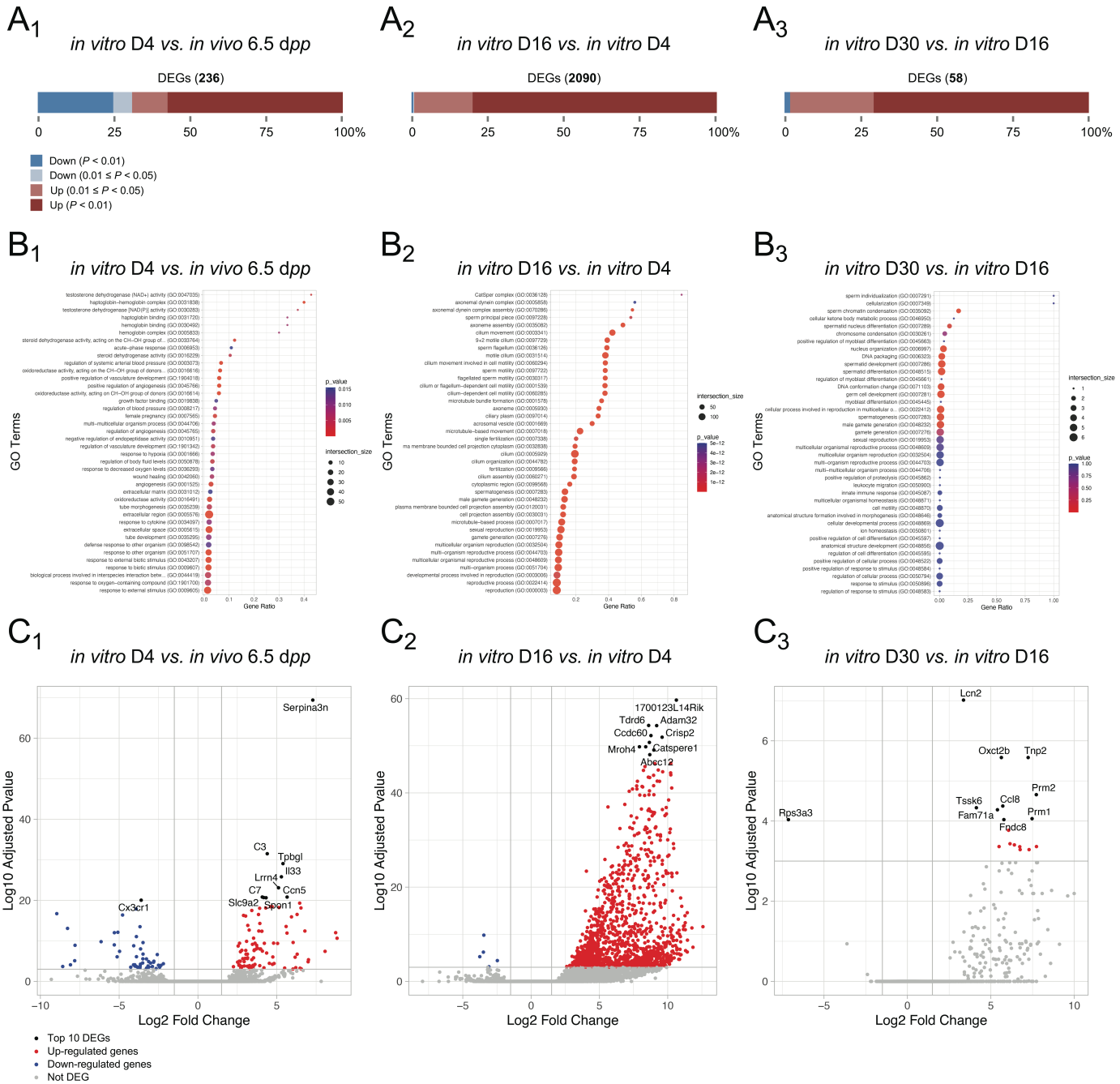

D1

Gene	log2-fold change	padj
Serpina3n	7.290392442	3.81E-70
C3	4.398405948	3.14E-32
Tpbgl	5.398050673	8.69E-30
Il33	5.297191589	1.55E-26
Lrrn4	5.115747952	7.98E-24
Ccn5	5.659965946	1.54E-21
C7	4.082993797	1.54E-21
Slc9a2	4.173599256	2.05E-21
Spon1	4.324510915	2.32E-21
Cx3cr1	-3.603404932	9.17E-21

D2

Gene	log2-fold change	padj
*1700123L14Rik**	10.63299052	2.01E-60
Tdrd6	8.603602312	4.86E-55
Adam32	9.191426689	5.28E-55
Ccdc60	8.773493307	6.86E-53
Crisp2	9.588885136	1.52E-52
Catspere1	8.657762597	1.94E-51
Mroh4	7.929953276	1.64E-50
Gm21269	8.390690487	1.64E-50
4930444F02Rik	8.985747662	7.91E-50
Abcc12	8.688149945	7.76E-49

D3

Gene	log2-fold change	padj
Lcn2	3.362252085	9.64E-08
Tnp2	7.236796178	2.61E-06
Oxct2b	5.631676490	2.61E-06
Prm2	7.726642269	2.20E-05
Ccl8	5.716861850	4.23E-05
Tssk6	4.133715345	4.67E-05
Fam71a	5.396506079	5.25E-05
Prm1	7.471844296	8.75E-05
Fndc8	5.782249903	9.24E-05
Rps3a3	-7.131679005	9.24E-05

Supplementary Figure S4. Gene-level differential expression analysis throughout the *in vitro* kinetics of the first spermatogenic wave. (**A**) The proportion of significantly DEGs are shown for the kinetic timepoints of D4 *vs*. 6.5 d*pp* (corresponding to D0) (**A_1**), D16 *vs*. D4 (**A_2**), and D30 *vs*. D16 (**A_3**). The number of DEGs for each comparison is available in order to have an appreciation of the difference in gene expression between the two conditions compared. (**$B_{1\text{-}3}$**) Corresponding GO enrichment dot plot. The 40 GO processes with the largest gene ratios are plotted in order of gene ratio. The size of the dots represents the number of genes in the significant DEGs associated with the GO Terms and the color of the dots represent the p_{adj} values. (**$C_{1\text{-}3}$**) Volcano plots compare the amount of gene expression change to the significance of that change (here plotted as the $\log_{10}$ transformation of the multiple test p_{adj} value),

with each point representing a single gene. The top 10 gene candidates are highlighted in black and by text labeling. The two marginal plots showing the distributions of the log_2-fold changes and negative log10 p_{adj} values are used to show cutoff choices and trade-offs. (**D_{1-3}**) Top ten DEGs with corresponding log_2-fold change and p_{adj} value.

*BioType Conflict, biotypes are flagged as conflicting when annotations from multiple sources for the same genome feature in the same strain are different (biotype annotations that differ among different strains for the equivalent genome feature are considered polymorphisms, not conflicts); DEGs, differentially expressed genes.

Main implications of principal top 10 DEGs

• ***In vitro* D4 *vs. in vivo* 6.5 d*pp. Serpina3n*** is a gene coding for a protease inhibitor cellular response to cytokine stimulus and well known to reduce apoptosis and inflammation. In addition, it was shown that Sertoli cells secrete Serpina3n that inhibits granzyme B-mediated apoptosis (Sipione *et al*., 2006). *Complement component 3* (*C3*) and *C7* encodes proteins that play central roles in the activation of complement system of the innate immune system (at middle and late steps of the complement cascade, respectively) (Janeway *et al*., 2001). Their activation are required for both classical and alternative complement activation pathways. *Il33* is a member of the cytokine family that is upregulated in non-immune cells in response to tissue injury (Liew *et al*., 2016) and is also an activator of the NF-κB signaling pathway (Miller *et al*., 2011). Recently, a study showed that *Il33* mRNA was not detectable in *in vivo* testes with sc-RNA-Seq strategy but was upregulated in interstitial somatic cells of *in vitro* cultured testes (Suzuki *et al*., 2021), supporting the inflammatory response occurring in non-immune cells. In addition, they found an upregulation of *Il33* in somatic cells of *in vitro* cultured testes. IL-33 activates inflammatory responses in cells expressing ST2L receptors, such as T cells, macrophages, mast cells, and innate lymphoid cells (Lu *et al*., 2015); therefore, IL-33 may also be involved in the inflammatory response in *in vitro* cultured testes. *Ccn5* gene encodes a member of the WNT1 inducible signaling pathway (WISP) protein subfamily, which belongs to the connective tissue growth factor (CTGF) family who are characterized by four conserved domains including an insulin-like growth factor-binding domain. Interestingly, testicular cortisol and WISP2 were involved in estrogen-regulated Sertoli cell proliferation (Berger *et al*., 2019). *Cx3cr1* is a gene associated with chemokine receptor activity. The protein is involved in the migration of circulating monocytes to non-inflamed tissues where they differentiate into macrophages and dendritic cells and acts as a negative regulator of angiogenesis, probably by promoting macrophage chemotaxis (Lokka *et al*., 2020).

• ***In vitro* D16 *vs. in vitro* D4.** The role of testis-specific *1700123L14Rik* gene (*Nup50B*) in spermatogenesis is currently unknown but its presence has already been reported by several studies within the mice testicular tissue and could be related to pachytene spermatocytes I (Wang *et al*., 2016). It has been previously reported that a *Nup50* knockout mouse is not viable but that fibroblasts derived from mouse embryos can be kept in culture (Smitherman *et al*., 2000). Although referred to as a pseudogene, *1700123L14Rik* is effectively expressed as indicated in the expression atlas (Papatheodorou *et al*., 2020) and the mouse genome database (Bult *et al*., 2019) and has been previously named *Nup50rel* (Smitherman *et al*., 2000). Nup50B might substitute the canonical *Nup50* paralog at least in mouse fibroblasts. Together, these data indicate that *Nup50* has a crucial function in nuclear pore complexes assembly during mitotic exit (Holzer *et al*., 2021). In mice, *Tdrd6* is known to be gametogenesis stage- and male-specific expressed, essential for spermiogenesis, directly in interaction with the chromatoid bodies structure components Mili and Miwi (Vasileva *et al*., 2009), and mediates early steps of spliceosome maturation in primary spermatocytes, more specifically in prophase I (Akpınar *et al*., 2017). *Adam32* is a gene predominantly expressed in the testis coding to sperm surface membrane protein that is involved in sperm-egg plasma membrane adhesion and fusion during fertilization (Nishimura *et al*, 2007; Choi *et al*., 2003). *Crisp2* (*Tpx-1*) is a gene specifically expressed in male haploid germ cells and associated with the regulation of some ion channels' activity and thereby regulate calcium fluxes during sperm capacitation and the acrosome reaction (Busso *et al*., 2007). *Catspere1* encodes a subunit of the pH-dependent CatSper calcium channel, a complex involved in Sperm cell hyperactivation and sperm motility (Chung *et al*., 2017). Despite its unknown functions,

Mroh4 shows testis-specific expression in EST Profile (UniGene) and Gene Expression Atlas (EMBLEBI). Within testicular cell types, *Mroh4* is enriched in spermatocytes and spermatids in mice (Zhou *et al*., 2017). *Gm21269* gene encodes the protein Gm21269, localized in the cytoplasm and nuclei of pachytene spermatocyte I during meiosis (Chadourne *et al*., 2021). *Abcc12* (*Mrp9*) is a member of the superfamily of ATP-binding cassette transporters. In mouse and boar sperm, MRP9 protein is clearly and exclusively localized in the sperm midpiece (Ono *et al*., 2007).

• ***In vitro* D30 *vs*. *in vitro* D16.** *Lcn2* encodes a protein that belongs to the lipocalin family that transport small hydrophobic molecules such as lipids, steroid hormones and retinoids (Kang *et al*., 2017; De La Chesnaye *et al*., 2020). *Tnp2* plays a key role in the replacement of histones to protamine in the elongating spermatids of mammalsin condensing spermatids, loaded onto the nucleosomes, where it promotes the recruitment and processing of protamines, which are responsible for histone eviction (Meistrich *et al*., 2003). *Oxct2b* is a haploid-specific gene regulated by a CRE-like element and bound to a testis-specific CREM isoform (Somboonthum *et al*., 2005). In mice, the transcription of *Prm1* and *Prm2* is known to initiates shortly after the completion of meiosis in round spermatids (Mali *et al*., 1989) and ceases about a week later in early elongated spermatids when all transcription stops (Kierszenbaum and Tres, 1975). Protamines substitute for histones in the chromatin of sperm during the haploid phase of spermatogenesis; they compact sperm DNA into a highly condensed, stable and inactive complex (Bao and Bedford, 2016). *Ccl8* is a chemokine gene coding to a protein involved in immunoregulatory and inflammatory processes (Van Coillie *et al*., 1999). *Tssk6* is a gene coding for a serine/threonine protein kinase that is required for postmeiotic chromatin remodeling and male fertility (Spiridonov *et al*., 2005). *Fam71a* is a testis-specific gene. As development stages progress, the number of gene transcripts increases and are at highest expression levels in adults. The action of the protein is not well known but, during, gene transcript levels of FAM71D is located in sperm flagella, increase dramatically throughout spermatogenesis and development of the testis, and is functionally involved in sperm motility (Ma *et al*., 2017). *Rps3a3* is a pseudogene coding for the ribosomal protein S3A3.

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