing during mouse sex determination. *Mol. Cell. Endocrinol.* 478, 84–96

424 36 Albrecht, K.H. and Eicher, E.M. (2001) Evidence That Sry Is Expressed in Pre-
425 Sertoli Cells and Sertoli and Granulosa Cells Have a Common Precursor. *Dev. Biol.*
426 240, 92–107

427 37 Harikae, K. *et al.* (2013) Heterogeneity in sexual bipotentiality and plasticity of
428 granulosa cells in developing mouse ovaries. *J. Cell Sci.* 126, 2834–2844

429 38 Tilmann, C. and Capel, B. (1999) Mesonephric cell migration induces testis cord
430 formation and Sertoli cell differentiation in the mammalian gonad. *Development*
431 126,

432 39 Romereim, S.M. and Cupp, A.S. (2016) Mesonephric Cell Migration into the

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

433 Gonads and Vascularization Are Processes Crucial for Testis Development. In
434 *Results and problems in cell differentiation* 58pp. 67–100
435 40 Ross, A.J. *et al.* (2003) , AMH induces mesonephric cell migration in XX gonads. ,
436 in *Molecular and Cellular Endocrinology*, 211, pp. 1–7
437 41 Brennan, J. *et al.* (2003) Pdgfr-alpha mediates testis cord organization and fetal
438 Leydig cell development in the XY gonad. *Genes Dev.* 17, 800–810
439 42 Miyabayashi, K. *et al.* (2013) Aristaless related homeobox gene, Arx, is implicated
440 in mouse fetal Leydig cell differentiation possibly through expressing in the
441 progenitor cells. *PLoS One* 8, e68050
442 43 Wijgerde, M. *et al.* (2005) Hedgehog Signaling in Mouse Ovary: Indian Hedgehog
443 and Desert Hedgehog from Granulosa Cells Induce Target Gene Expression in
444 Developing Theca Cells. *Endocrinology* 146, 3558–3566
445 44 Karpova, T. *et al.* (2015) Steroidogenic Factor 1 Differentially Regulates Fetal and
446 Adult Leydig Cell Development in Male Mice1. *Biol. Reprod.* 93, 83
447 45 Kelemen, O. *et al.* (2013) Function of alternative splicing. *Gene* 514, 1–30
448 46 Wahl, M.C. *et al.* (2009) The Spliceosome: Design Principles of a Dynamic RNP
449 Machine. *Cell* 136, 701–718
450 47 Baralle, F.E. and Giudice, J. (2017) Alternative splicing as a regulator of
451 development and tissue identity. *Nat. Rev. Mol. Cell Biol.* 18, 437–451
452 48 Hammes, A. *et al.* (2001) Two Splice Variants of the Wilms' Tumor 1 Gene Have
453 Distinct Functions during Sex Determination and Nephron Formation. *Cell* 106,
454 319–329
455 49 Rahmoun, M. *et al.* (2017) In mammalian foetal testes, SOX9 regulates expression

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

456 of its target genes by binding to genomic regions with conserved signatures. *Nucleic*
457 *Acids Res.* 45, 7191–7211

458 50 Chassot, A.-A. *et al.* (2008) Activation of beta-catenin signaling by Rspo1 controls
459 differentiation of the mammalian ovary. *Hum. Mol. Genet.* 17, 1264–77

460 51 Parma, P. *et al.* (2006) R-spondin1 is essential in sex determination, skin
461 differentiation and malignancy. *Nat. Genet.* 38, 1304–1309

462 52 Vainio, S. *et al.* (1999) Female development in mammals is regulated by Wnt-4
463 signalling. *Nature* 397, 405–409

464 53 Jeays-Ward, K. (2003) Endothelial and steroidogenic cell migration are regulated
465 by WNT4 in the developing mammalian gonad. *Development* 130, 3663–3670

466 54 Liu, C.-F. *et al.* (2010) Building pathways for ovary organogenesis in the mouse
467 embryo. *Curr. Top. Dev. Biol.* 90, 263–90

468 55 Jeays-Ward, K. *et al.* (2004) Wnt4 is required for proper male as well as female
469 sexual development. *Dev. Biol.* 276, 431–440

470 56 Garcia-Moreno, S.A. *et al.* (2018) *Epigenetic regulation of male fate commitment*
471 *from an initially bipotential system*, 468

472 57 Hiramatsu, R. *et al.* (2009) A critical time window of Sry action in gonadal sex
473 determination in mice. *Development* 136, 129–38

474 58 Nishino, K. *et al.* (2004) DNA methylation-mediated control of Sry gene expression
475 in mouse gonadal development. *J. Biol. Chem.* 279, 22306–22313

476 59 NISHINO, K. *et al.* (2011) Non-CpG Methylation Occurs in the Regulatory Region
477 of the Sry Gene. *J. Reprod. Dev.* 57, 586–593

478 60 Gierl, M.S.S. *et al.* (2012) GADD45G functions in male sex determination by

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

479 promoting p38 signaling and Sry expression. *Dev. Cell* 23, 1032–42

480 61 Kuroki, S. *et al.* (2013) Epigenetic regulation of mouse sex determination by the
481 histone demethylase Jmjd1a. *Science* (80-.). 341, 1106–1109

482 62 Tanaka, S.S. and Nishinakamura, R. (2014) Regulation of male sex determination:
483 genital ridge formation and Sry activation in mice. *Cell. Mol. Life Sci.* 71, 4781–802

484 63 Sekido, R. and Lovell-Badge, R. (2008) Sex determination involves synergistic
485 action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 453, 930–934

486 64 Gonen, N. *et al.* (2017) Normal Levels of Sox9 Expression in the Developing Mouse
487 Testis Depend on the TES/TESCO Enhancer, but This Does Not Act Alone. *PLoS*
488 *Genet.* 13, e1006520

489 65 Gonen, N. *et al.* (2018) Sex reversal following deletion of a single distal enhancer
490 of Sox9. *Science* 360, 1469–1473

491 66 Maatouk, D.M. *et al.* (2013) Disruption of mitotic arrest precedes precocious
492 differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4
493 mutant ovary. *Dev. Biol.* 383, 295–306

494 67 Minkina, A. *et al.* (2014) DMRT1 Protects Male Gonadal Cells from Retinoid-
495 Dependent Sexual Transdifferentiation. *Dev. Cell* 29, 511–520

496 68 Uhlenhaut, N.H. *et al.* (2009) Somatic Sex Reprogramming of Adult Ovaries to
497 Testes by FOXL2 Ablation. *Cell* 139, 1130–1142

498 69 Lindeman, R.E. *et al.* (2015) Sexual cell-fate reprogramming in the ovary by
499 DMRT1. *Curr. Biol.* 25, 764–771

500 70 Matson, C.K. *et al.* (2011) DMRT1 prevents female reprogramming in the postnatal
501 mammalian testis. *Nature* 476, 101–4

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

502 71 Lau, M.S. *et al.* (2017) Mutation of a nucleosome compaction region disrupts
503 Polycomb-mediated axial patterning. *Science* (80-.). 355, 1081–1084

504 72 Katoh-fukui, Y. *et al.* (1998) Male-to-female sex reversal in M33 mutant mice.
505 *Nature* 393, 688–692

506 73 Katoh-Fukui, Y. *et al.* (2012) Cbx2, a polycomb group gene, is required for Sry gene
507 expression in mice. *Endocrinology* 153, 913–24

508 74 Garcia-Moreno, S.A. *et al.* (2018) CBX2 is required during male sex determination
509 to repress female fate at bivalent loci. *bioRxiv* DOI: 10.1101/496984

510 75 Garcia-Moreno, S.A. *et al.* (2019) Gonadal supporting cells acquire sex-specific
511 chromatin landscapes during mammalian sex determination. *Dev. Biol.* 446, 168–
512 179

513 76 Maatouk, D.M. *et al.* (2017) Genome-wide identification of regulatory elements in
514 Sertoli cells. *Development* 144, 720–730

515 77 Bashamboo, A. *et al.* (2017) Anomalies in human sex determination provide unique
516 insights into the complex genetic interactions of early gonad development. *Clin.*
517 *Genet.* 91, 143–156

518 78 Wilhelm, D. *et al.* (2013) Mammalian Sex Determination and Gonad Development.
519 *Curr. Top. Dev. Biol.* 106, 89–121

520 79 Svingen, T. and Koopman, P. (2013) Building the mammalian testis: origins,
521 differentiation, and assembly of the component cell populations. *Genes Dev.* 27,
522 2409–26

523 80 Kreidberg, J.A. *et al.* (1993) WT-1 is required for early kidney development. *Cell*
524 74, 679–691

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

525 81 Miyamoto, N. *et al.* (1997) Defects of urogenital development in mice lacking
526 *Emx2*. *Development* 124, 1653–64

527 82 Fujimoto, Y. *et al.* (2013) Homeoproteins Six1 and Six4 regulate male sex
528 determination and mouse gonadal development. *Dev. Cell* 26, 416–430

529 83 Nef, S. *et al.* (2003) Testis determination requires insulin receptor family function
530 in mice. *Nature* 426, 291–295

531 84 Pitetti, J.-L. *et al.* (2013) Insulin and IGF1 Receptors Are Essential for XX and XY
532 Gonadal Differentiation and Adrenal Development in Mice. *PLoS Genet.* 9,
533 e1003160

534 85 Gregoire, E.P. *et al.* 21-Jul-(2018) , NRG1 signalling regulates the establishment of
535 Sertoli cell stock in the mouse testis. , *Molecular and Cellular Endocrinology*

536 86 Koopman, P. *et al.* (1991) Male development of chromosomally female mice
537 transgenic for Sry. *Nature* 351, 117–121

538 87 Chassot, A.-A. *et al.* (2012) WNT4 and RSPO1 together are required for cell
539 proliferation in the early mouse gonad. *Development* 139, 4461–72

540

1
2
3
4 541 **Figure legends:**

5
6
7 542 **Figure 1:**

8 543 (A) Schematic representation of a urogenital ridge showing the pronephros, mesonephros,
9 544 and metanephros. The gonads (in green) develop from the thickening of the coelomic
10 545 epithelium on the ventromedial surface of the mesonephros (adapted from [78]).

11 546 (B) Whole Tg(Nr5a1-GFP) mouse embryo at E10.5 (merge of bright-field and UV light).
12 547 GFP fluorescence is localized in the somatic cells of the developing gonads.
13
14 548

15 549 (C) Schematic representation of a transverse section of the genital ridge during gonadal
16 550 formation. The growth of the genital ridges occurs by the proliferation of the coelomic
17 551 epithelium (shown in green) and subsequently by the fragmentation of the underlying
18 552 basement membrane (pink dashed line), allowing the delamination of the proliferating CE
19 553 into the inner mesenchymal region of the mesonephros. Concurrently with the thickening,
20 554 the genital ridges are colonized by the migrating primordial germ cells (shown in purple).
21 555 (adapted from [79]).
22
23 556

24
25 557 **Figure 2:**

26 558 Description of the existing large-scale transcriptomic studies of mouse gonadal sex
27 559 determination. The blue and pink dots indicate the embryonic time points covered by the
28 560 studies (in XY and XX gonads respectively), while the pale coloured rectangles inform
29 561 about the method employed (microarrays, RNA-seq or single-cell (sc) RNA-seq). The
30 562 input cells of each studies is indicated on the right of the graph.
31
32 563

33
34 564 **Figure 3:**

35 565 Comparison of the information obtained from transcriptomic studies using bulk of whole
36 566 gonadal cells (left), bulk of sorted cell populations (center), or isolated single cells (right).
37 567

38
39 568 **Figure 4:**

40 569 (A) Dynamic of the accessibility of the cis-regulatory regions controlling *Sry* expression.
41 570 Prior to sex determination (E8.5), the cis-regulatory regions upstream to *Sry* gene present
42 571 high DNA CpG methylation and di-methylated state of lysine residue 9 on histone H3, a
43 572 histone modification associated with transcriptional repression. These marks prevent the
44 573 accessibility of the region for the transcription factors and thus inhibit *Sry* expression.
45 574 Around E11.5, the same regulatory regions appear demethylase and histones H3 are
46 575 demethylated with the action of JMJD1A. Thereby the transcription factors have access to
47 576 the cis-regulatory regions and trigger the expression of *Sry* [61].

48 577 (B) Over-expression of *Sox9* in XY is controlled by the TESCO enhancer regions but also
49 578 by the recently identified Enh13 regions, which is preferentially bound by SRY. Deletion
50 579 of the Enh13 is sufficient to cause male-to-female sex-reversal in XY mice [65].

51 580 (C) In XY mice, CBX2 prevents the activation of the *Lef1* gene via the Wnt pathway by
52 581 acting as a protector of the DNA compaction. This allows the stabilisation of the male
53 582 pathway and assure a normal testicular development. In XX and in XY mice lacking *Cbx2*,
54 583 the Wnt signalling activates *Lef1* gene expression, causing the up-regulation of the female
55 584 pathway and the development of ovaries [74].
56
57
58
59
60
61
62
63
64
65

585 **Tables:**

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

Genes	Full names	Phenotypes	References
<i>Gata4</i>	GATA-binding protein 4	Conditional knockout of <i>Gata4</i> induced at E8.75 impairs epithelial proliferation and basement membrane fragmentation. <i>Gata4</i> controls the expression of <i>Nr5a1</i> and <i>Lhx9</i> .	[2]
<i>Nr5a1</i> (<i>Ad4BP</i>, <i>Sf1</i>)	Nuclear receptor subfamily 5, group A, member 1	Constitutive knockout of <i>Nr5a1</i> causes the degeneration of the gonadal ridge by apoptosis and complete absence of both the adrenal glands and the gonads.	[10,11]
<i>Wt1</i>	Wilms' tumor 1	Constitutive induced mutation of <i>Wt1</i> leads to a disruption of the urogenital development, in particular to the absence of gonads, caused by causes increased cell death.	[48,80]
<i>Lhx9</i>	LIM homeobox 9	Constitutive <i>Lhx9</i> knockout mice show absence of gonads due to coelomic epithelial cell proliferation failure at E10.5.	[12]
<i>Emx2</i>	Empty spiracles homeobox 2	Constitutive <i>Emx2</i> 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]
<i>Six1</i> and <i>Six4</i>	SIX Homeobox 1 and 4	<i>Six1/Six4</i> double knockout results in smaller gonads with a male-to-female sex-reversal due to an impaired <i>Sry</i> expression. <i>Six1/Six4</i> regulates <i>Nr5a1</i> and <i>Zfp2</i> , a direct regulator of <i>Sry</i> .	[82]
<i>Insr</i> and <i>Igf1r</i>	Insulin receptor and IGF receptor 1	<i>Insr</i> and <i>Igf1r</i> double knockout affects the expression of <i>Nr5a1</i> 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]
<i>Numb</i>	NUMB endocytic adaptor protein	Conditional knockout of <i>Numb</i> and <i>Numbl</i> 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]
<i>Nrg1</i>	Neuregulin 1	Conditional <i>Nrg1</i> 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]

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

<i>Sry</i>	Sex determining region of the chromosome Y	<i>Sry</i> 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]
<i>Rspo1</i> and <i>Wnt4</i>	R-spondin 1 and Wingless-type MMTV integration site family, member 4	Constitutive knockout of <i>Wnt4</i> and <i>Rspo1</i> 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]

587

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.

Manuscript TIGS-D-18-00173

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, in mice as well as in human, 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"*

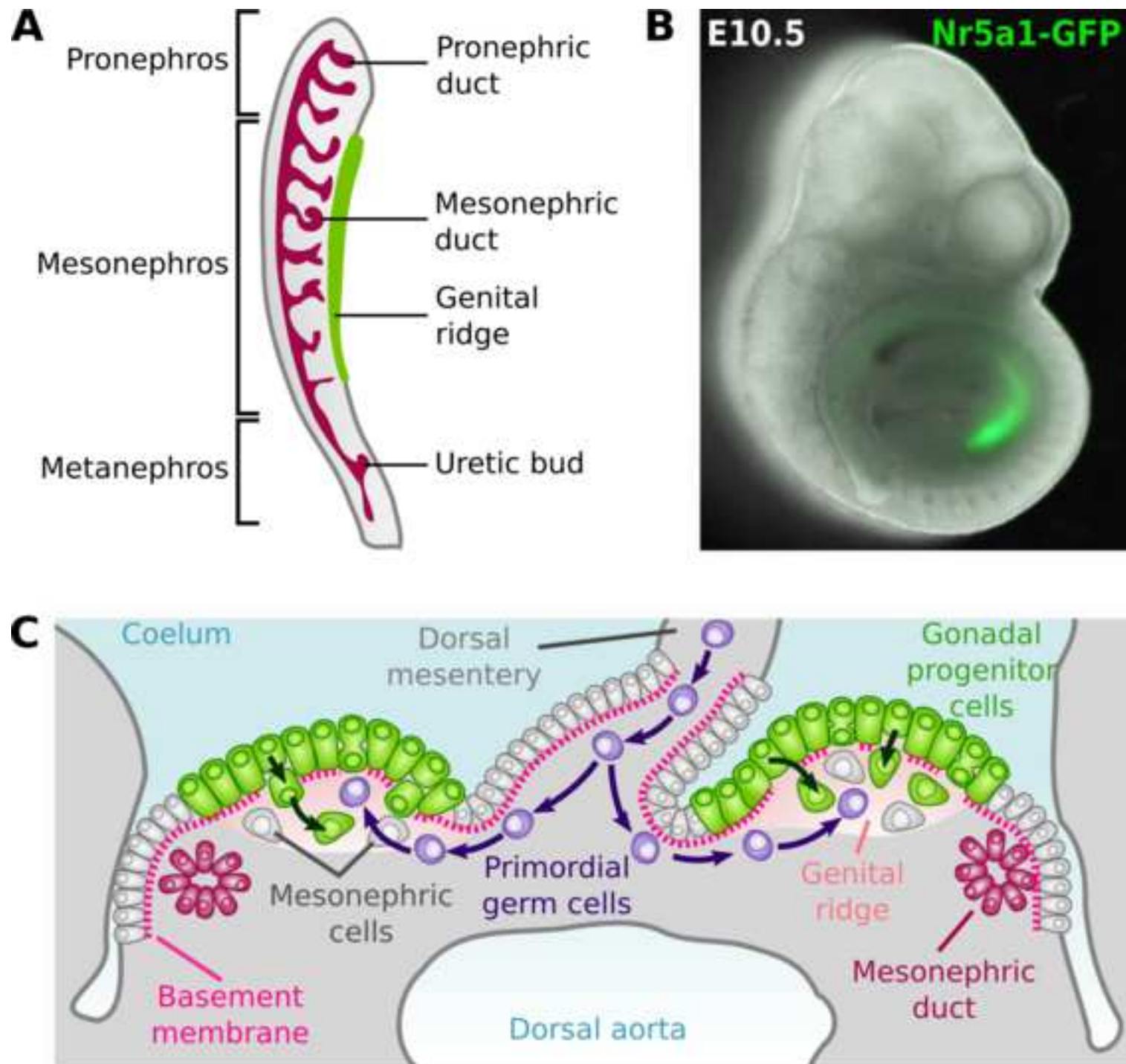
Response: done

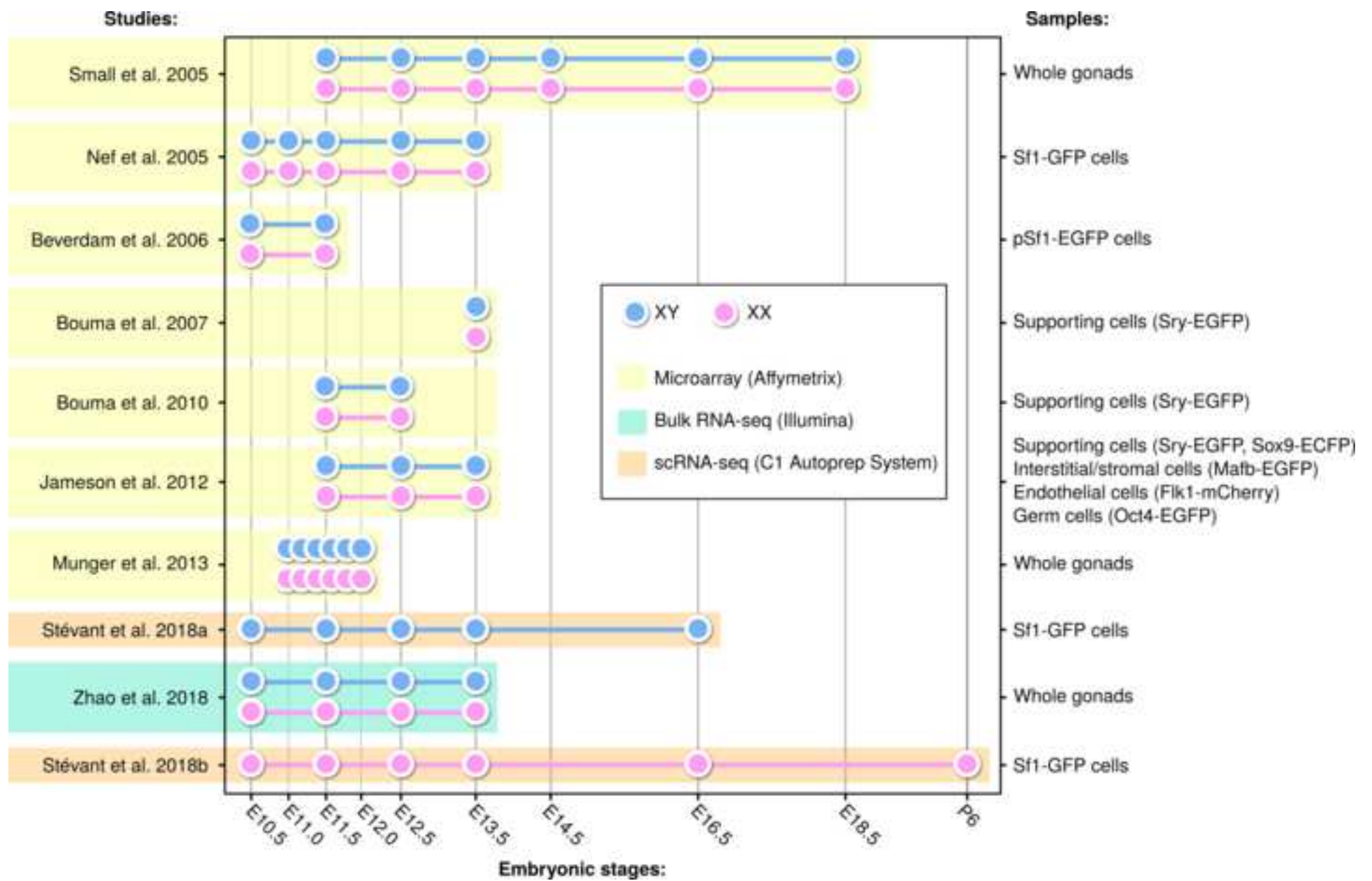
53) In the last "Outstanding Question":

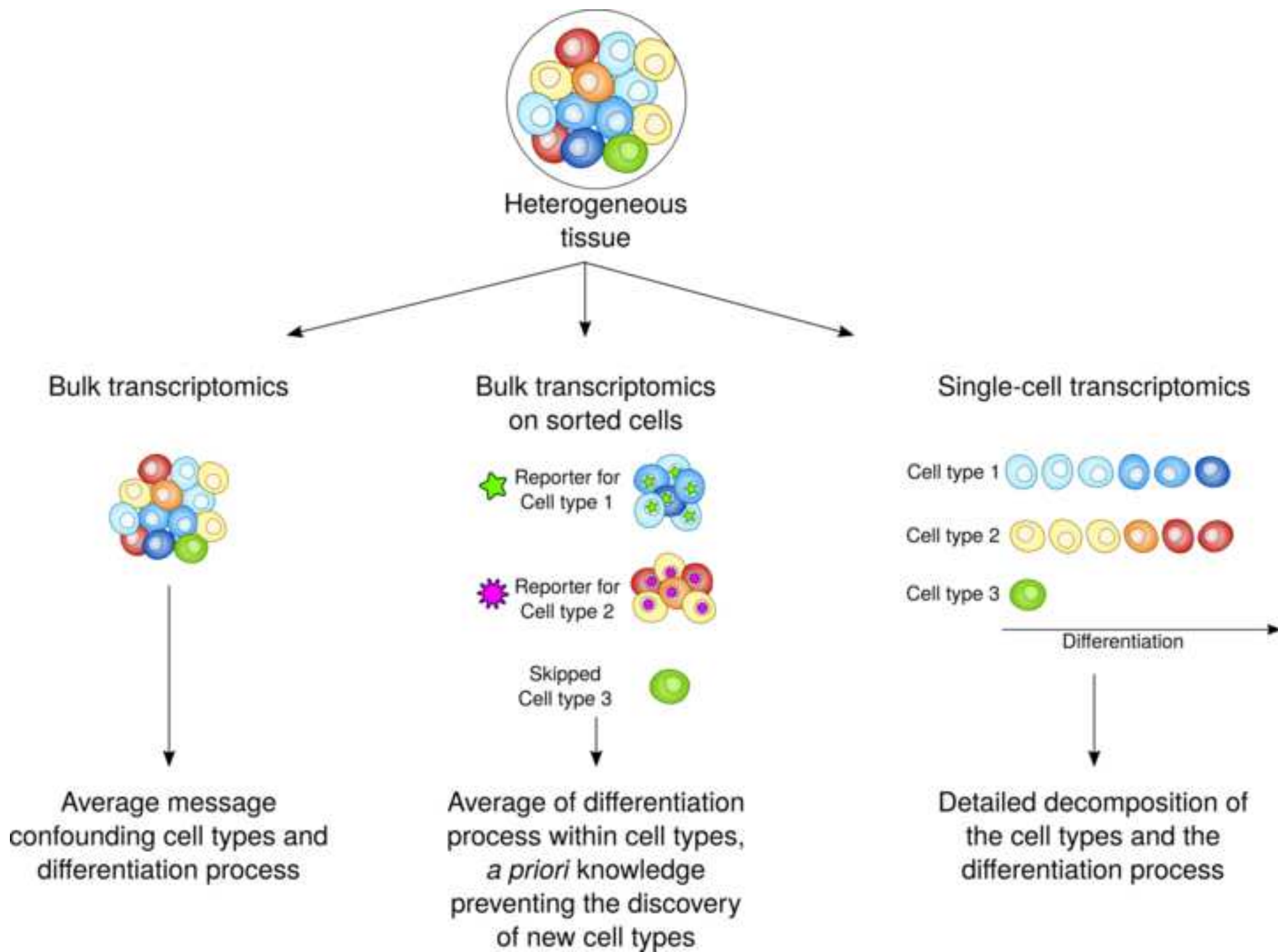
"To which extend genetic variation in intergenic regions...."

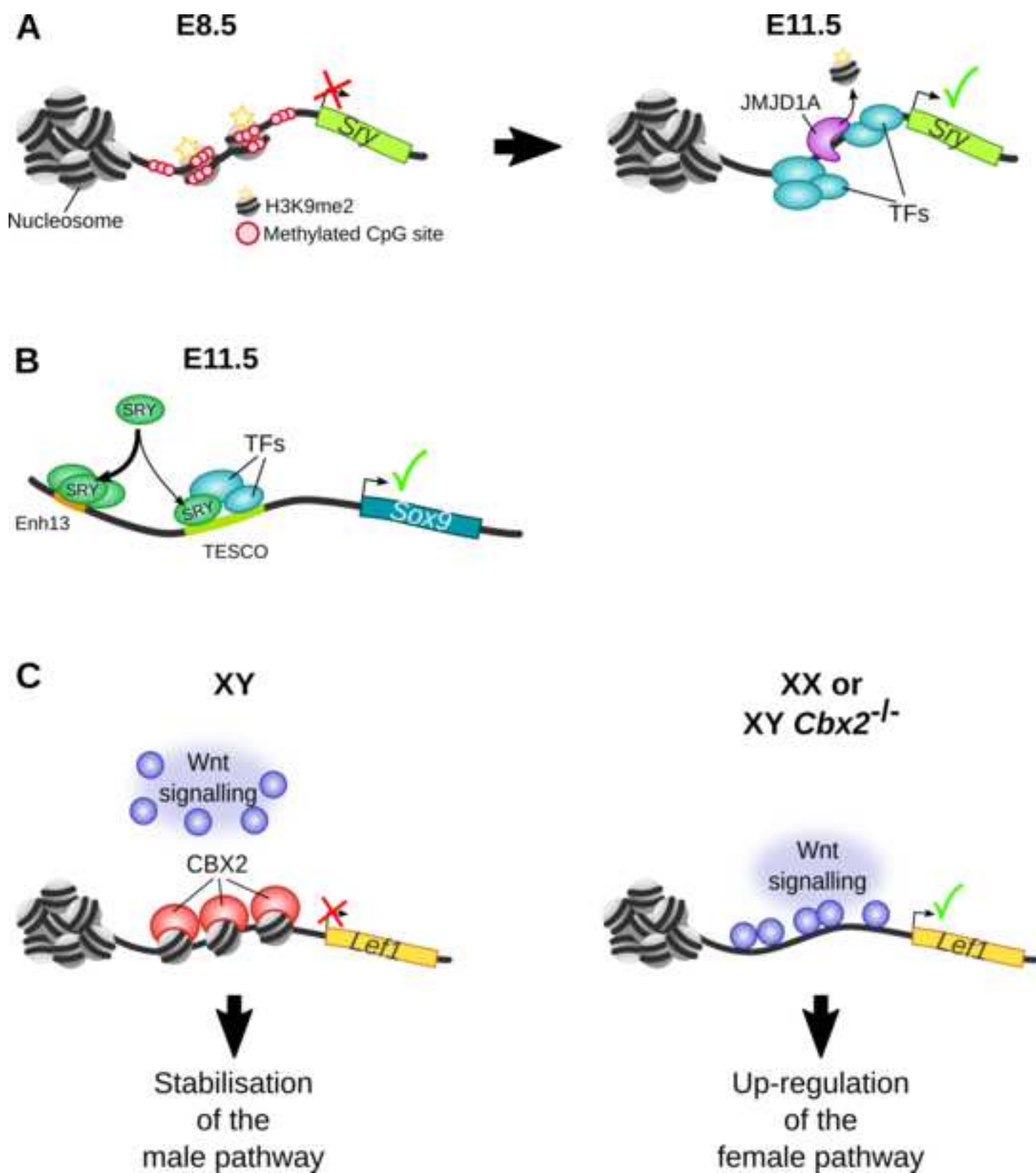
Change to : *"To what extent genetic...."*

Response: done












Click here to access/download
Supplementary Material
text box technical challenges v1.docx





Click here to access/download
Supplementary Material
Glossary v2.docx





[Click here to access/download](#)

Supplementary Material

[transc_dyn_sex_det_RV7 modif highlighted.docx](#)

