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## **RNA profiling of human testicular cells identifies syntenic lncRNAs associated with spermatogenesis**

Antoine D. Rolland, B Evrard, T A Darde, C Le Béguec, Y Le Bras, K Bensalah, S Lavoué, B Jost, M Primig, Nathalie Dejuçq-Rainsford, et al.

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1 **RNA profiling of human testicular cells identifies syntenic lncRNAs associated with**  
2 **spermatogenesis**

3

4 **Running title:** Syntenic lncRNAs expressed during spermatogenesis

5

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17

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21 **Abstract**

22 **STUDY QUESTION:** Is the noncoding transcriptional landscape during spermatogenesis  
23 conserved between human and rodents?

24 **SUMMARY ANSWER:** We identified a core group of 113 long noncoding RNAs (lncRNAs)  
25 and 20 novel genes dynamically and syntenically transcribed during spermatogenesis.

26 **WHAT IS KNOWN ALREADY:** Spermatogenesis is a complex differentiation process  
27 driven by a tightly regulated and highly specific gene expression program. Recently, several  
28 studies in various species have established that a large proportion of known lncRNAs are  
29 preferentially expressed during meiosis and spermiogenesis in a testis-specific manner.

30 **STUDY DESIGN, SIZE, DURATION:** To further investigate lncRNA expression in human  
31 spermatogenesis, we carried out a cross-species RNA profiling study using isolated testicular  
32 cells.

33 **PARTICIPANTS/MATERIALS, SETTING, METHODS:** Human testes were obtained  
34 from post-mortem donors (N=8, 51 years old on average) or from prostate cancer patients with  
35 no hormonal treatment (N=9, 80 years old on average) and only patients with full  
36 spermatogenesis were used to prepare enriched populations of spermatocytes, spermatids,  
37 Leydig cells, peritubular cells and Sertoli cells. To minimize potential biases linked to inter-  
38 patient variations, RNAs from two or three donors were pooled prior to RNA-sequencing  
39 (paired-end, strand-specific). Resulting reads were mapped to the human genome, allowing for  
40 assembly and quantification of corresponding transcripts.

41 **MAIN RESULTS AND THE ROLE OF CHANCE:** Our RNA-sequencing analysis of pools  
42 of isolated human testicular cells enabled us to reconstruct over 25,000 transcripts. Among  
43 them we identified thousands of lncRNAs, as well as many previously unidentified genes  
44 (novel unannotated transcripts) that share many properties of lncRNAs. Of note is that although

45 noncoding genes showed much lower synteny than protein-coding ones, a significant fraction  
46 of syntenic lncRNAs displayed conserved expression during spermatogenesis.

47 **LARGE SCALE DATA:**

48 Raw data files (fastq) and a searchable table (.xlsx) containing information on genomic features  
49 and expression data for all refined transcripts have been submitted to the NCBI GEO under  
50 accession number GSE74896.

51 **LIMITATIONS, REASONS FOR CAUTION:** Isolation procedures may alter the  
52 physiological state of testicular cells, especially for somatic cells, leading to substantial  
53 changes at the transcriptome level. We therefore cross-validated our findings with three  
54 previously published transcriptomic analyses of human spermatogenesis. Despite the use of  
55 stringent filtration criteria, i.e. expression cut-off of at least three fragments per kilobase of  
56 exon model per million reads mapped, fold-change of at least three and false discovery rate  
57 adjusted p-values of less than  $< 1\%$ , the possibility of assembly artefacts and false-positive  
58 transcripts cannot be fully ruled out.

59 **WIDER IMPLICATIONS OF THE FINDINGS:** For the first time, this study has led to the  
60 identification of a large number of conserved germline-associated lncRNAs that are potentially  
61 important for spermatogenesis and sexual reproduction. In addition to further substantiating  
62 the basis of the human testicular physiology, our study provides new candidate genes for male  
63 infertility of genetic origin. This is likely to be relevant for identifying interesting diagnostic  
64 and prognostic biomarkers and also potential novel therapeutic targets for male contraception.

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69 no competing financial interests.

70

71 **Keywords:** Human spermatogenesis; RNA profiling; novel unannotated transcripts; lncRNAs;  
72 expression conservation, synteny

73

## 74 **Introduction**

75 Over the last two decades genome-wide association studies (GWAS) have found very few  
76 significant hits that could explain male infertility (Tüttelmann *et al.*, 2007; Krausz *et al.*, 2015).  
77 Indeed causal genetic diagnoses in infertile males can be established in less than 30% of men  
78 in infertile couples and the etiology of altered spermatogenesis thus remains largely unclear  
79 (Tüttelmann *et al.*, 2018). In this context the noncoding counterpart of the genome is usually  
80 ignored in GWAS whereas accumulated evidence emphasizes that genetic variants in these  
81 regions can be a cause for missing heritability (Zhang and Lupski, 2015).

82 Because of the highly dynamic and complex expression program underlying spermatogenesis,  
83 which includes a great number of genes expressed in a testis-specific manner, this process has  
84 been the focus of numerous genomics studies (for review, see (Chalmel and Rolland, 2015)).  
85 Several RNA sequencing for expression quantification (RNA-seq) analyses in rodents and  
86 human have thus contributed to the identification of thousands of long noncoding RNAs  
87 (lncRNAs) expressed in the testis (Cabili *et al.*, 2011; Laiho *et al.*, 2013; Soumillon *et al.*, 2013;  
88 Chalmel *et al.*, 2014; Chocu *et al.*, 2014). However, such approaches have not been applied  
89 frequently in male infertility (Tüttelmann *et al.*, 2018) especially when considering the  
90 noncoding counterpart of the genome for genetic screening.

91 By definition, lncRNAs are a large class of noncoding RNAs more than 200 nucleotides  
92 in length. They are broadly classified as intergenic, intronic, or overlapping in the sense or  
93 antisense orientation according to their position relative to known protein-coding genes  
94 (Derrien *et al.*, 2012). Most lncRNAs are RNA polymerase II-transcribed and thus likely to be  
95 capped, polyadenylated, and spliced, like mRNAs (Cabili *et al.*, 2011; Guttman and Rinn,  
96 2012). Unlike mRNAs, however, lncRNAs exhibit unique cellular localization patterns highly  
97 correlated with the functions they perform in the cell. Known roles include the regulation of  
98 DNA methylation, histone modification, chromatin remodeling, and control of gene expression,

99 either in *cis* in the nucleus or in *trans* in the nucleus or the cytoplasm (for review, see Mercer  
100 and Mattick, 2013; Chen, 2016; Schmitz *et al.*, 2016). Common genomic features shared by  
101 lncRNAs include relatively short lengths, low exon numbers, low GC content, low sequence  
102 conservation (comparable to that of introns), low abundance, and high expression specificity.  
103 lncRNAs expressed within the testis share additional features, including peak expression at  
104 particular phases of germ cell differentiation, specifically during late steps of meiosis and  
105 spermiogenesis (Cabili *et al.*, 2011; Laiho *et al.*, 2013; Chalmel *et al.*, 2014). Furthermore, in  
106 rats, lncRNAs that are expressed most highly in meiotic spermatocytes have exons twice as  
107 long as those of lncRNAs or mRNAs expressed in other cell types or organs (Chalmel *et al.*,  
108 2014). While the relevance of these observations remains unknown, two recent studies assessed  
109 the functionality of testicular lncRNAs and revealed their critical roles during spermatogenesis  
110 in fly and mouse (Wen *et al.*, 2016; Hosono *et al.*, 2017; Wichman *et al.*, 2017).

111 In this study, we performed an in-depth characterization of the human testicular transcriptome.  
112 Our analysis of enriched adult testicular cells enabled us to reconstruct more than 25,000 “high-  
113 confidence” transcripts, including 1,368 lncRNAs and 511 novel unannotated transcribed  
114 regions (NUTs), whose low conservation, low protein-encoding potential, and absence of  
115 evidence at the protein level strongly suggest that they are novel lncRNAs. Finally, we  
116 investigated gene expression correlation between human and rodent spermatogenesis. Our  
117 finding that a core group of lncRNAs is transcribed syntenically during germ cell  
118 differentiation suggests they might play important roles in this process, thus deepening and  
119 broadening the molecular basis for understanding spermatogenesis (Chalmel *et al.*, 2007).  
120 Since those conserved lncRNAs also exhibit cell-specific expression, they represent excellent  
121 candidates for identifying diagnostic and prognostic biomarkers in azoospermic men and  
122 designing novel therapeutic options in male contraception. A graphical display of the

123 corresponding dataset is available *via* the ReproGenomics Viewer (<http://rgv.genouest.org>)

124 (Darde *et al.*, 2015, 2019).

125



## 126 **Materials and Methods**

### 127 Ethical considerations

128 Human adult testes were collected from multiorgan donors (N=8, 51 years old on average) and  
129 from prostate cancer patients (with no hormonal treatment) undergoing orchidectomy (N=9,  
130 80 years old on average), as detailed in Supplementary Table SI. The presence of full  
131 spermatogenesis was assessed by transillumination of freshly dissected seminiferous tubules  
132 (Nikkanen *et al.*, 1978), observation of spermatozoa and differentiated spermatids following  
133 tissue dissociation, as well as ploidy characterization of dissociated cells by cytometry and  
134 histology analysis of paraffin-embedded biopsies. All the required authorizations were  
135 obtained from the "Agence de la Biomédecine" (PFS09-015) (for deceased donors) and the  
136 "Comité de Protection des Personnes" (#02/31-407) (for cancer patients).

137 Prior to sequencing, RNAs from distinct donors were eventually pooled in an equimolar  
138 manner (Supplementary Table SI).

139

### 140 Sample isolation

141 *Leydig cells.* Human Leydig cells were isolated as previously described (Simpson *et al.*, 1987)  
142 with minor modifications (Willey *et al.*, 2003). Briefly, interstitial cells were recovered after  
143 collagenase digestion of the testicular parenchyma (0.5 mg/mL, 32°C, 45 minutes) and loaded  
144 onto a discontinuous density percoll gradient. After centrifugation at 600 g for 30 minutes, the  
145 cells were carefully recovered, washed with PBS, and allowed to plate for 2 days at 32°C in  
146 DMEM-F12 containing 10% fetal calf serum (FCS), fungizone (2.5 µg/mL), penicillin (50  
147 UI/mL), streptomycin (50 µg/mL), vitamin C (0.1 mM), vitamin E (10 µg/MI), insulin (10  
148 µg/mL), transferrin (10 µg/mL) and hCG (100 mIU/mL). The cells were then washed with PBS  
149 to remove nonadhering germ cells and were cultured for 3 more days in the same medium

150 without FCS. Cells were then harvested with a cell scraper, pelleted down, snap-frozen, and  
151 stored at -80°C until RNA extraction.

152

153 *Peritubular cells.* Human peritubular cells were isolated and cultured as described elsewhere  
154 (Albrecht *et al.*, 2006) with minor modifications. First, the testicular parenchyma underwent  
155 collagenase digestion (0.5 mg/mL, 32°C, 45 minutes). Individualized seminiferous tubule  
156 fragments (1-2 cm long) were selected, placed onto the surface of a plastic cell culture dish,  
157 and covered with 20-30 µL FCS. The FCS drops were allowed to start evaporating for 30  
158 minutes, and then DMEM-F12 containing 10% FCS, fungizone (2.5 µg/mL), penicillin (50  
159 UI/mL), and streptomycin (50 µg/mL) was slowly added to the culture dish. After 2 to 3 weeks  
160 of culture at 32°C, when peritubular cells had started to grow out of the seminiferous tubules,  
161 fragments were removed and the cells were allowed to grow for 2 more weeks. Cells were then  
162 harvested with a cell scraper, pelleted down, snap-frozen, and stored at -80°C until RNA  
163 extraction.

164

165 *Sertoli cells.* Isolated human primary Sertoli cells (Chui *et al.*, 2011) were purchased from  
166 Lonza (Walkersville, MD, USA) and cultured for 3 weeks at 32°C in Sertoli Cell Basal Medium  
167 (SeBM™, Lonza) containing FBS, fungizone, and gentamicin (SeGM™, Lonza). Cells were  
168 then harvested with a cell scraper, pelleted down, snap-frozen, and stored at -80°C until RNA  
169 extraction.

170

171 *Germ cells.* Pachytene spermatocytes and round spermatids were isolated according to  
172 previously described procedures (Guillaudeau *et al.*, 1996). Briefly, after collagenase digestion  
173 of testicular parenchyma (0.5 mg/mL, 32°C, 45 minutes), the seminiferous tubules were further

174 dilacerated with scalpels and finally redigested by trypsin (0.3 mg/mL, 32°C, 30 minutes). Cell  
175 suspensions were next washed with PBS, filtered on sheets of nylon gauze (300-, 100-, and 20-  
176 µm pore size) and on nylon wool before separation by an elutriation rotor (JE5 Beckman  
177 Instruments, Inc., Fullerton, CA, USA). Cells were then pelleted down, snap-frozen, and stored  
178 at -80°C until RNA extraction.

179

#### 180 RNA extraction, library construction, and RNA-Seq

181 Total RNA was extracted from tissues and cell pellets with the RNeasy mini Kit (Qiagen,  
182 Hilden, Germany), quantified with a NanoDrop™ 8000 spectrophotometer (Thermo Fisher  
183 Scientific, Waltham, MA, USA), and quality controlled with a 2100 Electrophoresis  
184 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Isolated cells and tissue samples  
185 from a total of 16 patients were investigated in duplicate and, in some cases, were pooled  
186 according to the availability of the material. Libraries of template molecules suitable for strand-  
187 specific high-throughput DNA sequencing were created by using “TruSeq Stranded Total RNA  
188 with Ribo-Zero Gold Prep Kit” (catalog # RS-122-2301; Illumina Inc., San Diego, CA, USA).  
189 Briefly, cytoplasmic and mitochondrial rRNA were removed from 500 ng of total RNA with  
190 biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads. After  
191 purification, the RNA was fragmented by using divalent cations at a high temperature. The  
192 cleaved RNA fragments were copied into first-strand cDNA with reverse transcriptase and  
193 random primers; second-strand cDNA synthesis followed, with DNA Polymerase I and RNase  
194 H. The double-stranded cDNA fragments were blunted with T4 DNA polymerase, Klenow  
195 DNA polymerase, and T4 polynucleotide kinase. A single ‘A’ nucleotide was added to the 3’  
196 ends of the blunt DNA fragments by using a Klenow fragment (3' to 5'exo minus) enzyme. The  
197 cDNA fragments were ligated to double-stranded adapters with T4 DNA Ligase. The ligated  
198 products were enriched by PCR amplification (30 sec at 98°C; [10 sec at 98°C, 30 sec at 60°C,

199 30 sec at 72°C] × 12 cycles; 5 min at 72°C). Excess PCR primers were removed by purification  
200 with AMPure XP beads (Beckman Coulter, Brea, CA, USA). Final cDNA libraries were  
201 quality-checked and quantified with a 2100 Electrophoresis Bioanalyzer (Agilent  
202 Technologies). The libraries were loaded in the flow cell at 7 pM concentration, and clusters  
203 were generated in the Cbot and sequenced in the Illumina Hiseq 2500 as paired-end 2×50 base  
204 reads following Illumina's instructions. Image analysis and base calling were performed with  
205 RTA 1.17.20 and CASAVA 1.8.2. Raw data files (fastq) and a searchable table (.xlsx)  
206 containing information on genomic features and expression data for all 25,161 refined  
207 transcripts have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession  
208 number GSE74896. All data are also conveniently accessible through the ReproGenomics  
209 Viewer (Darde *et al.*, 2015, 2019).

210

## 211 RNA-seq analysis

212 *Assembly of a unique set of human reference transcripts.* Ensembl (Yates *et al.*, 2016) and  
213 RefSeq (Pruitt *et al.*, 2014; Brown *et al.*, 2015) transcript annotations of the hg19 release of the  
214 human genome were downloaded from the University of California Santa Cruz (UCSC)  
215 genome browser website (Rosenbloom *et al.*, 2014) on November 4, 2014. Both transcript  
216 annotation files (GTF format) were subsequently merged into a combined set of nonredundant  
217 human reference transcripts (HRT), with Cuffcompare (Trapnell *et al.*, 2012). We also defined  
218 a nonredundant dataset of human splice junctions (HSJ) extracted from alignments of human  
219 transcripts and expressed sequence tags (ESTs) provided by UCSC.

220

221 *Mapping reads.* RNA-seq-derived reads from each sample duplicate were aligned  
222 independently with the hg19 release of the human genome sequence by TopHat (version 2.0.10)

223 (Trapnell *et al.*, 2009) using previously published approaches (Pauli *et al.*, 2012; Trapnell *et al.*, 2012; Chalmel *et al.*, 2014; Zimmermann *et al.*, 2015). Briefly, the TopHat program was  
224 run a first time for each RNA-seq fastq file with the HRT and HSJ datasets to improve read  
225 mapping. The resulting junction outputs produced by all TopHat runs were pooled and added  
226 to the HSJ dataset. TopHat was rerun a second time for each sample with the new HSJ dataset.  
227 The output of this second run comprised the final alignment (BAM format). Finally, BAM files  
228 corresponding to sample duplicates were subsequently merged and sorted with the samtools  
229 suite (Li *et al.*, 2009).

231 To compare RNA-seq data from different testicular cell populations across species  
232 appropriately, previously published rat and mouse RNA-seq datasets (Gan *et al.*, 2013;  
233 Soumillon *et al.*, 2013; Chalmel *et al.*, 2014) were reanalyzed with the same mapping protocol  
234 on the rn6 and mm9 releases of the rat and mouse genomes, respectively.

235

236 *Transcriptome assembly and quantification.* The transcriptome of each human testicular cell  
237 population was subsequently assembled, compared to known transcript annotations and  
238 quantified with the Cufflinks suite (version 2.2.1), with default settings applied (Trapnell *et al.*,  
239 2012). Briefly, the assembly step performed by Cufflinks using the merged alignment files  
240 yielded a set of ~51,000-380,000 transcript fragments (transfrags) for each testicular cell type  
241 (Supplementary Table SII). The Cuffcompare program was then used: first to define a  
242 nonredundant set of 778,012 assembled transcripts by tracking Cufflinks transfrags from all  
243 experimental conditions; and second to compare the resulting transcripts to the HRT dataset  
244 (i.e., known transcript annotations). Finally, the abundance of each transcript in each sample  
245 was assessed by Cuffdiff (within Cufflinks), expressed as fragments per kilobase of exon model  
246 per million reads mapped (FPKM). Abundance values were quantile-normalized to reduce  
247 systematic effects and to allow direct comparison between the individual samples.

248

249 *Refinement of assembled transcripts.* As suggested by Prensner *et al.* (2011) and Chalmel *et al.*  
250 (2014), we sequentially applied four filtering steps to discriminate the most robust transfrags  
251 from background noise (Supplementary Fig. S1). First, we selected 37,310 “detectable” or  
252 “expressed” transfrags, defined as those for which abundance levels exceeded 3 FPKM in at  
253 least one experimental condition (average value of sample duplicates). We next selected 33,562  
254 transcripts with a cumulative exon length  $\geq 200$  nt. Third, all transfrags that were not  
255 automatically annotated by Cuffcompare as a complete match (Cuffcompare class “=”),  
256 potentially novel isoform (“j”), unknown intronic (“i”, i.e. loci falling entirely within a  
257 reference intron and without exon-exon overlap with another known locus), intergenic (“u”),  
258 or antisense (“x”) isoforms were discarded, thereby leaving 28,253 transfrags for analysis.  
259 Finally, all transcript fragments that were annotated as either novel isoforms or novel genes  
260 (class codes “j”, “i”, “u” or “x”) and that did not have at least two exons (multiexon) were  
261 filtered out. Altogether, this strategy produced a high-confidence set of 25,161 transcripts  
262 meeting these refinement conditions and supporting total RNA molecules expressed in human  
263 testicular cells during spermatogenesis (Supplementary Fig. S1).

264

#### 265 Microarray data normalization

266 To monitor the expression level of the assembled mRNAs and lncRNAs in infertile men with  
267 non-obstructive azoospermia we integrated microarray data published by Malcher and  
268 colleagues (Malcher *et al.*, 2013). Raw data were downloaded from the NCBI GEO (Barrett *et al.*,  
269 2012) repository under the accession number GSE45885. The gene array data were  
270 normalized with the Robust Multi-Array Average method (Irizarry *et al.*, 2003) implemented  
271 in the statistical software R (version 3.5.1) using the Brainarray custom Chip Description Files

272 (CDF, version 23.0.0) so that intensity values are not summarized for each probe set but  
273 directly for each Entrez Gene ID (Dai *et al.*, 2005).

274

#### 275 Analysis of coding potential of the assembled transcripts

276 Before analyzing the protein-coding potential of the 25,161 high-confidence transcripts  
277 assembled in human testicular cell populations, we used TopHat's gffread tool to extract their  
278 DNA sequences. As already described (Chalmel *et al.*, 2014; Zimmermann *et al.*, 2015), the  
279 resulting nucleic sequences were classified as either coding or noncoding, according to an  
280 empirical integrative approach using four distinct predictive tools: Coding-Potential  
281 Assessment Tool (CPAT), HMMER, Coding Potential Calculator (CPC) and txCdsPredict  
282 (Kong *et al.*, 2007; Finn *et al.*, 2011; Kuhn *et al.*, 2013; Wang *et al.*, 2013a). Transcripts were  
283 considered protein-coding candidates if they had a coding probability  $>0.364$  in CPAT, an E-  
284 value  $<10^{-4}$  in HMMER (versus Pfam-A and -B), if they were classified as "coding" by CPC,  
285 or if they showed a txCdsPredict score  $>800$  (~90% predictive of protein-coding genes). Finally,  
286 transcripts were organized into five groups considered to have "Very High" (4/4 tools predict  
287 protein-coding potential), "High" (3/4), "Medium" (2/4), "Low" (1/4), or "No" (0/4) protein-  
288 coding potential according to whether their nucleic sequences were considered as protein-  
289 coding by four, three, two, one or none of the four predictive tools, respectively.

290

#### 291 Proteomics Informed by Transcriptomics strategy

292 To provide evidence at the protein level for assembled transcripts, we applied a Proteomics  
293 Informed by Transcriptomics (PIT) approach, as recently described by Evans and co-workers  
294 (Evans *et al.*, 2012). This approach relies on the query of tandem mass spectrometry (MS/MS)

295 proteomics spectra against a customized protein database derived from RNA-seq data of the  
296 same or similar samples.

297 *Assembly of a unique human reference proteome database.* The nucleic sequences of the  
298 25,161 high-confidence assembled transcript isoforms were translated into the three-first open  
299 reading frames with the EMBOSS's Transeq program (Rice *et al.*, 2000). Deduced amino acid  
300 sequences of at least 20 residues between two stop codons were defined as potential protein  
301 sequences. Finally, a nonredundant human reference proteome database was assembled by  
302 merging the UniProt (89,033 canonical and isoform sequences; release 2014\_08) (Pundir *et al.*,  
303 2015) and Ensembl (99,459 known and 50,117 predicted protein sequences; release-76) (Yates  
304 *et al.*, 2016) proteome databases with the set of predicted protein sequences.

305

306 *Protein identification.* For this experiment, we used a human adult testis MS/MS proteomics  
307 dataset available from the Human Proteome Map (Kim *et al.*, 2014). All analyses were  
308 performed with PeptideShaker (release 0.31.4) (Vaudel *et al.*, 2011), implemented in the  
309 Galaxy web-based genome analysis environment (Blankenberg *et al.*, 2010) (release 1.19.5.0),  
310 based on SearchGUI (release 1.19.5) (Vaudel *et al.*, 2011). First, the 46 raw data files (.raw)  
311 were downloaded from the PRIDE database under accession number PXD000561 and  
312 converted to mgf format with PeptideShaker. A concatenated target/decoy database was  
313 created by reversing the sequences from a target database with SearchGUI. Cross-linked  
314 peptide identification was thus carried out with X!Tandem, Open Mass Spectrometry Search  
315 Algorithm (OMSSA) and MS-GF+ (Vaudel *et al.*, 2011). We applied the parameters used by  
316 Pinto and coworkers (Pinto *et al.*, 2014): precursor ion tolerance units set at 10 ppm; fragment  
317 tolerance set at 0.05 Da; carbamidomethylation of cysteine defined as a fixed modification;  
318 oxidation of methionine defined as a variable modification; and only tryptic peptides with up  
319 to two missed cleavages were considered. All peptides with at least one validated peptide-



320 spectrum match (PSM) and a confidence interval greater than 80% were kept for further  
321 analyses. Finally, only identifications with a false discovery rate (FDR) < 1% indicated by the  
322 PeptideShaker validation method were considered.

323

#### 324 Statistical filtration and clustering analysis

325 *Statistical analysis.* The statistical filtration of the transfrags showing a differential expression  
326 (DE) across experimental samples was performed using AMEN (Chalmel and Primig, 2008)  
327 (Supplementary Fig. S3). First, we performed every pairwise comparison between  
328 experimental conditions and selected 23,687 transcript fragments yielding at least one fold-  
329 change greater or equal to 3.0 (average values of sample duplicates). A Linear Models for  
330 Microarray Data (LIMMA) statistical test (was finally used to identify 21,264 transcripts with  
331 significant abundance variations across samples (F-value adjusted with the FDR method:  $P \leq$   
332 0.05) (Smyth, 2004).

333

334 *Cluster analysis.* The 21,264 DE transfrags were next clustered into 11 expression patterns (P1-  
335 P11) by the k-means algorithm (Supplementary Fig. S3). The quality of the resulting k-means  
336 clusters was verified with Silhouette plots. The 11 resulting patterns were ordered according to  
337 peak expression levels in the different cell types. The 3,897 transfrags for which no significant  
338 differential expression was observed (fold-change <3 or  $P > 0.05$ ) were placed in a group term  
339 P0.

340

341 *Testis specificity analysis.* To filter transcripts expressed testis-specifically, we downloaded a  
342 tissue profiling dataset from the NCBI GEO under the accession number GSE45326 (Nielsen  
343 *et al.*, 2014). This experiment comprises 12 normal tissues including ovary, bladder, brain,

344 breast, colon, heart, kidney, liver, lung, muscle, prostate, and skin. To allow proper comparison  
345 with our own data, this dataset was re-analyzed with TopHat as described above. Next, the  
346 abundance of each transcript assembled in our dataset was assessed with Cuffquant and  
347 normalized with Cuffnorm (Trapnell *et al.*, 2012).

348 An empirical filtration approach based on abundance was applied to select transcripts reliably  
349 detected only in human testis. Assembled transcripts were considered testis-specific if their  
350 abundance was  $> 1$  FPKM in the testis and  $1 < \text{FPKM}$  in the 12 other tissues. To avoid selection  
351 of candidates with values close to the threshold, only those with an abundance at least three-  
352 fold higher in testis than in other tissues were retained.

353

#### 354 Quantification of syntenic transcripts in rodents

355 *Conversion of genome co-ordinates from humans to rodents.* Since potential orthologous loci  
356 in rodents of most human lncRNAs and novel loci identified in our study are probably unknown,  
357 co-ordinates of the 25,161 high-confidence assembled transcripts (GenePred format) were  
358 mapped to syntenic mouse (mm9) and rat (rn6) regions with UCSC's liftOver tool (parameters:  
359  $-\text{minMatch}=0.1$   $-\text{minBlocks}=0.5$ , as recommended by UCSC for cross-species conversion).

360

361 *Syntenic transcript quantification.* The abundance of each syntenic transcript in each rodent  
362 RNA-seq dataset (Gan *et al.*, 2013; Soumillon *et al.*, 2013; Chalmel *et al.*, 2014) was assessed  
363 with the Cufflinks suite (Pollier *et al.*, 2013). Abundance values (FPKM) were quantile-  
364 normalized to reduce systematic effects and to allow direct comparison between the individual  
365 samples.

366

367 *Identification of conserved and correlated loci.* *Rattus norvegicus* was selected as the reference  
368 rodent species since the human and rat datasets were produced with similar library preparation  
369 protocols (including an rRNA-depletion method) and comprise five similar testicular cell types  
370 (Leydig, peritubular, and Sertoli cells, spermatocytes, and round spermatids). A syntenic  
371 transcript was considered as “detectable” when its abundance value  $\geq 1$  FPKM in the rat.  
372 Finally, syntenic and detectable transcripts showing similar patterns in the rat (correlation  
373 coefficient  $\geq 0.8$  between the five common testicular cell types) defined the set of loci with a  
374 conserved and correlated expression profile between humans and rodents.

375

#### 376 Statistical tests

377 *Enrichment calculation.* AMEN (Chalmel and Primig, 2008) was used to calculate the Fisher  
378 exact probability, and the Gaussian hypergeometric test to identify significantly enriched terms  
379 from the gene ontology in the 11 expression patterns (P1-P11). A specific annotation term was  
380 considered enriched in a group of coexpressed genes if the *P* value was  $< 0.001$  and the number  
381 of genes in this cluster showing this annotation was  $> 3$ .

382

#### 383 Quantitative PCR experiments

384 Total RNA (2.5  $\mu\text{g}$ ) was first submitted to DNase treatment by the TURBO DNA-free™ Kit  
385 (Thermo Fisher Scientific). cDNA synthesis was performed on 800 ng of DNase-treated RNA,  
386 with the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories,  
387 Hercules, CA, USA), according to the manufacturer’s instructions. Quantitative PCR (qPCR)  
388 experiments were next performed on cDNA from isolated human Leydig cells (n=5),  
389 peritubular cells (n=2), Sertoli cells (n=2), pachytene spermatocytes (n=4), and round  
390 spermatids (n=4). Each gene was assessed in each sample in technical duplicates on 2 ng of

391 cDNA, by using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and  
392 CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories), according to the  
393 default program (95°C for 3 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s)  
394 including a melting curve step (65°C to 95°C with a 0.5°C increment and a hold time of 5 s  
395 before reading plate). Results were analyzed with the Bio-Rad CFX Manager. Previously  
396 published primers for GAPDH and RPLP0 were used for normalization purposes (Svingen *et*  
397 *al.*, 2014). Specific primers for candidate transcripts were designed with the Universal  
398 ProbeLibrary System Assay Design ([https://lifescience.roche.com/shop/products/universal-](https://lifescience.roche.com/shop/products/universal-probelibrary-system-assay-design)  
399 [probelibrary-system-assay-design](https://lifescience.roche.com/shop/products/universal-probelibrary-system-assay-design)). When intron-spanning primers could not be found, the  
400 Primer3 software (v4.0.0; <http://primer3.ut.ee/>) was used. Only primer pairs showing both a  
401 single peak in the melting curve analysis and an amplification efficiency between 95 and 105%  
402 were used in subsequent experiments (Supplementary Table SIII).

403

## 404 **Results**

405 Transcript assembly in human adult testicular cells identifies almost 500 new genes

406 We performed here an RNA-seq analysis of human adult testis, of meiotic and postmeiotic  
407 germ cells, as well as of Leydig, peritubular, and Sertoli cells, all originating from donors with  
408 apparent normal spermatogenesis (Supplementary Table SI). To cover their transcriptomes as  
409 broadly as possible, we sequenced ribosomal RNA-depleted total RNA rather than polyA  
410 RNAs that tend to be biased towards mRNA (Guttman *et al.*, 2013). We further reconstructed  
411 both known and unknown transcript isoforms according to an approach previously described  
412 (Chalmel *et al.*, 2014; Zimmermann *et al.*, 2015). In total, 77.3% of the reads that mapped to  
413 the human genome were further used to assemble, quantify, and refine a set of 25,161 “high-  
414 confidence” transcripts corresponding to 10,703 loci (Supplementary Table SII, and

415 Supplementary Fig. S1). Comparison to the human genome annotation (Ensembl and RefSeq  
416 combined, 204,222 nonredundant transcripts) showed that most of them correspond to known  
417 (8,777 transcripts, 34.9%) and novel (13,882, 55.2%) isoforms of annotated protein-coding loci,  
418 as well as to known (562, 2.2%) and novel (806, 3.2%) isoforms of annotated lncRNAs  
419 (Supplementary Fig. S2). Importantly, 511 transcripts (452 loci) appeared to be novel intronic  
420 (148, 0.59%), intergenic (193, 0.77%), and antisense (170, 0.68%) unannotated loci, referred  
421 to as NUTs (Fig. 1). An integrative approach combining a coding-potential prediction analysis  
422 and a PIT strategy (Evans *et al.*, 2012) further suggested that NUTs actually correspond to as-  
423 yet unidentified human lncRNAs (Supplementary Fig. S2). Use of isolated cells was critical  
424 for identifying these new loci, for which 83.6% would not have met the expression threshold  
425 criteria in total testis samples. Additionally, 623 remaining transcripts (2.5%) corresponded to  
426 other RNA types, such as pseudogenes and microRNAs, which were not further analyzed.

427

428 The dynamics of the testicular transcriptional landscape highlight the accumulation of  
429 lncRNAs during human spermiogenesis

430 To identify the transcripts preferentially expressed in each testicular cell population, we next  
431 performed a differential expression analysis. We found that 21,264 transcripts (84.5% of those  
432 assembled) showed significant differential expression, which we classified into 11 patterns  
433 (P1-P11) (Supplementary Fig. S3, and Fig. 2A): P1, P2, P3, and P4 comprise transcripts highly  
434 expressed, respectively, in all somatic cells, in Leydig cells, in peritubular cells and in Sertoli  
435 cells. P5 contains transcripts expressed in every cell type but spermatids. Transcripts in patterns  
436 P6 to P10 show gradual peak expression in differentiating germ cells from spermatocytes to  
437 spermatids. Finally, P11 is composed of transcripts with peak expression in the total testis.  
438 Importantly, the consistency of expression patterns was confirmed by different analyses  
439 (Supplementary Fig. S4-S10). These showed: the overall appropriate expression of known

440 testicular cell markers (Supplementary Fig. S4); a highly significant overlap with data from  
441 microarray analyses of testicular biopsies from infertile patients (Chalmel *et al.*, 2012; Malcher  
442 *et al.*, 2013) (Supplementary Fig. S5 and Supplementary Fig. S6) or from single-cell RNA-seq  
443 analysis of human adult spermatogenesis (Wang *et al.*, 2018) (Supplementary Fig. S7); the  
444 overrepresentation of relevant biological processes for each cluster (Supplementary Fig. S8);  
445 and the underrepresentation of X chromosome-derived genes in germ cell patterns P5 to P10  
446 as a reflection of meiotic sex chromosome inactivation (Turner, 2007) (Supplementary Fig.  
447 S9).

448 Next, we focused on known lncRNAs, and found that most (64.7%) were preferentially  
449 expressed in spermatids (P9 and P10,  $P < 10^{-177}$ ) (Fig. 2B). Similarly, the finding that most  
450 NUTs (69.6%) were also preferentially expressed during spermiogenesis (P9 and P10,  $P < 10^{-$   
451  $81$ ) (Fig. 2B) supports the idea that many of the NUTs are *bona fide* lncRNAs. The preferential  
452 expression of eight NUTs in spermatocytes and/or spermatids (Supplementary Fig. S10) was  
453 further validated with qPCR (Supplementary Table SIII).

454

#### 455 Expansion of the testis-specific repertoire of lncRNAs

456 Tissue-specific expression is often considered an indication that the genes involved play unique  
457 and important functions in a narrow range of biological processes. To ascertain in more detail  
458 the fraction of the testicular transcriptional landscape expressed only in this organ, we analyzed  
459 a tissue-profiling dataset including 12 types of normal human tissues (Nielsen *et al.*, 2014) and  
460 found that 16.5% (3515 / 21264) of the differentially expressed transcripts were detected only  
461 in testis. As expected, 91.4% were predominantly expressed in the germline, showing peak  
462 transcriptional induction in early spermatids (P9-P10, 2162/3515, 61.5%) and, to a lesser extent,  
463 in spermatocytes (P5-P8, 1051/3515, 29.9%). These RNA-seq data thus confirm and extend

464 earlier observations by showing that twice as many testis-specific transcripts are expressed  
465 during the haploid phase as during meiosis (Chalmel *et al.*, 2012). Consistent with their  
466 preponderant expression in meiotic and postmeiotic germ cells, 71% of the newly identified  
467 NUTs were also detected specifically in the testis. As already observed in the rat (Chalmel *et*  
468 *al.*, 2014), exons of a specific subset of lncRNAs expressed in meiotic and postmeiotic germ  
469 cells are longer than those of other coding and noncoding transcripts (Supplementary Fig. S11).

470

#### 471 Conserved testicular expression of syntenic mammalian lncRNAs

472 The core testicular transcriptome in mammals includes 18,847 “high-confidence” transcripts  
473 (~88.6%) located in genomic regions hypothesized to be homologous due to their shared  
474 synteny between humans and rodents (mice and rats) (Table I). The great majority of these  
475 syntenic transcripts (15,119 transcripts, 80.2%) were expressed in at least one type of testicular  
476 cell in rats. Furthermore, approximately half of the syntenic transcripts (8,457 transcripts,  
477 44.9%) displayed very similar expression patterns (correlation > 0.5) in human and rat, and  
478 therefore constitute a core set of evolutionary conserved loci involved in spermatogenesis (Fig.  
479 3). The present RNA-seq study broadens our insight into the conserved testicular expression  
480 program, since our previously published microarray analysis identified only 12.6% of coding  
481 genes in humans and rodents as being correlated (Chalmel *et al.*, 2007). Importantly, we were  
482 able to determine that the noncoding component of the core testicular transcriptome includes  
483 at least 113 lncRNAs and 20 NUTs (Table I). The expression pattern of syntenic mRNAs are  
484 far more often correlated than those of lncRNAs (45.8% versus 20.2%).

485

#### 486 **Discussion**

487 Gaining insight into the testicular transcriptional landscape is essential for a better  
488 understanding of genetic causes underlying infertility in men. It may also facilitate unraveling  
489 what is behind negative trends in several components of male reproductive health, including  
490 testicular cancer; and the genomic mechanisms involved in the genetic introgression that  
491 occurred during ancestral human admixtures and in which meiosis appears central (Jégou *et al.*,  
492 2017). The testis is undeniably the organ that expresses the highest number of genes in a tissue-  
493 specific manner, due primarily to the complex processes and associated factors that are required  
494 for male germ cell development (for reviews, see (Kleene, 2001; Eddy, 2002; Kimmins *et al.*,  
495 2004)). Some researchers consider, however, that the specificities of the spermatogenic cell  
496 expression program may also result from promiscuous or leaky transcription during and after  
497 meiosis, which would lead to the adventitious synthesis of nonfunctional transcripts (for review,  
498 see (Ivell, 1992)). Interestingly, the atypical patterns of gene expression in the testis have also  
499 been hypothesized to result from sexual selection, a distinct form of natural selection based on  
500 mate choice and competition for mating that acts at the level of the organism, cell, and molecule  
501 (for review, see (Kleene, 2005)). Sexual selection is notably responsible for the rapid evolution  
502 of often exaggerated reproductive traits in the competing sex, i.e., males in most species  
503 (Darwin, 1871; Hosken and House, 2011). This intense selection pressure to which male  
504 reproduction is subjected also triggers the rapid divergence of testicular genes, at both the  
505 expression and sequence levels (for review, see (Grath and Parsch, 2016)). Finally, together  
506 with potential leaky transcription in meiotic and postmeiotic germ cells, sexual selection is also  
507 responsible for making the testis a fertile ground for the birth of new genes, coding as well as  
508 noncoding, either from scratch or from pre-existing genes ((Xie *et al.*, 2012; Ruiz-Orera *et al.*,  
509 2015); for review, see (Kaessmann, 2010)). In this context, lncRNAs constitute a class of genes  
510 with a high origination rate and rapid turnover; most of them are consequently species- or  
511 lineage-specific, and their expression is highly enriched in the testis (Cabili *et al.*, 2011; Derrien



512 *et al.*, 2012; Necsulea *et al.*, 2014; Hezroni *et al.*, 2015); for review (Kapusta and Feschotte,  
513 2014)).

514 Several RNA-seq analyses have investigated the expression of lncRNAs during  
515 spermatogenesis in various animal species or used the testis as a model organ to identify new  
516 lncRNAs (Soumillon *et al.*, 2013; Necsulea *et al.*, 2014). Recently, two RNA-seq studies were  
517 performed using human male germ cells (Zhu *et al.*, 2016; Jan *et al.*, 2017). In those works,  
518 the expression of both mRNAs and lncRNAs was assessed in human spermatogonia,  
519 spermatocytes, and spermatids. However, since the analysis pipelines did not include transcript  
520 assembly, both studies failed to identify novel genes.

521 The study reported here is original because it aimed at identifying new transcript  
522 isoforms and unknown genes expressed during spermatogenesis in human testicular cells. Thus,  
523 the stringent quality criteria allowed us to assemble and quantify over 25,000 high-confidence  
524 transcripts. These included 11,627 and 766 new transcript isoforms for coding and for  
525 noncoding genes, respectively, as well as 511 completely novel unannotated multi-exon  
526 transcripts. Of particular interest is that more than 85% of these unknown genes showed  
527 preferential expression in spermatocytes and spermatids; this finding clearly illustrates that  
528 adult male germ cells constitute an important reservoir for gene discovery purposes (Chalmel  
529 *et al.*, 2014; Chocu *et al.*, 2014). It is also noteworthy that fewer than 15% of these transcripts  
530 would have met the expression cutoff we used if our analysis had included total testis only:  
531 most (~70%) were indeed identified because of their high expression in spermatids and, to a  
532 lower extent (~16%), in spermatocytes. This demonstrates the striking advantage of using  
533 isolated cells, in terms of sensitivity.

534 The 511 NUTs we identified actually correspond to 451 new human genes. Their virtual  
535 absence of protein-encoding potential strongly suggests that most, if not all, encode new  
536 lncRNAs. In agreement with previous reports in different species (Laiho *et al.*, 2013;

537 Soumillon *et al.*, 2013; Chalmel *et al.*, 2014), most lncRNAs showed preferential expression  
538 in human postmeiotic spermatids. Furthermore, a subset of lncRNAs that are expressed during  
539 meiosis have exons twice as long as other lncRNAs or mRNAs. These longer exons, of  
540 unknown functional relevance, appear to be a conserved phenomenon, since the same  
541 observation has been made for meiotic lncRNAs in rats (Chalmel *et al.*, 2014). As suggested  
542 previously (Naro *et al.*, 2017), one possible explanation could be that lncRNAs critical for  
543 spermatogenesis are stabilized for several days after their synthesis thanks to an intron retention  
544 program in meiotic spermatocytes.

545         Although many essential functions of lncRNAs in various biological processes have  
546 been demonstrated, the biological relevance of the massive expression of such RNAs during  
547 the metamorphosis of haploid spermatids into mature spermatozoa remains unknown. We  
548 cannot rule out the possibility that some of them are junk products of leaky transcription during  
549 meiosis. Nonetheless, their promoters' high degree of conservation — at least as conserved as  
550 protein-coding gene promoters — suggests strong selective constraints at the transcriptional  
551 level and important functions for these molecules (Carninci *et al.*, 2005; Necsulea *et al.*, 2014).  
552 Possible roles for certain spermatozoal lncRNAs during early embryonic development can also  
553 be proposed, given that sperm contain a complex population of transcripts that are delivered to  
554 the embryo upon fertilization (Ostermeier *et al.*, 2004; Jodar *et al.*, 2013; Sandler *et al.*, 2013).  
555 The preferential localization of lncRNAs in the vicinity of protein-coding genes involved in  
556 developmental processes points to a potential role for sperm lncRNAs in regulating expression  
557 of these genes (Ponjavic *et al.*, 2009; Cabili *et al.*, 2011; Chalmel *et al.*, 2014).

558         Finally, some lncRNAs may play fundamental roles during germ cell development  
559 itself (Wen *et al.*, 2016; Hosono *et al.*, 2017; Wichman *et al.*, 2017), even though predicting  
560 functions of lncRNAs on the basis of their sequence remains challenging. Their conservation  
561 at both the sequence and expression levels does, however, hint at important roles for some of

562 them (for review, see (Ulitsky, 2016)). In contrast to mRNAs, and as expected because of their  
563 intrinsic low sequence conservation, we identified syntenic regions for only 37% of human  
564 testicular lncRNAs. Among these, we defined a core group of 131 lncRNAs with syntenic  
565 transcription during spermatogenesis, which suggests they play key roles in germ cell  
566 development. The absence of conservation of the rest, at either the sequence or expression level,  
567 does not necessarily imply they are not functional: the vast majority of lncRNAs indeed have  
568 no homologs in species that diverged more than 50 million years ago (Necsulea *et al.*, 2014;  
569 Hezroni *et al.*, 2015). Additional work relying on expression data obtained in other hominins  
570 will be needed to clarify the functional contribution of such lineage-specific lncRNAs in human  
571 spermatogenesis.

572         That lncRNAs play important biological functions is now supported by many  
573 independent studies reporting their functional implications in almost all the investigated  
574 physiological and pathophysiological biological systems (Tao *et al.*, 2016), including  
575 spermatogenesis (Wen *et al.*, 2016; Hosono *et al.*, 2017; Wichman *et al.*, 2017). It is therefore  
576 important to note that lncRNAs are now presented as a novel class of diagnostic biomarkers  
577 and therapeutic targets for several disorders and pathologies (Lavorgna *et al.*, 2016; Arun *et*  
578 *al.*, 2018). On one hand, due to their cell-type specific expression pattern and their diversity of  
579 functions, GWAS of patients with non-obstructive azoospermia would obviously benefit from  
580 systematically screening for causal genetic variants in evolutionary-conserved lncRNAs  
581 expressed in the germline. On the other hand, those molecules could also be used as interesting  
582 diagnostic biomarkers, probing the presence or absence of specific testicular cell populations  
583 in infertile men with distinct spermatogenic arrests. They might also represent possible  
584 biomarkers that could help to determine the prognosis of hormonal therapy with  
585 hCG/recombinant FSH for infertile men with idiopathic nonobstructive azoospermia. Last but  
586 not least, advances in nucleic acid-based therapies are evolving at a steady rate and have already

587 shown success in several preclinical studies (Arun *et al.*, 2018). Such promising therapeutic  
588 approaches targeting lncRNAs critical for the male germ cell differentiation could pave the  
589 way towards exploring novel male contraceptive options that might be clinically relevant in  
590 the decade ahead (Lavorgna *et al.*, 2016). These examples are just some of the many clinical  
591 applications that could be made in our scientific field. Although many challenges remain to be  
592 addressed, especially regarding functional annotations of lncRNAs, the systematic  
593 characterization of the noncoding transcriptional landscape at play during the human  
594 spermatogenesis process is an indispensable prerequisite to such future directions. In this  
595 context, the novel and abundant data provided by the present study substantiates further the  
596 general basis without which deciphering the extremely complex mechanisms of normal and  
597 failed spermatogenesis in men will remain an utopic challenge.

598

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604

## 605 **Authors’ roles**

606 FC, ADR, and BJ designed the study and wrote the manuscript. FC and ADR supervised the  
607 research. FC prepared, analyzed, and interpreted RNA sequencing data. ADR and BJ prepared  
608 the testicular samples and interpreted sequencing data. BE prepared the testicular samples and  
609 validated expression data. YLB performed the PIT analysis. TAD contributed to the analysis

610 of the common genomic features shared by the assembled transcripts. CLB contributed to the  
611 cross-species data comparison. MP, NDR, BE, and YLB contributed to the manuscript. All  
612 authors approved the final version of the manuscript, and declare that they have no competing  
613 interests.

614

615

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621

## 622 **Conflict of interest**

623 There are no competing interests related to this study.

624

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- 869

870 **Figure legends**

871 **Figure 1** RNA-seq analysis of human testicular cells identifies new genes and transcript  
872 isoforms.

873 Classification of assembled transcripts according to their biotype and their status as known  
874 versus novel. Cuffcompare (Trapnell *et al.*, 2012) was used to compare the 25,161 refined  
875 transcripts with 362,647 reference transcripts to distinguish between known (Cufflinks class  
876 code “=”) and novel (class code “j”) isoforms of known coding and noncoding genes. This  
877 comparison also identified novel unannotated transcripts (NUTs) corresponding to potential  
878 new antisense (class code “x”), intronic (class code “i”) or intergenic (class code “u”) genes.

879 lncRNA: long noncoding RNA

880

881 **Figure 2** Expression dynamics across human testicular cells.

882 **A.** Expression profiles of differentially expressed (DE) transcripts. After statistical filtration,  
883 the 21,264 DE transcripts were clustered into 11 expression patterns (P1-11). The number of  
884 transcripts in each expression pattern is given at the top, and their median profile (red line) is  
885 plotted as well as the first and third quartiles (Q1 and Q3, gray shading). Samples marked in  
886 red and blue correspond to highest and lowest expression values, respectively. Samples marked  
887 in orange indicate a slightly lower expression level than maximum abundance, that is, have the  
888 second highest expression value. LC = Leydig cells; PC = peritubular cells; SC = Sertoli cells;  
889 Spc = spermatocytes; Spt = spermatids; TT = total testis. **B.** Frequency distribution of  
890 expression patterns according to transcript biotype. The percentage of transcripts from each of  
891 the 11 expression patterns (P1-11) is given for known (Cufflinks class code “=”) and novel (class  
892 code “j”) mRNAs and lncRNAs, as well as for intergenic (class code “u”), intronic (class code  
893 “i”), and antisense (class code “x”) NUTs.



894

895 **Figure 3** Syntenic expression during mammalian spermatogenesis.

896 Heatmap representation of 8,457 transcripts with conserved expression during mammalian  
897 spermatogenesis. Each line is a syntenic transcript/region, and each column a  
898 sample/experimental condition. The number of transcripts in each expression pattern (P1-P11)  
899 is given on the left and their relative expression levels in human (present study), rat (Chalmel  
900 *et al.*, 2014), and mouse (Gan *et al.*, 2013; Soumillon *et al.*, 2013) testicular samples are color-  
901 coded according to the scale bar (standardized abundance). Samples used for computing  
902 expression correlation between humans (present study) and rats (Chalmel *et al.*, 2014) are  
903 indicated in red. (p)Spg A/B = (pre)spermatogonia type A/B; (l/p)Spc = (leptotene/pachytene)  
904 spermatocytes; r/eSpt = round/elongated spermatids. FPKM: fragments per kilobase of exon  
905 model per million reads mapped

906

907 **Supplementary Figure S1** Strategy for refinement assembled transcripts.

908 After transcript reconstruction by Cufflinks, a three-layer filtration strategy was applied: First,  
909 only transcripts with an expression of  $\geq 3$  fragments per kilobase of exon model per million  
910 reads mapped (FPKM) in at least one experimental condition (average value of biological  
911 replicates) were considered. Second, transcripts with a length less than 200 nucleotides were  
912 filtered out. Third, novel transcript isoforms (Cuffcompare class “j”) and genes (classes “i”, “u”  
913 and “x”) were required to have at least two exons to be retained.

914

915 **Supplementary Figure S2** Coding potential evaluation of assembled transcripts.

916 First, the protein-encoding potential (PEP) of all 25,161 refined transcripts was evaluated by  
917 four distinct bioinformatics tools, *i.e.* CPAT, HMMER, CPC, and txCdsPredict (Kong *et al.*,

918 2007; Finn *et al.*, 2011; Kuhn *et al.*, 2013; Wang *et al.*, 2013b). Transcripts that were predicted  
919 as protein-coding by two to four tools or by zero or one tool were classified as having high or  
920 low PEP, respectively. Second, we used a proteomics informed by transcriptomics (PIT)  
921 strategy (Evans *et al.*, 2012) in which a testicular tandem mass spectrometry (MS/MS)  
922 proteomics dataset (Kim *et al.*, 2014) was queried against a custom protein database derived  
923 from sequences of assembled transcripts. The frequency distribution of transcripts showing  
924 high or low PEP and being evidenced (PIT+) or not (PIT-) at the protein level is given for  
925 different RNA biotypes. Nearly all mRNAs (97.4%) display a high PEP, and at least one high-  
926 confidence peptide was identified in human testis by MS for 67.9% of them. Most long  
927 noncoding RNAs (lncRNAs) (76.5%) show low PEP, and were rarely identified by the PIT  
928 strategy in the testis proteome. Like lncRNAs, 87.5% of novel unannotated transcripts (NUTs)  
929 display low PEP and were almost never (two of 511) identified in the PIT experiment.

930

931 **Supplementary Figure S3** Differential expression analysis of refined transcripts.

932 Refined transcripts were considered to be differentially expressed (DE) if they exhibited a fold-  
933 change  $\geq 3$  when all samples were compared to one other, and if they showed a significant  
934 expression difference according to a LIMMA statistical test with a false discovery rate-adjusted  
935 F-value of  $\leq 0.05$ . Finally, k-means clustering was used to group the 21,264 retained transcripts  
936 into 11 expression patterns (P1 to P11).

937

938 **Supplementary Figure S4** Expression profiles of the human testicular cell markers.

939 A false-color heatmap summarizes expression profiles of well-known markers for Leydig cells  
940 (green), peritubular cells (orange), Sertoli cells (red), spermatogonia (light blue),  
941 spermatocytes (blue), spermatids (violet), and germ cells (black). Each line corresponds to a

942 transcript and each column is a sample. Most Leydig cell markers showed peak expression in  
943 these cells (P2) or were either detected in all somatic cells (P1; NR5A1) or in total testis  
944 samples (P11; PTGDS and HSD17B3). One peritubular cell marker was preferentially  
945 expressed in these cells (P3; ACTA2) while a second was detected in all somatic cells (P1;  
946 LMOD1). Two Sertoli cell markers were detected in all somatic cells (P1), whereas expression  
947 for most specific markers for mature Sertoli cells peaked in total testis samples (P11). This  
948 could suggest that Sertoli cells undergo substantial dedifferentiation when cultured. The latter  
949 hypothesis would also explain why a robust marker for immature Sertoli cells showed peak  
950 expression in these cells (P4; KRT18). Finally, we found consistent expression profiles for all  
951 22 investigated germ cell markers, including known markers for spermatogonia (P6),  
952 spermatocytes (P5-P9), spermatids (P9-10), and germ cells in general (P6-7). LC = Leydig  
953 cells; PC = peritubular cells; SC = Sertoli cells; Spc = Spermatocytes; Spt = round spermatids.  
954 CCNA1 = cyclin A1; CLU = clusterin; CTSL = cathepsin L; CYP11A1 = cytochrome P450  
955 family 11 subfamily A member 1; DAZL = deleted in azoospermia like; DDX4 = DEAD-box  
956 helicase 4; DHCR7 = 7-dehydrocholesterol reductase; DHH = desert hedgehog signaling  
957 molecule; FGFR3 = fibroblast growth factor receptor 3; GATA4 = GATA binding protein 4;  
958 HSD11B1 = hydroxysteroid 11-beta dehydrogenase 1; HSD17B3 = hydroxysteroid 17-beta  
959 dehydrogenase 3; IGF1 = insulin like growth factor 1; INHA = inhibin subunit alpha; INHBB  
960 = inhibin subunit beta B; KRT18 = keratin 18; LDHC = lactate dehydrogenase C; LMOD1 =  
961 leiomodulin 1; MAGEA4 = MAGE family member A4; MEI1 = meiotic double-stranded break  
962 formation protein 1; MEIOB = meiosis specific with OB-fold; MNS1 = meiosis specific  
963 nuclear structural 1; NR5A1 = nuclear receptor subfamily 5 group A member 1; PHF13 = PHD  
964 finger protein 13; PRM1 = protamine 1; PRM2 = protamine 2; PSAP = prosaposin; PTGDS =  
965 prostaglandin D2 synthase; SOX9 = SRY-box 9; SPO11 = SPO11 initiator of meiotic double  
966 stranded breaks; STAR = steroidogenic acute regulatory protein; SYCP1 = synaptonemal

967 complex protein 1; SYCP2 = synaptonemal complex protein 2; SYCP3 = synaptonemal  
968 complex protein 3; TEX101 = testis expressed 101; TF = transferrin; TNP1 = transition protein  
969 1; TNP2 = transition protein 2; TSPO = translocator protein; TXNDC8 = thioredoxin domain  
970 containing 8; VCAM1 = vascular cell adhesion molecule 1; WT1 = WT1 transcription factor.

971

972 **Supplementary Figure S5** Correlating testicular expression data across technologies.

973 Over-/Underrepresentation of genes from the 11 expression patterns (RNA-seq data) with those  
974 from 13 testicular expression clusters (Microarray data) published by Chalmel and coworkers  
975 are shown (Chalmel *et al.*, 2012). The names of expression patterns (P1-P11) and the  
976 corresponding numbers of genes are indicated on top of each column, while those for  
977 expression clusters (C1-C13) are shown on the left. Each expression cluster is associated with  
978 specific testicular cell populations, including prepubertal testicular cells (C1), Leydig and  
979 peritubular cells (C2-4), Sertoli cells (C5-7), and germ cells (C8-13). Numbers of loci as  
980 observed and expected are given within color-coded rectangles: Red and blue indicate over-  
981 and underrepresentation, respectively, according to the scale bar. Numbers in bold indicate  
982 significantly over-/underrepresented terms. Genes peaking in somatic cells in the RNA-seq  
983 dataset (P1-P4) are significantly overrepresented in clusters C1-C5. P11 (peak expression in  
984 total testis samples) shows high enrichment in C5-C7. P5-P10 (progressive peak expression  
985 through spermatocytes to spermatids) display a gradual enrichment with clusters C8-C13.  
986 Prepub. = prepubertal; LC = Leydig cells; PC = Peritubular cells; SC = Sertoli cells.

987

988 **Supplementary Figure S6** Correlating RNA-seq data from isolated testicular cells with  
989 microarray data from patients with non-obstructive azoospermia.

990 A heatmap displaying the relative expression levels of known transcripts, including 21,409  
991 mRNAs and 102 lncRNAs, as determined in both our RNA-seq analysis of isolated human  
992 testicular cells (left) and a microarray analysis of patients with non-obstructive azoospermia  
993 (NOA) (Malcher et al., 2013) (right) is presented. Each line corresponds to a transcript, and  
994 each column corresponds to an individual sample (left) or to the average of sample replicates  
995 (right). Transcripts are organized according to expression patterns defined in the present study.  
996 Relative expression levels are color-coded according to the scale bars. Transcripts showing  
997 highest expression in isolated spermatocytes and spermatids show a progressive decreasing  
998 signal in biopsies from patients with spermatogenetic arrests at the post-meiotic stage up to  
999 Sertoli-cell only syndrome. Conversely, transcripts overexpressed in isolated somatic cells also  
1000 exhibited strongest expression in biopsies depleted of germ cells. SPC = Spermatocytes; SPT  
1001 = Spermatids; TT = Total testis; POST = spermatogenesis arrested at the post-meiotic stage;  
1002 MEI = spermatogenesis arrested at the meiotic stage; PRE = spermatogenesis arrested at the  
1003 pre-meiotic stage; SCOS = Sertoli cell-only syndrome.

1004

1005 **Supplementary Figure S7** Correlating RNA-seq data from isolated testicular cells with single-  
1006 cell RNA-seq data from patients with normal spermatogenesis or with NOA.

1007 A heatmap displaying the relative expression levels of known transcripts, including 21,696  
1008 mRNAs and 455 lncRNAs, as determined in both our RNA-seq analysis of isolated human  
1009 testicular cells (left) and a single-cell RNA-seq analysis of human testicular cells from patients  
1010 with normal spermatogenesis or with NOA (Wang *et al.*, 2018) (right) is presented. Each line  
1011 corresponds to a transcript, and each column corresponds to an individual sample (left) or to  
1012 the average of several single cells (right). Transcripts are organized according to expression  
1013 patterns defined in the present study. Relative expression levels are color-coded according to

1014 the scale bars. This comparison show a high consistency of expression profiles obtained from  
1015 these two different approaches. UMI = Unique Molecular Identifier.

1016

1017 **Supplementary Figure S8** Gene ontology term enrichment analysis.

1018 Overrepresented biological processes associated with genes from the 11 expression patterns  
1019 (P1-P11) are shown. The names of expression patterns are indicated on top of each column.  
1020 Numbers of loci as observed and expected are given within color-coded rectangles: Red and  
1021 blue indicate over- and underrepresentation, respectively, according to the scale bar. Numbers  
1022 in bold indicate significantly overrepresented terms. P1 is enriched for biological processes  
1023 such as *carbohydrate metabolic process*, *protein glycosylation*, and *vesicle-mediated transport*.  
1024 P2 is associated with gene ontology (GO) terms related to androgen synthesis (*cholesterol*  
1025 *transport*, *steroid biosynthetic process*). P3 is significantly associated with *tube morphogenesis*,  
1026 *angiogenesis*, and *muscle structure development*. P4 is enriched in terms associated with  
1027 *neuron projection* and *pyruvate metabolism*; the latter is essential for providing lactate and  
1028 pyruvate to developing germ cells (Kaiser *et al.*, 2005). P5 is associated with terms related to  
1029 RNA processing, splicing, and transport. P6 was enriched for *piRNA metabolic process*,  
1030 *chromatin organization*, and *DNA methylation involved in gamete generation*. P6-7 is  
1031 associated with *meiotic nuclear division* and *DNA repair*. P7-P9 are over-represented in GO  
1032 terms related to flagellum formation such as *cilium assembly* and *cilium movement*. Most  
1033 germline expression patterns (P6-P10) are enriched in *reproduction* and *spermatogenesis*.

1034

1035 **Supplementary Figure S9** Testicular gene expression and sex chromosomal localization. An  
1036 ideogram of the X (panel A) and Y (panel B) chromosomes as well as the localization of  
1037 transcripts from the 11 expression patterns P1-P11 are shown. For each expression pattern, the

1038 chromosomal positions of transcripts are displayed as vertical lines that are color-coded  
1039 according to their corresponding biotype (blue = protein-coding; red = lncRNAs; violet = NUT;  
1040 gray = other biotypes). Numbers of loci as observed and expected are given within color-coded  
1041 rectangles: Red and blue indicate over- and underrepresentation, respectively, according to the  
1042 scale bar. Numbers in bold indicate significant over-/underrepresentation ( $P$  value  $\leq 0.05$ ). P1  
1043 is enriched whereas P5 and P7-P8 are depleted for X-linked genes. A substantial transcriptional  
1044 reactivation of the sex chromosomes is observed in P9-P10.

1045 **Supplementary Figure S10** Quantitative PCR validation of eight NUTs.

1046 Histograms represent expression profiles of candidate genes (+/- SEM) relative to GAPDH  
1047 mRNA levels. These experiments confirm the expression profiles of selected transcripts and,  
1048 more importantly, validate the existence of these newly identified genes.

1049

1050 **Supplementary Figure S11** A subgroup of meiotic lncRNAs have longer exons.

1051 A. Classification of lncRNAs and NUTs according to their genomic features. All 21,264 DE  
1052 transcripts underwent multicomponent analysis followed by model-based clustering according  
1053 to typical genomic features of lncRNAs: expression level (Max. abundance), expression  
1054 specificity (Shannon entropy), sequence conservation, percentage of GC content, number ( $N^\circ$ )  
1055 of exons, cumulative (Cum.) exon length, average (Av.) exon size, and protein-encoding  
1056 potential (PEP). Gray dots indicate mRNAs and colored dots lncRNAs and NUTs. This resulted  
1057 in the classification of lncRNAs and NUTs into eight subgroups (clusters 1-8), including cluster  
1058 6, containing transcripts that are much longer because their exons are longer.

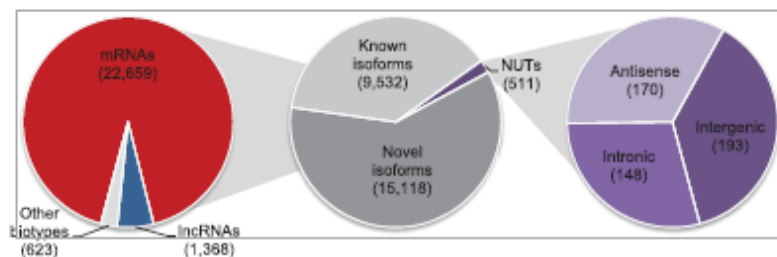
1059 B. Violin plot representation of selected genomic features for all differentially expressed  
1060 mRNAs and lncRNAs as well as for a subgroup (cluster 6) of lncRNAs and NUTs: sequence  
1061 conservation (phastCons score), number of exons, transcript length (in nucleotides, nt) and

1062 exon length (nt). Transcript size in cluster 6 is significantly larger than that of other lncRNAs  
1063 ( $P < 3.10^{-74}$ , Wilcoxon signed-rank test) or of known mRNAs (2275 nt;  $P < 2.10^{-11}$ ). These  
1064 transcripts have a number of exons similar to that of other lncRNAs, while their exon length is  
1065 more than five times that of known mRNAs ( $P < 7.10^{-89}$ ) and of other lncRNAs (302 nt;  $P <$   
1066  $6.10^{-68}$ ). Evolutionary sequence conservation is also lower in noncoding transcripts than in  
1067 mRNAs ( $P < 9.10^{-48}$ ).

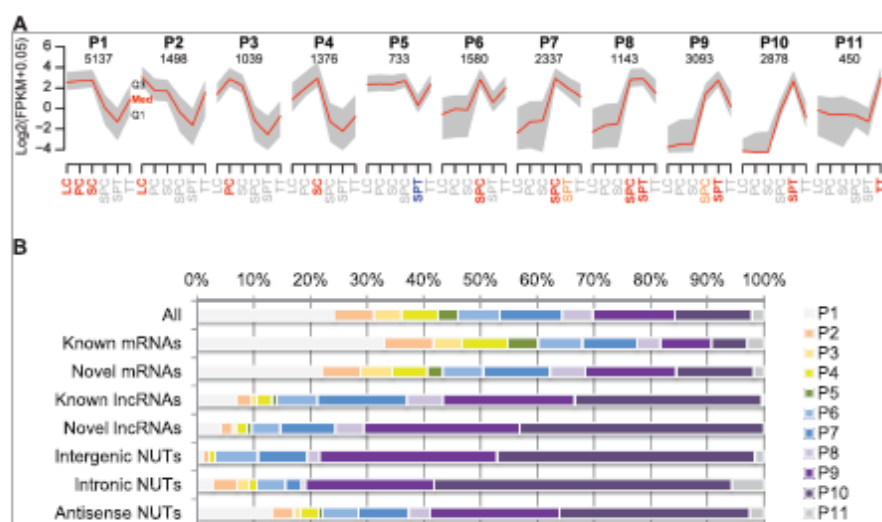
1068 C. Overrepresentation of lncRNAs and NUTs from cluster 6 across expression patterns. The  
1069 enrichment ( $-\log[p\text{-value}]$ , hypergeometric test) is shown and the number of lncRNAs and  
1070 NUTs from cluster 6 reported in brackets for each expression pattern (P1-P11). Cluster 6 is  
1071 significantly associated with lncRNAs preferentially transcribed during meiosis (P6).



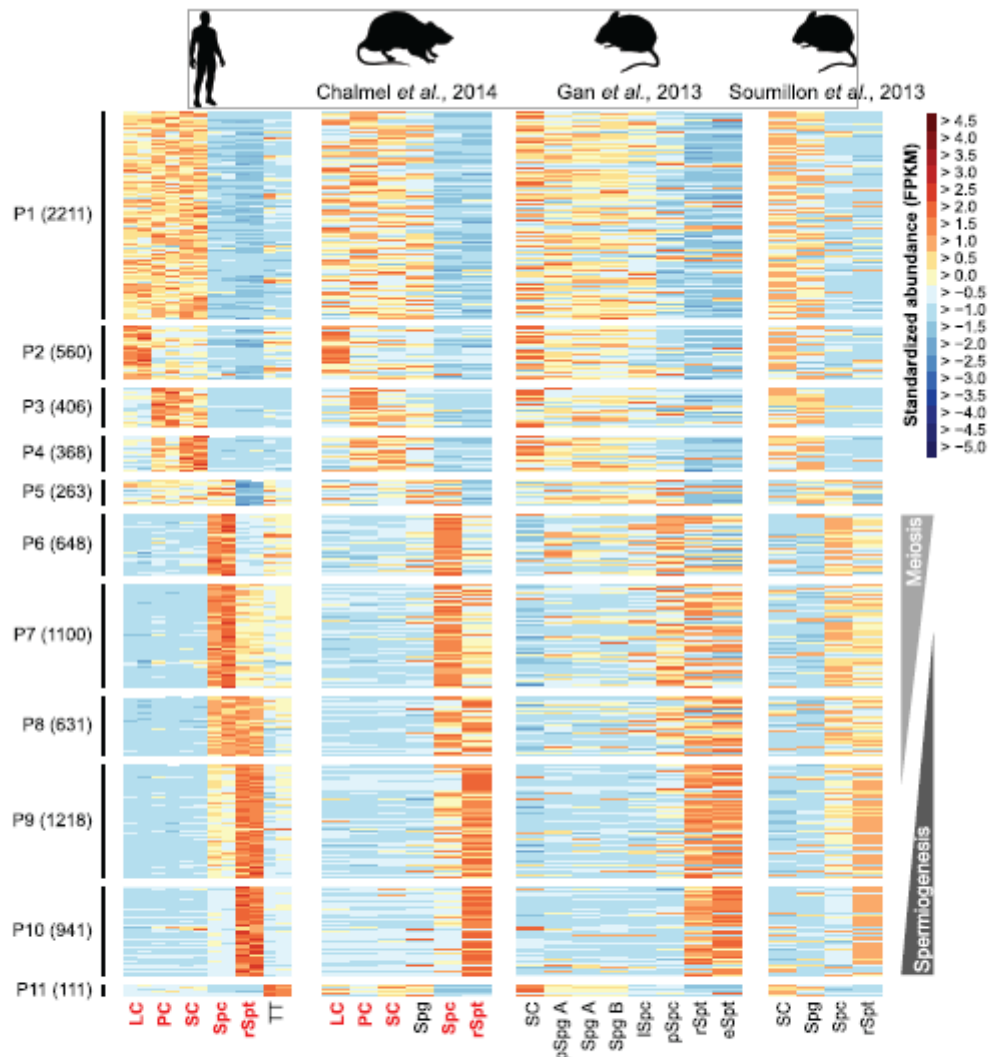
## Figures



**Figure 1** RNA-seq analysis of human testicular cells identifies new genes and transcript isoforms. Classification of assembled transcripts according to their biotype and their status as known versus novel. Cuffcompare (Trapnell *et al.*, 2012) was used to compare the 25 161 refined transcripts with reference transcripts to distinguish between known (Cufflinks class code '=') and novel (class code 'j') isoforms of known coding and noncoding genes. This comparison also identified NUTs corresponding to potential new antisense (class code 'x'), intronic (class code 'i') or intergenic (class code 'u') genes. lncRNA: long noncoding RNA.



**Figure 2** Expression dynamics across human testicular cells. **A.** Expression profiles of DE transcripts. After statistical filtration, the 21 264 DE transcripts were clustered into 11 expression patterns (P1–P11). The number of transcripts in each expression pattern is given at the top, and their median profile (red line) is plotted as well as the first and third quartiles (Q1 and Q3, gray shading). Samples marked in red and blue correspond to highest and lowest expression values, respectively. Samples marked in orange indicate a slightly lower expression level than maximum abundance, that is, have the second highest expression value. LC = Leydig cells; PC = peritubular cells; SC = Sertoli cells; Spc = spermatocytes; Spt = spermatids; TT = total testis. **B.** Frequency distribution of expression patterns according to transcript biotype. The percentage of transcripts from each of the 11 expression patterns (P1–P11) is given for known (Cufflinks class code '=') and novel (class code 'j') mRNAs and lncRNAs, as well as for intergenic (class code 'u'), intronic (class code 'i'), and antisense (class code 'x') NUTs.



**Figure 3 Syntenic expression during mammalian spermatogenesis.** Heatmap representation of 8457 transcripts with conserved expression during mammalian spermatogenesis. Each line is a syntenic transcript/region, and each column a sample/experimental condition. The number of transcripts in each expression pattern (P1–P11) is given on the left and their relative expression levels in human (present study), rat (Chalmel et al., 2014) and mouse (Gan et al., 2013; Soumillon et al., 2013) testicular samples are color-coded according to the scale bar (standardized abundance). Samples used for computing expression correlation between humans (present study) and rats (Chalmel et al., 2014) are indicated in red. (p)Spg A/B = (pre)spermatogonia type A/B; (l/p)Spc = (leptotene/pachytene) spermatocytes; r/eSpt = round/elongated spermatids. FPKM: fragments per kilobase of exon model per million reads mapped

# Table

**Table 1** Sequence and expression conservation of testicular genes.

	Differentially expressed	Syntenic regions	Syntenic & detected	Syntenic & correlated
<b>Total</b>	21 264	18 847	15 119	8457
<b>mRNAs</b>	18 915	17 848	14 652	8179
<b>lncRNAs</b>	1303	479	181	113
<b>NUTs</b>	484	179	46	20
Other biotypes	562	341	240	145

Statistics of sequence conservation in the rat (Syntenic regions), expression detection in the rat samples (Syntenic and detected), and expression conservation in the rat (Syntenic and correlated) are reported for all DE human transcripts and for distinct transcript biotypes. lncRNA, long noncoding RNA; NUT, novel unannotated transcribed region.