# Dynamics of the Transcriptional Landscape During Human Fetal Testis and Ovary Development

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# Dynamics of the transcriptional landscape during human fetal testis and ovary development

**Running title**: Human fetal gonad transcriptomes

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## Abstract

**STUDY QUESTION**: Which transcriptional program triggers sex differentiation in bipotential gonads and downstream cellular events governing fetal testis and ovary development in humans?

**SUMMARY ANSWER**: The characterisation of a dynamically-regulated protein-coding and noncoding transcriptional landscape in developing human gonads of both sexes highlights a large number of potential key regulators that show an early sexually dimorphic expression pattern.

**WHAT IS KNOWN ALREADY**: Gonadal sex differentiation is orchestrated by a sexually dimorphic gene expression program in XX and XY developing fetal gonads. A comprehensive characterisation of its noncoding counterpart offers promising perspectives for deciphering the molecular events underpinning gonad development and for a complete understanding of the aetiology of disorders of sex development in humans.

**STUDY DESIGN, SIZE, DURATION**: To further investigate the protein-coding and noncoding transcriptional landscape during gonad differentiation, we used RNA-sequencing (RNA-seq) and characterised the RNA content of human fetal testis (N=24) and ovaries (N=24) from 6 to 17 postconceptional week (PCW), a key period in sex determination and gonad development.

**PARTICIPANTS/MATERIALS, SETTING, METHODS**: First trimester fetuses (6-12 PCW) and second trimester fetuses (13-14 and 17 PCW) were obtained from legally-induced normally-progressing terminations of pregnancy. Total RNA was extracted from whole human fetal gonads and sequenced as paired-end 2x50 base reads. Resulting sequences were mapped to the human genome, allowing for the assembly and quantification of corresponding transcripts.

**MAIN RESULTS AND THE ROLE OF CHANCE**: This RNA-seq analysis of human fetal testes and ovaries at seven key developmental stages led to the reconstruction of 22,080 transcripts differentially expressed during testicular and/or ovarian development. In addition to 8,935 transcripts

displaying sex-independent differential expression during gonad development, the comparison of testes and ovaries enabled the discrimination of 13,145 transcripts that show a sexually dimorphic expression profile. The latter include 1,479 transcripts differentially expressed as early as 6 PCW, including 39 transcription factors, 40 long noncoding RNAs and 20 novel genes. Despite the use of stringent filtration criteria (expression cut-off of at least 1 fragment per kilobase of exon model per million reads mapped, fold-change of at least 2 and false discovery rate adjusted p-values of less than < 1%) the possibility of assembly artefacts and of false-positive differentially expressed transcripts cannot be fully ruled out.

**LARGE SCALE DATA:** Raw data files (fastq) and a searchable table (.xlss) containing information on genomic features and expression data for all refined transcripts have been submitted to the NCBI GEO under accession number GSE116278.

**LIMITATIONS, REASONS FOR CAUTION**: The intrinsic nature of this bulk analysis, i.e. the sequencing of transcripts from whole gonads, does not allow direct identification of the cellular origin(s) of the transcripts characterised. Potential cellular dilution effects (e.g. as a result of distinct proliferation rates in XX and XY gonads) may account for a few of the expression profiles identified as being sexually dimorphic. Finally, transcriptome alterations that would result from exposure to pre-abortive drugs cannot be completely excluded. Although we demonstrated the high quality of the sorted cell populations used for experimental validations using quantitative RT-PCR, it cannot be totally excluded that some germline expression may correspond to cell contamination by, for example, macrophages.

**WIDER IMPLICATIONS OF THE FINDINGS**: For the first time, this study has led to the identification of a thousand of protein-coding and noncoding candidate genes showing an early, sexually dimorphic, expression pattern that have not previously been associated with sex differentiation. Collectively, these results increase our understanding of gonad development in

humans, and contribute significantly to the identification of new candidate genes involved in fetal gonad differentiation. The results also provide a unique resource that may improve our understanding of the fetal origin of testicular and ovarian dysgenesis syndromes, including cryptorchidism and testicular cancers.

**STUDY FUNDING/COMPETING INTEREST(S)**: This work was supported by the French National Institute of Health and Medical Research (Inserm), the University of Rennes 1, the French School of Public Health (EHESP), the Swiss National Science Foundation [SNF n° CRS115_171007 to B.J.], the French National Research Agency [ANR n° 16-CE14-0017-02 and n°18-CE14-0038-02 to F.C], the Medical Research Council [MR/L010011/1 to PAF] and the European Community's Seventh Framework Programme (FP7/2007-2013) [under grant agreement no 212885 to PAF] ] and from the European Union’s Horizon 2020 Research and Innovation Programme [under grant agreement no 825100 to PAF and SMG]. There are no competing interests related to this study.

**Keywords:** Human gonad development; fetal testis; fetal ovary; sex differentiation; disorders of sex development; transcriptional profiling; novel unannotated transcripts; long noncoding RNAs; bulk RNA-sequencing; proteomics informed by transcriptomics.

## Introduction

Mammalian ovary and testis development is a unique process compared to other organ development, both developing from a bipotential organ which commits to a different fate following gonadal sex determination and ending up as entirely different organs. This decisive turning point guides the gonad toward one of the two developmental pathways, both including specific cell type development and proliferation. Proper differentiation of these cell lineages determines the reproductive health of the future being. In humans, the gonadal primordium arises from the thickening of the coelomic epithelium at the surface of the mesonephros around 4$^{th}$ postconceptional week (PCW, corresponding to 6 weeks of gestation/amenorrhea), and contains several precursor cell types, notably precursors of supporting and steroidogenic cell lineages, as well as primordial germ cells (PGC) (Wilhelm *et al.*, 2013). The expression of the Y-linked transcription factor sex-determining region Y (SRY) during the 6$^{th}$ PCW in supporting cell precursors of the genital ridge triggers the expression of the SRY-box transcription factor 9 (SOX9) transcription factor which subsequently promotes a highly orchestrated gene expression program (Koopman *et al.*, 1991; Vidal *et al.*, 2001; Sekido and Lovell-Badge, 2008; Li *et al.*, 2014; Rahmoun *et al.*, 2017). These molecular events activate the commitment of Sertoli cells, leading to testis cord formation, the appearance of a fetal Leydig cell from 7 PCW onwards, and production of male hormones (androgens, insulin-like 3 protein and anti-Müllerian hormone) that are essential for embryo masculinization. In the absence of SRY the R-spondin 1 (RSPO1)/Wnt family member 4 (WNT4)/β-catenin pathway and forkhead box L2 (FOXL2) induce another complex cascade of transcriptional events, giving rise to fetal ovaries marked by the differentiation of pre-granulosa cells and the commitment of germ cells into meiosis from 10 PCW onwards (Vainio *et al.*, 1999; Schmidt, 2004; Uda *et al.*, 2004; Ottolenghi *et al.*, 2007; Chassot *et al.*, 2008; Liu *et al.*, 2009; Le Bouffant *et al.*, 2010; Childs *et al.*, 2011). To ensure the appropriate commitment to a given fate, the sex-specific pathways antagonize each other to repress the alternative fate (Kim and Capel,

2006; Chang *et al.*, 2008; Maatouk *et al.*, 2008; Wilhelm *et al.*, 2009; Kashimada *et al.*, 2011; Jameson *et al.*, 2012a; Greenfield, 2015; Bagheri-Fam *et al.*, 2017). Sex differentiation therefore stems from a critical moment triggering a complex transcriptional landscape that governs gonad specification and organogenesis. Both male and female expression programs are highly dynamic and complex, but many blank areas remain in the map of our understanding, thus preventing full understanding of most disorders of sex development (DSDs) (Eggers *et al.*, 2016). In particular, the noncoding counterpart of the transcriptome is likely to play critical roles in the physiology and the pathophysiology of the human developing gonads (Wu *et al.*, 2016).

Many dedicated studies have investigated *in situ* the gonadal expression pattern of specific human genes and/or proteins known to play important roles in sex determination or gonad development (Hanley *et al.*, 1999, 2000; de Santa Barbara *et al.*, 2001; Ostrer *et al.*, 2007; Mamsen *et al.*, 2017). In addition, several valuable genome-wide expression studies have used microarray technology to analyze whole fetal gonads (Small *et al.*, 2005; Fowler *et al.*, 2009; Houmard *et al.*, 2009; Rolland *et al.*, 2011; Munger *et al.*, 2013; del Valle *et al.*, 2017; Mamsen *et al.*, 2017) or isolated fetal gonadal cell populations (Nef *et al.*, 2005; Beverdam and Koopman, 2006; Bouma *et al.*, 2007, 2010) in several mammalian species, including human. Recently single-cell transcriptomic analyses of thousands of cells have opened up a new window onto gonadal cell lineages during fetal life (Guo *et al.*, 2015, 2017; Li *et al.*, 2017; Stévant *et al.*, 2018, 2019). Although the latest technologies hold great potential for further investigations, they are currently limited in their ability to study alternative splicing, decipher the noncoding expression program or discover novel genes (Haque *et al.*, 2017). Alternatively, “bulk” RNA-sequencing (RNA-seq) can be used to circumvent these issues (Gkountela *et al.*, 2015). Several landmark studies have performed RNA-seq in the mouse to investigate the transcriptome of the fetal Leydig cells (McClelland *et al.*, 2015; Inoue *et al.*, 2016), the regulome of the key gonadal transcription factor SOX9 (Rahmoun *et al.*, 2017), and the

transcriptome of the developing fetal testes and ovaries at key developmental stages (Zhao *et al.*, 2018). Despite the limited availability of normal fetal human gonads, RNA-seq analyses of gonad development in humans represent a very precious resource for our understanding.

In the present study, we performed a strand-specific, ribo-depleted RNA sequencing approach to unravel the protein-coding and noncoding transcriptional landscape of human developing fetal testes and ovaries from 6 to 17 PCW. Selected time-points were chosen to encompass the time window from early transcription of the SRY gene in the male to differentiation and primary sex-specific control of the cell lineages in both gonads. This analysis allowed us to identify a complex sexually and non-sexually dimorphic expression program driving gonad development, with a focus on non-coding genes as well as new unannotated genes that had not previously been described. In particular we have highlighted a core set of transcripts showing a sex-biased expression after the onset of SRY expression within the whole gonad during the $6^{th}$ PCW that likely plays a critical role in fetal gonad differentiation, and potentially for sex determination in humans. Our study significantly expands knowledge of human gonadogenesis and provides a rich source of data for geneticists and clinicians working in the field of DSDs.

## Materials and Methods

### Ethical considerations and sample collection

#### *First trimester fetuses*

Human fetuses (8-14 GW) were obtained from legally-induced normally-progressing terminations of pregnancy performed in Rennes University Hospital. Tissues were collected with women's written informedconsent, in accordance with the legal procedure agreed by the National Agency for Biomedical research (#PFS09-011) and the approval of the Local ethics committee of Rennes Hospital (# 11-48). The termination of pregnancy was induced using a standard combined mifepristone and misoprostol protocol, followed by aspiration. Gestational age was determined by ultrasound, and further confirmed by measurement of foot length for mathematical estimation of fetal development (Evtouchenko *et al.*, 1996; O'Shaughnessy *et al.*, 2019). The gonads were recovered and dissected free of mesonephros in ice-cold phosphate-buffered saline (PBS) using a binocular microscope (Olympus SZX7, Lille, France). The sex of the gonad was determined by morphological criteria, except for fetuses younger than 7 PCW, for which a PCR was performed on genomic DNA using primers specific for SRY (ACAGTAAAGGCAACGTCCAG; ATCTGCGGGAAGCAAACTGC) (Friel *et al.*, 2002) as well as for amelogenin X-linked (AMELX) and amelogenin Y-linked (AMELY) (CTGATGGTTGGCCTCAAGCCTGTG; GTGATGGTTGGCCTCAAGCCTGTG) (Akane *et al.*, 1992).

#### *Second trimester fetuses*

Human fetuses (13-14 and 17 PCW) were obtained from pregnant women after legally induced abortions at the Aberdeen Pregnancy Counselling Service. The collection of fetal material was approved by the National Health Service (NHS) Grampian Research Ethics Committees (REC

04/S0802/21). In all cases, women seeking elective terminations of pregnancy were recruited with full written, informed consent by nurses working independently of the study at the Aberdeen Pregnancy Counselling Service. Maternal data and medications used were recorded. Only fetuses from normally progressing pregnancies (determined at ultrasound scan prior to termination) from women over 16 years of age were collected following termination induced by a standard combined mifepristone and misoprostol protocol, as detailed previously (O'Shaughnessy *et al.*, 2007; Fowler *et al.*, 2008). Fetuses were transported to the laboratory within 30 min of delivery, weighed, sexed and the crown-rump length recorded. Fetal tissues were snap-frozen in liquid nitrogen and stored at -80°C.

RNA extraction, library construction and RNA-sequencing

Total RNA was extracted from human fetal gonads using the RNeasy mini Kit (Qiagen, Hilden, Germany), quantified using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and quality controlled using a 2100 Electrophoresis Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA extracts with a high quality RNA integrity number (average value = 9.8 - ranging from 8.6 to 10) were included. Libraries suitable for strand-specific high throughput DNA sequencing were then constructed, essentially as previously described (Jégou *et al.*, 2017; Rolland *et al.*, 2019) using "TruSeq Stranded Total RNA with Ribo-Zero Gold Prep Kit" (catalog # RS-122-2301, Illumina Inc., San Diego, CA, USA). The libraries were finally loaded in the flow cell at 7 pM concentration and clusters were generated in the Cbot and sequenced in the Illumina Hiseq 2500 as paired-end 2x50 base reads following Illumina's instructions. Image analysis and base calling were performed using RTA 1.17.20 and CASAVA 1.8.2.

## Read mapping, transcript assembly and quantification with the Tuxedo suite

### *Assembly of a unique set of human reference transcripts*

Ensembl (Yates *et al.*, 2016) and RefSeq (Pruitt *et al.*, 2014; Brown *et al.*, 2015) transcript annotations (GTF format) of human genome (release hg19) were downloaded from the UCSC (Speir *et al.*, 2016) in June 2015, and were merged with Cuffcompare (Pollier *et al.*, 2013). This non-redundant annotation was then used as the human reference transcripts (HRT) as previously published (Chalmel *et al.*, 2014). A non-redundant dataset of human splice junctions (HSJ) was also extracted from alignments of human transcripts and expressed sequence tags on the human genome as provided by UCSC.

### *Read mapping*

Reads from each individual sample were aligned to the hg19 release of the human genome with TopHat2 (version 2.0.12) (Trapnell *et al.*, 2012) using previously published approaches (Pauli *et al.*, 2012; Trapnell *et al.*, 2012; Chalmel *et al.*, 2014; Zimmermann *et al.*, 2015). Briefly, TopHat2 program was first run for each fastq file, using HRT and HSJ datasets to improve read mapping. Exonic junction outputs were then merged and added to the HSJ set. TopHat2 was next run a second time using the new HSJ dataset to produce a final alignment file (BAM format) for each. Finally, BAM files corresponding to the same experimental condition (fetal testes or ovaries, at a given time-point) were merged and sorted with the Samtools suite (version 2.19.0) (Li *et al.*, 2009).

### *Transcriptome assembly and quantification*

The Cufflinks suite (version 2.2.1, default settings) (Pollier *et al.*, 2013) was used to assemble transcript fragments (or transfrags) for each experimental condition based on merged BAM files. Resulting assembled transcripts were further merged into a non-redundant set of transfrags which were further compared to the HRT dataset with the Cuffcompare program. Finally, Cuffquant was

used to estimate abundance of each transcript in each individual sample as fragments per kilobase of exon model per million reads mapped (FPKM), and the normalization of the expression values across samples was performed by Cuffnorm.

*Refinement of assembled transcripts*

A four-step strategy was used to filter out dubious transcripts as previously described (Prensner *et al.*, 2011; Chalmel *et al.*, 2014) (Fig. S1). First, based on the Cuffcompare comparison, we only selected transfrags defined as complete match ('='), potentially novel isoform ('j'), falling entirely within a reference intron ('i') or an intergenic region ('u'), or showing exonic overlap with reference on the opposite strand ('x'). Next, assembled transcripts of less than 200 nucleotides or that were undetectable (<1 FPKM) were discarded. Finally, all transfrags that were annotated as either novel isoforms or novel genes (class codes "j", "i", "u" or "x") and that did not harbor at least two exons (multi-exonic) were filtered out.

*Principal component analysis*

Principal component analysis (PCA) was performed based on the expression values of refined transcripts with the FactoMineR package to graphically evaluate the distribution of sequenced samples (Lê *et al.*, 2008).

Coding potential analysis of assembled transcripts

DNA sequences of the refined transcripts were extracted with TopHat's gffread tool. As recommended in (Chocu *et al.*, 2014; Zimmermann *et al.*, 2015), the protein-coding potential of each transcript was estimated with an empirical integrative approach based on four predictive tools: Coding-Potential Assessment Tool (CPAT, coding probability > 0.364) (Wang *et al.*, 2013), HMMER (E-value < 10-4) (Finn *et al.*, 2011), Coding Potential Calculator (CPC, class "coding")

(Kong *et al.*, 2007) and txCdsPredict (score >800) (Kuhn *et al.*, 2013). Finally, we considered assembled transcripts to likely encode for proteins if their nucleic sequences were considered as protein-coding by at least two predictive tools.

Proteomics informed by transcriptomics strategy

As previously published in (Chocu *et al.*, 2014; Rolland *et al.*, 2019), we made use of a Proteomics Informed by Transcriptomics (PIT) approach (Evans *et al.*, 2012) to provide evidence at the protein level for assembled transcripts. First, we assembled a customized non-redundant protein database by merging the UniProt (Pundir *et al.*, 2015) and the Ensembl (Yates *et al.*, 2016)(downloaded 2015/10) proteome databases together with the set of predicted proteins derived from the assembled transcripts. Briefly, the refined transcript sequences were translated into the three-first open reading frames with the EMBOSS's Transeq program (Rice *et al.*, 2000) and only the amino acid sequences of at least 20 residues were selected.

We next made use of the human fetal gonad MS/MS proteomics datasets available from the Human Proteome Map (Kim *et al.*, 2014). First, 131 raw data files (corresponding to three fetal ovary samples and two fetal testis samples) were downloaded from the PRIDE database (accession number PXD000561) (Vizcaíno *et al.*, 2016) and converted into mgf format with ProteoWizard (Adusumilli and Mallick, 2017). Subsequent analyses were performed with SearchGUI (Vaudel *et al.*, 2011) (version 3.2.20) and PeptideShaker (Vaudel *et al.*, 2015)(version 1.16.8). A concatenated target/decoy database was created from the enriched reference proteome with SearchGUI. Cross-peptide identification was then performed with X!Tandem, Open Mass Spectrometry Search Algorithm (OMSSA), and MSGF+ tools, with these following parameters: precursor ion tolerance units set at 10 ppm; fragment tolerance set at 0.05 Da; carbamidomethylation of cysteine defined as a

fixed modification; oxidation of methionine and acetylation of protein N-term defined as a variable modification; only tryptic peptides with up to two missed cleavages; and minimum peptide length set to six amino acids. All peptides with at least one validated peptide spectrum match (PSM) and a confidence score greater than 80% with PeptideShaker, were kept for further analyses. Finally, only identifications with a false discovery rate (FDR) <1% were selected.

Statistical transcript filtration

The set of sexually dimorphic transcripts (SDTs) was defined by filtering transfrags that exhibited a $\geqslant$ 2-fold difference between the male and female gonads (using median expression values of sample replicates) in at least one of the seven developmental stages. A Linear Models for Microarray Data (LIMMA) statistical test was then used to identify transcripts displaying significant changes between male and female gonads (F-value adjusted with a FDR $\leqslant$ 5%) (Smyth, 2004). Among SDTs, those showing a differential expression as early as 6 PCW were designated as the set of early SDTs (or early-SDTs). In addition, the set of developmental regulated transcripts (DRTs) were also selected by isolating transfrags that exhibited a $\geqslant$ 2-fold difference between two developmental stages during either male or female gonad development. Similar to the selection of SDTs, a F-value adjusted with a FDR $\leqslant$ 5% was then used to identify candidates showing significant variations across developmental stages. Finally, the difference between the sets of DRTs and SDTs allowed us to discriminate non-sexually dimorphic transcripts (NSDTs) corresponding to the set of transfrags showing a developmentally regulated expression pattern across fetal gonad development, but no significant differential expression between male and female gonads.

## Cluster and functional analyses

The resulting SDTs and NSDTs were then clustered into fourteen (named P1-14) and six (Q1-6) expression patterns with the unsupervised hierarchical clustering on principle components (HCPC) algorithm, respectively (Lê *et al.*, 2008). These clusters were then ordered according to peak expression levels across developmental stages in testes first and then ovaries. Gene Ontology (GO) term enrichments were estimated with the Fisher exact probability, using a Gaussian hypergeometric test implemented in the Annotation Mappin Expression and Network suite (AMEN) (Chalmel and Primig, 2008). A GO term was considered significantly associated with a given expression pattern if the FDR-corrected P value was $\leqslant$5% and the number of genes bearing this annotation was $\geqslant$5.

## Transcription factors and their related target genes

To get an insight into potentially important regulators that might be involved in early human gonad development or sex determination, transcriptional factors and their targets were extracted from public databases, the Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining database (TRRUST) (Han *et al.*, 2015) and Transcription Factor encyclopedia (Yusuf *et al.*, 2012).

## FACS sorting and quantitative PCR validation

### *Single cell dissociation and cell sorting*

Single cell suspensions were obtained from gonads by a standard enzymatic and mechanic digestion procedure. Gonads were cut into small pieces and digested in 0.25% Trypsin-0.02% EDTA (#T4049, Sigma-Aldrich) and 0.05 mg/ml DNase (#DN25, Sigma-Aldrich, Missouri, USA) for 10 min at 37 °C. Trypsin digestion was stopped by adding 10% fetal bovine serum in M199 media and samples

were centrifuged at 350 g for 5 min at 37°C. Dispersed cells were resuspended in PBS and counted on a Malassez hemocytometer after labeling of dead cells with Trypan blue. For testes, a plasma membrane labelling of cord cells was performed using a mouse FITC-conjugated anti-human epithelial antigen (clone Ber EP4; Dako # F0860, diluted 1:100) associated with a labelling of germ cells with a mouse R-Phycoerythrin-coupled anti-human KIT proto-oncogene, receptor tyrosine kinase (KIT/CD117) (clone 104D2, BioLegend, San Diego, CA, USA, # 313204, diluted 1:100) for 30 min at room temperature. For ovaries, germ cells were labeled with the mouse R-Phycoerythrin-coupled anti-human KIT/CD117 as described above. Cells were sorted by a flow cytometer cell sorter FACSAriaII (BD Biosciences, New Jersey, USA) equipped with Diva software. Cells were collected in PBS, centrifuged at 500g for 45 min at 4°C and pelleted cells were stored at -80°C until RNA extraction.

*Quantitative RT-PCR*

RNA was extracted from cell pellets with PicoPure RNA Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Total RNAs (100 ng) were reverse transcribed with iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) and quantitative PCR was performed using the iTaq© universal SYBR green supermix (Biorad) according to manufacturer's instructions in a Cfx384 OneTouch Real-Time PCR system (Biorad). The following amplification program was used: an initial denaturation of 3 min at 95°C, 40 cycles of 10 sec denaturation at 95°C and 30 sec at 62°C for annealing and extension. Dissociation curves were produced using a thermal melting profile performed after the last PCR cycle. Primer pairs flanking introns were designed in order to avoid amplification of contaminating genomic DNA whenever possible and they were aligned onto human RefSeq transcripts using primer-BLAST to check for their specificity. Furthermore, only those primers that produced a single peak during the melting curve step (i.e. with peak temperature

variance of no more than 0.5°C) were considered and their efficiency was evaluated using serial dilutions of cDNA templates (Table I). Ribosomal protein lateral stalk subunit P0 (RPLP0) and ribosomal protein S20 (RPS20) mRNA, used as internal controls for normalization purposes, were initially validated in human adult testis gonad (Svingen *et al.*, 2014) and further used for human fetal gonads (Jørgensen *et al.*, 2018). Results were calculated with Bio-Rad CFX Manager 3.1 using the ΔΔCT method as n-fold differences in target gene expression, relative to the reference gene and calibrator sample, which comprises an equal mixture of all the tested samples for a given organ.

Immunohistochemistry and immunofluorescence

Upon collection, additional gonads (seven testes and five ovaries) were fixed either in Bouins fluid fixative or paraformaldehyde 4% (w/v) for 1 to 2 hours, embedded in paraffin using standard procedures and cut into 5 µm-thick sections. After dewaxing and rehydration, slides were treated for antigen retrieval with pre-heated 10 mM citrate buffer, pH 6.0 at 80°C for 40 min before cooling at room temperature . Sections were blocked for 1 h at room temperature with 4% bovine serum albumin in PBS before the overnight incubation at 4°C with the primary antibody diluted in Dako antibody diluent (Dako Cytomation, Trappes, France). Antibodies and conditions are described in Table II. Secondary antibodies were goat anti-rabbit biotinylated antibody (E0432, Dako, diluted 1:500); or rabbit anti-mouse biotinylated antibody (E0464, Dako, diluted 1:500). Sections were developed with streptavidin-horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories Inc.) and counterstained with hematoxylin. Stained sections were examined and photographed under light microscopy (Olympus BX51). For cryosectioning, additional paraformaldehyde-fixed gonads were cryopreserved in PBS-sucrose 20%, embedded in NEG50TM (Allan-Richard Scientific, Thermo

353 Fisher Scientific) and cut in 8 µm-thick sections. Thawed sections were treated for antigen retrieval
354 with citrate buffer, as described, when necessary (for WT1 transcription factor, WT1; neurexin 3,
355 NRXN3; contactin 1, CNTN1; and lin-28 homolog A, LIN28), rinsed in PBS and incubated
356 overnight at 4°C with primary antibody. Rinsed sections were incubated with the ad hoc fluorescent
357 secondary antibodies (1:500). The second primary antibody was subsequently incubated overnight at
358 4°C followed by the corresponding secondary antibody. Secondary antibodies were either 488 or 594
359 Alexa Fluor conjugated antibodies made in chicken for rabbit- and mouse-hosted primary antibodies,
360 and in donkey for Contactin 1 (CNTN1) primary antibody (Invitrogen). Sections were mounted in
361 prolong Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA, USA; Thermo Fisher
362 Scientific). Slides were examined and photographed with an AxioImager microscope equipped with
363 an AxioCam MRc5 camera and the ZEN software (Zeiss, Le Pecq, France).

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## Results

### Expression profiling of fetal gonads identifies more than 300 new genes in the human genome

To investigate the expression program governing gonad differentiation in humans, we performed a RNA-seq analysis on fetal testes *(n*=24) and ovaries (*n*=24), covering seven developmental stages from 6 to 17 PCW (i.e. from 8 to 19 gestational weeks) (Fig. 1A). Following read mapping and transcript reconstruction (Supplementary Table SI), a stringent refinement strategy selected a "high-confidence" set of 35,194 transcripts expressed in human fetal gonads (Supplementary Fig. S1). A PCA of these expression data provided a first hint on the biological relevance of our dataset: The first three components indeed appeared strongly correlated with the developmental stage (dimensions 1 and 3) and the genetic sex (dimension 2) of samples (19.7% and 15.3% of variance, respectively) (Fig. 1B and C). A hierarchical clustering based on the 35 first PCA dimensions explaining 90% of the total variance of the data further confirms the reliable distribution of the samples according to the sex and the developmental stage (Fig. 1D).

The comparison of reconstructed transcripts with RefSeq and Ensembl reference annotations identified known (13,673; 38.9%) and novel (18,718; 53.2%) isoforms of annotated protein-coding genes as well as known (529, 1.5%) and novel (680, 1.9%) isoforms of annotated long noncoding RNAs (lncRNAs). Importantly this comparison also identified 318 novel unannotated transcripts (NUTs) corresponding to new intronic (81, 0.2%), intergenic (164, 0.5%) or antisense (73, 0.2%) as yet uncharacterized genes in the human genome. The comparison of genomic and expression features highlighted significant differences between mRNAs, lncRNAs and NUTs (Supplementary Fig. S2). As expected, lncRNAs are expressed at lower levels than mRNAs, are more specifically expressed during single stages of development, and have lower sequence conservation, length, number of exons and GC content. Interestingly, this "non-coding" trend is exacerbated by NUTs, as they have a lower

abundance, conservation, length, number of exons and GC content as well as a more restricted expression than lncRNAs and mRNAs (Supplementary Fig. S2).

We next combined results from a coding potential analysis and from a proteomics informed by transcriptomics approach (PIT) (Evans *et al.*, 2012) to characterize the protein-encoding potential of novel isoforms and loci identified above. As expected, 98.5% of mRNAs (known and novel isoforms) displayed a high protein-encoding potential, and 56% of them were supported at the protein level thanks to at least one identified peptide (Fig. 1E). On the other hand, only 48.1% of lncRNAs (known and novel isoforms) were predicted to have a high protein-encoding potential (PEP), and as little as 8.5% were identified during the PIT analysis, usually through a single peptide identification (Fig. 1E). When NUTs were finally evaluated, they mostly displayed features similar to lncRNAs: 76.1% of them were predicted to have a low protein-encoding potential while only 0.4% were confirmed at the protein level (Fig. 1E). Besides, 94 lncRNAs (including 53 with two or more peptides identified) and one NUT were both predicted to have a high protein-encoding potential and demonstrated at the protein level.

## Transcriptome dynamics during human gonad development define sexually and non-sexually dimorphic expression programs

We next focused on genes with dynamic expression patterns during gonad development. Several steps of statistical filtration led to the selection of 13,145 transcripts (7,633 genes) that were differentially-expressed between testes and ovaries (called SDT), most of which (10,521 transcripts, 6,587 genes) were also developmentally-regulated (Fig. 1F). Notably, 8,935 transcripts (5,961 genes) were differentially-expressed during gonad development but did not show significant difference between sexes (NSDT).

We further classified SDT into 14 clusters (termed P1-P14) according to their preferential expression pattern (Fig. 2A and B). Patterns P1-P7 include transcripts showing peak expression in fetal testes at 6 to 7 PCW (P1; *SRY*; doublesex and mab-3 related transcription factor 1,*DMRT1*), at 7 PCW (P2; *SOX9*, desert hedgehog signalling molecule, *DHH*; inhibin subunit beta B, *INHBB*), at 9 to 12 PCW (P4; cytochrome P450 family 11 subfamily A member 1, *CYP11A1*; cytochrome P450 family 17 subfamily A member 1, *CYP17A1*; steroidogenic acture regulatory protein, *STAR*; luteinizing hormone/choriogonadotropin receptor, *LHCGR*; insulin like 3, *INSL3*), at 13 to 17 PCW (P6; actin alpha 2, *ACTA2;* prostaglandin D2 synthase, *PTGDS*) or with a broader expression pattern throughout testis development (P3; nuclear receptor subfamily 5 group A member 1, *NR5A1*; nuclear receptor subfamily 0 group B member 1, *NR0B1;* and P5; WT1 transcription factor, *WT1;* claudin 11 *CLDN11*). Similarly, transcripts belonging to patterns P8-P14 are preferentially expressed in fetal ovaries and display peak expression at 7 PCW (P8; *RPSO1*, anti-Mullerian hormone receptor type 2, *AMHR2*; nanog homeobox, *NANOG*), at 7-9 PCW (P9; POU class 5 homeobox 1, *POU5F1*; developmental pluripotency associated 2 and 4, *DPPA2*/*4;* lin-29 homolog A and B, *LIN28A*/*B*), at 12 PCW (P11; *FOXL2*, deleted in azoospermia like, *DAZL;* piwi like RNA-mediated gene silencing 2, *PIWIL2*), at 13-14 PCW (P12; MET proto-oncogene, receptor tyrosine kinase, *MET;* delta like canonical Notch ligand 4, *DLL4;* X inactive specific transcript, *XIST;* vascular endothelial growth factor A, *VEGFA*), at 17 PCW (P13; meiotic double-stranded break formation protein 1, *MEI1;* meiosis specific with OB-fold, *MEIOB;* SPO11 initiator of meiotic double stranded breaks, *SPO11;* synaptonemal complex protein 1 to 3, *SYCP1-3;* and P14; folliculogenesis specific bHLH transcription factor, *FIGLA;* NOBOX oogenesis homeobox, *NOBOX;* spermatogenesis and oogenesis specific basic helix-loop-helix 1, *SOHLH1*) or with a broader expression throughout ovarian development (P10; DNA meiotic recombines 1, *DMC1;* empty spiracles homeobox 2, *EMX2;* lymphoid enhancer binding factor 1 *LEF1;* Wnt family member 2B, *WNT2B*). We also

evaluated the functional relevance of expression patterns by a GO term enrichment analysis (Fig. 2C). Several broad biological processes related to organogenesis and/or cell differentiation were enriched in various testis-associated (P1, P2, P5, P6) and ovary-associated (P8) patterns. More precisely, expression pattern P4 was found to be enriched in genes involved in steroidogenesis while several processes associated with meiosis and female germ cell development were found to be enriched in P13 and P14, which is consistent with the differentiation and development of Leydig cells in fetal testes from 7 PCW onwards and with the commitment of ovarian germ cells into meiosis from 12 PCW onwards, respectively. Finally, we investigated the distribution of RNA biotypes and found that lncRNAs and NUTs were significantly enriched in expression patterns P12 to P14 (Fig. 2D).

The 8,935 NSDTs were also classified according to peak expression into six expression patterns (termed Q1-Q6) and include transcripts expressed at early stages of gonad differentiation (6-7 PCW; Q1 and Q2; including *WT1*, GATA binding protein 4, *GATA4*), following sexual differentiation (7-9 PCW; Q3 and Q4; *DMRT1*, SRY-box transcription factor 8, *SOX8*) or at later stages of gonad development (12-17 PCW; Q5 and Q6; nuclear receptor subfamily 6 group A member 1, *NR6A1*) (Fig. 3A-C). Finally, when investigating the distribution of RNA biotypes within co-expression groups, we found that lncRNAs and NUTs were enriched in expression patterns Q1 and Q6 (Fig. 3D). While these transcripts are also likely to include factors with important roles during gonadal differentiation and development, they were not further investigated in this study. All data, however, are available through the ReproGenomics Viewer (RGV) genome browser (http://rgv.genouest.org/) (Darde *et al.*, 2015, 2019) and are also available as a searchable table (.xlss) containing information on genomic features and expression data for all refined transcripts (submitted to the NCBI GEO under accession number GSE116278).

459 A complex transcriptional program governing early gonadal differentiation

460 In order to highlight new candidate genes that could be involved in early gonadal differentiation, we
461 focused our analysis on 1,479 SDTs showing a significant differential expression in fetal gonads as
462 early as 6 PCW (Supplementary Fig. S3). Most of these early-SDTs (61.7%) logically belong to
463 early expression patterns P1, P2, P8 and P9. This set of genes is composed of important actors
464 including *SRY* (P1), *SOX9*, *DHH*, patched 1 (*PTCH1*) and cytochrome P450, family 26 subfamily b
465 polypeptide 1 (*CYP26B1*) (P2), LIM homeobox 9 (*LHX9*) (P6), activin A receptor type 1B
466 (*ACVR1B*) (P8), *AMHR2* (P9) or *FOXL2* (P11) which demonstrates the relevance of this filtration for
467 selecting important factors in sex differentiation (Supplementary Fig. S3B and S3C). In addition to
468 well-known transcription factors, such as SRY, SOX9, LHX9, or FOXL2, 174 early SDTs
469 correspond to 131 genes encoding transcriptional regulators that should also play a critical role in the
470 establishment of this complex sexually dimorphic expression program (Supplementary Table SII).
471 Although the proportion of early-SDTs in the distinct expression patterns P1-P14 according to their
472 coding status are generally similar to those of SDT (Supplementary Fig. S3D), it is important to note
473 that this set of candidates includes 40 lncRNAs and 20 NUTs which may be involved in early steps
474 of gonad differentiation.

475

476 Distinct cellular expression patterns of newly identified genes involved in human sex determination

477

478 To investigate further the cellular origin of selected candidates, we performed immunohistochemistry
479 experiments as well as quantitative PCR (qPCR) on FACS-sorted cells. The successful enrichment of
480 Sertoli cells (hEpA+/KIT-), germ cells (KIT+) and interstitial cells (hEpA-/KIT-) from 6–7 PCW
481 testes was notably validated by the expression of *SOX9*, KIT proto-oncogene, receptor tyrosine

kinase (*KIT*) and nuclear receptor subfamily 2 group F member 2 (*NR2F2*), respectively (Supplementary Fig. S4A-C), while that of germ cells (KIT+) and somatic cells (KIT-) from 6–8 PCW and 10–12 PCW ovaries was confirmed by the high expression levels of *KIT*, *FOXL2* or *NR2F2*, respectively (Supplementary Fig. S4D-F). We first investigated genes that display expression profiles similar to that of *SRY* (P1; high expression and clear sexual dimorphism in 6 PCW testes), such as Wnt ligand secretion mediator (*WLS*), C-X-C motif chemokine ligand 14 (*CXCL14*) and C-C motif chemokine receptor 1 (*CCR1*) (Fig. 4A). While *WLS* was mainly expressed in Sertoli cells it was also substantially expressed in interstitial cells, whereas *CXCL14* was only expressed in Sertoli cells (Fig. 4A). In contrast, the expression of *CCR1* was mainly detected in germ cells. We also analysed genes with an expression profile similar to *SOX9* (P2; clear sexual dimorphism and peak of expression in 7 PCW testes), such as EPH receptor B1 (*EPHB1*), fetal and adult testis expressed 1 (*FATE1*), MAGE family member B1 (*MAGEB1*), erb-b2 receptor tyrosine kinase 3 (*ERBB3*), Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1 (*CITED1*) and a NUT antisense to CITED1 (TCONS_00249587) (Fig. 4B). We found that *EPHB1*, *FATE1*, *MAGEB1* and *ERBB3* were indeed expressed in Sertoli cells and to a lesser extent in interstitial cells, while SRY-box transcription factor 10 (*SOX10)* was expressed at similar levels in Sertoli cells and interstitial cells (Fig. 4B). Interestingly, we found that both *CITED1* and its potential antisense RNA were specifically and simultaneously expressed in Sertoli cells. Consistently with qPCR results, SOX10, EPHB1, MAGEB1, and FATE1 proteins were indeed all found in cord cells at the histological level, but exhibited varying ratios of expression in Sertoli and germ cells (Fig. 4C). For instance, SOX9 was expressed only in Sertoli cells, whereas MAGEB1 was clearly expressed in germ cells as well.

We also investigated the cell distribution of genes preferentially expressed in ovary (P8-P9) (Fig. 5A-D). First, several of them show a higher differential expression as early as 6 PCW, and were

preferentially expressed in somatic cells, including neurexin 3 (*NRXN3*), contactin 1 (*CNTN1*) and SET nuclear proto-oncogene (*SET*), or specifically in somatic cells such as the NUT *TCONS_00153406* (Fig. 5A). In agreement, immunolabeling showed NRXN3 protein in the nucleus of cells surrounding KIT+ germ cells, with a pattern very similar to that of WT1 (Fig. 5D). Interestingly, CNTN1 protein was found in a subset of epithelial cells surrounding LIN28+ germ cells in 6 PCW ovaries, or in ovarian cords adjacent to the mesonephric-gonadal junction (Fig. 5D) but not in the surface epithelium. Several genes show higher levels of differential expression at later stages in gonad development (Fig. 5B-C). Some of those genes, such as neuropeptide Y (*NPY*), SRY-box transcription factor 4 (*SOX4*) and the novel transcript *TCONS_00224470*, were preferentially expressed in somatic cells, as was *RSPO1* (Fig. 5B). In contrast, others displayed patterns typical of germline-associated expression patterns, including *POU5F1* (a well-known germ cell marker) and three NUTs, *TCONS_00113718*, *TCONS_00055038* and *TCONS_00042565*, which were highly expressed in female germ cells from 7 to 12 PCW (Fig. 5C).

519 **Discussion**

520 Unravelling the molecular sequence of events involved in gonadogenesis and sex determination is
521 essential in order to understand DSDs. Although a significant number of studies have already
522 examined the sexually dimorphic expression program driving gonad development in animal models
523 (Beverdam and Koopman, 2006), its characterization remains elusive in humans. Three studies have
524 investigated the transcriptome of the developing gonads from 5.7 to 10 PCW (Mamsen et al., 2017;
525 del Valle et al., 2017) and of ovarian primordial follicle formation from 13 to 18 PCW (Fowler *et al.*,
526 2009) in humans. However, they were based on microarray technologies, thus restricting the gene set
527 studied and limiting the characterization of non-coding transcripts and the identification of new
528 genes. Our study is among the first to capitalize on the power of the "bulk" RNA-seq technology to
529 perform an in-depth characterization of the dynamic transcriptional landscape of whole human fetal
530 gonads, from early differentiation (i.e. 6 PCW) up to Leydig cell transition in the testis and primary
531 follicle formation in the ovary (i.e. 17 PCW), at both the protein-coding and non-coding levels. In
532 particular our results identify transcriptional regulators, lncRNAs and novel genes (NUTs) that show
533 an early sexually dimorphic expression pattern and could therefore play important regulatory roles
534 from sex determination onwards. Nevertheless, as in any model, including animals that are sacrificed
535 with anesthesia or $CO_2$ or in the case of spontaneous abortions where the development of the embryo
536 or foetus can be disturbed, it should be borne in mind that there is a small chance that some
537 transcriptional alterations might result from exposure to pre-abortive drugs. Collectively this work
538 constitutes a rich resource for the community by providing new information regarding the early
539 molecular events that could be involved in both normal sex differentiation and DSDs.

540 Our study confirms and complements previous findings accumulated in humans and other species
541 (Nef *et al.*, 2005; Beverdam and Koopman, 2006; Jameson *et al.*, 2012b; Zhao *et al.*, 2018; Planells
542 *et al.*, 2019). For instance our dataset validates the onset of *SRY* transcription prior to 6 PCW

(detected at 5.5 PCW in (Mamsen *et al.*, 2017)) but also demonstrates the over-expression of *SOX9* in the testis as early as 6 PCW (previously reported at only 6.8 PCW in (Mamsen *et al.*, 2017)), which may reflect the higher sensitivity of RNA-seq as compared to microarrays (Mantione *et al.*, 2014). Altogether our transcriptional profiling allowed us to identify over 33,000 transcripts expressed in human developing fetal gonads, including mRNAs and lncRNAs as well as unknown genes. Although our analysis was mostly focused on SDTs, a set of almost 9,000 transcripts showing similar expression profiles in testes and ovaries (NSDTs) was also identified despite major cell composition differences between the two gonad types, especially at later developmental stages. These transcripts therefore represent valuable information on critical molecular factors underlying or required for the development of both XX and XY gonads, including for instance WT1 and its multiple isoforms required at different stages (Hastie, 2017).

We then focused our analysis on 1,479 SDTs showing sexual dimorphism as early as 6 PCW, including more than 1,000 candidate genes that have not previously been associated with sex differentiation. It is noteworthy that most of these early dimorphic profiles are likely to result from true differential transcriptional regulation rather than from dilution effects, as the cell composition of XX and XY fetal gonads at this stage remains highly analogous. To further highlight new promising candidates that might be involved in the regulation of this complex expression program we next focused on the 131 genes encoding transcription factors and showing an early SDT pattern (Supplementary Table SII). Among them, the cAMP responsive element modulator (CREM), a well-known regulator of gene expression programming of post-meiotic germ cells in the adult testis (Hogeveen and Sassone-Corsi, 2006), is preferentially expressed in XY gonads at 6 PCW. We also found that one of its target genes, the tachykinin precursor 1 (*TAC1*) (Qian *et al.*, 2001), is over-expressed in the fetal ovary at this early developmental stage suggesting that CREM might negatively regulate TAC1 in the human fetal testis. While its role during sex determination remains

unknown, TAC1 encodes several neuropeptides belonging to the tachykinin family that are critical for many biological processes (Dehlin and Levick, 2014; Sun and Bhatia, 2014; Sorby-Adams *et al.*, 2017). GLI family zing finger 1 (*GLI1)* is also an interesting candidate as it is preferentially expressed in the fetal testis as early as 6 PCW (Mamsen *et al.*, 2017) and encodes a transcription factor known to regulate the expression of the secreted frizzled related protein 1 (SFRP1) (Kim *et al.*, 2010). Since SFRP1 is critical for fetal testis development in the mouse (Warr *et al.*, 2009), acting through its suppression of Wnt signalling (Kim *et al.*, 2010), this could suggest a potential important role for both *GLI1* and *SFRP1* during sex differentiation in humans.

Mamsen and collaborators deduced from their microarray experiment that the onset of steroidogenesis in male gonads occurred at 7.5 PCW (Mamsen *et al.*, 2017). We also consistently found that the expression of genes involved in steroidogenesis increased drastically in testes from 7 PCW onwards. However, several genes, such as *CYP17A1*, *CYP11A1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*HSD3B2*) and hydroxysteroid 17-beta dehydrogenase 3 (*HSD17B3*), actually exhibited sexual dimorphism as early as 6 PCW, suggestive of an earlier induction of the molecular networks underlying Leydig cell differentiation. A more likely explanation could be that even if they are expressed only in Leydig cells later in development, (pre-)Sertoli cells may also express such factors in early stages. This would be in line with the described co-operation between these two cell types for the synthesis of androgens in the mouse fetal testis (O'Shaughnessy *et al.*, 2000; Shima *et al.*, 2013) at 6 PCW. Among male-biased early-SDTs we also identified expression patterns similar to that of *SRY*, such as for the Wnt ligand secretion mediator (*WLS*) and for the C-C motif chemokine receptor 1 (*CCR1*). *WLS*, which we found to be preferentially expressed in fetal Sertoli cells, is an important mediator of Wnt secretion (Bänziger *et al.*, 2006; Das *et al.*, 2012), suggesting a potential role in promoting sex determination. We found *CCR1* to be preferentially expressed in germ cells. This was rather surprising since a sexually-

dimorphic expression pattern at such an early developmental stage (i.e. as early as 6PCW) is expected to result from primary changes in expression in somatic cells as they commit to their male or female fates. CCR1 encodes a chemokine receptor thought to be implicated in stem cell niche establishment and maintenance and it has already been shown to be expressed in postnatal gonocytes in the mouse (Simon *et al.*, 2010). Although we demonstrated the high quality of the sorted cell populations used in the current study, it cannot be totally excluded that the germline expression of *CCR1* may indeed correspond to a contamination by KIT-expressing somatic cells such as macrophages. Many other candidate genes display a *SOX9*-like expression pattern suggesting that some of them could also be important for Sertoli cell differentiation. Among these candidate genes, several are already known to be important for gonad development or fertility in humans and/or mice, such as such as FATE1, MAGEB1 or SOX10. Interestingly, while we found the expression pattern of SOX10 to be conserved between human and mouse (i.e. with strong preferential expression in young fetal testes), that of SOX8 was not. Instead, we found SOX8 to be expressed in a similar manner in human fetal testes and ovaries, with peak expression between 6 and 7 PWC followed by subsequent downregulation. While this expression profile is clearly not incompatible with a role during early testis differentiation as in the mouse (Schepers *et al.*, 2003), it also suggests a potential broader involvement in development of both XX and XY gonads in humans. The role of other candidates, such as the Erb-b2 receptor tyrosine kinase 3 (*ERBB3*) and the EPH receptor B1 (*EPHB1*), remains unknown during early gonad development. The expression of *ERBB3* has been reported in mouse PGCs in the genital ridge suggesting that the ErbB signalling might contribute to control of growth and survival of PGCs (Toyoda-Ohno *et al.*, 1999). The expression of *EPHB1* has never been reported in the fetal testis but is involved in angiogenesis and neural development (Pasquale, 2005).

Due to the limited number of known markers for distinct fetal ovarian somatic cells, the association of female-biased expression patterns (P8-P14) with specific cell populations remains challenging at the whole gonad level. We found that several PGC markers, such as *KIT*, *POU5F1*, *NANOG* or *LIN28A*, are over-expressed in the ovary, compared with the testis, as early as 6 PCW. This is in line with the fact that PGCs proliferate at a higher rate than somatic cells in the human fetal ovary (Bendsen *et al.*, 2003; Lutterodt *et al.*, 2009; Mamsen *et al.*, 2010). Experimental investigations allowed us to identify candidate genes associated with the ovarian somatic cell lineages, such as SRY-box 4 (*SOX4*), SET nuclear proto-oncogene (*SET*), contactin 1 (*CNTN1*), neurexin 3 (*NRXN3*) and neuropeptide Y (*NPY*). This set of genes holds great promise as potential key factors for female sex determination and ovary differentiation. *SOX4* encodes a transcription factor with a high mobility group box domain and its expression has already been described in supporting cells of the mouse gonads, although without evident sexual dimorphism (Zhao *et al.*, 2017). *SET*, for which we demonstrate a highly sexually dimorphic expression as early as 6 PCW, is implicated in transcriptional regulation through epigenomic modifications and has been associated with polycystic ovary syndrome (Jiang *et al.*, 2017). Other candidate genes expressed in somatic cells appear to be implicated in neurogenesis, such as *NRXN3*, *NPY* and *CNTN1* (Sutton *et al.*, 1988; Markiewicz *et al.*, 2003; Bizzoca *et al.*, 2012; Harkin *et al.*, 2016). *NRXN3* is expressed at a very weak level in the human fetal brain between 8 and 12 PCW (Harkin *et al.*, 2016) , i.e. 2 weeks after a high transcriptional induction in the fetal ovary at 6 PCW, which may indicate an independent role of the gene in both processes. NPY is already known to control female reproductive processes at the hypothalamus level, and to have a direct action on ovarian cell proliferation and apoptosis in prepubertal gilts (Sirotkin *et al.*, 2015). In contrast, *CNTN1* encodes a neuronal cell adhesion molecule that has never been described in reproductive-related processes, but seems to be a key factor in the development of many cancers (Chen *et al.*, 2018). The role of these three candidates in

female developing gonads remains unknown. All of the above mentioned male-biased (*WLS*, *CCR1*, *ERBB3*, *EPHB1*, *CITED1* and *asCITED1*) and female-biased (*SOX4*, *SET*, *NRXN3* and *NPY*) candidate genes would require further functional experiments to untangle their role during gonad development.

One of the most original contributions of our study is to unravel the non-coding counterpart of the fetal gonadal transcriptome. To the best of our knowledge, this is the first study to address this issue in humans. As mentioned before, we assembled 1,209 lncRNAs and 318 NUTs expressed in developing fetal gonads. The statistical comparison of their genomic features and a protein-encoding analysis strongly suggest that the vast majority of NUTs corresponds to newly identified lncRNAs. However, based on the PIT approach and the protein-encoding analyses, a small fraction (6.2%) of the identified noncoding transcripts are good candidates for novel protein-coding genes as they were confirmed at the protein level. Our RNA-seq analysis also contributed to the identification of 680 antisense lncRNAs, including one located on the opposite strand of the Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1 (*CITED1*). Both sense and antisense (*asCITED1*, *TCONS_00249587*) transcripts showed a preferential, highly correlated expression in fetal Sertoli cells as early as 6 PCW. In the mouse, *Cited1* has been reported to be a potential target of *Sry* (Li *et al.*, 2014) and is specifically expressed in the adult testis (Fagerberg *et al.*, 2014). Our results suggest that *asCITED1* might contribute to the regulation of *CITED1*, and could therefore be implicated in early testis development in humans. We also report an accumulation of lncRNAs in expression patterns associated with female meiosis (P13-P14). This result is line with similar observations that have been made in adult germ cells from meiosis onwards (Cabili *et al.*, 2011; Laiho *et al.*, 2013; Chalmel *et al.*, 2014; Rolland *et al.*, 2019). This phenomenon thus seems to be conserved in both male and female germ cells, which suggests that lncRNAs might also play critical roles in human fetal oocytes. Furthermore, we observed that the vast majority of noncoding early-

662 SDTs (16/20 NUTs, and 28/40 lncRNAs) were preferentially expressed in fetal ovaries, which may
663 reflect a specific non-coding transcriptional program at play during early ovary development. Further
664 investigation allowed us to identify that early, female-biased NUTs were preferentially expressed in
665 germ cells (*TCONS_00042656*, *TCONS_00055038*, *TCONS_00113718*) although some were also
666 expressed in somatic cells (*TCONS_00153406* and *TCONS_00224470*). Additional functional
667 analysis will be essential to elucidate the role of these germline and somatic candidates in the
668 physiology of the fetal developing gonads and in the aetiology of DSDs. DSDs indeed comprise
669 heterogeneous conditions affecting the genital system, with a wide range of phenotypes. The
670 management of these disorders is globally improved by genetic diagnosis, as it leads to a more
671 accurate prognosis and prediction of the long-term outcome. Recently, recommendations from the
672 European Cooperation in Science and Technology state that genetic diagnosis should preferentially
673 use whole exome sequencing of a panel of candidate genes, while whole genome sequencing should
674 be restricted to suspected oligo- or poly-genic DSDs (Audí *et al.*, 2018). Nevertheless, the majority
675 of genetic testing remains inconclusive as most causative genes involved in DSDs have not yet been
676 identified (Alhomaidah *et al.*, 2017). Although many challenges remain to understand the
677 implications of lncRNAs during gonad development in humans, their functional roles in almost all
678 investigated biological systems are now supported by several studies (Cheng *et al.*, 2016; Tao *et al.*,
679 2016), including during gonad development (Rastetter *et al.*, 2015; Taylor *et al.*, 2015; Winge *et al.*,
680 2017) and for gonadal functions (Ohhata *et al.*, 2011; Bao *et al.*, 2013; Taylor *et al.*, 2015; Watanabe
681 *et al.*, 2015; Wen *et al.*, 2016; Hosono *et al.*, 2017; Wichman *et al.*, 2017; Jégu *et al.*, 2019).
682 Genome-wide association studies of patients with DSD would therefore greatly benefit from
683 screening for new causal genetic variants in lncRNAs expressed early in sex determination. In this
684 context our resource will assist geneticists to refine and complete the required panel of disease

candidate genes by including non-coding genes involved in testicular and ovarian dysgenesis syndromes with a fetal origin, including cryptorchidism and testicular cancers.

Single-cell technologies now open new avenues for the genomic characterization of biological systems, including the study of cellular heterogeneity. When compared to bulk approaches, single-cell transcriptomics allows transcriptional signatures to be robustly assigned to specific cell types. In this fast-evolving field, however, distinct available technologies have specific advantages and limitations, and may be favoured depending on the scientific question (Baran-Gale *et al.*, 2018). For instance, high-throughput systems that enable the analysis of several thousands of cells, including the mature and popular droplet-based high-throughput system from 10x Genomics, are needed in order to study discrete cell populations and/or to accurately reconstruct cell differentiation processes. On the other hand these systems suffer from a relatively low sensitivity and specificity: they only capture a partial fraction of the transcriptome of each individual cell (~2-4,000 genes per cell). Furthermore, by focusing on either the 3' or the 5' extremity of RNA molecules, they do not allow the reconstruction of transcript isoforms or the discovery of new genes. In the near future, increased sensitivity of droplet-based methods, combined with long-read sequencing technologies, will provide accurate transcriptome information at the isoform level and at a single-cell resolution (Byrne *et al.*, 2019), hopefully at an affordable price. In the meantime, bulk RNA-seq and current single-cell technologies remain highly complementary. The current study will support and complement future single-cell experiments aimed at reconstructing cell lineage progression in fetal gonads.

Overall, our study comprehensively describes the dynamic transcriptional landscape of the fetal gonads at seven key developmental stages in humans. This work discovered extensive sexually and non-sexually dimorphic expression changes, not only of protein-coding genes but also of lncRNAs and novel genes that are triggered early during gonad differentiation. This rich resource significantly

extends existing state of the art knowledge and constitutes an invaluable reference atlas for the field of reproductive sciences and sex determination in particular.

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**Authors' roles**

FC, ADR, SMG and BJ designed the study. FC, ADR and EL wrote the manuscript. FC and ADR supervised the research. EL and FC prepared, analysed, and interpreted data. ADR, SMG, IC, MBM, PF, PAF, SLP, and BJ prepared the samples and interpreted sequencing data. BE, ADR and SMG validated expression data. SMG, PF, PAF and BJ contributed to the manuscript. All authors approved the final version of the manuscript.

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**Conflict of interest**

There are no competing interests related to this study.

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1138

**Figure legends**

**Figure 1** Sample collection and assessment of homogeneity.

(A) Human fetal gonads used in this study were collected at seven developmental stages, i.e. at 6, early 7, late 7, 9, 12, 13–14 and 17 postconceptional week (PCW). The number of replicates is indicated for each stage and sex. An overview of the main differentiation processes within human fetal testes and ovaries during the studied time window is also provided. PGC = primordial germ cell; LC = Leydig cell. The panel (B) displays the correlation ($R^2$) of the first 10 dimensions of principal component analysis (PCA) with the development stage and the genetic sex. The PCA was performed on expression data from 35,194 refined transcripts across all 48 human fetal gonads. Red values represent significant correlations (p-value $\leqslant$ 1%). (C) A scatter plot represents the position of each sample along the first two dimensions. Smaller dots represent samples, and are linked to bigger dots that represent the average expression of transcripts across replicates. The histogram represents the percentage of information carried by each dimension of the PCA. Testis samples are colored in blue, while ovaries are in red. Time point of each condition is provided in PCW. e7 = early 7 PCW; l7 = late 7 PCW. The variability between the samples is mainly explained by their age of development (dimension 1) and by their genetic sex (dimension 2). The two arrows highlight the divergence of gonads transcriptomes, from a common origin (at 6 PCW) to their distinct fate. (D) A dendrogram shows the hierarchical relationship between the 48 samples. The hierarchical clustering is based on the 35 first PCA dimensions explaining 90% of the total variance of the data. Male samples are colored in blue, female samples are in red. (E) Coding potential analysis of refined transcripts. The combined results of the Protein-Encoding Potential (PEP) and the Proteomics Informed by Transcriptomic (PIT) strategies, i.e. Low or High PEP transcripts with (PIT+) or without (PIT-) identified peptide(s) are represented for each transcript biotype. (F) Two statistical filtrations were used to select differentially-expressed transcripts. First, we performed an “intra-sex”

comparison in which all developmental stages were compared to each other during testis development on the one hand, and during ovarian development on the other hand (Fold-change ⩾2 in at least one comparison). Second, we performed an "inter-sex" comparison in which testes and ovaries were compared at each developmental stage (Fold-change ⩾ 2 in at least one comparison). Subsequently, a linear models for microarray data (LIMMA) statistical test was performed on both sets of transcripts to select those with significant expression variation across replicates [false discovery rate (FDR)-adjusted F-value of ⩽ 0.05]. A total of 13,145 transcripts that display "inter-sex" expression variations were defined as "Sexually dimorphic transcripts" (SDT), while 8,935 developmentally-regulated transcripts that do not exhibit sexual dimorphism were defined as "Non-sexually dimorphic transcripts" (NSDT).

**Figure 2** Sexually dimorphic expression patterns during human gonad development.

**(A)** Heatmap representation of 13,145 SDTs, distributed into 14 expression patterns (P1 to P14), across seven developmental stages for both testes and ovaries. Each row corresponds to a transcript, and each column an experimental condition, *i.e.* the average of testes or ovaries from a given PCW. The standardized abundance of transcripts is color-coded according to the scale bar, red corresponding to the highest expression level, blue to the lowest. **(B)** Repartition of known markers involved in gonad differentiation and development within SDT expression patterns. Note that several isoforms of a given transcript can be assembled and display distinct expression. **(C)** Gene ontology (GO) terms found to be enriched (BH corrected p-value <0.05) in each expression pattern. **(D)** Transcript biotypes and isoform status proportion in SDT (pie chart) and within each cluster of differentially expressed transcripts (barplot). The comparison of the 13,145 SDT with the human reference transcriptome by Cuffcompare (Pollier *et al.*, 2013) classified them as known isoform

(class code "="), novel isoforms (class code "j"), novel unannotated transcripts (NUTs) in intronic regions (class code "i"), intergenic regions ("u"), antisense of known transcripts (class code "x") or other ambiguous biotypes. Proportion of mRNAs, long non-coding (lnc)RNAs and NUTS in SDT clusters is given. Total number of transcripts in each cluster is indicated on the right side of the plot. An enrichment analysis using a hypergeometric strategy highlighted a significant accumulation of lncRNAs and NUT in the P12, P13 and P14 cluster (p-value <0.05) compared to their distribution within the 14 SDT clusters.

**Figure 3** Non-sexually dimorphic expression patterns during human gonad development.

**(A)** Heatmap representation of 8,935 NSDTs, distributed into six expression patterns (Q1 to Q6), across seven developmental stages for both testes and ovaries. Each row is a transcript, and each column is an experimental condition, i.e. the average of testes or ovaries from a given PCW. The standardized abundance of transcripts is color-coded according to the scale bar, red corresponding to the highest expression level, blue to the lowest. **(B)** Repartition of known markers involved in gonad differentiation and development within SDT expression patterns. Note that several isoforms of a given can be assembled and display distinct expression. **(C)** GO terms found to be enriched (BH corrected p-value <0.05) in each expression pattern. **(D)** The comparison of the 8,935 NSDT with the human reference transcriptome by Cuffcompare (Pollier *et al.*, 2013) classified them as known isoform (class code "="), novel isoforms (class code "j"), novel unannotaed transcripts (NUTs) in intronic regions (class code "i"), intergenic regions ("u"), antisense of known transcripts (class code "x") or other ambiguous biotypes. Proportions of mRNAs, lncRNAs and NUTS in NSDT (pie chart) and within NSDT clusters are given (barplot). The total number of transcripts in each cluster is indicated on the right side of the barplot. An enrichment analysis using a hypergeometric strategy

highlighted a significant accumulation of lncRNAs and NUTs in Q1 and Q6 clusters (p-value <0.05) compared to their distribution within the six NSDT clusters.

**Figure 4** Cellular investigation of early-SDTs that are over-expressed in fetal testis.

Expression levels (line graphic) and quantitative RT-PCR (histograms) of genes from expression pattern P1 and P2, which exhibit a higher differential expression **(A)** at 6 PCW, such as sex determining region Y (*SRY*), wntless Wnt ligand secretion mediator (*WLS*), C-X-C motif chemokine ligand 14 (*CXCL14*) and C-C motif chemokine receptor 1 (*CCR1*), and **(B)** at 7 PCW, such as SRY-box 9 (*SOX9*), SRY-box 10 (*SOX10*), EPH receptor B1 (*EPHB1*), MAGE family member B1 (*MAGEB1*), fetal and adult testis expressed 1 (*FATE1*), erb-b2 receptor tyrosine kinase 3 (*ERBB3*), Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 1 (*CITED1*) and novel unannotated transcript antisense of CITED1 (*TCONS_00249587*). Expression levels from RNA-sequencing (RNA-seq) as a function of age are depicted as blue lines for the testis and pink lines for the ovaries. Each point represents the mean fragments per kilobase of exon model per million reads mapped (FPKM) ± SEM of the levels measured in four (12 PCW and younger) and two different gonads (13-14 and 17 PCW). Quantitative PCR was performed on the different testicular sorted cell populations of germ cells (KIT+, red bars) Sertoli cells (hEpA+, green bars) and other cells (KIT-/ hEpA-, grey bars). Each column shows a pool of sorted cells from five fetal (6.9-7.3 PCW) testes. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *RPLP0* and *RPS20*. **(D)** Representative immunohistochemistry of SOX9, SOX10, KIAA1210, EPHB1, MAGEB1 and FATE1 on a 7.1 PCW testis. Arrows indicate germ cells (GC). Scale bar: 50 µM.

**Figure 5** Cellular investigation of early-SDTs that are over-expressed in fetal ovary.

Expression levels (line graphic) and quantitative RT-PCR (histograms) of genes from expression pattern P8 and P9, which exhibit a higher differential expression **(A)** at 6 PCW, such as Neurexin 3 (*NRXN3*), contactin 1 (*CNTN1*) and SET nuclear proto-oncogene (*SET*), **(B)** at 7 PCW, such as R-spondin 1 (*RSPO1*), neuropeptide Y (*NPY*), SRY-box 4 (*SOX4*) and NUT *TCONS_00224470*, and **(C)** or later on, as POU class 5 homeobox 1 (*POU5F1*) and NUTs *TCONS_00113718*, *TCONS_00055038* and *TCONS_00042565*. Expression levels from RNA-seq as a function of age are depicted as blue lines for the testis and pink lines for the ovaries. Each point represents the mean FPKM ± SEM of the levels measured in four (12 PCW and younger) and two different gonads (13-14 and 17 PCW). Quantitative RT-PCR was performed on the ovarian sorted cell populations of germ cells (KIT+, pink bars) and other cells (KIT-, grey bars). Each column shows a pool of sorted cells from seven early differentiating (6.7-8.7 PCW, 7–9 PCW) and three fetal (10.6-11.7 PCW, 10–12 PCW) ovaries. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes *RPLP0* and *RPS20*. **(D)** Immunofluorescence for NRXN3 (green) and KIT (red), LIN28 (green) and CNTN1 (red), and WT1 (green) and KIT (red), in early differentiating ovaries (6-6.6 PCW). Scale bar: 100 µM.

**Supplementary Figure S1** Refinement strategy of assembled transcripts.

Following transcript reconstruction with Cufflinks, a refinement strategy was performed to discard sequencing and assembly artefacts: only transcripts with an expression of $\geqslant$ 1 FPKM in at least one experimental condition (average value of biological replicates) were considered; transcripts with a length of less than 200 nucleotides were discarded; novel transcript isoforms (Cuffcompare class “j”)

and genes (classes “i”, “u” and “x”) were required to harbor at least two exons. FPKM = fragments per kilobase of exon model per million reads mapped.

**Supplementary Figure S2** Genomic and expression features comparison.

Violin plot representation of selected expression and genomic features for all expressed mRNAs and long noncoding (lncRNAs) and novel unannotated transcripts (NUTs): **(A)** maximum abundance (log), **(B)** Shannon entropy, **(C)** sequence conservation (phastCons score), **(D)** cumulative exons size (log), **(E)** number of exons (log), and **(F)** GC content (%GC).

**Supplementary Figure S3** Early sexually dimorphic expression patterns during human gonad development.

**(A)** Heatmap representation of 1,479 early sexually dimorphic transcripts (early-SDTs), distributed into 14 expression patterns (P1 to P14), across seven developmental stages for both testes and ovaries. Each row is a transcript, and each column is an experimental condition, i.e. the average of testes or ovaries from a given a gestational week (GW). The standardized abundance of transcripts is color-coded according to the scale bar, red corresponding to the highest expression level, blue to the lowest. **(B)** Repartition of known markers involved in gonad differentiation and development within early-SDT expression patterns. Note that several isoforms of a given gene can be assembled and display distinct expression. **(C)** Gene ontology (GO) terms found to be enriched (BH corrected p-value <0.05) in each expression pattern. **(D)** The comparison of the 1,479 early-SDT with the human reference transcriptome by Cuffcompare (Pollier *et al.*, 2013) classified them as known isoform (class code “=”), novel isoforms (class code “j”), novel unannotated transcripts (NUTs) in intronic regions (class code “i”), intergenic regions (“u”), antisense of known transcripts (class code “x”) or

other ambiguous biotypes. Proportion of mRNAs, lncRNAs and NUTS in early-SDTs (pie chart) and within each cluster is given (barplot). Total number of transcripts in each cluster is indicated on the right side of the barplot.

**Supplementary Figure S4** Testicular and ovarian cell-sorting by flow cytometry.

**(A)** Representative immunofluorescence of hEpA-FITC (green) staining of cord cells and KIT-PE (red) staining of germ cells in testis sections of a 7 PCW old embryo. **(B)** Representative Side (SSC) versus Forward (FSC) scatter plot showing hEpA/Mast/stem cell growth factor receptor Kit (KIT) dot plots according to size (FSC-H) and cellular granularity (SSC-H). Example of gating strategy for flow cytometry sorting of Sertoli cells (hEpA+/KIT-, green dots), germ cells (hEpA-/KIT+, red plots) and other cells types (hEpA-/KIT-, grey plots). **(C)** Quantitative RT-PCR of KIT proto-oncogene, receptor tyrosine kinase (KIT), nuclear receptor subfamily 2 group F member 2 (NR2F2)and SRY-box transcription factor 9 (SOX9) was performed on the different sorted cell populations of germ cells (KIT+, red bars) Sertoli cells (hEpA+, green bars) and other cells (KIT-/ hEpA-, grey bars). Each column shows a pool of sorted cells from five fetal (6.9-7.3 PCW) testes. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes ribosomal protein lateral stalk subunit P0 (RPLP0) and ribosomal protein S20 (RPS20). **(D)** Representative immunofluorescence of KIT-PE (red) staining of germ cells in sections of an ovary at 11 PCW. **(E)** SSC versus FSC scatter plot showing KIT dot plots according to size (FSC-H) and cellular granularity (SSC-H). Example of gating strategy for flow cytometry sorting of germ cells (KIT+, red plots) and other cells types (KIT-, grey plots). **(F)** Quantitative RT-PCR of KIT, NR2F2 and forkhead box L2 (FOXL2) was performed on the different sorted cell populations of germ cells (KIT+, red bars) and other cells (KIT-, grey bars). Each column shows a pool of sorted cells from seven early differentiating (6.7-8.7 PCW, 7–9 PCW) and three fetal (10.6-11.7 PCW, 10–

12 PCW) ovaries. Each bar represents the mean ± SEM of the fold change in target gene expression relative to the reference genes RPLP0 and RPS20. Scale bars: 100 µM.

## Table legends

**Table I. List of primers that were used for q-PCR experiments.**

**Table II. Antibodies used for immunofluorescence and immunohistochemistry.**

**Supplementary Table SI. Statistics for read mapping and transcript assembly.**

**Supplementary Table SII. Early sexually dimorphic transcripts encoding transcription factors.**

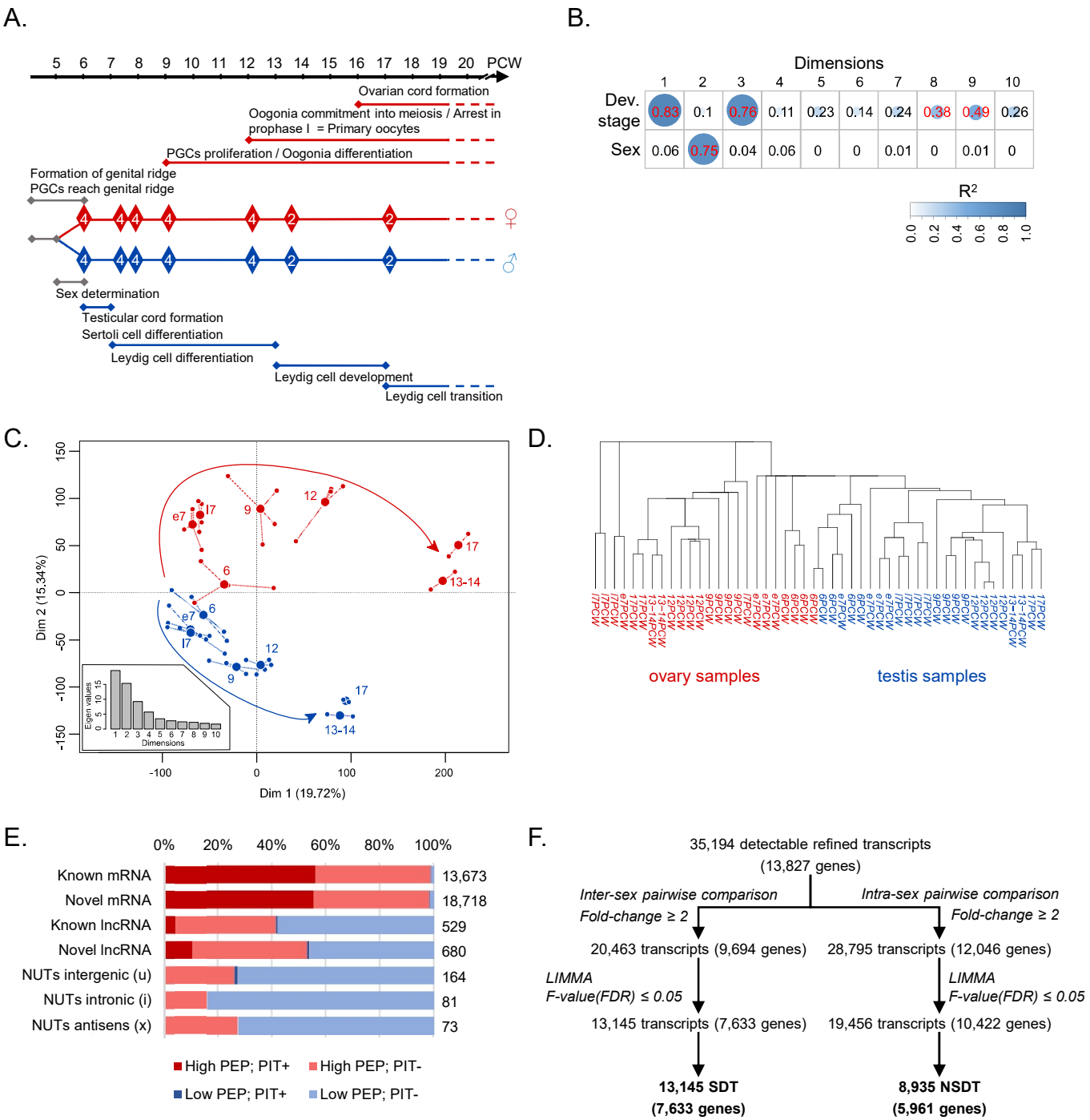

A.
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 PCW
Ovarian cord formation
Oogonia commitment into meiosis / Arrest in prophase I = Primary oocytes
PGCs proliferation / Oogonia differentiation
Formation of genital ridge
PGCs reach genital ridge
Sex determination
Testicular cord formation
Sertoli cell differentiation
Leydig cell differentiation
Leydig cell development
Leydig cell transition
B.
Dimensions
1 2 3 4 5 6 7 8 9 10
Dev. stage 0.83 0.1 0.76 0.11 0.23 0.14 0.24 0.38 0.49 0.26
Sex 0.06 0.75 0.04 0.06 0 0 0.01 0 0.01 0
R²
0.0 0.2 0.4 0.6 0.8 1.0
C.
Dim 2 (15.34%)
Dim 1 (19.72%)
Eigen values
Dimensions
D.
ovary samples
testis samples
E.
0% 20% 40% 60% 80% 100%
Known mRNA 13,673
Novel mRNA 18,718
Known lncRNA 529
Novel lncRNA 680
NUTs intergenic (u) 164
NUTs intronic (i) 81
NUTs antisens (x) 73
High PEP; PIT+
High PEP; PIT-
Low PEP; PIT+
Low PEP; PIT-
F.
35,194 detectable refined transcripts
(13,827 genes)
Inter-sex pairwise comparison
Fold-change ≥ 2
Intra-sex pairwise comparison
Fold-change ≥ 2
20,463 transcripts (9,694 genes)
28,795 transcripts (12,046 genes)
LIMMA
F-value(FDR) ≤ 0.05
LIMMA
F-value(FDR) ≤ 0.05
13,145 transcripts (7,633 genes)
19,456 transcripts (10,422 genes)
13,145 SDT
(7,633 genes)
8,935 NSDT
(5,961 genes)

Fig. 1

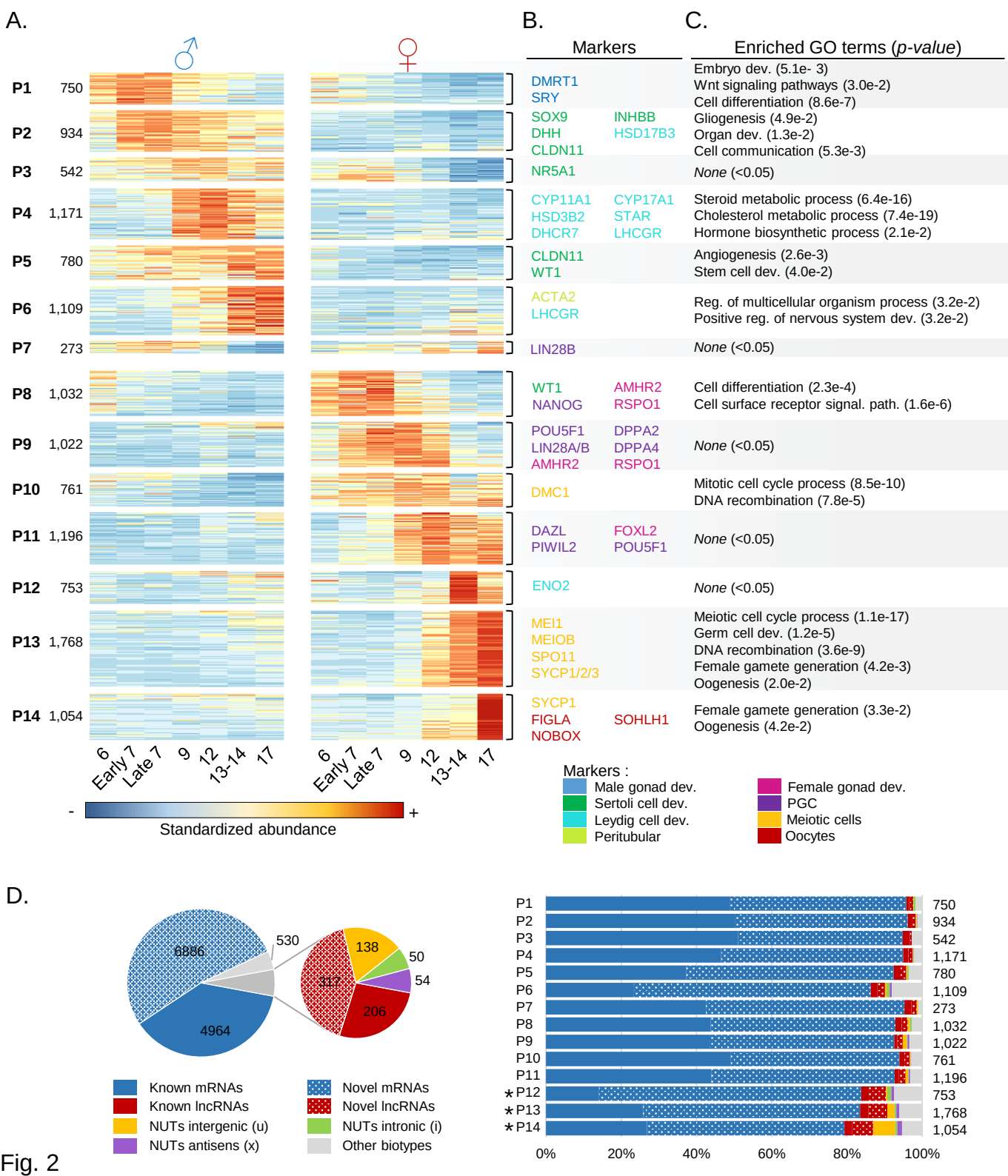

A.
B.
C.
Markers
Enriched GO terms (p-value)
P1 750
DMRT1
SRY
Embryo dev. (5.1e- 3)
Wnt signaling pathways (3.0e-2)
Cell differentiation (8.6e-7)
P2 934
SOX9
DHH
CLDN11
INHBB
HSD17B3
Gliogenesis (4.9e-2)
Organ dev. (1.3e-2)
Cell communication (5.3e-3)
P3 542
NR5A1
None (<0.05)
P4 1,171
CYP11A1
HSD3B2
DHCR7
CYP17A1
STAR
LHCGR
Steroid metabolic process (6.4e-16)
Cholesterol metabolic process (7.4e-19)
Hormone biosynthetic process (2.1e-2)
P5 780
CLDN11
WT1
Angiogenesis (2.6e-3)
Stem cell dev. (4.0e-2)
P6 1,109
ACTA2
LHCGR
Reg. of multicellular organism process (3.2e-2)
Positive reg. of nervous system dev. (3.2e-2)
P7 273
LIN28B
None (<0.05)
P8 1,032
WT1
NANOG
AMHR2
RSPO1
Cell differentiation (2.3e-4)
Cell surface receptor signal. path. (1.6e-6)
P9 1,022
POU5F1
LIN28A/B
AMHR2
DPPA2
DPPA4
RSPO1
None (<0.05)
P10 761
DMC1
Mitotic cell cycle process (8.5e-10)
DNA recombination (7.8e-5)
P11 1,196
DAZL
PIWIL2
FOXL2
POU5F1
None (<0.05)
P12 753
ENO2
None (<0.05)
P13 1,768
MEI1
MEIOB
SPO11
SYCP1/2/3
Meiotic cell cycle process (1.1e-17)
Germ cell dev. (1.2e-5)
DNA recombination (3.6e-9)
Female gamete generation (4.2e-3)
Oogenesis (2.0e-2)
P14 1,054
SYCP1
FIGLA
NOBOX
SOHLH1
Female gamete generation (3.3e-2)
Oogenesis (4.2e-2)
6
Early 7
Late 7
9
12
13-14
17
-
+
Standardized abundance
Markers :
Male gonad dev.
Sertoli cell dev.
Leydig cell dev.
Peritubular
Female gonad dev.
PGC
Meiotic cells
Oocytes
D.
6886
530
4964
317
138
50
54
206
Known mRNAs
Known lncRNAs
NUTs intergenic (u)
NUTs antisens (x)
Novel mRNAs
Novel lncRNAs
NUTs intronic (i)
Other biotypes
P1 750
P2 934
P3 542
P4 1,171
P5 780
P6 1,109
P7 273
P8 1,032
P9 1,022
P10 761
P11 1,196
*P12 753
*P13 1,768
*P14 1,054
0%
20%
40%
60%
80%
100%

Fig. 2

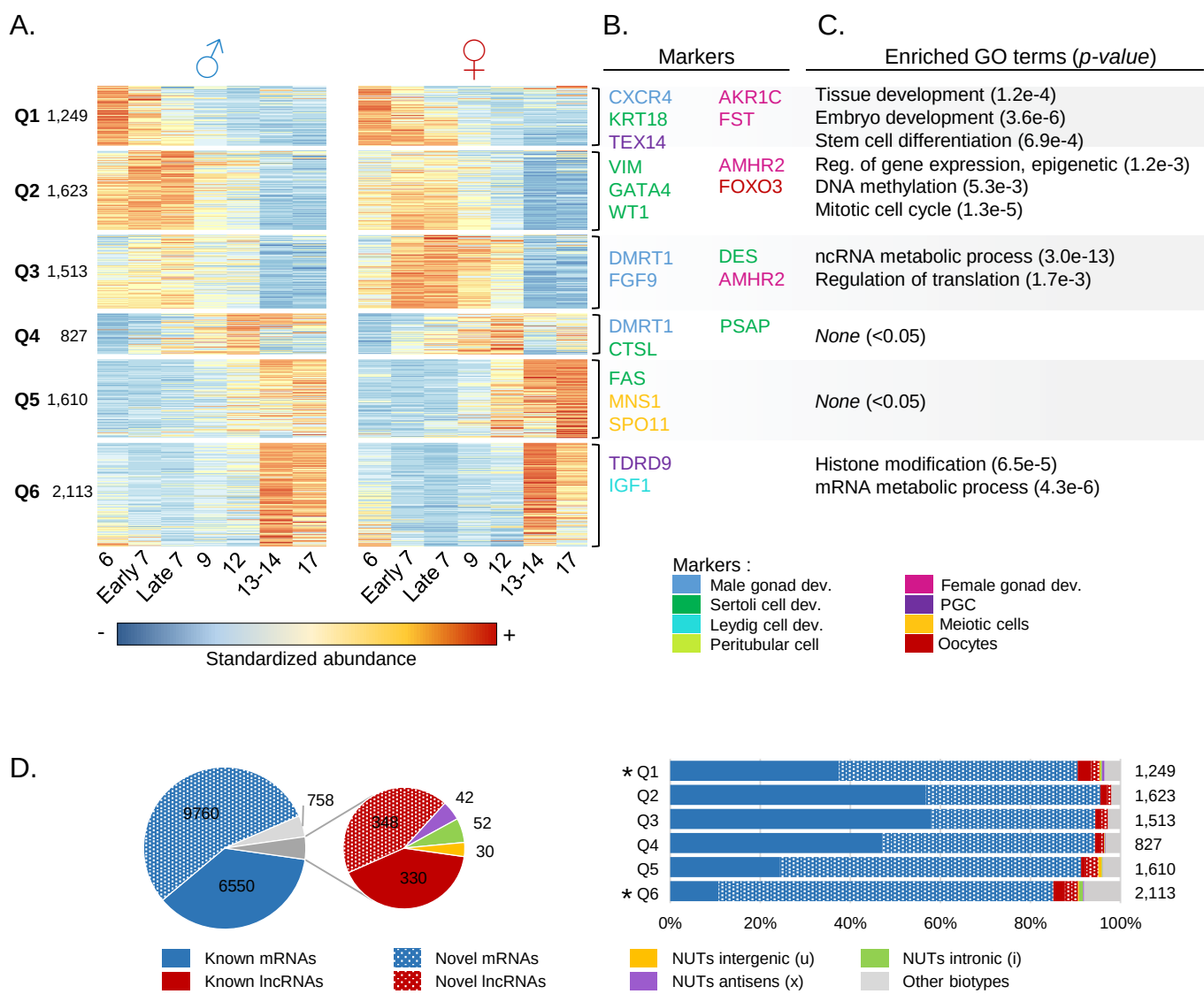
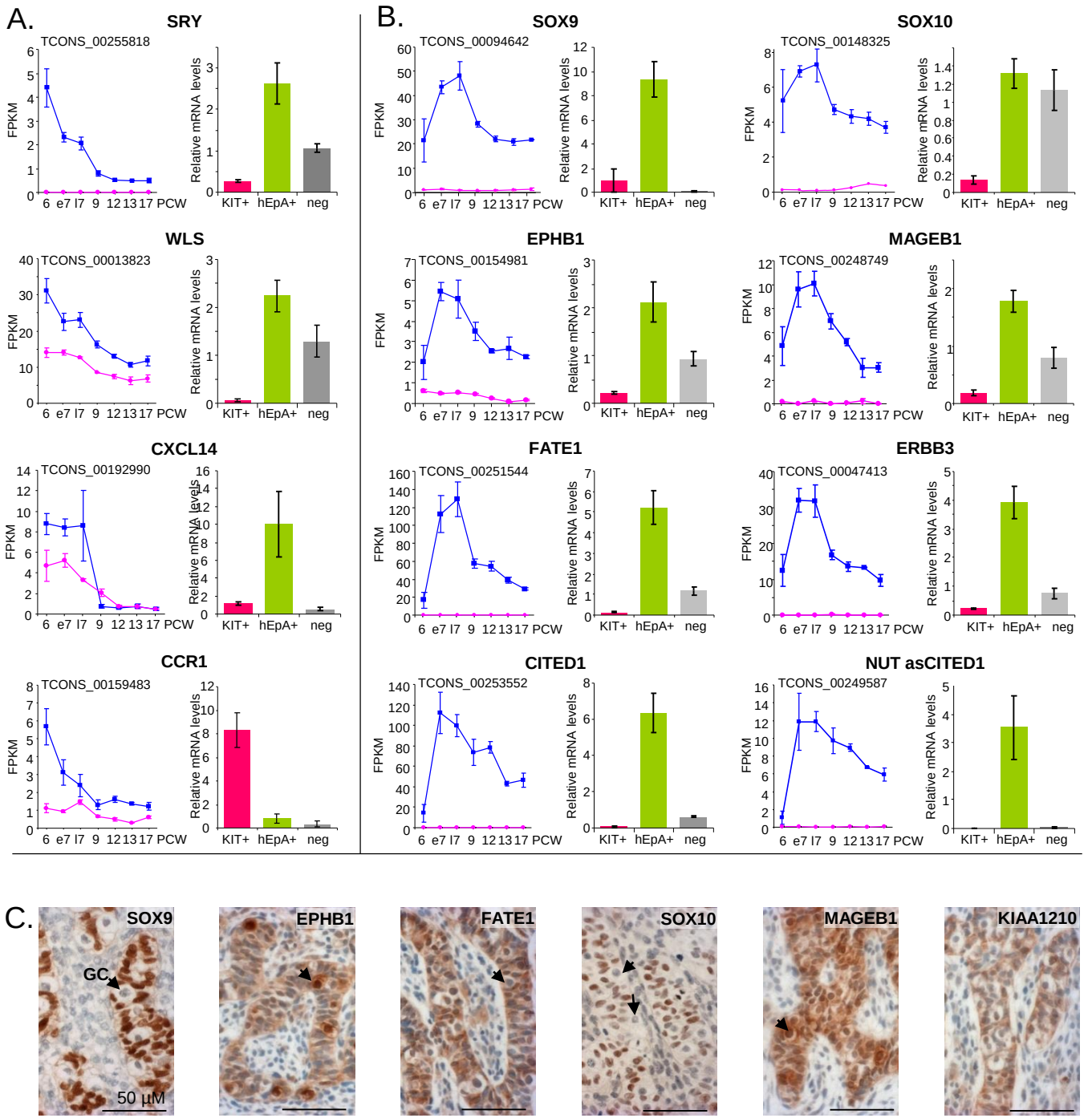
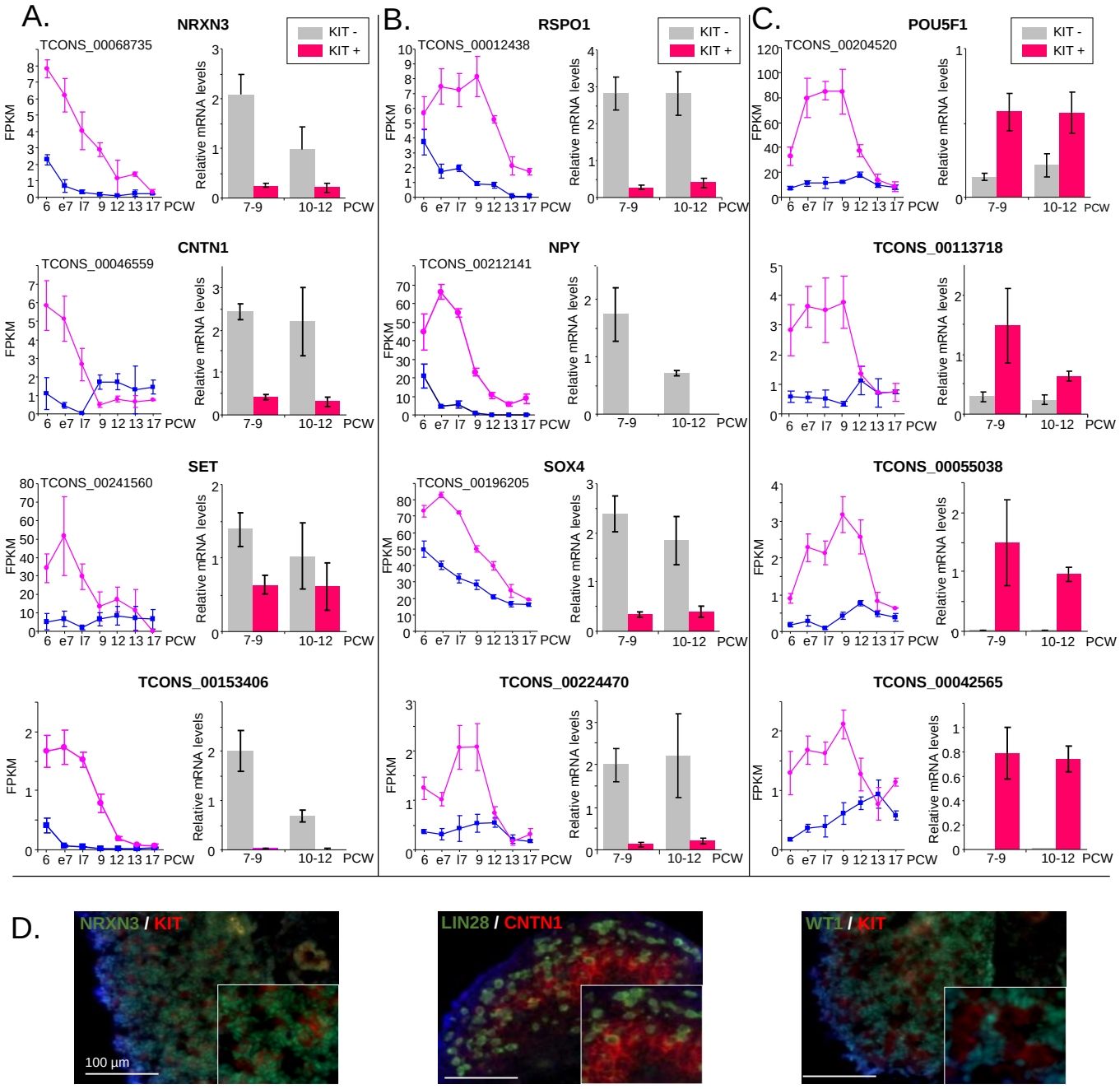

A.
B.
C.
Markers
Enriched GO terms (p-value)
Q1 1,249
Q2 1,623
Q3 1,513
Q4 827
Q5 1,610
Q6 2,113
CXCR4
KRT18
TEX14
AKR1C
FST
Tissue development (1.2e-4)
Embryo development (3.6e-6)
Stem cell differentiation (6.9e-4)
VIM
GATA4
WT1
AMHR2
FOXO3
Reg. of gene expression, epigenetic (1.2e-3)
DNA methylation (5.3e-3)
Mitotic cell cycle (1.3e-5)
DMRT1
FGF9
DES
AMHR2
ncRNA metabolic process (3.0e-13)
Regulation of translation (1.7e-3)
DMRT1
CTSL
PSAP
None (<0.05)
FAS
MNS1
SPO11
None (<0.05)
TDRD9
IGF1
Histone modification (6.5e-5)
mRNA metabolic process (4.3e-6)
6
Early 7
Late 7
9
12
13-14
17
Standardized abundance
Markers :
Male gonad dev.
Sertoli cell dev.
Leydig cell dev.
Peritubular cell
Female gonad dev.
PGC
Meiotic cells
Oocytes
D.
9760
758
6550
348
42
52
30
330
Known mRNAs
Known lncRNAs
Novel mRNAs
Novel lncRNAs
* Q1
Q2
Q3
Q4
Q5
* Q6
1,249
1,623
1,513
827
1,610
2,113
0%
20%
40%
60%
80%
100%
NUTs intergenic (u)
NUTs antisens (x)
NUTs intronic (i)
Other biotypes

Fig. 3


A.
SRY
TCONS_00255818
WLS
TCONS_00013823
CXCL14
TCONS_00192990
CCR1
TCONS_00159483
B.
SOX9
TCONS_00094642
SOX10
TCONS_00148325
EPHB1
TCONS_00154981
MAGEB1
TCONS_00248749
FATE1
TCONS_00251544
ERBB3
TCONS_00047413
CITED1
TCONS_00253552
NUT asCITED1
TCONS_00249587
FPKM
Relative mRNA levels
6 e7 l7 9 12 13 17 PCW
KIT+ hEpA+ neg


C.
SOX9
GC
50 μM
EPHB1
FATE1
SOX10
MAGEB1
KIAA1210

Fig. 4


A.
NRXN3
TCONS_00068735
CNTN1
TCONS_00046559
SET
TCONS_00241560
TCONS_00153406
B.
RSPO1
TCONS_00012438
NPY
TCONS_00212141
SOX4
TCONS_00196205
TCONS_00224470
C.
POU5F1
TCONS_00204520
TCONS_00113718
TCONS_00055038
TCONS_00042565
KIT -
KIT +
FPKM
Relative mRNA levels
6 e7 l7 9 12 13 17 PCW
7-9
10-12 PCW


D.
NRXN3 / KIT
100 µm
LIN28 / CNTN1
WT1 / KIT

Fig. 5

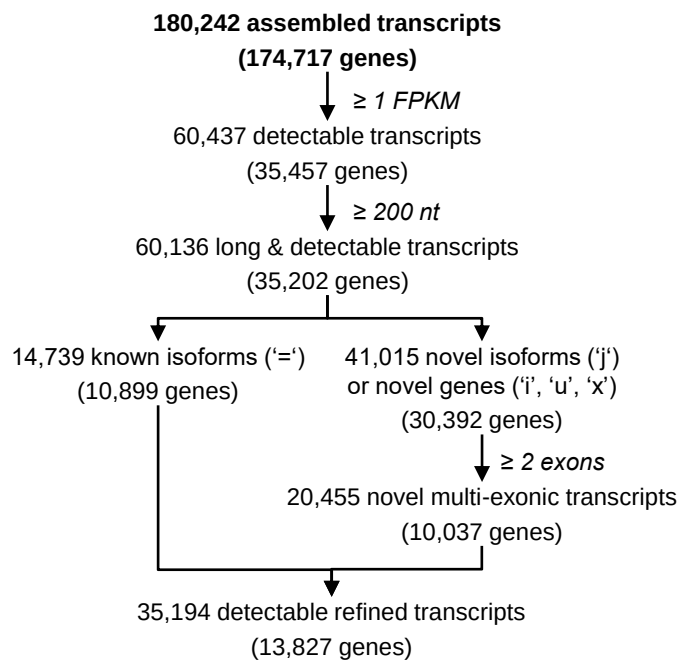

180,242 assembled transcripts
(174,717 genes)
≥ 1 FPKM
60,437 detectable transcripts
(35,457 genes)
≥ 200 nt
60,136 long & detectable transcripts
(35,202 genes)
14,739 known isoforms (‘=‘)
(10,899 genes)
41,015 novel isoforms (‘j‘)
or novel genes (‘i’, ‘u’, ‘x’)
(30,392 genes)
≥ 2 exons
20,455 novel multi-exonic transcripts
(10,037 genes)
35,194 detectable refined transcripts
(13,827 genes)

Fig. S1

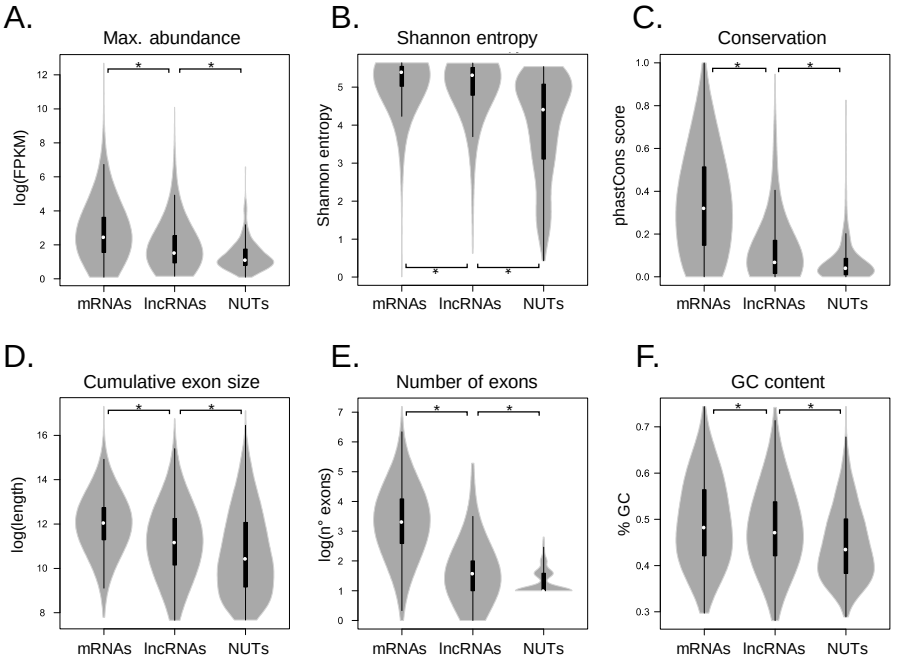
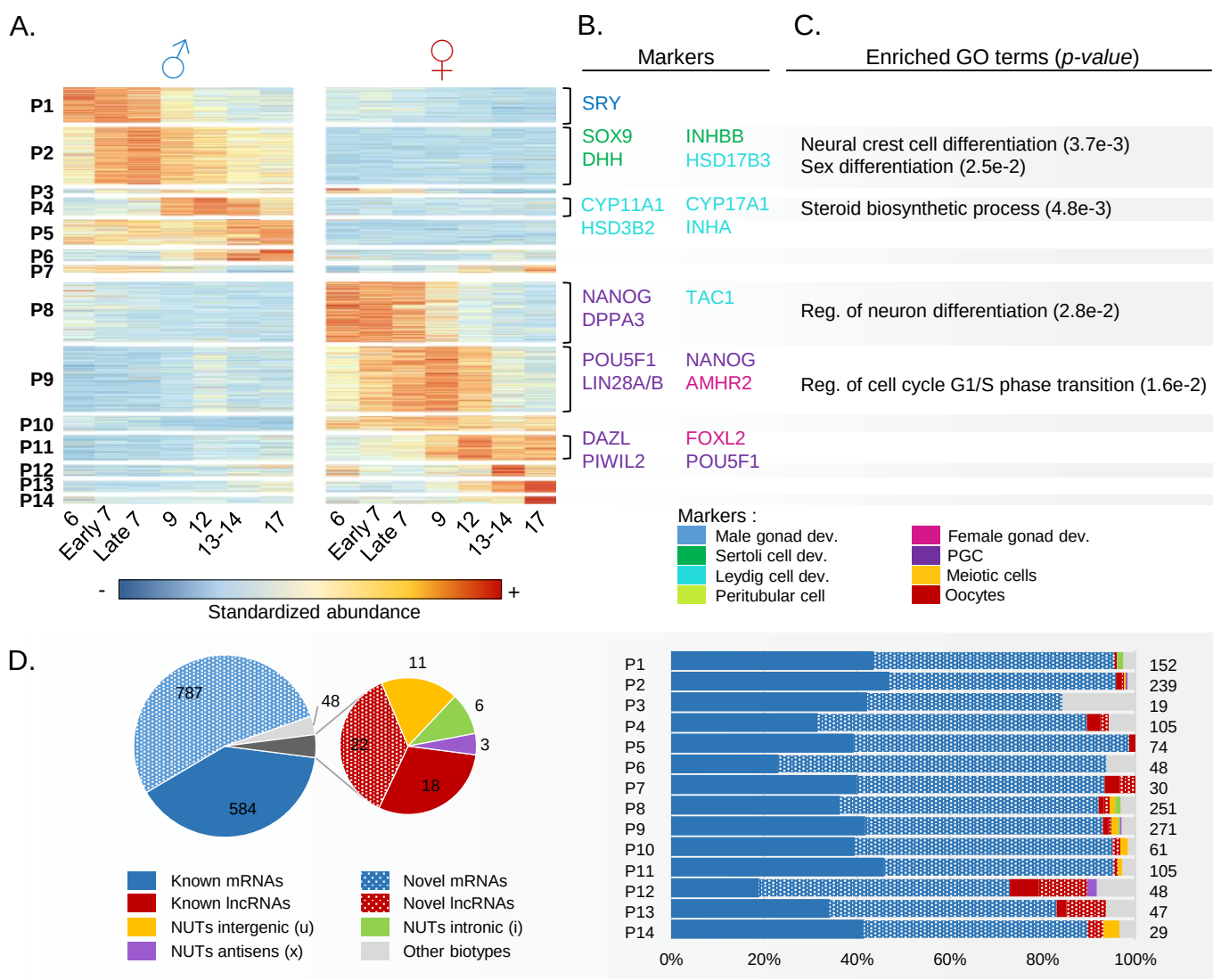

A.
Max. abundance
log(FPKM)
mRNAs
lncRNAs
NUTs
B.
Shannon entropy
Shannon entropy
C.
Conservation
phastCons score
D.
Cumulative exon size
log(length)
E.
Number of exons
log(n° exons)
F.
GC content
% GC

Fig. S2


A.
B.
C.
Markers
Enriched GO terms (p-value)
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
SRY
SOX9
DHH
INHBB
HSD17B3
Neural crest cell differentiation (3.7e-3)
Sex differentiation (2.5e-2)
CYP11A1
HSD3B2
CYP17A1
INHA
Steroid biosynthetic process (4.8e-3)
NANOG
DPPA3
TAC1
Reg. of neuron differentiation (2.8e-2)
POU5F1
LIN28A/B
NANOG
AMHR2
Reg. of cell cycle G1/S phase transition (1.6e-2)
DAZL
PIWIL2
FOXL2
POU5F1
6
Early 7
Late 7
9
12
13-14
17
Standardized abundance
Markers :
Male gonad dev.
Sertoli cell dev.
Leydig cell dev.
Peritubular cell
Female gonad dev.
PGC
Meiotic cells
Oocytes


D.
787
584
48
11
6
3
22
18
Known mRNAs
Known lncRNAs
NUTs intergenic (u)
NUTs antisens (x)
Novel mRNAs
Novel lncRNAs
NUTs intronic (i)
Other biotypes
P1 152
P2 239
P3 19
P4 105
P5 74
P6 48
P7 30
P8 251
P9 271
P10 61
P11 105
P12 48
P13 47
P14 29
0%
20%
40%
60%
80%
100%

Fig. S3

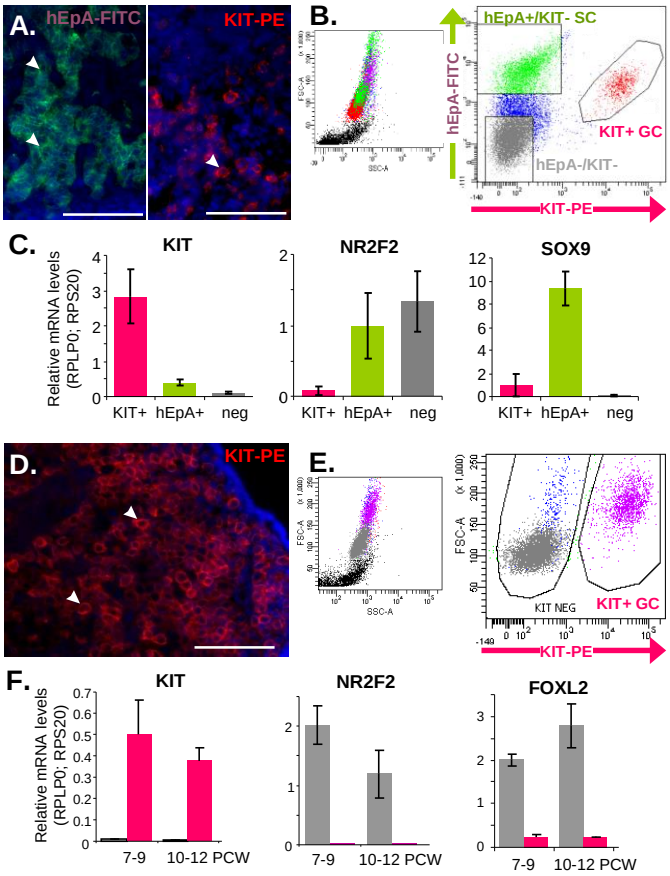

A.
hEpA-FITC
KIT-PE
B.
hEpA+/KIT- SC
hEpA-FITC
KIT+ GC
hEpA-/KIT-
KIT-PE
C.
KIT
NR2F2
SOX9
Relative mRNA levels (RPLP0; RPS20)
KIT+ hEpA+ neg
D.
KIT-PE
E.
FSC-A
SSC-A
KIT NEG
KIT+ GC
KIT-PE
F.
KIT
NR2F2
FOXL2
Relative mRNA levels (RPLP0; RPS20)
7-9 10-12 PCW

Fig. S4

**Table 1. List of primers that were used for q-PCR experiments**

| Gene | Forward (5'-3') |
|---|---|
| CCR1 | CAGAAAGCCCCAGAAACAAA |
| CITED1 | TGCACTTGATGTCAAGGGTG |
| CNTN1 | TTGGGAAGATGGTAGCTTGG |
| CXCL14 | ATGAAGCCAAAGTACCCGCA |
| EPHB1 | AGAGGAGGGAAAAGGACCAGG |
| ERBB3 | ACAGCCCCAGATCTGCAC |
| FATE1 | GGCAATTTCCAAGGCATACG |
| FOXL2 | GCGAAGTTCCCGTTCTACGA |
| KIT | TTCTTACCAGGTGGCAAAGG |
| MAGEB1 | CTATGGGGAACCCCGTAAGT |
| NPY | CTACATCAACCTCATCACCAGG |
| NR2F2 | GCCATAGTCCTGTTCACCTCA |
| NRXN3 | GCTGAGAACAACCCCAATA |
| POU5F1 | TACTCCTCGGTCCCTTTCC |
| RPLP0 | TCTACAACCCTGAAGTGCTTGAT |
| RPS20 | AACAAGCCGCAACGTAAAATC |
| RSPO1 | ACACTTCCCAGCATCTGAGACCAA |
| SET | AAATCAAATGGAAATCTGGAAAGG |
| SOX10 | AAGCCTCACATCGACTTCGG |
| SOX4 | GACCTGCTCGACCTGAACC |
| SOX9 | AACGCCTTCATGGTGTGG |
| SRY | ACAGTAAAGGCAACGTCCAG |
| TCONS_00042565 | GCGGCCCTAAGACAAAGAAC |
| TCONS_00055038 | CCCACCTTCCTCCTTCCTG |
| TCONS_00113718 | GCCCAACCACAGAAGGTTT |
| TCONS_00153406 | TCTACTTGTTTCTGGAGCTGAAG |
| TCONS_00224470 | CCTGGGCTACACTGGTCTTT |
| TCONS_00249587 | GGGAGAAAAGTAGCCCCAAG |
| WLS | CCTTGGTTCCAATTCATGCT |

| Reverse (5’-3’) | Size (bp) | Reference |
|---|---|---|
| GGTGTTTGGAGTTTCCATCC | 80 | Primer 3 |
| GTTGTAGGAGAGCCTATTGG | 201 | PMID:22703800 |
| TGATAACAAGGGTTCCAGTGC | 116 | PMID: 26855587 |
| TCTCGTTCCAGGCGTTGTAC | 148 | PMID: 24700803 |
| GGTTTCCCACGGCATCTC | 183 | PMID: 24121831 |
| GTTGGGCGAATGTTCTCATC | 78 | PMID: 23991224 |
| CTAGTCTGCGCCACTGCATC | 68 | PMID: 17761949 |
| CTCGTTGAGGCTGAGGTTGT | 75 | Primer BLAST |
| AAATGCTTTCAGGTGCCATC | 209 | PMID: 21668453 |
| GGTTTCAGCATAGGCTCTCG | 130 | Primer 3 |
| TCACCACATTGCAGGGTCT | 133 | PMID: 27722841 |
| AATCTCGTCGGCTGGTTG | 131 | PMID: 24318875 |
| ATGCTGGCTGTAGAGCGATT | 179 | PMID: 28013231 |
| CAAAAACCCTGGCACAAACT | 131 | PMID: 24743772 |
| CAATCTGCAGACAGACACTGG | 96 | PMID: 24743772 |
| ACGATCCCACGTCTTAGAACC | 166 | PMID: 24743772 |
| TGCTGAACAGGATGGGAAGAAGGT | 146 | PMID: 23617070 |
| AAAGAAGCTCTCTGGTTCCTCATG | 101 | PMID: 22677993 |
| TCCATGTTGGACATTACCTCGT | 67 | PMID: 23338937 |
| CCGGGCTCGAAGTTAAAATCC | 107 | PMID: 28535514 |
| TCTCGCTCTCGTTCAGAAGTC | 124 | Primer3 |
| ATCTGCGGGAAGCAAACTGC | 293 | PMID: 11869379 |
| TCTGACCAGAAAATCGCTTC | 104 | Primer3 |
| TCAGTGCAGAAGAGCCCAA | 86 | Primer3 |
| CTGGGACAGGATGGAGAGG | 97 | Primer3 |
| TGCAGTAACATCCTCCTCCTC | 120 | Primer3 |
| GAGCACCTCATTCTTGGCT | 70 | Primer3 |
| GAAGCAAATGGAGAGACGGA | 111 | Primer 3 |
| TTCAGTCCACTCAGCAAACG | 138 | Primer3 |

**Table 2. Antibodies used for immunofluorescence and immunohistochemistry.**

| Antigen | | HIER |
|---|---|---|
| hEpA-FITC | Mouse FITC-coupled anti-human epithelial antigen | - |
| CNTN1 | Contactin 1 | Ci |
| EPHB1 | ephrin type-B receptor 1 | Ci |
| ERBB3 | HER3/ErbB3 (D22C5) XP® Rabbit mAb | Ci |
| FATE1 | fetal and adult testis expressed 1 | Ci |
| KIAA1210 | KIAA1210 | Ci |
| KIT/CD117-PE | mouse R-Phycoerythrin-coupled anti-human KIT/CD117; clone 104D2 | - |
| LIN28 | lin-28 homolog A | Ci |
| MAGEB1 | melanoma-associated antigen B1 | Ci |
| NRXN3 | neurexin 3 | Ci |
| SOX9 | SRY (sex determining region Y)-box9 | Ci |
| SOX10 | SRY (sex determining region Y)-box 10 | Ci |
| WT1 | Wilms tumor 1 | Ci |

| Dilution | Antibody supplier and product number |
|---|---|
| 1 :100 | Dako, F0860 |
| 1 :100 (IF) | R&D systems, AF904 |
| 1:2500- 1:5000 | Sigma, HPA067740 |
| 0,215277778 | Cell Signaling Tech., #12708 |
| 1:1000-1:2500 | Sigma, HPA034604 |
| 1:200- 1:500 | Sigma, HPA048322 |
| 1 :100 | BioLegend, 313204 |
| 1 :100 (IF) | Abcam, Ab46020 |
| 1:100 - 1:250 | Sigma, HPA001193 |
| 1 :200 / 1 :100 (IF) | Sigma, HPA0002727 |
| 0,111111111 | Millipore, AB5535 |
| 1:200- 1:500 | Sigma, HPA068898 |
| 1:100 (IF) | Santa-Cruz Biotech, sc-192 |

**Table S1. Statistics for read mapping and transcript assembly.**

Average number of raw and mapped reads, percentage of mapping and number of assembled transcripts are indicated for each experimental condition. PCW = post coitum week. The number of non-redundant assembled transcripts is also provided during testicular and ovarian developments, as well as the overall number of non-redundant assembled transcripts in this study.

| | PCW | 6 | e7 | l7 | 9 | 12 | 13-14 | 17 | Total |
|---|---|---|---|---|---|---|---|---|---|
| Fetal testis | n° samples | 4 | 4 | 4 | 4 | 4 | 2 | 2 | 24 |
| | Average sequenced pairs of read | 50 163 366 +- 5 540 241 | 43 637 558 +- 3 217 370 | 45 033 799 +- 4 891 256 | 44 578 922 -6124741 | 61 408 394 +- 31 392 665 | 46 992 435 +-3 747 882 | 47 295 757 +- 2 131 643 | 339 110 230 |
| | Average mapped pairs of read | 45 088 608 +- 4 567 496 | 39 066 943 +-2 877 937 | 38 915 843 +- 6 718 006 | 36 500 690 +- 3 675 842 | 46 586 645 +- 20 642 653 | 40 120 429 +- 2 994 901 | 38 721 188 +- 4 093 627 | 285 000 344 |
| | % of mapped reads | 89,9 | 89,5 | 86,4 | 81,9 | 75,9 | 85,4 | 81,9 | 84,4 |
| | n° reconstructed transcripts | 164 931 | 129 802 | 103 431 | 116 903 | 107 148 | 164 652 | 147 287 | 142 953 |
| | | | | | | | | | |
| Fetal ovary | n° samples | 4 | 4 | 4 | 4 | 4 | 2 | 2 | 24 |
| | Average sequenced pairs of read | 51 756 993 +- 3 476 980 | 42 038 738 +- 3 950 805 | 43 167 327 +- 6 221 180 | 45 461 320 +- 8 410 709 | 43 085 238 +- 6 565 924 | 47 163 616 +- 3 195 188 | 38 044 232 +- 2 869 127 | 310 717 463 |
| | Average mapped pairs of read | 46 047 326 +- 3 200 842 | 38 087 267 +-3 581 721 | 33 349 134 +- 3 167 555 | 37 688 289 +- 10 017 251 | 34 516 893 +- 5 232 477 | 38 186 847 +- 1 789 162 | 33 236 072 +- 2 927 283 | 261 111 827 |
| | % of mapped reads | 89 | 90,6 | 77,3 | 82,9 | 80,1 | 81 | 87,4 | 84 |
| | n° recontructed transcripts | 186 499 | 121 800 | 93 201 | 12 817 | 112 165 | 191 983 | 174 783 | 168 195 |
| | **n° of non-redundant reconstructed transcripts** | | | | | | | | **180 242** |

**Table SII . Early sexually dimorphic transcri**

Early SDT genes encoding transcription factors
genes (column E) are reported. Known interactic
including TRRUST (Han *et al.* , 2015) and the T
associations with sex reversal, DSDs or testis/ov

| Gene symbol | Ensemble Gene IDs |
|---|---|
| ADAMTS19 | ENSG00000145808 |
| BACH2 | ENSG00000112182 |
| BCL11B | ENSG00000127152 |
| BIN1 | ENSG00000136717 |
| BRDT | ENSG00000137948 |
| BRIP1 | ENSG00000136492 |
| CBFA2T2 | ENSG00000078699 |
| CDX1 | ENSG00000113722 |
| CDYL | ENSG00000153046 |
| CHD9 | ENSG00000177200 |
| CITED1 | ENSG00000125931 |
| **CREBBP** | ENSG00000005339 |
| CREM | ENSG00000095794 |
| CUL4B | ENSG00000158290 |
| CUX2 | ENSG00000111249 |
| DEPDC7 | ENSG00000121690 |
| ELF4 | ENSG00000102034 |
| ESR1 | ENSG00000091831 |
| ESR2 | ENSG00000140009 |
| ETV4 | ENSG00000175832 |
| ETV5 | ENSG00000244405 |
| FOSL2 | ENSG00000075426 |
| FOXH1 | ENSG00000160973 |
| FOXI3 | ENSG00000214336 |
| FOXL2 | ENSG00000183770 |
| GABPB2 | ENSG00000143458 |
| GATAD2A | ENSG00000167491 |
| GLI1 | ENSG00000111087 |
| GRIP1 | ENSG00000155974 |
| HEY2 | ENSG00000135547 |
| HIC2 | ENSG00000169635 |
| HIST1H1T | ENSG00000187475 |
| HIVEP2 | ENSG00000010818 |
| IRX1 | ENSG00000170549 |
| KDM4C | ENSG00000107077 |
| KLF16 | ENSG00000129911 |

| | |
|---|---|
| KLF4 | ENSG00000136826 |
| KLF8 | ENSG00000102349 |
| LARP1B | ENSG00000138709 |
| LBX2 | ENSG00000179528 |
| LHX2 | ENSG00000106689 |
| LHX9 | ENSG00000143355 |
| LIN28A | ENSG00000131914 |
| LIN28B | ENSG00000187772 |
| MACF1 | ENSG00000127603 |
| MAEL | ENSG00000143194 |
| MBNL2 | ENSG00000139793 |
| MED23 | ENSG00000112282 |
| MTA1 | ENSG00000182979 |
| MYBL2 | ENSG00000101057 |
| MYCL | ENSG00000116990 |
| NANOG | ENSG00000111704 |
| NANOGP1 | ENSG00000176654 |
| NCOA1 | ENSG00000084676 |
| NCOR1 | ENSG00000141027 |
| **NFAT5** | ENSG00000102908 |
| NFATC2 | ENSG00000101096 |
| NFE2L3 | ENSG00000050344 |
| NFKB2 | ENSG00000077150 |
| NKRF | ENSG00000186416 |
| NKX3-1 | ENSG00000167034 |
| NR6A1 | ENSG00000148200 |
| NRG1 | ENSG00000157168 |
| NRK | ENSG00000123572 |
| ONECUT1 | ENSG00000169856 |
| PHB2 | ENSG00000215021 |
| PHF8 | ENSG00000172943 |
| PLXNC1 | ENSG00000136040 |
| POU5F1 | ENSG00000204531 |
| POU5F1B | ENSG00000212993 |
| **PPARG** | ENSG00000132170 |
| PRDM1 | ENSG00000057657 |
| RAD51 | ENSG00000051180 |
| RBM26 | ENSG00000139746 |
| RBMX | ENSG00000147274 |
| REST | ENSG00000084093 |
| RFX2 | ENSG00000087903 |
| RNF125 | ENSG00000101695 |
| RP11-313J2.1 | ENSG00000215146 |
| SALL1 | ENSG00000103449 |

| | |
|---|---|
| SALL4 | ENSG00000101115 |
| SAMD11 | ENSG00000187634 |
| SAP18 | ENSG00000150459 |
| SETDB2 | ENSG00000136169 |
| SF3A2 | ENSG00000104897 |
| SMAD9 | ENSG00000120693 |
| SMARCA1 | ENSG00000102038 |
| **SOX10** | ENSG00000100146 |
| **SOX9** | ENSG00000125398 |
| SP6 | ENSG00000189120 |
| **SRY** | ENSG00000184895 |
| TBX1 | ENSG00000184058 |
| TCF25 | ENSG00000141002 |
| **TCF3** | ENSG00000071564 |
| TEAD4 | ENSG00000197905 |
| TFAP2C | ENSG00000087510 |
| TOX | ENSG00000198846 |
| UBTF | ENSG00000108312 |
| VENTX | ENSG00000151650 |
| WHSC1 | ENSG00000109685 |
| ZBTB1 | ENSG00000126804 |
| ZBTB7C | ENSG00000184828 |
| ZC3H11A | ENSG00000058673 |
| ZC3HAV1 | ENSG00000105939 |
| ZFP30 | ENSG00000120784 |
| **ZFP42** | ENSG00000179059 |
| ZFY | ENSG00000067646 |
| ZFYVE20 | ENSG00000131381 |
| ZKSCAN8 | ENSG00000198315 |
| ZMYND11 | ENSG00000015171 |
| ZMYND8 | ENSG00000101040 |
| ZNF138 | ENSG00000197008 |
| ZNF208 | ENSG00000160321 |
| ZNF213 | ENSG00000085644 |
| ZNF217 | ENSG00000171940 |
| ZNF227 | ENSG00000131115 |
| ZNF232 | ENSG00000167840 |
| ZNF281 | ENSG00000162702 |
| ZNF385A | ENSG00000161642 |
| ZNF41 | ENSG00000147124 |

| | |
|---|---|
| ZNF468 | ENSG00000204604 |
| ZNF560 | ENSG00000198028 |
| ZNF607 | ENSG00000198182 |
| ZNF638 | ENSG00000075292 |
| ZNF66 | ENSG00000160229 |
| ZNF676 | ENSG00000196109 |
| ZNF729 | ENSG00000196350 |
| ZNF76 | ENSG00000065029 |
| ZNF90 | ENSG00000213988 |
| ZNF93 | ENSG00000184635 |
| ZNF98 | ENSG00000197360 |

**ɒtion factors.**

(columns A-C), their corresponding expression pattern(s) (column D) and their early SDT target
ɔns between transcription factors and their known target genes were extracted from public databases
ranscription Factor encyclopedia (Yusuf *et al.* , 2012). Gene names in red, green or blue have known
ɜary cancer, respectively.

| **Assembled transcripts IDs** | **Expression patterns** | **eSDT target genes** |
|---|---|---|
| TCONS_00184938 | P8 | - |
| TCONS_00206773;TCONS_00206776;TCONS_00206777 | P8 | - |
| TCONS_00073504 | P8 | - |
| TCONS_00130031;TCONS_00130033 | P9 | - |
| TCONS_00004335 | P14 | - |
| TCONS_00098944 | P7 | - |
| TCONS_00136955 | P9 | - |
| TCONS_00185901 | P11 | **PPARG**;POSTN |
| TCONS_00195284 | P8 | - |
| TCONS_00084978 | P3 | - |
| TCONS_00249587;TCONS_00253552;TCONS_00253553;TCONS_00253554;TCONS_00253556 | P2 | - |
| TCONS_00087391 | P11 | RAD51 |
| TCONS_00022167 | P1 | ACE;TAC1;G6PD |
| TCONS_00254445;TCONS_00254446 | P1 | - |
| TCONS_00049582 | P8 | - |
| TCONS_00033456 | P11 | - |
| TCONS_00254729 | P5 | - |
| TCONS_00201882 | P12 | PMAIP1;GREB1;ESR1;PLAC1 |
| TCONS_00072230 | P11 | - |
| TCONS_00097882;TCONS_00097883 | P9 | - |
| TCONS_00165043 | P9 | - |
| TCONS_00115256 | P6 | CLU |
| TCONS_00236474 | P9 | - |
| TCONS_00128881 | P9 | - |
| TCONS_00163150 | P11 | FOXL2;**CYP17A1**;**CYP11A1** |
| TCONS_00005817 | P13 | - |
| TCONS_00107359;TCONS_00107362 | P9 | - |
| TCONS_00047499 | P2 | **SOX9**;SFRP1 |
| TCONS_00053783 | P8 | - |
| TCONS_00200804 | P2 | - |
| TCONS_00145356;TCONS_00145358 | P9 | - |
| TCONS_00204095 | P13 | - |
| TCONS_00208943 | P5 | - |
| TCONS_00180488;TCONS_00180489 | P8 | - |
| TCONS_00237196 | P12 | - |
| TCONS_00110133 | P8 | - |

| | | |
|---|---|---|
| TCONS_00245945 | P9 | LAMA3;LAMA1;NANOG;PFKP;IFITM3 |
| TCONS_00249352 | P9 | - |
| TCONS_00170683 | P9 | - |
| TCONS_00128298 | P8 | - |
| TCONS_00241252 | P11 | - |
| TCONS_00007929 | P6 | - |
| TCONS_00001570;TCONS_00001571 | P9 | - |
| TCONS_00199732 | P9 | - |
| TCONS_00002167 | P11 | - |
| TCONS_00006676 | P13 | - |
| TCONS_00060407 | P2 | - |
| TCONS_00208410 | P9 | - |
| TCONS_00070138 | P13 | ESR1 |
| TCONS_00137452 | P9 | MYBL2 |
| TCONS_00012583;TCONS_00012585 | P9 | - |
| TCONS_00044962;TCONS_00044963 | P8;P9 | POU5F1 |
| TCONS_00044969 | P9 | - |
| TCONS_00115064 | P11 | - |
| TCONS_00096460;TCONS_00096465 | P5;P1 | **PPARG**;IGFBP3;ESR1 |
| TCONS_00085918 | P7 | CCL2 |
| TCONS_00140516;TCONS_00140524 | P8 | ENPP2 |
| TCONS_00212178 | P9 | - |
| TCONS_00024595 | P2 | - |
| TCONS_00254399 | P8 | - |
| TCONS_00232099;TCONS_00232101 | P5;P2 | ESR1;**ACTG2** |
| TCONS_00246680;TCONS_00246681 | P11 | - |
| TCONS_00226393 | P8 | - |
| TCONS_00250221 | P8 | - |
| TCONS_00080027 | P8 | - |
| TCONS_00051081 | P2 | - |
| TCONS_00253265 | P13 | - |
| TCONS_00048895 | P2 | - |
| TCONS_00204519;TCONS_00204520 | P9 | NANOG;**ZFP42** |
| TCONS_00230146 | P9 | - |
| TCONS_00149760 | P9 | CAV1;KLF4;GSTA2 |
| TCONS_00199766;TCONS_00199790 | P9 | - |
| TCONS_00075018 | P8 | - |
| TCONS_00063928 | P1 | - |
| TCONS_00254939 | P12 | **KIT** |
| TCONS_00168163;TCONS_00168164 | P8 | TAC1;LIN28A;GABRB3;KCNQ2 |
| TCONS_00110433 | P13 | - |
| TCONS_00101424 | P9 | - |
| TCONS_00027617 | P9 | - |
| TCONS_00088597 | P10 | - |

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|---|---|---|
| TCONS_00140537;TCONS_00140540;TCONS_00140543;TCONS_00140544 | P9 | SALL4;POU5F1 |
| TCONS_00000033;TCONS_00000037 | P1 | - |
| TCONS_00056892 | P8 | - |
| TCONS_00058144;TCONS_00058145;TCONS_00058146 | P8 | - |
| TCONS_00106327;TCONS_00106328;TCONS_00106329 | P2;P5 | - |
| TCONS_00061933 | P7 | - |
| TCONS_00254705 | P2 | - |
| TCONS_00148324;TCONS_00148325 | P2 | MPZ;PLP1;GJB1;**EDNRB** |
| TCONS_00094642 | P2 | KLF4;COL2A1;HAPLN1;COL9A1;PRAME;**SOX10** |
| TCONS_00098228 | P1 | - |
| TCONS_00255818 | P1 | **SOX9**;**PROM1** |
| TCONS_00145221 | P2 | - |
| TCONS_00086893;TCONS_00086894 | P2 | - |
| TCONS_00110107;TCONS_00110108;TCONS_00110111 | P13;P9 | POU5F1 |
| TCONS_00044600 | P9 | - |
| TCONS_00137982;TCONS_00137983 | P9 | ESR1 |
| TCONS_00233028 | P5 | - |
| TCONS_00097957 | P8 | - |
| TCONS_00025976 | P8 | - |
| TCONS_00166063 | P9 | - |
| TCONS_00067741 | P1 | - |
| TCONS_00104942 | P2 | - |
| TCONS_00008281 | P6 | - |
| TCONS_00223757 | P3 | - |
| TCONS_00112190 | P12 | - |
| TCONS_00173057;TCONS_00173058;TCONS_00173059 | P8;P9 | - |
| TCONS_00255507;TCONS_00255508;TCONS_00255510 | P2;P5 | - |
| TCONS_00158407 | P3 | - |
| TCONS_00196743 | P10 | - |
| TCONS_00020765 | P14 | - |
| TCONS_00140371 | P4 | - |
| TCONS_00213671 | P8 | - |
| TCONS_00111586;TCONS_00111591 | P9 | - |
| TCONS_00083148 | P4 | - |
| TCONS_00140595;TCONS_00140597;TCONS_00140603 | P9;P8 | - |
| TCONS_00108714 | P12 | - |
| TCONS_00095744 | P9 | - |
| TCONS_00018508 | P1 | - |
| TCONS_00053148 | P8 | - |
| TCONS_00253003 | P5 | - |

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|---|---|---|
| TCONS_00113462 | P8 | - |
| TCONS_00110665 | P9 | - |
| TCONS_00112218 | P9 | - |
| TCONS_00117137 | P9 | - |
| TCONS_00107451 | P10 | - |
| TCONS_00111593;TCONS_00111597 | P13;P9 | - |
| TCONS_00107534 | P9 | - |
| TCONS_00197324 | P12 | - |
| TCONS_00107416 | P11 | - |
| TCONS_00107411 | P10 | - |
| TCONS_00111603 | P11 | - |