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Sertoli-cell-specific knockout of connexin 43 leads to multiple alterations in testicular gene expression in prepubertal mice

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SUMMARY

A significant decline in human male reproductive function has been reported for the past 20 years but the molecular mechanisms remain poorly understood. However, recent studies showed that the gap junction protein connexin-43 (CX43; also known as GJA1) might be involved. CX43 is the predominant testicular connexin (CX) in most species, including in humans. Alterations of its expression are associated with different forms of spermatogenic disorders and infertility. Men with impaired spermatogenesis often exhibit a reduction or loss of CX43 expression in germ cells (GCs) and Sertoli cells (SCs). Adult male transgenic mice with a conditional knockout (KO) of the *Gja1* gene [referred to here as connexin-43 (*Cx43*)] in SCs (SC*Cx43*KO) show a comparable testicular phenotype to humans and are infertile. To detect possible signaling pathways and molecular mechanisms leading to the testicular phenotype in adult SC*Cx43*KO mice and to their failure to initiate spermatogenesis, the testicular gene expression of 8-day-old SC*Cx43*KO and wild-type (WT) mice was compared. Microarray analysis revealed that 658 genes were significantly regulated in testes of SC*Cx43*KO mice. Of these genes, 135 were upregulated, whereas 523 genes were downregulated. For selected genes the results of the microarray analysis were confirmed using quantitative real-time PCR and immunostaining. The majority of the downregulated genes are GC-specific and are essential for mitotic and meiotic progression of spermatogenesis, including *Stra8, Dazl* and members of the DM (*dsx* and *map-3*) gene family. Other altered genes can be associated with transcription, metabolism, cell migration and cytoskeleton organization. Our data show that deletion of *Cx43* in SCs leads to multiple alterations of gene expression in prepubertal mice and primarily affects GCs. The candidate genes could represent helpful markers for investigators exploring human testicular biopsies from patients showing corresponding spermatogenic deficiencies and for studying the molecular mechanisms of human male sterility.

INTRODUCTION

A significant decline in human male reproductive function associated with diseases of the male genital tract (azoospermia, cryptorchidism, testicular cancer) has been reported for the past two decades (Asklund et al., 2004; Povey and Stocks, 2010; Pointis et al., 2011). In addition, recent studies revealed that intercellular junctions, specifically gap junctions, might be structures of interest and be involved in this phenomenon (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Pointis et al., 2011).

Gap junction channels connect neighboring cells in nearly all tissues; they permit an exchange of metabolites, signaling molecules and ions, and regulate numerous physiological functions such as cell proliferation, differentiation and apoptosis (Goodenough et al., 1996). Each channel is composed of two hemichannels, called connexons, separately contributed by the two participating cells. The connexons themselves are hexamers formed by self assembly of six connexins (CXs) (Bruzzone et al., 1996). To date, the family of CX genes consists of 20 members in the mouse and 21 members in the human genome (Sohl and Willecke, 2004).

In addition to their classical role, recent studies have reported on the channel-independent functions of gap junctions (Stout et al., 2004). These functions comprise, for example, extrinsic guidance of migrating cells owing to CX-mediated cell adhesion, as well as intracellular processes. Among the connexins, CX43 in particular has been shown to exert effects on migration by interfering with receptor signaling, cytoskeletal remodeling and tubulin dynamics (Kameritsch et al., 2011). Undocked gap junction hemichannels might also exert physiological roles because connexons are involved in: the release of, for example, ATP and NAD^+, Ca^{2+} wave propagation, cell-volume control and the passage of survival signals within the tissue (Spray et al., 2006; Pointis et al., 2010). CXs might also function as scaffold proteins for the attachment of cytoplasmic partner molecules (Huang et al., 1998) and as signaling molecules (Kardami et al., 2007). CX43 interactions with the cytoskeleton have been suggested by several previous studies. The CX43 C-terminus has been shown to bind TJP1 (Giepmans et al., 2001a), a scaffold protein that facilitates linkage of the membrane with the actin cytoskeleton (Itoh et al., 1997; Hartsock and Nelson, 2008). CX43 can also directly bind to tubulin (Giepmans et al., 2001b). In a very

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recent study, CX43 was shown to modulate cell migration by regulating microtubule dynamics associated with abnormal actin stress fiber organization (Francis et al., 2011).

CX43 is the predominant testicular CX in most species, including humans (Risley et al., 1992; Steger et al., 1999; Batias et al., 2000; Bravo-Moreno et al., 2001; Brehm et al., 2002). In adult men, CX43 protein localization has been investigated in testes both with normal spermatogenesis and associated with different forms of spermatogenic impairment (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Roger et al., 2004; Brehm et al., 2006; Steiner et al., 2011). As in rodent testes, CX43 immunoreactivity was generally present between Leydig cells (LCs) (Pérez-Armendariz et al., 1994) and, within the seminiferous epithelium, CX43 was found to form an integral component of the Sertoli cell (SC)-SC junctional complexes that represent the anatomical base of the blood-testis barrier (BTB) (Risley et al., 1992). Further components of the BTB, in addition to the CX-based gap junctions, are cadherin (CDH)-based adherens junctions and occludin (OCLN)-based tight junctions (Pointis et al., 2005). There is further evidence that CX43 plays a role in the coordination of changes in SC junctional permeability as well as in SC and germ cell (GC) differentiation (Pelletier, 1995). Furthermore, the synchronization of GC proliferation and differentiation is mediated through the gap junctional network (Decrouy et al., 2004).

Within the normal human (and murine) seminiferous epithelium, CX43 immunoreactivity is localized between SCs and GCs (spermatogonia and primary spermatocytes) and along the SC-SC junctional complexes (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003). Some information is available concerning the presence of gap junctions and expression of CX43 in seminiferous tubules of pathologic human testes. By means of freeze fracture, no gap junctions were detected in feminized human testes (Nagano and Suzuki, 1976) and the presence of atypical testicular gap junctions was observed in infertile patients (Bigliardi and Vegni-Talluri, 1977). Gap-junction-like cell-membrane specializations were rare in hypospermatogenic and aspermatogenic testes (Schleiermacher, 1980). In addition, testicular histology of these infertile adult patients frequently reveals spermatogenic defects that are associated with heterogeneous SC populations. For example, tubules showing (1) spermatogenic arrest at the spermatogonial level and those with (2) SC-only (SCO) syndrome are characterized by immature populations of SCs expressing fetal differentiation markers, such as anti-Muellerian-hormone (AMH) and cytokeratin 18 (CK18) (Bergmann and Kliesch, 1994), and exhibit a reduction or even loss of CX43 in GCs and SCs (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003). Finally, alterations of CX43 expression have been correlated to the development of human carcinoma in situ (CIS) of the testis and testicular tumors (Brehm et al., 2002; Roger et al., 2004; Brehm et al., 2006).

Taken together, there now exist many studies and review articles (e.g. Pointis and Segretain 2005; Pointis et al., 2005; Sridharan et al., 2007b; Pointis et al., 2010; Gilleron et al., 2011; Weider et al., 2011a; Pointis et al., 2011) that support functional roles of CX43 in the regulation of human spermatogenesis: (1) CX43 forms intercellular channels between SCs and proliferating GCs, indicating that this CX is involved in the physiological maturation and proliferation processes of these cell types; (2) alterations of CX43 expression are correlated with testicular disorders in men; for example, individuals with CIS and seminoma, the most frequent type of human GC tumors, show a reduction of CX43 expression in SCs and tumor cells. In CIS tubules, the reduction of CX43 expression was shown to be regulated at the transcriptional level and accompanied with a dedifferentiation of SCs (Brehm et al., 2006); (3) moreover, possible mutations in the human *CX43* gene have been discussed because a recent study working with a mutant mouse model of oculodentodigital dysplasia (ODDD) showed impaired spermatogenesis, supporting the possibility of subfertility in ODDD human males (Gregory et al., 2011). However, to date, there is still a lack of information on the molecular mechanisms resulting in the impairment of human male reproductive function through CX disruption.

To further investigate the role of CX43, several knockout (KO) and knock-in (KI) mice have been generated. General KO of the *Cx43* gene leads to cardiac malformation and prenatal death; thus, a functional analysis of spermatogenesis was not possible in this KO mouse line (Reaume et al., 1995). Nevertheless, a severe GC loss in the embryonic tissue has been detected, indicating an indispensable role of CX43-mediated cell coupling for GC development (Juneja et al., 1999). Testes of *Cx43*-null mutant embryos neither allow a normal GC proliferation nor differentiation when grafted under the kidney capsule of adult males (Roscoe et al., 2001). However, the detailed mechanisms that lead to sterility of the male *Cx43* mice in the mentioned studies are still unknown (Pointis et al., 2005).

To study the effects of a deletion of *Cx43* on spermatogenesis, a conditional SC-specific KO of the *Cx43* gene (SC*Cx43*KO) has been generated and revealed that *Cx43* expression in SCs is an absolute requirement for normal testicular development and initiation of spermatogenesis (Brehm et al., 2007; Sridharan et al., 2007a). Compared with the generalized KO (Reaume et al., 1995), SC*Cx43*KO mice are viable, but infertile (Brehm et al., 2007; Sridharan et al., 2007a). Weighing of animals and testes displays no differences in the body weight between SC*Cx43*KO and wild type (WT); however, testes were significantly smaller in the KO males. Furthermore, adult SC*Cx43*KO mice show a significantly reduced number of GCs per tubule, whereas the mean number of SCs per tubule is significantly higher compared with WT (Brehm et al., 2007). Interestingly, histological analysis of adult SC*Cx43*KO mice revealed similar spermatogenic alterations as seen in infertile humans, such as an arrest of spermatogenesis at the level of spermatogonia, or SCO syndrome (Brehm et al., 2007; Sridharan et al., 2007a).

The number of GCs that can be supported by each SC is fixed for each species. This species-specific ratio of GCs to SCs is determined prepubertally by proliferation and apoptosis (Rodriguez et al., 1997; Sharpe et al., 2003). A disruption of cell-cell communication is thought to lead to increased apoptosis and GC loss, demonstrating the significant role of testicular CX to maintain the survival of GCs by regulating intercellular communications between GCs and adjacent supporting cells (Lee et al., 2006b). Dye-coupling studies demonstrated that CX43 participates in the coupling between SCs and between SCs and GCs, with the dye transfer from SCs to GCs being mostly unidirectional (Decrouy et al., 2004). Thus, the observed phenotype in adult SC*Cx43*KO mice might, for instance, be due to: (1) a disrupted direct connection; for example, a disturbed flow of signaling molecules and high-energy metabolites from SCs to

GCs via gap junction channels containing CX43 or (2) channel-independent functions.

To get a deeper insight into the molecular mechanisms and possible signaling pathways leading to the observed histological phenotype and infertility in adult SC*Cx43*KO mice, a genome-wide gene expression profiling of 8-day-old WT and SC*Cx43*KO mice was performed. Mice of that age have been chosen because (1) the morphology of the testis was still comparable between the two genotypes and (2) murine spermatogenesis has been initiated, indicated by the first appearance of type B spermatogonia (Bellvé et al., 1977).

RESULTS

GC number is significantly reduced in 8-day-old SC*Cx43*KO mice

Comparison of testis weight revealed no significant differences between WT and SC*Cx43*KO mice on day 8 post partum (p.p.). However, morphological and histological examination detected obvious differences in the composition of intratubular cells between the two genotypes (Fig. 1A-D). GC number was three times higher in testes of WT mice, whereas the number of SCs (and LCs) was not affected by the deletion of *Cx43* (Figs 1, 2).

Differential gene expression in WT and KO mice

Microarray analysis revealed 658 significantly regulated genes [$P \leq 0.05$ and $-2 \geq$fold change (FC)≥ 2]. Of these, 135 genes showed higher gene expression values, whereas 523 displayed lower gene expression values in SC*Cx43*KO mice compared with WT mice. Annotation was possible for 475 downregulated and 106 upregulated genes. Table 1 gives an overview of the highest ranked genes and the functional category they belong to. All significantly regulated genes can be found in supplementary material Table S1A,B.

Hierarchical clustering of the 658 significantly regulated genes shows two clusters clearly differing between WT and KO mice (Fig. 3). Within each cluster, the gene expression profiles of the biological replicates of WT1-3 and KO1-3 mice were homogenous, indicating reproducibility of the microarray results. Within the WT group, two subclusters are present, i.e. WT1 versus WT2/3. This small difference in the expression profile can be explained because WT2 and 3 were from the same litter, whereas WT1 was another litter from the same mouse.

Gene ontology (GO) of the differentially regulated genes revealed clusters of biological categories representing functional annotation groups. The upregulated genes were assigned to three clusters, involved in chemotaxis, cell migration and cytoskeleton organization (Table 2). The downregulated genes could be assigned to 16 clusters, with the majority of these genes being involved in spermatogenesis, cell cycle/meiosis, transcription, sexual differentiation, cell migration, DNA modification and translation (Table 2). The detailed functional annotation clusters can be found in supplementary material Table S2A,B.

Pathway analysis for functions with the differentially regulated genes resulted in spermatogenesis as a top function. The 29 genes within spermatogenesis were mainly downregulated and involved in subfunctions such as meiosis of male GCs or expression of RNA, highlighting the influence of the KO gene *Cx43* on the different aspects of spermatogenesis (Fig. 4).

Additionally, pathway analysis revealed ten networks each containing interactions of nine or more genes from the list of differentially regulated genes (supplementary material Table S3). The top network contained the KO gene *Cx43* and genes that were overall involved in infectious disease, gene expression and antigen presentation. The network is depicted in Fig. 5A, showing the interaction of *Cx43* with these genes. Remarkably, most of the genes in the network involving *Cx43* are downregulated and involved in gametogenesis, gene activation, quantity of gap junctions, cell division, cell differentiation and cell migration.

Further interactions of *Cx43* are indicated in the predefined canonical 'GC-SC junction pathway' (Fig. 5B). It is shown that interaction between SCs and GCs can be supported by protein complexes and/or proteins such as CDHE- or CDHN-CTNNB1 (CDHE/N-CTNNB1), PONSIN-AFADIN-NECTIN2, ITGα6β1, TNFR and TGFβR.

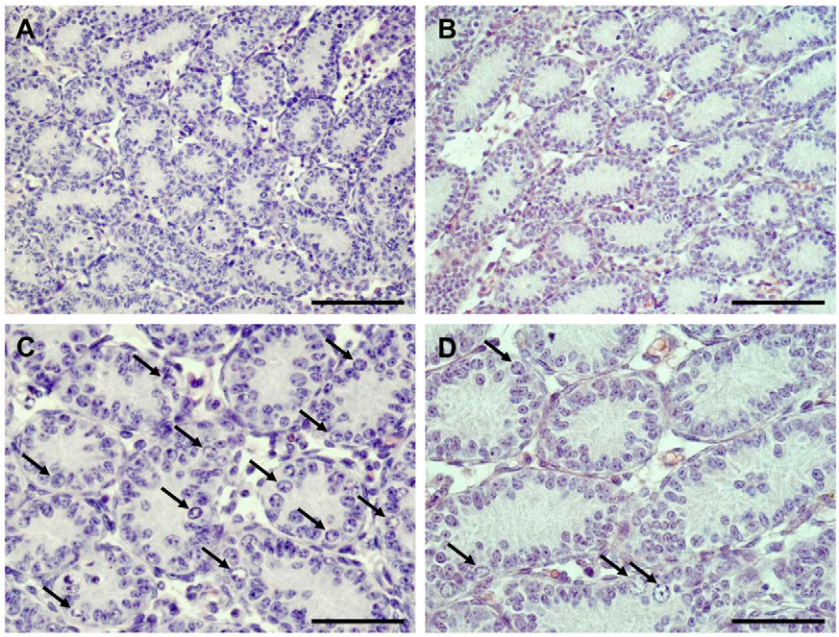

Fig. 1. Representative histology of WT and SC*Cx43*KO mouse testis at day 8 p.p. H&E staining of a WT mouse testis (A,C) shows SC and type A and B spermatogonia (arrows). By contrast, in seminiferous cords of an SC*Cx43*KO mouse (B,D), only single GCs (arrows) are detectable. Scale bars: (A,B) 100 µm; (C,D) 50 µm.

Table 1. Overview of the highest ranked up- and downregulated genes

Gene symbol	Entrez ID	Gene name	*t*-test *P*-value	RP *P*-value	FC KO/WT
Upregulation (FC>3.0)					
Cell migration/chemotaxis					
Acta1	11459	actin, alpha 1, skeletal muscle	0.01	0.00	11.99
Pcp4	18546	Purkinje cell protein 4	0.00	0.00	59.79
Ccl17	20295	chemokine (C-C motif) ligand 17	0.00	0.00	3.02
Ccl9	20308	chemokine (C-C motif) ligand 9	0.02	0.00	3.13
S100a9	20202	S100 calcium binding protein A9 (calgranulin B)	0.00	0.00	3.02
Tns1	21961	tensin 1	0.02	0.00	3.11
Mid1	17318	midline 1	0.03	0.00	3.52
Metabolism					
Adh7	11529	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	0.01	0.00	3.62
Adpgk	72141	ADP-dependent glucokinase	0.00	0.00	21.50
Receptors					
Arhgef12	69632	Rho guanine nucleotide exchange factor (GEF) 12	0.01	0.00	6.02
Dkk2	56811	dickkopf homolog 2 (Xenopus laevis)	0.00	0.00	6.01
Esr2	13983	estrogen receptor 2 (beta)	0.04	0.00	3.01
Vmn1r208	171252	vomeronasal 1 receptor 208	0.03	0.00	3.55
Protein transport					
Cadps	27062	Ca^{2+}-dependent secretion activator	0.01	0.00	3.21
Kpna2	16647	karyopherin (importin) alpha 2	0.00	0.00	7.36
Defense response					
Defb9	246079	defensin beta 9	0.02	0.00	3.66
Downregulation (FC<–5.7)					
Spermatogenesis					
Stra8	20899	stimulated by retinoic acid gene 8	0.03	0.00	–26.70
Sohlh1	227631	spermatogenesis and oogenesis specific basic helix-loop-helix 1	0.00	0.00	–6.98
Dazl	13164	deleted in azoospermia-like	0.04	0.00	–6.04
Rnf17	30054	ring finger protein 17	0.01	0.00	–9.64
Reproductive system development/cell cycle					
Hnf1b	21410	HNF1 homeobox B	0.00	0.00	–6.32
Lhx8	16875	LIM homeobox protein 8	0.01	0.00	–7.42
Sox3	20675	SRY-box containing gene 3	0.00	0.00	–13.30
Tex11	83558	testis expressed gene 11	0.02	0.00	–15.44
Piwil2	57746	piwi-like homolog 2 (Drosophila)	0.03	0.00	–5.89
Taf7l	74469	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.03	0.00	–6.66
Sycp2	320558	synaptonemal complex protein 2	0.01	0.00	–8.90
Eaf2	106389	ELL associated factor 2	0.00	0.00	–6.85
Hormad1	67981	HORMA domain containing 1	0.02	0.00	–7.68
Regulation of transcription					
Hmx1	15371	H6 homeobox 1	0.04	0.00	–20.53
Otx1	18423	orthodenticle homolog 1 (Drosophila)	0.03	0.00	–9.38
Utf1	22286	undifferentiated embryonic cell transcription factor 1	0.02	0.00	–7.27
Snai3	30927	snail homolog 3 (Drosophila)	0.00	0.00	–6.11
Grhl1	195733	grainyhead-like 1 (Drosophila)	0.01	0.00	–6.83
Pou3f3	18993	POU domain, class 3, transcription factor 3	0.00	0.00	–6.13
Zfy2		zinc finger protein 2, Y linked (Zfy2)	0.00	0.00	–5.84
Dmrtb1	56296	DMRT-like family B with proline-rich C-terminal, 1	0.04	0.00	–68.15
Dmrtc2	71241	doublesex and mab-3 related transcription factor like family C2	0.00	0.00	–7.31
Transport					
Crabp1	12903	cellular retinoic acid binding protein I	0.00	0.00	–12.69
Clic6	209195	chloride intracellular channel 6	0.02	0.00	–6.92
Immune response					
Zbp1	58203	Z-DNA binding protein 1	0.00	0.00	–9.70

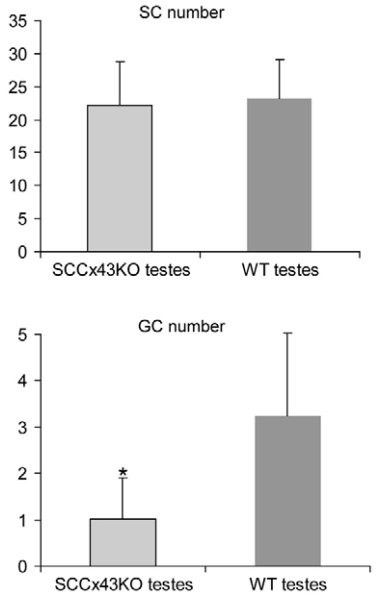

Fig. 2. Comparison of SC and GC numbers of WT and SC*x43*KO mouse testis at day 8 p.p. Average number of SCs and GCs per cord, comparing the two genotypes at day 8 p.p. GC number is significantly reduced in KO mice. Error bars represent standard deviation (s.d.). The asterisk indicates the statistical significant difference ($P<0.05$) between the WT and SC*x43*KO mice. Seminiferous cords of WT mice contain 3.24±1.78 GCs and 23.17±5.92 SCs. Cords of KO mice have 1.02±0.89 GCs and 22.17±6.64 SCs.

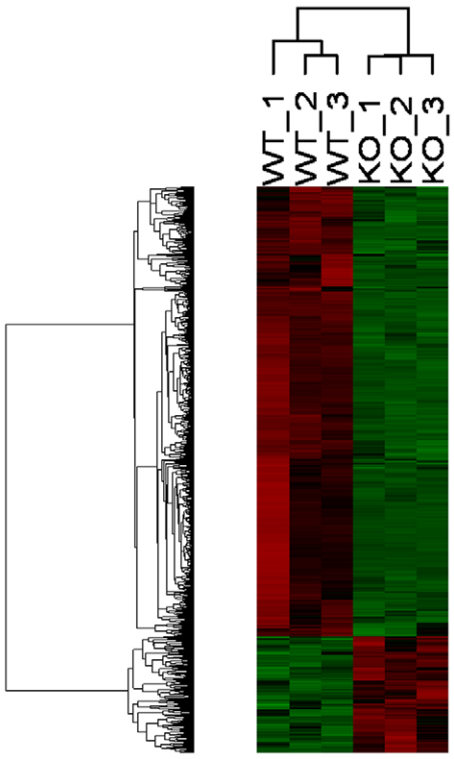

Fig. 3. Hierarchical clustering of 658 significantly regulated genes. Genes are depicted in rows and samples in columns. Green indicates downregulation; red indicates upregulation.

When overlaying gene expression data onto the canonical pathway, only the interaction via CDHE/N-CTNNB1 is affected by downregulation of *Cdh1*. The putative connections of CX43 and the canonical pathway are given as orange arrows in Fig. 5B. CX43 can be related to this canonical pathway by interaction with ACTB, TJP1 and with a complex consisting of proto-oncogene products SRC and MTMR2.

The family of CX genes comprises 20 members in the mouse (in the groups *Gja-Gje*). To assess whether other junction genes, especially the members of the gap junction family, were also affected from the KO of *Cx43*, the gene expression of those junction genes available on the microarray was determined (supplementary material Table S4). Beside *Cx43*, none of the remaining junction genes reached statistical ($P\leq0.05$) or biological ($-2\geq FC\geq2$) significance. Nevertheless, *Gja6*, *Gjb3*, *Gjc1*, *Gjd3* and *Tjp3* were biologically downregulated (FC ranging from −1.3 to −1.8), with *Gjb3*, *Gjc1* and *Gjd3* being statistically significant ($P\leq0.05$). The interaction of the junction genes with *Cx43* is depicted in Fig. 5C.

The majority of genes associated with spermatogenesis are GC-specific

The majority of the differentially expressed genes can be associated with spermatogenesis. The majority of these genes are exclusively expressed in GCs (Table 3).

Validation of the microarray data by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was applied to validate the microarray data. In total, 19 candidate genes, showing different ranges of values (up, down, unchanged), were selected to be analyzed. Among the chosen genes, some are mainly associated with the BTB formed by SCs, such as *Cdh2*, *Cld11*, *Ocln* and *Tjp1*. Other genes were selected for their FC value or their importance in spermatogenesis. After applying the calculated correction factors, the qRT-PCR data were mostly consistent with the microarray results, displaying a significant correlation between the log ratios of the considered genes in the qRT-PCR and the corresponding log ratios in the microarray analysis with correlation coefficient of 0.86 and significance P-value <0.01. Relative mRNA levels of *Sohlh1*, *Ddx25*, *Cdkn2a*, *Stra8*, *Dmrt6*, *Sox3*, *Dazl*, *Cx43*, *Piwil2*, *Tex11* and *Sycp2* were lower in the KO mice compared with WT animals (Fig. 6).

Confirmation of *Cx43* deletion by immunohistochemistry

CX43 immunostaining at day 8 p.p. revealed no immunoreactivity in seminiferous cords of SC*x43*KO mice (Fig. 7), whereas, in seminiferous cords of the prepubertal WT males, a typical immature and diffuse adluminal signal was detectable.

Immunohistochemistry confirmed qRT-PCR results at protein level

Selecting two highly downregulated GC-specific genes [*Stra8* (Fig. 8) and *Dazl* (Fig. 9)] and one gene associated with the BTB and with no significant alteration at mRNA level [*Ocln* (Fig. 10)], we applied immunohistochemistry (IHC) to examine their level of protein expression.

In seminiferous cords of the 8-day-old WT mice, many spermatogonia and first preleptotene spermatocytes showed an

Table 2. Functional annotation clustering of up- and downregulated genes

Functional group	Genes	*P*-value range min.	*P*-value range max.
Upregulated genes			
Chemotaxis	*Robo1*, *S100a9*, *Zic1*, *Apbb1*, *Ccl17*, *Ccl6*, *Themis*, *Ccl9*, *Cfi*, *Ltb*	0.002	0.063
Cell migration	*Vav3*, *Tns1*, *Robo1*, *Mdga1*, *S100a9*, *Esr2*, *Apbb1*	0.005	0.041
Cytoskeleton organization	*Acta1*, *S100a9*, *Cnn1*, *Apbb1*, *Tacc2*	0.046	0.186
Downregulated genes			
Spermatogenesis	*Adad1*, *Asz1*, *Ccnb1ip1*, *Chrna7*, *Clgn*, *D1pas1*, *Dazl*, *Ddx25*, *Dmc1*, *Dnd1*, *Figla*, *Foxl2*, *Ggt1*, *Hmga1*, *Mael*, *Morc1*, *Msh4*, *Ovol1*, *Piwil2*, *Rad51c*, *Rec8*, *Rnf17*, *Sohlh1*, *Sohlh2*, *Sox3*, *Spata19*, *Spata5*, *Stra8*, *Sycp2*, *Sycp3*, *Taf7l*, *Tcfl5*, *Tdrd1*, *Tex11*, *Tex15*, *Tle3*, *Zbtb16*, *Zpbp*, *Rad51c*	4.43E–14	2.48E–12
Cell cycle/meiosis	*Ccnb1ip1*, *Cdkn2a*, *Clgn*, *Cpeb1*, *Dmc1*, *Fanci*, *Fsd1*, *Hells*, *Hormad1*, *Hormad2*, *Mael*, *Msh4*, *Nek3*, *Ovol1*, *Piwil2*, *Rad51*, *Rad51c*, *Rbm38*, *Rec8*, *Smc1b*, *Stra8*, *Suv39h2*, *Sycp1*, *Sycp2*, *Sycp3*, *Tdrd1*, *Tex11*, *Tex15*, *Trnp1*, *Uba3*, *Uhmk1*	2.36E–15	0.004
Transcription	*Cbx2*, *Cdkn2a*, *Dazl*, *Eaf2*, *Ehf*, *Fgf8*, *Foxl2*, *Gabpa*, *Glis1*, *Grhl3*, *Gtpbp4*, *Hells*, *Hmga1*, *Hmx1*, *Hnf1b*, *Htatip2*, *Irf6*, *Isl1*, *Krt17*, *Lin28a*, *Mael*, *Nfib*, *Ovol1*, *Ovol2*, *Pax8*, *Piwil2*, *Pou2f2*, *Pou3f3*, *Rictor*, *Rnf128*, *Sall4*, *Satb1*, *Shh*, *Six2*, *Spink12*, *Stra8*, *Tdrd1*, *Utf1*, *Zbtb16*, *Zbtb42*	0.001	0.041
Sexual differentiation	*Ascl2*, *Ccnb1ip1*, *Cdh1*, *Chrna7*, *Col4a3bp*, *Dmc1*, *Fgf8*, *Foxf1a*, *FoxlL2*, *Gabpa*, *Cx43*, *Hnf1b*, *Kif3a*, *Krt8*, *Lhx8*, *Msh4*, *Ovol2*, *Sall4*, *Shh*, *Six2*, *Sohlh1*, *Spint1*, *Stra8*, *Sycp2*, *Tex11*, *Tex15*	0.003	0.049
Cell migration	*Asz1*, *Chrna7*, *Dab1*, *Fgf8*, *Cx43*, *Isl1*, *Krt14*, *Krt17*, *Lhx2*, *Lhx6*, *Mesp1*, *Ovol2*, *Pou3f3*, *Reln*, *Shh*, *Taf7l*, *Tnn*	0.004	0.091
DNA modification and translation	*Arih1*, *Asb9*, *Ate1*, *Dazl*, *Dnahc12*, *Fbxo15*, *Foxl2*, *Hells*, *Herc3*, *Krt17*, *Lin28a*, *Mael*, *Oas2*, *Piwil2*, *Psma8*, *Psmb9*, *Rffl*, *Rictor*, *Rnf128*, *Satb1*, *Siah1b*, *Stra8*, *Suv39h2*, *Tdrd1*, *Uba3*, *Uba6*, *Ube2e3*, *Uchl1*, *Usp12*, *Usp18*, *Usp37*, *Usp45*, *Zyg11b*	0.004	0.181

Clusters with at least one significantly overrepresented GO category ($P<0.05$) have been taken into account. For upregulated genes, three clusters could be determined and for downregulated genes the clusters have been summarized into five representative main groups.

intensive staining for STRA8 (Fig. 8A). By contrast, only single cells of the SC*Cx43*KO mice revealed an immunoreactivity for STRA8 (Fig. 8B). Note the immunonegative spermatogonia in Fig. 8B (arrows). In adult WT mice, a stage-specific immunoreaction for STRA8 can be seen (Fig. 8C), whereas no immunoreaction at all can be detected in seminiferous tubules that show an arrest of spermatogenesis at the level of spermatogonia (arrow) in adult mutants (Fig. 8D).

Regarding the distribution pattern of DAZL, there is also an obvious difference between the two genotypes (Fig. 9A-D). The number of stained cells is drastically reduced in 8-day-old SC*Cx43*KO mice, confirming results obtained by qRT-PCR. Note the immunonegative spermatogonia in Fig. 9B (arrows). In adult mice, a clear immunoreaction for DAZL can be seen in spermatogonia and spermatocytes (Fig. 9C). By contrast, in seminiferous tubules showing an arrest of spermatogenesis at the level of spermatogonia (arrows) in adult SC*Cx43*KO mice, not even spermatogonia show a DAZL immunostaining (Fig. 9D, arrows).

OCLN represents an integral SC membrane protein and is involved in the formation of the BTB. Deletion of *Cx43* in SCs results in no obvious changes in OCLN protein synthesis and localization when comparing 8-day-old WT (Fig. 10A) and mutant (Fig. 10B) animals.

Evaluation of DMRT7 protein synthesis using immunofluorescence

Immunofluorescence (IF) revealed no immunoreactivity for DMRT7 in most seminiferous tubules in 15-day-old KO mice (Fig. 11B), whereas the WT mice showed a specific staining in most pachytene spermatocytes (Fig. 11A). In adult WT males, a specific staining can be detected in nearly all spermatocytes (Fig. 11C), whereas only paraffin autofluorescence is seen in KO tubules (Fig. 11D). The insert in Fig. 11C shows paraffin autofluorescence in interstitial LCs in negative controls.

DISCUSSION

The prevalence of human couple infertility is extremely high and in many countries affects one in seven couples (Sharpe, 2010). Although the nature and exact proportion of the predominant cause of the problem remains controversial, the World Health Organization (WHO) reports that, in nearly 40% of cases the cause can be attributed to the female, in 20% to the male and in 25% to both; however, in 15% the cause still remains unknown (WHO, 1987). On the basis of these figures, the incidence of human male factor infertility in the general population is approximately 7%, making it more prevalent than type 1 and 2 diabetes mellitus combined, and this proportion is expected to rise. With at least one third of infertile men being diagnosed as having idiopathic infertility associated with impaired spermatogenesis, there is an urgent need to better understand the status of male reproductive health, especially in Europe and industrialized countries (EU Strategic Research Plan, 2010).

Impaired spermatogenesis (with e.g. an arrest of spermatogenesis at different stages and/or SCO syndrome) can result from a variety of causes. For example, genetic defects like Klinefelter syndrome and cryptorchidism exhibit a fivefold increased risk for the development of testicular cancer, which represents the most

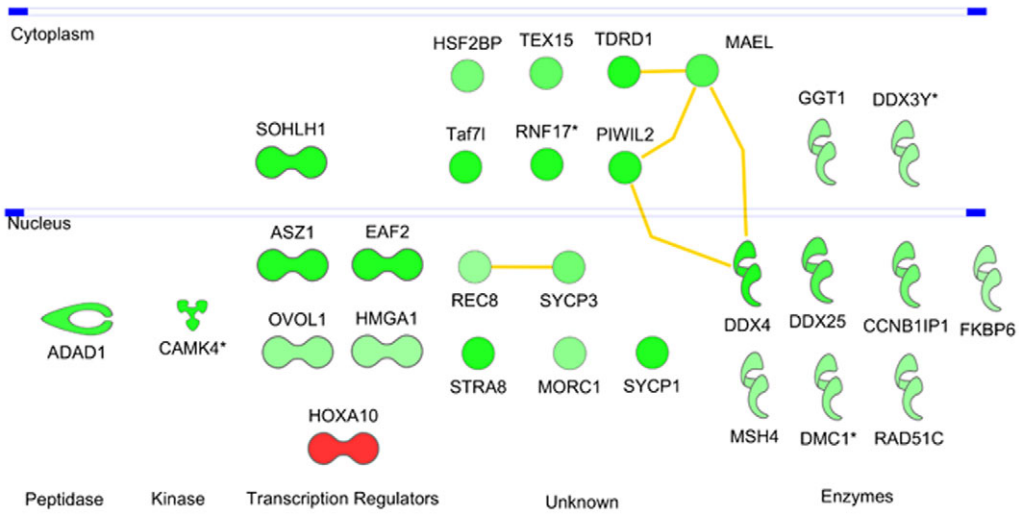

Fig. 4. Interaction network of functions involved in spermatogenesis. Analysis of all regulated genes in IPA revealed spermatogenesis as a top function. Known protein-protein interactions for the involved genes are highlighted by yellow lines. Colors represent the FC in expression of the genes. Red, significant upregulation with FC>2; green, significant downregulation with FC<–2. Genes in dark green show higher FC compared with genes in light green (e.g. *Sohlh1* with FC –7.0 and *Rec8* with FC –2.4).

common cancer among men (Møller and Skakkebaek, 1999; Skakkebaek et al., 2001; Asklund et al., 2004). In approximately 25% of these men, an aberrant spermatogenic appearance is also evident in the 'assumed normal' contralateral testis. In a series of prospective and well-standardized studies over the past 10 years, it has been found that the prevalence of an abnormally low sperm count in young men is as high as 15-20% (Jørgensen et al., 2006; Andersson et al., 2008). Testicular biopsies of these men often reveal mixed atrophy showing seminiferous tubules with qualitatively normal spermatogenesis directly adjacent to tubules with spermatogenic arrest and/or SCO syndrome (Steger et al., 1996; Steger et al., 1998).

Recent studies revealed that the gap junction protein CX43 seems to be involved in different forms of spermatogenic disorders (Steger et al., 1999; Brehm et al., 2002; Defamie et al., 2003; Steiner et al., 2011) and in the pathogenesis of human testicular germ cell tumors (Brehm et al., 2002; Steiner et al., 2011), so a transgenic mouse model (SC*Cx43*KO) has been established to investigate cellular and molecular mechanisms that are essential for male GC differentiation and to identify aberrant signaling pathways in 'male factor infertility due to impaired spermatogenesis' (Brehm et al., 2007; Sridharan et al., 2007a).

In the present study, using the microarray technology and after applying the calculated correction factors, several candidate genes possibly involved in the observed reduction of GC number, in preventing the initiation of spermatogenesis and thus in the disturbed progression beyond mitosis of the remaining GCs in adult SC*Cx43*KO mice have been detected. These genes (connected with a CX43 deficiency) could also represent helpful markers for investigators exploring the molecular mechanisms of human male sterility. GO analysis revealed chemotaxis, cell migration and cytoskeleton organization as the major functional clusters affected within the upregulated genes. Interestingly, genes associated to the group of cell migration are also highly present within the group of downregulated genes, indicating an important role for CX43-meditated communication in this functional gene class.

Within the seminiferous epithelium, CX43 is thought to be important for both the SC-SC functional synchronization and the SC-GC crosstalk (Pointis et al., 2010). Here, direct contact-dependent junctional pathways play a pivotal role in the regulation of GC and SC proliferation and differentiation, and the maintenance of the male phenotype (Mruk and Cheng, 2004). In addition to (intermingled) CX43 gap junctions, the other junctions are composed of specialized proteins implicated in cell adhesion, the regulation of paracellular diffusion, the establishment and maintenance of cell polarity, the regulation of cell attachment, and the regulation of actin proteins (Cheng and Mruk, 2002; Derangeron et al., 2009; Cheng et al., 2011). CX43 interactions with the cytoskeleton have been suggested by several previous studies. The C-terminus of CX43 has been shown to bind TJP1 (Giepmans et al., 2001a), facilitating the linkage of the membrane with the actin cytoskeleton (Itoh et al., 1997; Hartsock and Nelson, 2008). CX43 can also directly bind to tubulin (Giepmans et al., 2001b). In a very recent work, CX43 was shown to modulate cell migration by regulating microtubule dynamics associated with abnormal actin stress fiber organization (Francis et al., 2011). In this context, in the present study, *Tuba3* [encoding for TUBA3 (FC –4.5)] and different Krt genes (*Krt14*, *17*, *24* and *8*; encoding for keratins) were significantly downregulated, implying alterations in cytoskeleton dynamics.

During embryonal development, human SCs contain both CK18 and vimentin; however, only the latter persists in adult testis (Franke et al., 1979) and CK18 expression nearly disappears after the 20th week of gestation. CK18 intermediate filaments are completely lost around birth, thereby signaling the earliest transition of SCs to a mature state. CK18 expression, therefore, indicates a state of undifferentiation (Rogatsch et al., 1996; Franke et al., 2004). Interestingly, co-expression of vimentin together with CK18 occurs in SCs of adult testes under pathological conditions

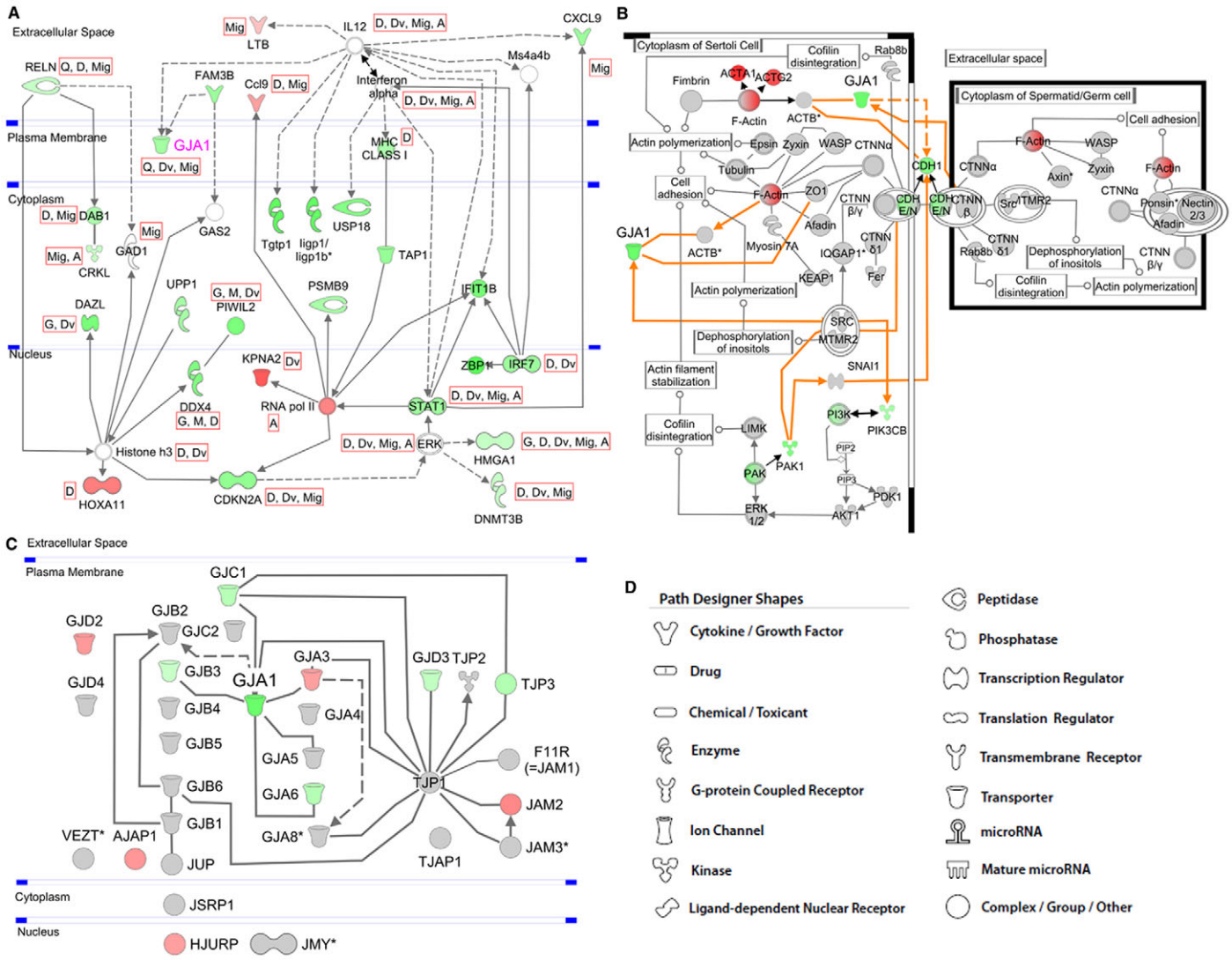

Fig. 5. The interaction networks and canonical pathways were generated with IPA. A description of the used symbols is given in D. The symbols are colored according to the FC of gene expression in KO versus WT. A solid line represents a direct relationship, and a dashed line an indirect relationship between molecules. An asterisk denotes the presence of two or more probes on the chip for the respective gene. (A) Interaction network of *Cx43* (*Gja1*) with genes deriving from the top network of differentially regulated genes. Genes and/or molecules are connected via indirect or direct connections. The gene symbols are colored according to their FC: red, significant upregulation with FC>2; green, significant downregulation with FC<–2. Some relevant functions have been selected and assigned to the respective genes symbols. These are: G, gametogenesis; A, activation of gene(s); Q, quantity of gap junctions; M, meiosis; D, differentiation; Dv, division; Mig, migration of cells. (B) Canonical GC-SC junction pathway. The predefined canonical pathway 'GC-SC junction' has been expanded by adding *Cx43* (*Gja1*) and its relations to pathway-related genes (orange lines). Only the section in which GJA1 is involved is shown. Red, significant upregulation with FC>2; green, significant downregulation with FC<–2. (C) Network of members of the family of CX genes and other junction-related genes. Genes with an FC higher than 1.2 are colored in red, and genes with an FC lower than –1.2 are colored in green. (D) Symbols used for graphical presentation of pathways.

(Steger et al., 1996; Kliesch et al., 1998) concomitant with a reduction or loss of CX43 (Brehm et al., 2002). Moreover, in a recent study, tubulin was identified as a 14-3-3β binding partner, and thus is essential for cell adhesion within the seminiferous epithelium, cell polarity of SCs and normal spermatogenesis (Graf et al., 2011).

Further GO groups include strongly downregulated genes involving spermatogenesis, cell cycle/meiosis, transcription, sexual differentiation, cell migration, and DNA modification and translation, confirming a requirement for *Cx43* expression of these features.

Interestingly, among the group of downregulated genes that can be associated with spermatogenesis, a very high percentage of GC-specific genes were present (Ruggiu et al., 1997; Dai et al., 1998; Reber and Cereghini, 2001; Sapiro et al., 2002; Saunders et al., 2003; Li et al., 2005; Raverot et al., 2005; Ballow et al., 2006; Pangas et al., 2006; Cheng et al., 2007; Lin et al., 2007; Anderson et al., 2008; Zhou et al., 2008). This was surprising, because an SC-specific gene

Table 3. List of differentially expressed GC-specific genes associated with spermatogenesis

Gene	Testicular localization	Publication	KO-model-derived functions in male spermatogenesis	Publication
Ovol1	Primary and secondary spermatocytes	Dai et al., 1998	Regulation of pachytene progression of male GCs	Li et al., 2005
Spag6	Sperm	Sapiro et al., 2002	Maintenance of sperm flagellar motility and structural integrity of mature sperm	Sapiro et al., 2002
Sohlh1	Prespermatogonia, spermatogonia	Ballow et al., 2006	Regulation of spermatogonial differentiation into spermatids	Ballow et al., 2006
Lhx8	GC	Pangas et al., 2006	Possible role in regulation of spermatogonial differentiation into spermatocytes	Pangas et al., 2006; Ballow et al., 2006
Dazl	Spermatogonia type B, spermatocytes	Ruggiu et al., 1997	Progression through meiotic prophase and/or GC differentiation	Saunders et al., 2003
Stra8	Spermatogonia type A and B, preleptotene spermatocytes, early leptotene spermotocytes	Zhou et al., 2008	Regulation of meiotic initiation	Anderson et al., 2008
Hnf1b	GC	Reber and Cereghini, 2001	Homozygous vHNF1 mice die before gastrulation	Reber and Cereghini, 2001
Sox3	Spermatogonia	Raverot et al., 2005	Differentiation of spermatogonia and/or initiation and progression of meiosis	Raverot et al., 2005
Zpbp2	Sperm	Lin et al., 2007	Morphological normal sperm formation and/or fertility	Lin et al., 2007
Taf7l	Spermatogonia, spermatocytes, round spermatids	Cheng et al., 2007	Development of normal sperm count and motility	Cheng et al., 2007
Tex11	GC	Wang et al., 2001	Essential for spermatid development and completion of spermatogenesis	Tsai-Morris et al., 2004; Yang et al., 2008
Sycp2	GC	Wang et al., 2001	Differentiation of zygotene-like spermatocytes into normal pachytene spermatocytes (synaptonemal complex assembly and chromosomal synapsis during male meiosis)	Yang et al., 2006; Yang et al., 2008
Piwil2	GC	Beyret and Lin, 2011	Spermatocytes without PIWI proteins are arrested in the pachytene stage	Beyret and Lin, 2011
Ddx25	GC	Gutti et al., 2008	Post-transcriptional regulator of spermatogenesis (as a component of mRNP particles), controls apoptosis in spermatocytes of adult mice	Gutti et al., 2008

deletion would be expected first to lead to an altered expression pattern related to this cell population. However, our results can be explained by the juxtaposition and dependency of the two cell types, which is interrupted by the deletion of the *Cx43* gene. As shown by dye transfer studies, the coupling between SCs and GCs is unidirectional from SCs to GCs (Decrouy et al., 2004). Together with the observation that the gene expression of mainly GCs is influenced, our results might lead to the assumption that the interruption of poorly characterized mediators of signals from SCs to GCs affect primarily the normal development of early GC populations, preventing the initiation of spermatogenesis. Examples for such GC-specific genes are depicted in Table 3. The physiological relevance of the genes identified by the microarray study is underscored by the finding that at least eight downregulated genes have already been shown to be essential for normal spermatogenesis. Using qRT-PCR, IHC and IF, we were able to verify the microarray results for selected genes of interest, such as *Stra8*, *Dazl* and *Dmrt7*.

Stra8 (FC –26.70) is a vertebrate-specific factor expressed in GCs in response to retinoic acid (RA) (Anderson et al., 2008). STRA8 protein expression at day 8 p.p. is restricted to the spermatogonia and first preleptotene spermatocytes (Zhou et al., 2008). RA is known to be essential for normal spermatogenesis. The actions of RA are mediated through six distinct ligand-dependent transcription factors, including three RA receptors (RARα, β and γ) and three retinoid X receptors (Zhou et al., 2008). It is well known that RA stimulates the expression of *Cx43* (Batias et al., 2000; Tanmahasamut and Sidell, 2005; Vine and Bertram, 2005). A recent publication by Chung et al. further demonstrated that a deletion of RARα leads to an alteration of gap-junction-based cell coupling (Chung et al., 2010). KO studies of the *Stra8* gene revealed its importance for functional spermatogenesis: its deletion leads to infertility of male and female mice. In male KO mice, infertility is caused by a failure in meiotic initiation, blocking the transition into the meiotic prophase (Anderson et al., 2008). The close relation between *Cx43* and *Stra8* is affirmed by their overlapping regulatory mechanisms, their common requirement during GC development and the dependency of *Stra8* expression on *Cx43* expression. Whether this interaction is causally related or the result of altered interactions between GCs and SCs leading to a loss of signaling molecules that are essential for *Stra8* transcription and STRA8 protein synthesis in GCs requires further investigation. However, in a very recent study a close relationship between RA signaling, STRA8 expression and the control of meiotic entry in the human fetal gonad was shown (Childs et al., 2011).

Dazl (FC –6.04), also established as a GC-specific gene, can be detected in the cytoplasm of type B spermatogonia and less abundantly in pachytene, preleptotene and zygotene spermatocytes

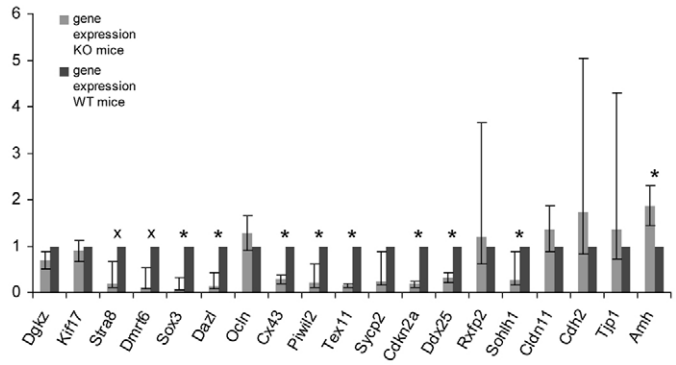

Fig. 6. Relative gene expression of WT mice versus SC*Cx43*KO mice after applying the calculated correction factors. Bars represent mean±s.d. mRNA levels of three independent qRT-PCR experiments. Calculations were realized determining *Actb* and *Hsp90ab1* as housekeeping genes and applying the ΔΔC(t) method. The genes *Sox3*, *Dazl*, *Cx43*, *Piwil2*, *Tex11*, *Ddx25*, *Cdkn2a* and *Sohlh1* were significantly downregulated (*$P<0.05$), whereas the downregulation of *Stra8* and *Dmrt6* was close to significant (x). The only significant upregulation was detected for *Amh*.

(Ruggiu et al., 1997). Like *Stra8*, KO studies revealed a pivotal role for this gene in spermatogenesis. Interestingly, the nature of the *Dazl* KO is very similar to the observed one in SC*Cx43*KO mice. In both murine lines the males are infertile and exhibit a reduced number of GCs, and the remaining GCs show an arrest of spermatogenesis, precluding progression through meiotic prophase (Saunders et al., 2003). A comparison of significantly regulated genes in 5- and 7-day-old testes of WT and *Dazl* KO mice with results from the present study showed that just two genes were significantly regulated in both experiments, namely *Ovol1* and *Nfx2* (Maratou et al., 2004). These results could lead to the conclusion that there might be a synergistic action of *Dazl* and *Cx43*, rather than a shared pathway leading to the meiotic failure observed in the two mutant mouse lines. Moreover, *Dazl* has been demonstrated recently to have a significant contribution in the differentiation of embryonic stem cells into pre- and post-meiotic GCs (Kerr and Cheng, 2010), and humans with spermatogenic failures showed significantly lower levels of *Dazl* transcripts compared with men with normal spermatogenesis (Lin et al., 2001). Furthermore, these data provide evidence for an important role of *Nxf2* and *Ovol1* for GC development beyond mitosis.

The DM family of genes encode a widely conserved transcription factor family; these proteins are involved in sexual differentiation at least along three phyla (Murphy et al., 2007). Mammals express at least seven DM domain genes (*Dmrt1-7*) (Kim et al., 2003). *Dmrt1* is known to be essential for sexual differentiation in mammals (Raymond et al., 2001), whereas little information is available for *Dmrt6* [also known as *Dmrtb1* (FC –68.15)]. Kim et al. detected a very high expression of *Dmrt2*, *5*, *6* and *7* in adult testis (Kim et al., 2007). Because it is known that *Dmrt7* [also known as *Dmrtc2* (FC –7.31)] is essential for male meiosis and spermatogenesis, we explored its protein expression comparing WT and SC*Cx43*KO mice. Because DMRT protein is restricted to pachytene spermatocytes (and sperm), 15-day-old mice were used for this experiment. In these SC*Cx43*KO mice, only single spermatocytes were present and showed a weak signal for DMRT7 protein, suggesting that the reduction of gene expression of DM family members observed in 8-day-old KO males proceeds and is finally abrogated in older KO mice, particularly because *Dmrt* mRNA has also been detected in spermatogonia (Kawamata et al., 2007). This hypothesis was confirmed by DMRT7 IF in adult KO mice. Based on the fact that at least two members of the DM-domain family (*Dmrt6* and *Dmrt7*) show an altered expression pattern, it is very probable that this gene family displays a contribution to the disturbed spermatogenesis and GC loss observed in adult SC*Cx43*KO mice. Furthermore, *Dmrt1* has been linked with human testicular germ cell tumor development (Looijenga et al., 2006) and susceptibility (Kanetsky et al., 2011).

Beyond the genes confirmed by qRT-PCR and IHC or IF, the expression of several more genes, for example *Sohlh1* (FC –6.98), *Sox3* (FC –13.30), *Sycp2* (FC –8.90) and *Piwil2* (FC –5.89), was significantly altered in our study and might also be interesting new candidate genes for comparative human investigations. *Sohlh1* and *Sox3* single KO mice show an arrest of spermatogenesis at the level of spermatogonia and, comparable to the present mouse model, their deletion leads to a disruption of GC differentiation beyond early developmental stages and to infertility (Raverot et al., 2005; Ballow et al., 2006). By contrast, the deletion of *Sycp2* and *Piwil2* leads to a spermatogenic arrest from zygotene to early pachytene stages (Kuramochi-Miyagawa et al., 2004; Yang et al., 2006). The Piwi family genes play essential roles in stem cell self-renewal and gametogenesis (Lee et al., 2006a). Thus, it might be possible that *Cx43* acts as a molecular upstream promoter of *Piwil2* that is in turn known to be able to modulate the expression of stem-cell-specific genes, including *Stra8* or *Cx45*. These genes require further examination to elucidate their possible role in the phenotype of adult SC*Cx43*KO mice and their connection to CX43.

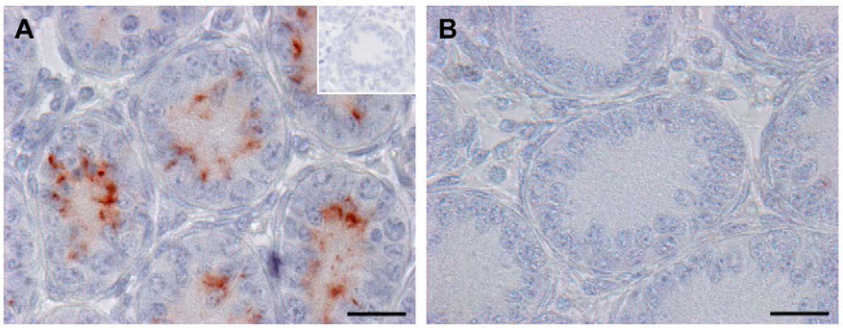

Fig. 7. Confirmation of *Cx43* deletion by IHC. In seminiferous cords of WT mice (A) a typical prepubertal diffuse adluminal staining pattern for CX43 can be seen. By contrast, cords of SC*Cx43*KO mice show no CX43 immunoreactivity at all (B). Insert in A: negative control. Scale bars: 20 μm.

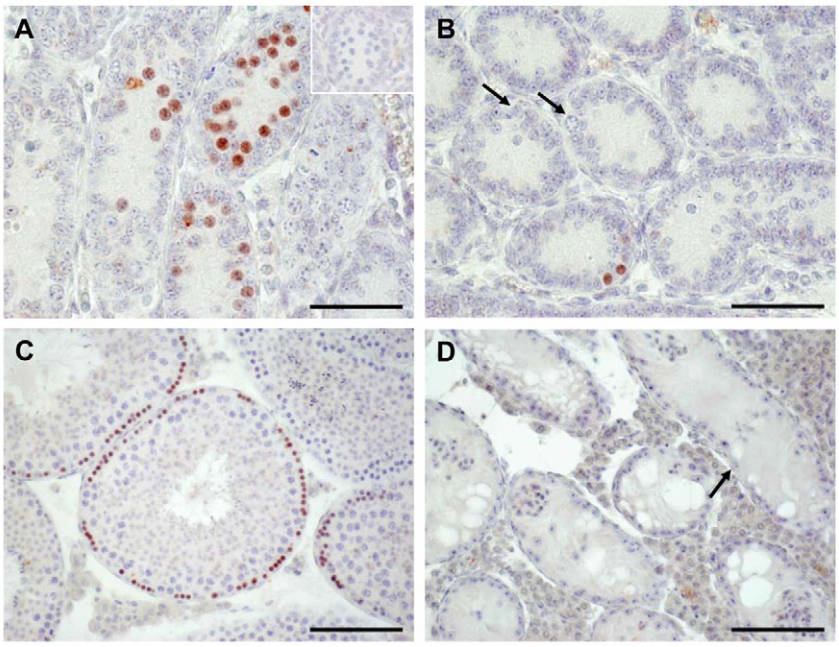

Fig. 8. IHC confirmed the findings obtained by microarray analysis and qRT-PCR for *Stra8* (GCs). The occurrence of STRA8 protein revealed obvious differences between WT (A,C) and SCC*x43*KO (B,D) mice. In seminiferous cords of the 8-day-old WT mice, many spermatogonia and first preleptotene spermatocytes show an intensive staining for STRA8 (A). By contrast, only single cells of the SCC*x43*KO mice revealed an immunoreactivity for STRA8 (B). Note the immunonegative spermatogonia in B (arrows). In adult WT mice, a stage-specific immunoreaction for STRA8 can be seen (C), whereas no immunoreaction at all can be detected in seminiferous tubules of adult mutants showing an arrest of spermatogenesis at the level of spermatogonia (arrow, D). Insert in A: representative negative control for STRA8 experiments. Scale bars: (A,B) 50 μm; (C,D) 100 μm.

CX43 gap junctions between SCs are further known to form an intercellular communication network within the seminiferous epithelium corresponding to an initial 'syncytium-like organization' that allows the tubular coordination of SC metabolism and, indirectly, via metabolic and signaling coupling, the synchronization of GC proliferation and differentiation. At present, it cannot totally be excluded that the failure to initiate spermatogenesis and the observed GC deficiency in adult SCC*x43*KO mice reflects an altered state of SC maturation and a sign for functional immaturity of the SC, as proposed by Sridharan et al. (Sridharan et al., 2007a). Thus, impaired GC development as early as day 8 p.p. might represent underlying abnormalities in SCs, because there exists a reciprocal regulation of SC and GC differentiation, and functional SCs are a prerequisite for normal spermatogenesis (Sharpe et al., 2003; Griswold, 1995). Also, seminiferous tubules of adult humans with different forms of spermatogenic impairment house immature SC populations (Steger et al., 1996). Thus, a disturbed maturation of nursing SCs might consequently result in an asynchronous and altered differentiation of SC-dependent GCs, leading to alterations of candidate gene expression in GCs of SCC*x43*KO mice (and infertile men).

Multiple genes that are essential for BTB formation, such as *Ocln*, *Tjp1* or *Cld11*, were subjected to qRT-PCR analysis (Saitou et al., 2000). In agreement with the microarray data, examination of their

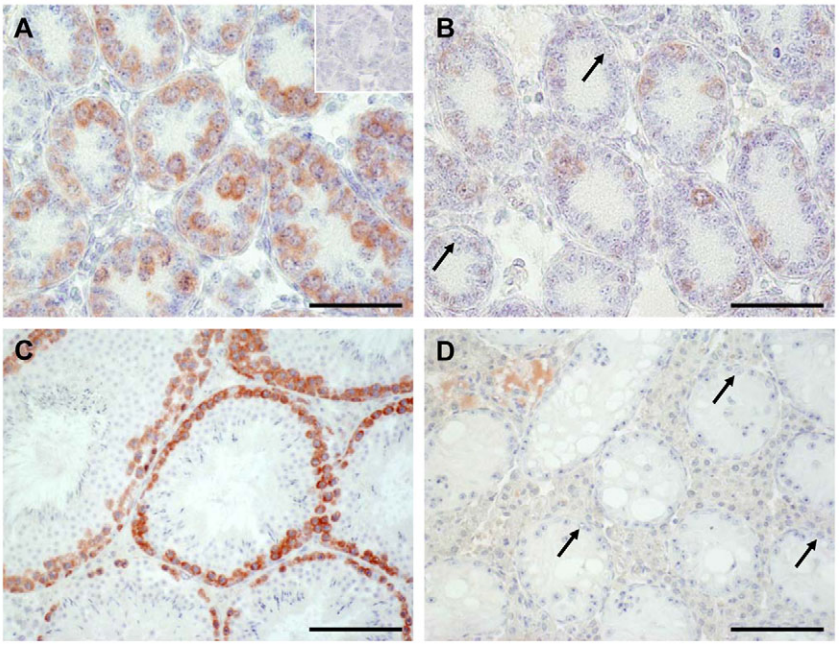

Fig. 9. IHC confirmed also the findings obtained by microarray analysis and qRT-PCR for *Dazl* (GCs). The occurrence of GC-specific DAZL protein revealed obvious differences between WT (A,C) and SCC*x43*KO (B,D) mice. The number of stained cells is drastically reduced in 8-day-old SCC*x43*KO mice (B), confirming results obtained by qRT-PCR. Note immunonegative spermatogonia in B (arrows). In adult mice, a clear immunoreaction for DAZL can be seen in spermatogonia and spermatocytes (C). By contrast, in adult SCC*x43*KO mice not even spermatogonia (arrows) show a DAZL immunostaining (D). Insert in A: representative negative control for DAZL experiments. Scale bars: (A,B) 50 μm; (C,D) 100 μm.

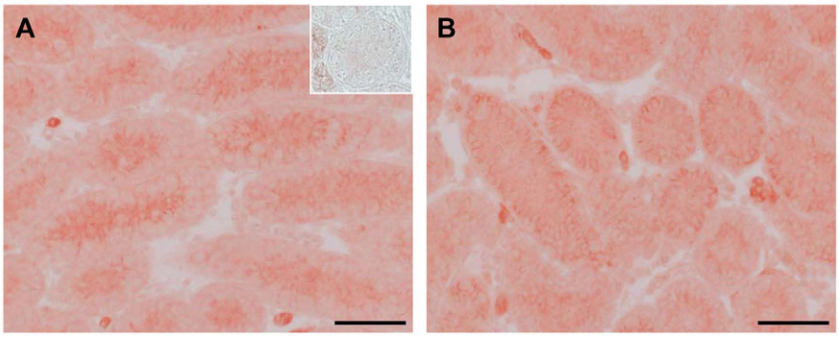

Fig. 10. IHC (without counterstaining) also approved the findings obtained by microarray analysis and qRT-PCR for *Ocln* (SCs). No differences for OCLN are detectable comparing WT (A) and SC*Cx43*KO (B) mice. Insert in A: representative negative control for OCLN experiments. Note faint unspecific binding in LCs. Scale bars: 50 µm.

mRNA expression on day 8 p.p. revealed no significant differences between WT and SC*Cx43*KO mice, although most genes associated with this barrier showed a higher transcription rate in KO mice. Using IHC, localization of OCLN protein seemed to be similar in WT and SC*Cx43*KO mice, implying no functional alterations.

Direct evidence for a possible relationship between CX43, junction dynamics and spermatogenesis derives from Carette et al. (Carette et al., 2010). By using SC*Cx43*KO mice, specific anti-*Cx43* siRNA and gap junction blockers in the SerW3 SC line, this study demonstrated that CX43-based gap junctions participate in the regulation of adherens and tight junction protein expression. Interestingly, increased protein levels of CDHN, β-CTNNB1 and OCLN, but decreased TJP1 levels, were observed in adult KO mice compared with their WT littermates (Carette et al., 2010). Because no significant alterations in *Tjp1* and *Ocln* gene expression profiles have been detected in the present study at day 8 p.p., the observed alterations in TJP1 and OCLN levels in adult KO mice seem to occur after postnatal day 8, together with a functional BTB formation but possibly associated with (1) an impairment in the dynamic process of opening and closing of this barrier and/or (2) permanent BTB closure as suggested by the authors. The close relationship between gap and adherens junctions has been demonstrated in studies investigating CDHE, and it was shown that CDHE (*Cdh1*; FC –2.8 in the present study) was able to influence the intracellular trafficking and function of both CX26 and CX43 (Hernandez-Blazquez et al., 2001). In the human testis, an altered expression of TJP1 and TJP2, and a dysfunction of claudin-11, have been demonstrated in SCs associated with CIS (Fink et al., 2006; Fink et al., 2009).

Because it has been further demonstrated that CX43 immunolocalization shifts from the luminal to the basal region of the seminiferous epithelium concomitant with BTB formation (Bravo-Moreno et al., 2001), it may be speculated that CX43 has a functional role in the compartmental reorganization of the seminiferous epithelium during puberty. Thus, it is possible that loss of CX43 in SCs results in an altered organization of the seminiferous epithelium leading to (1) morphological, structural and/or metabolic disorganization, (2) altered synchronization of SC-GC metabolism and (3) altered maturation during the first waves of spermatogenesis. This again could result in impaired spermatogenesis in adult SC*Cx43*KO mice.

In contrast to adult SC*Cx43*KO mice, in which the number of SCs per tubule cross-section is significantly higher in comparison to WT mice (Brehm et al., 2007; Sridharan et al., 2007a), no

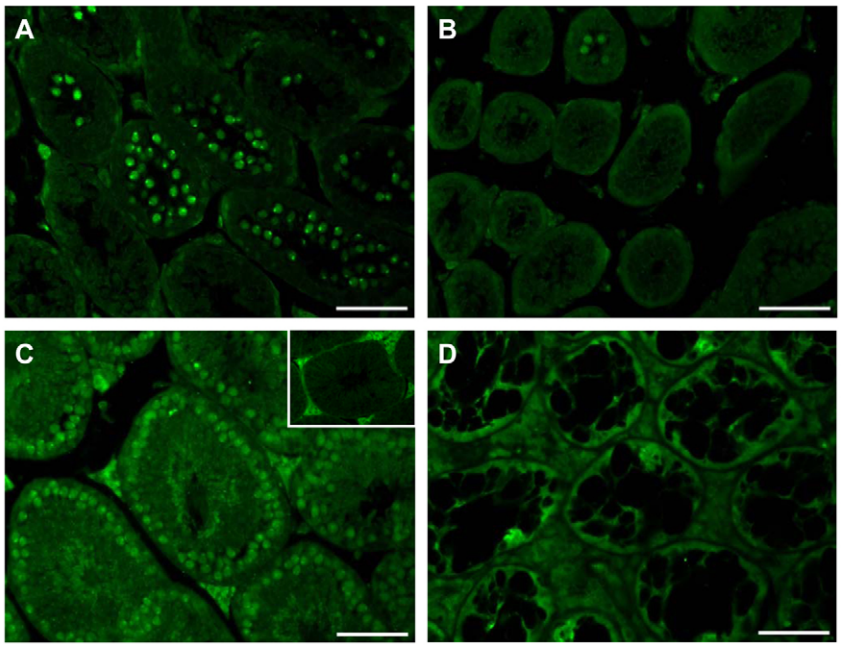

Fig. 11. IF for DMRT7. WT (A,C) and SC*Cx43*KO (B,D) on day 15 p.p. (A,B) and in adulthood (C,D) were compared. Results showed a higher number of DMRT7 immunopositive cells in pubertal WT testis compared with SC*Cx43*KO mice (A,B). In adult WT males, a specific staining can be detected in nearly all spermatocytes (C), whereas only paraffin autofluorescence is seen in KO tubules (D). Insert in C: note paraffin autofluorescence in interstitial LCs in negative controls. Scale bars: 50 µm.

significant difference concerning the SC number between 8-day-old KO and WT mice can be found. In rodents, SC proliferation takes place during the fetal period until 3 weeks p.p., with a maximum at around 2 days before birth (Orth, 1982). With puberty the mitotic activity stops, SCs mature and the final number of SCs is established. Owing to the Cre mice used in this study (*Amh*-Cre; Cre-expression under the control of the *Amh* promoter), possible alterations do mainly occur in our SC*Cx43*KO mice during the time period with the highest mitotic activity and proliferation of SCs. This fact supports the assumption of a remaining proliferation potential (capacity) of SCs in adult SC*Cx43*KO mice, as suggested by Sridharan et al. (Sridharan et al., 2007b).

By contrast, the GC number was significantly decreased as early as day 8 p.p. in SC*Cx43*KO mice, similar to results seen in adult KO mice. These results are supported by other published data obtained by grafting the testes of *Cx43*-deficient mice under the kidney capsule of WT mice, showing that the GC population fails to expand postnatally in the absence of CX43 (Roscoe et al., 2001). In the last decade, different *Cx43* KO mice have been generated and the importance of *Cx43* to male gametogenesis has been shown: e.g. there is a 50% depletion of primordial GCs in fetal male mice lacking the *Cx43* gene (Juneja et al., 1999) and a significant reduction of GCs in adult SC*Cx43*KO mice (Brehm et al., 2007; Sridharan et al., 2007a) (and in the present study). It was further demonstrated that primordial GCs are already gap-junction intercellular-communication-competent cells and that GC deficiency in *Cx43* KO embryos might arise from an increased apoptosis by abnormal p53 activation (Francis and Lo, 2006). A recent in vitro study confirms the involvement of CX43 gap junctions in the regulation of GC number by controlling spermatogonia survival rather than proliferation (Gilleron et al., 2009). To answer the question of whether the number of GCs is already altered before postnatal day 8 and whether the migration of GCs is altered, new breedings to obtain younger KO mice have been performed. Preliminary results comparing KO and WT mice at day 2 p.p. showed that already at this very early postnatal age the number of GCs per tubule cross-section is drastically reduced in KO mice compared with WT. To investigate even earlier (fetal) developmental ages, for the investigation of primordial GC migration, a study design similar to that of Francis and Lo (Francis and Lo, 2006) and new breeding strategies are planned. These data imply an important role for *Cx43* in SCs on maintaining the number of GCs by regulating CX43 gap junctions. Whether CX43 from SCs is involved in GC growth by controlling primordial GC and/or spermatogonia survival rather than their proliferation, as shown by Gilleron et al. (Gilleron et al., 2009), remains to be elucidated for SC*Cx43*KO mutants in future studies. In this context, it was shown that a low spermatogenic efficiency in infertile men showing an arrest of spermatogenesis at the level of spermatogonia was not only due to postmeiotic events, but also to a decrease in the mitotic activity of spermatogonia (Steger et al., 1998). And, finally, it was demonstrated that many infertile individuals face, besides meiotic defects, the problem of reduced numbers of spermatogonia and spermatogonial stem cells (Hentrich et al., 2011).

Our data reveal that (1) there are considerable differences in the gene expression patterns of SC*Cx43*KO and WT mice at day 8 p.p. (and these differences have a negative impact on protein synthesis in young and adult KO mice), (2) SC-specific deletion of *Cx43* seems to effect primarily GCs and (3) expression of important GC-specific genes seems to be *Cx43* associated and/or regulated. Because several of the altered genes have been shown to be essential for normal (mitotic and meiotic) progression of GC development and initiation of spermatogenesis, it was additionally demonstrated that corresponding proteins are altered in adult mutants. Thus, these genes and their proteins could be involved in the developmental and histological disorder observed in adult SC*Cx43*KO mice (and in similar human spermatogenic defects).

In conclusion, observed changes in gene expression in the mutant mice suggest a role for SC-GC crosstalk via CX43 gap junctions. However, in addition to its classical role in direct intercellular communication, observed alterations in KO gene expression might also be due to (1) a loss of CX43 hemichannel function in SCs and consequently within the seminiferous epithelium, (2) a loss of direct or indirect interaction with other junctional proteins and/or (3) a loss of interaction or binding of CX43 in SCs to corresponding cytoskeleton partners. Thus, loss of CX43 hemichannels in SCs, which might act as a paracrine conduit to spread factors that modulate the fate of SCs themselves, and GCs can lead to their maturational arrest.

Changes in candidate gene expression patterns could have also been caused by (4) a loss of direct *Cx43* effects on gene transcription, because it was demonstrated in a study working with fetal *Cx43*-null mutants that the loss of the *Cx43* gene is able to exert profound effects on the expression pattern of other testicular CX genes. In fetal testes of *Cx43* KO mice, only four CX mRNAs were detectable (*Cx26*, *Cx37*, *Cx40* and *Cx45*), compared with normal fetal WT testes exhibiting eight CX members (Juneja, 2003). In the present study, a 'close to significant' downregulation of *Gja6* (coding for CX33) was detected, confirming its close association with CX43 (Fiorini et al., 2004). It is finally also possible that (5) a disturbed maturation of nursing SCs might consequently result in alterations in: differentiation of SC-dependent GCs, candidate gene expression in GCs of SC*Cx43*KO mice and the observed GC numbers.

A time-course study comparing SC*Cx43*KO and WT mice from day 6, 10, 12 and 14 p.p. would be useful in confirming the role of these candidate genes and could lead to the identification of altered pathways before and during the first waves of spermatogenesis. Moreover, current studies are comparing histological phenotypes of impaired spermatogenesis and significantly altered candidate genes in SC*Cx43*KO mice with corresponding deficiencies in men by using human testicular biopsies. In addition, these biopsies will be investigated for possible mutations in the *Cx43* gene in SCs or GCs using UV-laser-assisted cell picking (microdissection approach).

It is for all these reasons that the established transgenic SC*Cx43*KO mouse model provides an interesting tool to study and understand the causes of impaired spermatogenesis because it is directly relevant to different corresponding cases of human subfertility and sterility.

METHODS

Mice

Breeding strategy, genotyping and confirmation of *Cx43* gene loss by β-galactosidase IHC of WT and homozygous SC*Cx43*KO mice were carried out as described previously (Brehm et al., 2007). For

all experiments, 8-day-old mice were used (n=3 per genotype), except for IF where 15-day-old mice were utilized (n=3 per genotype), because DMRT7 protein is known to be first detectable from day 13.5 onwards. Additionally, for STRA8 and DAZL IHC and for DMRT7 IF, adult WT and SC*Cx43*KO mice (n=3 per genotype) were investigated. Three KO mice and two WT mice were littermates, one WT mouse was from another litter but from the same parents. Genotyping showed that the used KO animals were all 'homozygously floxed *Cx43* and *Cre* positive' mice, whereas the WT mice (littermates) were '*Cx43* homoflox but *Cre* negative'. Within the WT group (microarray), two subclusters were present, i.e. WT1 versus WT2/3. This small difference in the expression profile can be explained because WT2 and 3 were from the same litter whereas WT1 was from another litter but from the same mouse (parents). Animal experiments were approved by the animal rights committee at the regional commission of Giessen, Germany (decision V54-19c 20/15 c GI 18/1) and the ethics commission at the University of Giessen, Germany (decision 56/05).

Tissue retrieval and treatment

Mice were anesthetized and sacrificed by intraperitoneal injection of an overdose cocktail of ketamine hydrochloride (Medistar, Holzwickede, Germany) and xylazine (Serumwerk Bernburg, Bernburg, Germany). The body and testis weights were defined. Both testes were removed immediately. One testis was snap-frozen in liquid nitrogen and then stored at –80°C until RNA extraction, the other was placed in Bouin's solution overnight for IHC and hematoxylin and eosin staining (H&E), followed by paraffin embedding. For IF the second testis was placed in formalin for 2 hours before paraffin embedding.

Histochemical techniques

For histological evaluation and IHC, 5-µm slices from all KO and WT mice were sectioned. Sections 1, 10, 20, 30, 40 and 50 were stained with H&E according to standard techniques; sections 2, 11, 21, 31, 41 and 51 were used for β-galactosidase IHC. With the latter sections and method, the mean number of SCs (nuclei) per tubule cross-section was determined, avoiding in this way that single spermatogonia mimic and displace SCs, resulting in reproducible SC numbers per tubule cross-section. Then, the number of SCs and GCs per tubule cross-section was counted for at least 25 tubules per section and the average number was calculated to compare the real cell composition of WT and SC*Cx43*KO mice. This quantification method was published previously (Brehm et al., 2007; Weider et al., 2011b). Quantitative microscopic analysis was performed using a Leica DM LB microscope (Leica, Wetzlar, Germany) at a magnification of 400×. Statistical evaluation was done using BMDP Statistical Software Package (Statistical Solutions Ltd, Cork, Ireland).

On the basis of this data, a correction factor of 1/(ØGC number) was calculated for GC-specific genes and a factor of 1/(ØGC+ØSC number) for genes expressed in GCs and SCs. This implied a factor of approximately 1/3 for WT and 1/1 for SC*Cx43*KO mice for genes exclusively expressed in GCs and a factor of ca. 1/26 for WT and 1/24 for SC*Cx43*KO mice for genes expressed in both GCs and SCs. The obtained factor was used to correct the data of the microarray analysis by the number of GCs for genes expressed only in GCs or in both cell compartments.

RNA extraction

To carry out microarray analysis, one testis of three mice of each genotype was used. Total RNA was prepared using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Microarray analysis

Gene expression profiling

RNA was subjected to transcription and hybridization as follows: target preparation was done using the MessageAmp II Kit (Ambion, Applied Biosystems) following the original protocol. Briefly: 1 µg total RNA was used in cDNA synthesis reaction with a poly-A binding primer containing the T7-polymerase promoter. Resulting cDNA was transcribed into cRNA in one round amplification in the presence of 11-Bio-UTP. Double-stranded cDNA and biotin-labeled cRNA were purified using the mini columns included in the kit. The eluted cRNA was quantified with the NanoDrop (NanoDrop Technologies, Rockland, DE) and a quality profile with the Agilent 2100 bioanalyzer (Agilent Technologies) was made. Portions of 20 µg cRNA were subjected to fragmentation in the presence of Mg^{2+}. Subsequently, 10 µg fragmented cRNA (target) was loaded onto CodeLink Mouse Whole Genome Microarray glass slides containing 36.227 probe sets (Applied Microarrays, Tempe, AZ) and hybridized for 18 hours in a Minitron shaker incubator (Infors AG, Bottmingen, Germany) at 37°C/300 rpm (n=2 arrays per sample). Washing and dyeing with Cy-5-coupled streptavidin was done according to the original protocol for CodeLink arrays (Applied Microarrays, Tempe, AZ) and the arrays were scanned using a GenePix 4000 B scanner and GenePix Pro 4.0 Software (Axon Instruments, Arlington, TX).

Spot signals of CodeLink bioarrays were quantified using CodeLink System Software consisting of Batch Submission (V2.2.27) and Expression Analysis (V2.2.25) (GE Healthcare) as outlined in the user's manual. CodeLink Expression Software 1.21 generated background corrected raw as well as median centered intra-slide normalized data. The intra-slide normalized data were used for further analysis. The software automatically calculated thresholds for intra-slide normalized intensities for each array and flagged genes as TRUE when the gene intensity was higher than the threshold or FALSE when the intensity was lower than the threshold. The present call of a microarray was given as the ratio of genes flagged as TRUE/total number of genes on microarray. Microarrays subjected to data analysis showed a mean present call of 83%, indicating a high number of genes above threshold, i.e. being flagged as TRUE. Furthermore, the software flagged each gene value as GOOD, EMPTY, POOR, NEG or MSR, defining different quality measures as outlined in the user's manual. Only gene values flagged as GOOD or EMPTY were used in the following analysis workflow:

(1) Definition of groups: Group 1: WT mice [testes of three WT mice at day 8 p.p. (three biological replicates), each on two microarrays (two technical replicates)]. Group 2: SC*Cx43*KO mice [testes of three KO mice at day 8 p.p. (three biological replicates), each on two microarrays (two technical replicates)]. A total of 12 microarrays (six per group) were subjected to analyses.

(2) Removal of genes with a high number of missing values or of values being flagged as FALSE: genes with missing values ≥50% of all arrays in a group were excluded from the dataset. Genes that

were flagged as FALSE in >50% of arrays in each group were also excluded from the dataset. A total of 26,044 probe sets remained after quality control.

(3) Gene expression values deviating more than three times from the group median were classified as outliers and have been removed.

(4) Imputation of remaining missing values: remaining missing values were imputed using sequential K-nearest neighbor (SKNN) imputation (Kim et al., 2004) with k=5.

(5) Normalization of imputed dataset: imputed dataset was normalized using quantile normalization in R (Bolstad et al., 2003) and logged to base 2.

(6) Array outlier detection: dissimilarity matrices of the normalized dataset were generated in AVADIS-Pride (Gwadry et al., 2005) to determine outlier arrays within the dataset. No outlier arrays were identified in the dataset.

(7) Correction for differing GC numbers: to exclude that the differentially gene expression was due to the differing numbers of GCs in the two genotypes, microarray data were normalized with the correcting factor obtained by the statistical evaluation of the morphometrical data. There was nearly no difference in the FC of gene expression either with or without adjustment for cell numbers.

(8) Statistical analysis of microarrays: for each gene, the mean value of all technical replicates of a mouse and the FC thereof was calculated in MAYDAY (Battke et al., 2010). To identify differentially regulated genes between WT and SC*Cx43*KO mice, the dataset was subjected to a two-class rank statistics [Rank products (RP)] as described below (Breitling et al., 2004; Breitling and Herzyk, 2005). Additionally, the Student's t-test was applied. For each gene, a significance level of P-value<0.05 was determined for both tests combined with an FC of at least twofold. A differentially regulated gene was considered to be significant when the P-value in the t-test was less than 0.05, the P-value in RP was less than 0.05 and the FC was equal to/higher than 2 or equal to/less than 2.

(9) Annotation of genes: significantly regulated genes were annotated using the web-based annotation tools SOURCE (Diehn et al., 2003) and the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 2008) (Dennis et al., 2003) as described in the manual.

(10) Hierarchical cluster analysis: hierarchical cluster analysis of the significantly genes was performed in dCHIP using Euclidean distance and centroid linkage.

(11) Enriched functional categories: enriched functional categories within the differentially regulated genes were determined using DAVID (Dennis et al., 2003) version 2.0. Based on statistical methods, significance levels were calculated that describe the overrepresentation of functional categories by a list of genes – the differentially regulated genes in our case. If a GO category or biological process is significantly overrepresented, which means that a relatively high number of genes assigned to the respective function is present in the selection, this will result in a low P-value. In functional annotation clustering, GO categories are grouped according to the genes from the list of regulated genes that have been assigned to them. An enrichment score is calculated for each cluster, based on the proportion of significantly overrepresented biological processes. Clusters with an enrichment score above 1.2 have been taken into account. Those clusters represented at least one significantly enriched biological process (i.e. with a P-value of less than 0.05).

(12) Pathway analysis: a relationship between genes and their connection to existing regulatory pathways was examined using Ingenuity Pathway Analysis (IPA), a web-based entry tool developed by Ingenuity Systems, Inc. (http://www.ingenuity.com).

Complete data are available at the Gene Expression Omnibus (GEO) database under the accession number GSE23431. This study adhered to the MIAME standards (Brazma et al., 2001).

cDNA synthesis and qRT-PCR

Changes in mRNA levels of altered genes identified by the CodeLink Mouse Whole Genome Bioarray were confirmed using qRT-PCR. Three biological replicates per genotype were processed. DNAse treatment was performed according to manufacturer's instructions using the RNase-Free DNase Set (Qiagen, Hilden, Germany). cDNA was synthesized from 15 µl total RNA (adjusted to 200 nmol/30 µl) using MultiScribe Reverse Transcriptase (Applied Biosystems Inc., Darmstadt, Germany). Purification of cDNA was realized by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Specific primer pairs were created using Beacon Designer Software (Bio-Rad, München, Germany). Before use in the main experiment, primers were first tested in normal PCR to

Table 4. Primer sequences used in qRT-PCR experiments

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Actb	TTCCTTCTTGGGCATGGAGT	TACAGGTCTTTGCGGATGTC
Hsp90ab1	AAGAGAGCAAGGCAAAGTTTGAG	TGGTCACAATGCAGCAAGGT
Dgkz	CCTGGAGGTCATCGGCTTCAC	TCTCGGCACTGCGTCAATCG
Kif17	ACTCCTCCCGTTCCCACTCC	GCCAGGTCTACCAGGTTGAGC
Stra8	CGCCTTCGCAGACCTCACC	GCTCGCTCCCTGGCTGTC
Sox3	GCCCTGGAGAACCCCAAGATG	ACCGCACGCAGTCGCTTG
Tex11	CATCTCTGTGCGTCTTACGTTGC	ATTCCGCTTGCTTGCTTCTATGC
Sycp2	GGCTGACCCATCGTGGACAG	ACACCCGCAGACACTTTAGGC
Dmrt6	CGACTACGGGCATCCTCTGAG	GACGCAGGCAGGGCACTG
Sohlh1	CAGGAGGCGGATCTCGTTGAG	ATGCTGTGGGCAAGCTGGAG
Rxfp2	TCCGTGGGCGTCTTTGACATC	GCAGCAGCACCGAGACCTC
Tjp1	CCCTACCAACCTCGGCCTT	AACGCTGGAAATAACCTCGTTC
Ocln	ATCCTGTCTATGCTCATTATTGTG	CTGCTCTTGGGTCTGTATATCC
Cldn11	CGTCATGGCCACTGGTCTCT	GGCTCTACAAGCCTGCACGTA
Cdh2	TGGCAATCAAGTGGAGAACC	ATCCGCATCAATGGCAGTG
Cdkn2a	TGGTGAAGTTCGTGCGATCCC	GGTGCGGCCCTCTTCTCAAG
Dazl	GGAGGCCAGCACTCAGTCTTC	AGCCCTTCGACACACCAGTTC
Cx43	ACAGCGGTTGAGTCAGCTTG	GAGAGATGGGGAAGGACTTGT
Amh	CCAACGACTCCCGCAGCTC	CTTCCCGCCCATGCCACTC
Ddx25	CTGCCAGACACGTCGAAATGC	TCGCTGCTCCACTGTGAGTTC
Piwil2	CAGAAGACTCCAGCCCACCAC	TCAAGACCCATGCCACGGAAC

Actb, actin beta; *Hsp90ab1*, heat shock protein 90 kDa alpha, class B member 1; *Dgkz*, diacylglycerol kinase zeta; *Kif17*, kinesin family member 17; *Stra8*, stimulated by retinoic acid gene 8; *Sox3*, SRY-box containing gene 3; *Tex11*, testis expressed gene 11; *Sycp2*, synaptonemal complex protein 2; *Dmrt6*, DMRT-like family B with proline-rich C-terminal, 1; *Sohlh1*, spermatogenesis and oogenesis specific basic helix-loop-helix 1; *Rxfp2*, relaxin/insulin-like family peptide receptor 2; *Tjp1*, tight junction protein 1; *Ocln*, occludin; *Cldn11*, claudin 11; *Cdh2*, cadherin 2; *Cdkn2a*, cyclin-dependent kinase inhibitor 2A; *Dazl*, deleted in azoospermia-like; *Cx43*, gap junction protein, alpha 1; *Amh*, anti-Muellerian hormone; *Ddx25*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 25; *Piwil2*, piwi-like homolog 2 (Drosophila).

TRANSLATIONAL IMPACT

Clinical issue

Although a significant decline in human male reproductive function has been reported for the past 20 years, the molecular mechanisms responsible remain poorly understood. Recent studies indicate that the gap junction protein connexin-43 (CX43) – the predominant testicular connexin in most species, including humans – might be involved. For example, many individuals with impaired spermatogenesis exhibit a reduction or loss of CX43 expression in germ cells and Sertoli cells (SCs; part of the seminiferous tubule). It has been shown that adult male transgenic mice with a conditional knockout of the *Gja1* gene (also referred to as *Cx43*; encoding CX43) in SCs (SC*Cx43*KO) show a testicular phenotype similar to such individuals and are infertile.

Results

Here, the authors sought to identify possible signaling pathways and molecular mechanisms underlying the testicular phenotype and failure to initiate spermatogenesis in SC*Cx43*KO mice. They used microarray analysis to compare testicular gene expression of 8-day-old SC*Cx43*KO and wild-type (WT) mice. This analysis revealed that 658 genes were significantly regulated in testes of SC*Cx43*KO mice. Of these genes, 135 were upregulated and 523 genes were downregulated. Most downregulated genes, including *Stra8, Dazl* and members of the DM (*dsx* and *map-3*) gene family, are germ-cell-specific and essential for mitotic and meiotic progression of spermatogenesis. Other genes altered are associated with transcription, metabolism, cell migration and cytoskeletal organization. These data show that deletion of *Cx43* in SCs leads to changes in the expression of multiple genes in prepubertal mice, and affects primarily germ cells.

Implications and future directions

These data indicate that, among the different transgenic mouse models that are used to elucidate the multiple mechanisms underlying human male infertility, SC*Cx43*KO mice provide a unique model for identifying candidate genes in germ cells. The candidate genes identified in this study could represent useful markers in investigations of human testicular biopsies from patients with spermatogenic deficiencies, and for studying the molecular mechanisms of human male infertility.

confirm that only one specific band could be produced. Afterwards, a qRT-PCR with a dilution series was performed to detect the efficiency of the qRT-PCR reaction and to prove that only one product can be detected within the melting curve. A representative number of PCR products were sequenced additionally. Only primers passing the multiple testing without interceptions finally were used. qRT-PCR was performed using IQ SYBR Green Supermix and CFX96 Real-Time System (Bio-Rad, München, Germany). Per sample, 1 µl cDNA was used for amplification. Specific forward and reverse primers (Table 4) were added to the Mastermix. Cycling conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 1 minute. After 95°C for 10 seconds melt curve was produced with 65°C to 95°C and an increment of 0.5°C every 5 seconds. *Actb* and *Hsp90ab1* were used as housekeeping genes. All experiments included negative controls lacking cDNA and were carried out as triplicates. Based on the ΔΔC(t) method, evaluation of the qRT-PCR was realized using the CFX Manager Software (Bio-Rad, Germany).

IHC and IF

In order to confirm whether the alterations at RNA level are reflected in protein synthesis, IHC and IF was applied. Slides were dewaxed, rehydrated and microwaved in sodium citrate buffer, pH 6.0. Tissue sections were treated with 3% H_2O_2, blocked with 5% bovine serum albumin to prevent nonspecific binding, followed by incubation with primary antibody at 4°C overnight. Primary antibodies were: (1) rabbit anti-STRA8 (stimulated by retinoic acid gene 8; 1:250; kindly provided by Michael Griswold, Pullman, Washington, DC), (2) rabbit anti-CX43 (1:100; New England Biolabs GmbH, Frankfurt, Germany), (3) rabbit anti-OCLN (1:500; Invitrogen, Karlsruhe, Germany) and (4) mouse anti-DAZL antiserum (deleted in azoospermia-like Dazla; 1:250; kindly provided by Howard Cooke, Edinburgh, UK). The slides were covered with a corresponding (compatible) biotinylated secondary antibody (goat anti-rabbit, goat anti-mouse or rabbit anti-goat, from DAKO, Hamburg, Germany) at room temperature (RT) for 1 hour, followed by the application of peroxidase-conjugated streptavidine (VECTASTAIN Elite ABC Standard Kit; Peroxidase, Biologo, Kronshagen, Germany) for 1 hour at RT. Peroxidase activity was visualized by the Peroxidase Substrat Kit AEC (Biologo, Kronshagen, Germany). For IF, formalin-fixed testes were used. After removal of the paraffin wax, rehydration and cooking in sodium citrate buffer with pH 6.0, slices were blocked with 5% goat serum for 1 hour and incubated overnight with the primary rabbit anti-DMRT7 antibody (1:200; kindly provided by David Zarkower, Minneapolis, MN). Exposure of the slices to a biotinylated secondary antibody (goat anti-rabbit; DAKO, Hamburg, Germany) at RT for 1 hour was followed by a 1 hour incubation with NeutrAvidin, DyLight 488 Conjugated (Fisher Scientific GmbH, Schwerte, Germany) and covering with Aqua Polymouth (Polysciences, Inc., Warrington, PA). Negative controls for both IHC and IF were performed by omitting the primary antibody.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

S.G., H.H., T.C., M.B. and R.B. were involved in conception and design of the experiments and co-wrote the manuscript; S.G. and H.H. analyzed the data; S.G. performed most of the experiments, e.g. breeding of SC*Cx43*KO mice and corresponding littermates, H&E stainings, RNA extraction, cDNA synthesis, qRT-PCR and IHC or IF; S.T. performed the microarray analysis; M.M. and K.F. performed statistical analysis and determined the used correction factors; F.G. provided the AMH-Cre mice for the breeding experiments; K.W. prepared figures, tables and supplemental data for publication; all authors read and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Supplemental Table 1a

ACCN	Gene Symbol	Gene Name	T-Test p-value	RP p-value	FC KO|WT
NM_009606.1	*Acta1*	actin, alpha 1, skeletal muscle	0.01	0.00	12.0
NM_009610.1	*Actg2*	actin, gamma 2, smooth muscle, enteric	0.01	0.00	2.2
NM_009626.2	*Adh7*	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	0.01	0.00	3.6
NM_028121.2	*Adpgk*	ADP-dependent glucokinase	0.00	0.00	21.5
AI574175.1	*AI574175*	expressed sequence AI574175	0.03	0.00	2.1
NM_009731.1	*Akr1b7*	aldo-keto reductase family 1, member B7	0.03	0.00	2.5
NM_177275.2	*Amigo3*	amphoterin induced gene and ORF 3	0.01	0.00	2.5
NM_029898.1	*Ankrd55*	ankyrin repeat domain 55	0.03	0.00	2.1
NM_009685.1	*Apbb1*	amyloid beta (A4) precursor protein-binding, family B, member 1	0.01	0.00	2.5
NM_007473.3	*Aqp7*	aquaporin 7	0.02	0.00	2.7
AI506359.1	*Arhgef12*	Rho guanine nucleotide exchange factor (GEF) 12	0.01	0.00	6.0
AK044114.1	*B230208B08Rik*	RIKEN cDNA B230208B08 gene	0.03	0.00	2.5
BM938798.1	*Cadps*	Ca2+-dependent secretion activator	0.01	0.00	3.2
NM_011332.2	*Ccl17*	chemokine (C-C motif) ligand 17	0.00	0.00	3.0
NM_009139.1	*Ccl6*	chemokine (C-C motif) ligand 6	0.04	0.00	2.7
NM_011338.1	*Ccl9*	chemokine (C-C motif) ligand 9	0.02	0.00	3.1
NM_007686.1	*Cfi*	complement component factor i	0.00	0.00	2.2
NM_172621.1	*Clic5*	chloride intracellular channel 5	0.00	0.00	2.5
NM_009922.2	*Cnn1*	calponin 1	0.03	0.00	2.2
NM_031396.1	*Cnnm1*	cyclin M1	0.01	0.00	2.5
BX528891.1	*Cnnm1*	cyclin M1	0.00	0.00	2.3
NM_027024.2	*Cst13*	cystatin 13	0.01	0.00	2.0
NM_009978.1	*Cst8*	cystatin 8 (cystatin-related epididymal spermatogenic)	0.00	0.00	2.1
NM_007817.1	*Cyp2f2*	cytochrome P450, family 2, subfamily f, polypeptide 2	0.01	0.00	2.6
NM_139219.1	*Defb9*	defensin beta 9	0.02	0.00	3.7
BB343266.2	*Dip2b*	DIP2 disco-interacting protein 2 homolog B (Drosophila)	0.02	0.00	2.1
NM_020265.3	*Dkk2*	dickkopf homolog 2 (Xenopus laevis)	0.00	0.00	6.0
AK020222.1	*Dnhd1*	dynein heavy chain domain 1	0.03	0.00	2.1
NM_176933.3	*Dusp4*	dual specificity phosphatase 4	0.03	0.00	2.1
AK087736.1	*E330013P06*	hypothetical protein E330013P06	0.01	0.00	4.2
NM_133362.1	*Erdr1*	erythroid differentiation regulator 1	0.00	0.00	4.1

NM_010157.3	*Esr2*	estrogen receptor 2 (beta)	0.04	0.00	3.0
AK078610.1	*Fam155a*	family with sequence similarity 155, member A	0.01	0.00	5.3
NM_173446.1	*Fam155a*	family with sequence similarity 155, member A	0.02	0.00	2.6
CF578283.1	*Gpc6*	glypican 6	0.01	0.00	2.2
NM_010352.1	*Gsg1*	germ cell-specific gene 1	0.03	0.00	2.3
BC082537.1	*Hepacam*	hepatocyte cell adhesion molecule	0.03	0.00	2.8
NM_175189.3	*Hepacam*	hepatocyte cell adhesion molecule	0.01	0.00	2.2
CK334862.1	*Hist1h2ae*	histone cluster 1, H2ae	0.02	0.00	2.1
NM_008263.1	*Hoxa10*	homeobox A10	0.01	0.00	5.2
NM_010450.1	*Hoxa11*	homeo box A11	0.00	0.00	4.8
NM_008273.1	*Hoxd11*	homeobox D11	0.01	0.00	3.0
BY762577.1	*Ikzf3*	IKAROS family zinc finger 3	0.00	0.00	2.0
BB245041.2	*Kctd8*	potassium channel tetramerisation domain containing 8	0.05	0.00	2.7
NM_026324.1	*Kirrel3*	kin of IRRE like 3 (Drosophila)	0.04	0.00	2.1
BI106231.1	*Kpna2*	karyopherin (importin) alpha 2	0.00	0.00	7.4
NM_010701.1	*Lect1*	leukocyte cell derived chemotaxin 1	0.05	0.00	2.1
AK086355.1	*Lsamp*	limbic system-associated membrane protein	0.03	0.00	2.1
NM_008518.1	*Ltb*	lymphotoxin B	0.00	0.00	2.1
AK004706.1	*Mdga1*	MAM domain containing glycosylphosphatidylinositol anchor 1	0.00	0.00	2.2
BE943834.1	*Meis2*	Meis homeobox 2	0.05	0.00	2.1
NM_010825.2	*Meis2*	Meis homeobox 2	0.00	0.00	2.1
AK030913.1	*Mid1*	midline 1	0.04	0.00	3.7
CK330720.1	*Mid1*	midline 1	0.03	0.00	3.5
BI694853.1	*Mid1*	midline 1	0.00	0.00	2.3
AK034278.1	*Mrap2*	melanocortin 2 receptor accessory protein 2	0.05	0.00	2.1
NM_013602.2	*Mt1*	metallothionein 1	0.01	0.00	4.7
NM_175418.3	*Mybpc1*	myosin binding protein C, slow-type	0.02	0.00	2.8
NM_175418.3	*Mybpc1*	myosin binding protein C, slow-type	0.00	0.00	2.6
NM_175418.3	*Mybpc1*	myosin binding protein C, slow-type	0.02	0.00	2.0
NM_146969.1	*Olfr1243*	olfactory receptor 1243	0.01	0.00	2.1
NM_146334.1	*Olfr1330*	olfactory receptor 1330	0.00	0.00	2.0

BC082538.1	*P4ha3*	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III	0.04	0.00	2.1
NM_008791.2	*Pcp4*	Purkinje cell protein 4	0.00	0.00	59.8
AK035507.1	*Pgm5*	phosphoglucomutase 5	0.00	0.00	2.4
AI929868.1	*Plcb1*	phospholipase C, beta 1	0.03	0.00	3.0
NM_023127.1	*Polr2k*	polymerase (RNA) II (DNA directed) polypeptide K	0.00	0.00	4.4
BQ175159.1	*Ppm1h*	protein phosphatase 1H (PP2C domain containing)	0.02	0.00	2.1
AK079637.1	*Ppp1r3g*	protein phosphatase 1, regulatory (inhibitor) subunit 3G	0.01	0.00	2.9
NM_008926.2	*Prkg2*	protein kinase, cGMP-dependent, type II	0.00	0.00	2.6
BE980253.1	*Rab27b*	RAB27b, member RAS oncogene family	0.02	0.00	2.1
BM244450.2	*Ralgapa1*	Ral GTPase activating protein, alpha subunit 1	0.02	0.00	2.5
NM_021477.3	*Rbfox1*	RNA binding protein, fox-1 homolog (C. elegans) 1	0.04	0.00	2.4
NM_181596.2	*Retnlg*	resistin like gamma	0.03	0.00	2.4
AK079512.1	*Rgs10*	regulator of G-protein signalling 10	0.01	0.00	3.4
AK122245.2	*Rimbp2*	RIMS binding protein 2	0.01	0.00	2.9
AK038787.1	*Robo1*	roundabout homolog 1 (Drosophila)	0.02	0.00	2.1
AA726096.1	*Rpl10a-ps2*	ribosomal protein L10A, pseudogene 2	0.01	0.00	2.6
NM_133982.1	*Rpp25*	ribonuclease P 25 subunit (human)	0.01	0.00	2.3
NM_009114.1	*S100a9*	S100 calcium binding protein A9 (calgranulin B)	0.00	0.00	3.0
BF453892.1	*Serinc3*	serine incorporator 3	0.02	0.00	2.0
NM_009144.1	*Sfrp2*	secreted frizzled-related protein 2	0.02	0.00	3.0
AK012380.1	*Shisa9*	shisa homolog 9 (Xenopus laevis)	0.04	0.00	2.0
NM_020258.2	*Slc37a2*	solute carrier family 37 (glycerol-3-phosphate transporter), member 2	0.02	0.00	2.1
NM_133924.1	*Snx21*	sorting nexin family member 21	0.04	0.00	3.2
NM_009262.2	*Spock1*	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1	0.01	0.00	5.0
NM_001001332.1	*Stfa1*	stefin A1	0.00	0.00	4.1
NM_025288.1	*Stfa3*	stefin A3	0.01	0.00	2.6
NM_017465.1	*Sult2b1*	sulfotransferase family, cytosolic, 2B, member 1	0.02	0.00	2.9

BE949104.1	*Tacc2*	transforming, acidic coiled-coil containing protein 2	0.04	0.00	2.8
NM_153801.1	*Tecrl*	trans-2,3-enoyl-CoA reductase-like	0.04	0.00	2.3
AA920804.1	*Themis*	thymocyte selection associated	0.03	0.00	2.0
NM_177794.2	*Tmem26*	transmembrane protein 26	0.02	0.00	4.2
NM_009394.2	*Tnnc2*	troponin C2, fast	0.00	0.00	2.7
AK053112.1	*Tns1*	tensin 1	0.02	0.00	3.1
CA874096.1	*Trps1*	trichorhinophalangeal syndrome I (human)	0.04	0.00	2.1
NM_020505.1	*Vav3*	vav 3 oncogene	0.02	0.00	2.7
NM_134218.1	*Vmn1r208*	vomeronasal 1 receptor 208	0.03	0.00	3.5
AU067815.1	*Xkr4*	X Kell blood group precursor related family member 4	0.03	0.00	2.0
AK048587.1	*Zfp182*	zinc finger protein 182	0.02	0.00	2.2
BF581959.1	*Zfp949*	zinc finger protein 949	0.01	0.00	2.8
NM_009573.3	*Zic1*	zinc finger protein of the cerebellum 1	0.02	0.00	2.9
AK013590.1	*2900024J01Rik*	RIKEN cDNA 2900024J01 gene	0.04	0.00	3.4
AK077867.1	*2900097C17Rik*	RIKEN cDNA 2900097C17 gene	0.00	0.00	6.8
NM_183126.1	*6030498E09Rik*	RIKEN cDNA 6030498E09 gene	0.04	0.00	2.0
AK034241.1	*A930018M24Rik*	RIKEN cDNA A930018M24 gene	0.02	0.00	3.9
AK032000.1			0.01	0.00	11.8
BC038320.1			0.00	0.00	7.3
AW494725.1			0.05	0.00	4.5
BU700460.1			0.01	0.00	3.8
AK078237.1			0.01	0.00	3.3
BG095502.1			0.01	0.00	2.9
AK083974.1			0.01	0.00	2.8
BM229163.2			0.01	0.00	2.7
AK012039.1			0.02	0.00	2.6
CB847142.2			0.01	0.00	2.5
BM234787.2			0.02	0.00	2.5
AK017987.1			0.01	0.00	2.5
AK084170.1			0.00	0.00	2.5
BM205157.2			0.01	0.00	2.4
BQ174503.1			0.01	0.00	2.4
BB450465.2			0.01	0.00	2.4
BB804671.1			0.01	0.00	2.3
BG070526.2			0.01	0.00	2.3
BE952863.1			0.02	0.00	2.3
AK077881.1			0.04	0.00	2.2
AK038705.1			0.01	0.00	2.2
AV297662.1			0.05	0.00	2.2
BX512545.1			0.04	0.00	2.2
AI585482.1			0.03	0.00	2.2
AK018644.1			0.04	0.00	2.1
BB315069.2			0.03	0.00	2.1

AK087305.1			0.02	0.00	2.1
BB268463.2			0.01	0.00	2.0
BC062894.1			0.02	0.00	2.0

Supplemental Table 1b

ACCN	Gene Symbol	Gene Name	T-Test p-value	RP p-value	FC KO|WT
NM_027669.1	*41153*	septin 12	0.00	0.00	-2.6
U43892.1	*Abcb7*	ATP-binding cassette, sub-family B (MDR/TAP), member 7	0.02	0.00	-2.1
NM_009350.1	*Adad1*	adenosine deaminase domain containing 1 (testis specific)	0.00	0.00	-4.5
AK015063.1	*Adad2*	adenosine deaminase domain containing 2	0.03	0.00	-4.5
NM_009623.1	*Adcy8*	adenylate cyclase 8	0.03	0.00	-4.9
NM_172923.1	*AI118078*	expressed sequence AI118078	0.00	0.00	-2.2
BC042709.1	*Aim1l*	absent in melanoma 1-like	0.00	0.00	-5.1
NM_009647.2	*Ak4*	adenylate kinase 4	0.00	0.00	-2.1
AK006382.1	*Akap13*	A kinase (PRKA) anchor protein 13	0.02	0.00	-2.0
BY714766.1	*Als2cr11*	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 11 (human)	0.00	0.00	-3.6
NM_175200.2	*Als2cr11*	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 11 (human)	0.02	0.00	-7.4
NM_144524.1	*Angel1*	angel homolog 1 (Drosophila)	0.02	0.00	-2.0
NM_178263.1	*Ankrd27*	ankyrin repeat domain 27 (VPS9 domain)	0.03	0.00	-2.1
BC096609.1	*Ankrd34b*	ankyrin repeat domain 34B	0.05	0.00	-2.4
NM_009677.3	*Ap1g1*	adaptor protein complex AP-1, gamma 1 subunit	0.04	0.00	-2.1
NM_018790.1	*Arc*	activity regulated cytoskeletal-associated protein	0.00	0.00	-2.8
NM_009705.1	*Arg2*	arginase type II	0.00	0.00	-4.9
AI840762.1	*Arhgap44*	Rho GTPase activating protein 44	0.03	0.00	-3.2
NM_019927.1	*Arih1*	ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (Drosophila)	0.03	0.00	-2.0
AK041237.1	*Arl5b*	ADP-ribosylation factor-like 5B	0.00	0.00	-2.6
AK081550.1	*Armc6*	armadillo repeat containing 6	0.01	0.00	-2.3
NM_027027.1	*Asb9*	ankyrin repeat and SOCS box-containing 9	0.00	0.00	-2.5
NM_008554.2	*Ascl2*	achaete-scute complex homolog 2 (Drosophila)	0.00	0.00	-2.3
NM_023729.2	*Asz1*	ankyrin repeat, SAM and basic leucine zipper domain containing 1	0.02	0.00	-5.1
NM_001029895.1	*Ate1*	arginyltransferase 1	0.01	0.00	-2.1

BC018510.1	*Atf7ip2*	activating transcription factor 7 interacting protein 2	0.04	0.00	-2.4
AK016180.1	*Atf7ip2*	activating transcription factor 7 interacting protein 2	0.00	0.00	-2.9
BC018510.1	*Atf7ip2*	activating transcription factor 7 interacting protein 2	0.02	0.00	-3.1
BI692011.1	*Atp10b*	ATPase, class V, type 10B	0.01	0.00	-4.5
AA612185.1	*Baz1a*	bromodomain adjacent to zinc finger domain 1A	0.00	0.00	-2.5
BC013712.1	*BC013712*	cDNA sequence BC013712	0.01	0.00	-2.1
NM_145357.1	*BC023105*	cDNA sequence BC023105	0.03	0.00	-3.7
NM_153544.2	*BC030867*	cDNA sequence BC030867	0.01	0.00	-2.0
NM_177567.2	*BC049762*	cDNA sequence BC049762	0.00	0.00	-5.7
NM_172521.1	*BC125332*	cDNA sequence BC125332	0.01	0.00	-12.9
NM_177772.2	*Bpil2*	bactericidal/permeability-increasing protein-like 2	0.01	0.00	-9.5
BE957236.1	*C030009O12Rik*	RIKEN cDNA C030009O12 gene	0.02	0.00	-9.3
BQ174675.1	*C1ql1*	complement component 1, q subcomponent-like 1	0.00	0.00	-2.6
AK082309.1	*C230036F13Rik*	RIKEN cDNA C230036F13 gene	0.02	0.00	-4.7
AK082498.1	*C230057A21Rik*	RIKEN cDNA C230057A21 gene	0.01	0.00	-2.1
AK021218.1	*C330022B21Rik*	RIKEN cDNA C330022B21 gene	0.01	0.00	-2.4
AK082869.1	*C430002E04Rik*	RIKEN cDNA C430002E04 gene	0.00	0.00	-5.5
AK049571.1	*C430042M11Rik*	RIKEN cDNA C430042M11 gene	0.00	0.00	-2.5
NM_009793.1	*Camk4*	calcium/calmodulin-dependent protein kinase IV	0.00	0.00	-5.3
X62537.1	*Cbx2*	chromobox homolog 2 (Drosophila Pc class)	0.00	0.00	-2.3
BC006583.1	*Ccdc136*	coiled-coil domain containing 136	0.03	0.00	-2.2
NM_177616.2	*Ccdc157*	coiled-coil domain containing 157	0.02	0.00	-2.1
NM_144527.2	*Ccdc21*	coiled-coil domain containing 21	0.04	0.00	-2.3
BC058624.1	*Ccdc36*	coiled-coil domain containing 36	0.00	0.00	-2.9
NM_175430.2	*Ccdc40*	coiled-coil domain containing 40	0.03	0.00	-2.3
BG066504.2	*Ccnb1ip1*	cyclin B1 interacting protein 1	0.00	0.00	-3.2
NM_021893.2	*Cd274*	CD274 antigen	0.03	0.00	-2.5
NM_021893.2	*Cd274*	CD274 antigen	0.02	0.00	-2.7
NM_009864.1	*Cdh1*	cadherin 1	0.01	0.00	-2.8
NM_007662.1	*Cdh15*	cadherin 15	0.03	0.00	-3.2

NM_009877.1	*Cdkn2a*	cyclin-dependent kinase inhibitor 2A	0.00	0.00	-4.4
NM_009882.2	*Cebpz*	CCAAT/enhancer binding protein zeta	0.02	0.00	-2.0
NM_176844.3	*Chrna5*	cholinergic receptor, nicotinic, alpha polypeptide 5	0.01	0.00	-2.5
BQ555385.1	*Chrna7*	cholinergic receptor, nicotinic, alpha polypeptide 7	0.02	0.00	-2.2
BC094889.1	*Chrnb4*	cholinergic receptor, nicotinic, beta polypeptide 4	0.02	0.00	-3.8
NM_009904.1	*Clgn*	calmegin	0.00	0.00	-4.5
NM_172469.1	*Clic6*	chloride intracellular channel 6	0.02	0.00	-6.9
NM_023420.1	*Col4a3bp*	collagen, type IV, alpha 3 (Goodpasture antigen) binding protein	0.00	0.00	-4.5
NM_030052.2	*Cox7b2*	cytochrome c oxidase subunit VIIb2	0.01	0.00	-2.8
NM_007755.1	*Cpeb1*	cytoplasmic polyadenylation element binding protein 1	0.04	0.00	-3.1
NM_013496.1	*Crabp1*	cellular retinoic acid binding protein I	0.00	0.00	-12.7
X90648.1	*Crkl*	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	0.02	0.00	-2.1
AW492342.1	*Crxos1*	Crx opposite strand transcript 1	0.01	0.00	-3.6
NM_008599.1	*Cxcl9*	chemokine (C-X-C motif) ligand 9	0.02	0.00	-3.7
NM_145548.1	*Cyp2j13*	cytochrome P450, family 2, subfamily j, polypeptide 13	0.02	0.00	-2.6
NM_177307.2	*Cyp4f39*	cytochrome P450, family 4, subfamily f, polypeptide 39	0.02	0.00	-3.1
J04847.1	*D1Pas1*	DNA segment, Chr 1, Pasteur Institute 1	0.01	0.00	-2.3
NM_175326.2	*D330045A20Rik*	RIKEN cDNA D330045A20 gene	0.00	0.00	-4.4
NM_010014.2	*Dab1*	disabled homolog 1 (Drosophila)	0.00	0.00	-3.1
NM_010021.2	*Dazl*	deleted in azoospermia-like	0.04	0.00	-6.0
AK019495.1	*Ddx10*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	0.02	0.00	-2.6
NM_013932.2	*Ddx25*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 25	0.03	0.00	-4.1
NM_010029.1	*Ddx4*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	0.03	0.00	-5.4
BC013672.1	*Ddx60*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	0.00	0.00	-2.5
BB513971.2	*Ddx60*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	0.04	0.00	-2.8
NM_177857.1	*Dennd2c*	DENN/MADD domain containing 2C	0.00	0.00	-2.4

NM_144804.1	*Depdc7*	DEP domain containing 7	0.03	0.00	-2.0
NM_010059.1	*Dmc1*	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	0.00	0.00	-2.5
AF020191.1	*Dmrtb1*	DMRT-like family B with proline-rich C-terminal, 1	0.04	0.00	-68.2
NM_027732.1	*Dmrtc2*	doublesex and mab-3 related transcription factor like family C2	0.00	0.00	-7.3
AK053672.1	*Dmxl2*	Dmx-like 2	0.02	0.00	-2.9
BF011365.1	*Dnahc12*	dynein, axonemal, heavy chain 12	0.02	0.00	-2.5
NM_008299.1	*Dnajb3*	DnaJ (Hsp40) homolog, subfamily B, member 3	0.03	0.00	-2.1
NM_173383.1	*Dnd1*	dead end homolog 1 (zebrafish)	0.00	0.00	-2.5
NM_001003960.1	*Dnmt3b*	DNA methyltransferase 3B (Dnmt3b), transcript variant 2	0.00	0.00	-2.1
NM_029761.2	*Dok5*	docking protein 5	0.02	0.00	-2.1
NM_010074.2	*Dpp4*	dipeptidylpeptidase 4	0.00	0.00	-2.6
NM_028615.1	*Dppa2*	developmental pluripotency associated 2	0.00	0.00	-2.9
NM_139218.1	*Dppa3*	developmental pluripotency-associated 3	0.01	0.00	-3.4
AK014832.1	*Dscaml1*	Down syndrome cell adhesion molecule-like 1	0.03	0.00	-2.7
NM_023742.1	*Dtx2*	deltex 2 homolog (Drosophila)	0.00	0.00	-2.6
NM_019819.2	*Dusp14*	dual specificity phosphatase 14	0.00	0.00	-2.1
NM_029352.3	*Dusp9*	dual specificity phosphatase 9	0.00	0.00	-3.1
AI451538.1	*E330020D12Rik*	Riken cDNA E330020D12 gene	0.04	0.00	-2.0
BC004721.1	*Eaf2*	ELL associated factor 2	0.00	0.00	-6.9
CA450918.1	*Efcab10*	EF-hand calcium binding domain 10	0.03	0.00	-2.0
NM_028916.1	*Efhc2*	EF-hand domain (C-terminal) containing 2	0.00	0.00	-3.2
NM_020596.1	*Egr4*	early growth response 4	0.02	0.00	-2.8
NM_007914.2	*Ehf*	ets homologous factor	0.00	0.00	-2.7
NM_021300.1	*Ehox*	ES cell derived homeobox containing gene (Ehox)	0.00	0.00	-2.5
NM_007939.1	*Epha8*	Eph receptor A8	0.02	0.00	-2.1
NM_181548.2	*Eras*	ES cell-expressed Ras	0.00	0.00	-5.3
NM_015774.2	*Ero1l*	ERO1-like (S. cerevisiae)	0.04	0.00	-2.5
NM_025276.2	*Evpl*	envoplakin	0.00	0.00	-2.3
NM_007974.2	*F2rl1*	coagulation factor II (thrombin) receptor-like 1	0.00	0.00	-2.5
NM_175449.3	*Fam26f*	family with sequence similarity 26, member F	0.03	0.00	-2.6

NM_020622.1	*Fam3b*	family with sequence similarity 3, member B	0.05	0.00	-3.8
AK077262.1	*Fam83g*	family with sequence similarity 83, member G	0.00	0.00	-3.0
NM_145946.1	*Fanci*	Fanconi anemia, complementation group I	0.02	0.00	-2.0
AK082809.1	*Fbxo15*	F-box protein 15	0.04	0.00	-2.0
AK007274.1	*Fbxw27*	F-box and WD-40 domain protein 27	0.00	0.00	-2.1
NM_053072.2	*Fgd6*	FYVE, RhoGEF and PH domain containing 6	0.01	0.00	-2.2
NM_023304.1	*Fgf22*	fibroblast growth factor 22	0.01	0.00	-2.9
NM_010205.1	*Fgf8*	fibroblast growth factor 8	0.00	0.00	-3.3
NM_012013.1	*Figla*	folliculogenesis specific basic helix-loop-helix	0.04	0.00	-4.1
NM_033571.1	*Fkbp6*	FK506 binding protein 6	0.00	0.00	-2.0
NM_174993.1	*Fmr1nb*	fragile X mental retardation 1 neighbor	0.00	0.00	-5.3
NM_001007580.1	*Fndc3c1*	fibronectin type III domain containing 3C1	0.00	0.00	-2.2
NM_010426.1	*Foxf1a*	forkhead box F1a	0.00	0.00	-2.4
BB437522.2	*Foxf1a*	forkhead box F1a	0.00	0.00	-2.6
NM_012020.1	*Foxl2*	forkhead box L2	0.03	0.00	-2.3
NM_183178.1	*Fsd1*	fibronectin type 3 and SPRY domain-containing protein	0.00	0.00	-3.5
NM_031261.1	*Fthl17*	ferritin, heavy polypeptide-like 17 (Fthl17)	0.00	0.00	-3.4
M74515.1	*Gabpa*	GA repeat binding protein, alpha	0.01	0.00	-2.5
NM_008069.3	*Gabrb1*	gamma-aminobutyric acid (GABA) A receptor, subunit beta 1	0.02	0.00	-6.5
NM_010252.3	*Gabrg1*	gamma-aminobutyric acid (GABA) A receptor, subunit gamma 1	0.00	0.00	-6.7
NM_172693.2	*Galnt12*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12	0.01	0.00	-2.7
NM_172451.1	*Galnt6*	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6	0.01	0.00	-3.4
NM_018734.2	*Gbp3*	guanylate binding protein 3	0.00	0.00	-7.7
NM_008620.2	*Gbp4*	guanylate binding protein 4	0.02	0.00	-5.5
NM_029509.2	*Gbp8*	guanylate-binding protein 8	0.02	0.00	-4.6
NM_013528.2	*Gfpt1*	glutamine fructose-6-phosphate transaminase 1	0.02	0.00	-3.3
NM_008116.1	*Ggt1*	gamma-glutamyltransferase 1	0.00	0.00	-2.6

NM_010288.2	*Gja1*	gap junction protein, alpha 1	0.00	0.00	-3.4
NM_147221.1	*Glis1*	GLIS family zinc finger 1	0.00	0.00	-2.7
BU937304.1	*Gm12260*	histone cluster 1, H3 pseudogene	0.02	0.00	-2.3
BB652115.1	*Gm1679*	predicted gene 1679	0.00	0.00	-2.1
AI449690.1	*Gm3858*	predicted gene 3858	0.02	0.00	-3.9
BE199336.1	*Gm4841*	predicted gene 4841	0.02	0.00	-5.9
AI196437.1	*Gm4951*	predicted gene 4951	0.00	0.00	-8.6
AI587854.1	*Gm4984*	predicted pseudogene 4984	0.01	0.00	-2.8
BY756231.1	*Gm949*	predicted gene 949	0.00	0.00	-5.1
AA718109.1	*Gm960*	predicted gene 960	0.00	0.00	-2.5
NM_008141.2	*Gnat2*	guanine nucleotide binding protein, alpha transducing 2 (Gnat2)	0.00	0.00	-3.1
NM_022422.3	*Gng13*	guanine nucleotide binding protein 13, gamma (Gng13)	0.00	0.00	-4.2
NM_026680.2	*Golt1a*	golgi transport 1 homolog A (S. cerevisiae)	0.01	0.00	-4.3
NM_029674.1	*Got1l1*	glutamic-oxaloacetic transaminase 1-like 1	0.01	0.00	-2.5
NM_145890.1	*Grhl1*	grainyhead-like 1 (Drosophila)	0.05	0.00	-3.8
NM_145890.1	*Grhl1*	grainyhead-like 1 (Drosophila)	0.01	0.00	-6.8
NM_001013756.1	*Grhl3*	grainyhead-like 3 (Drosophila)	0.01	0.00	-3.6
AK036014.1	*Grik3*	glutamate receptor, ionotropic, kainate 3	0.00	0.00	-3.7
NM_031391.1	*Gtf2a1*	general transcription factor II A, 1	0.00	0.00	-2.7
NM_027000.2	*Gtpbp4*	GTP binding protein 4	0.01	0.00	-2.1
CN834482.1	*Gtsf1*	gametocyte specific factor 1	0.00	0.00	-3.6
NM_178747.2	*Gulo*	gulonolactone (L-) oxidase	0.01	0.00	-2.9
NM_010394.2	*H2-Q7*	histocompatibility 2, Q region locus 7	0.02	0.00	-3.0
NM_146101.1	*Habp2*	hyaluronic acid binding protein 2	0.01	0.00	-2.7
NM_008234.2	*Hells*	helicase, lymphoid specific	0.02	0.00	-3.6
NM_010416.1	*Hemt1*	hematopoietic cell transcript 1 (Hemt1)	0.00	0.00	-6.1
AK017531.1	*Herc3*	hect domain and RLD 3	0.00	0.00	-2.6
CF425980.1	*Hfm1*	HFM1, ATP-dependent DNA helicase homolog (S. cerevisiae)	0.02	0.00	-3.6
AF117382.1	*Hic2*	hypermethylated in cancer 2	0.01	0.00	-2.6
NM_016660.1	*Hmga1*	high mobility group AT-hook 1	0.00	0.00	-2.2
NM_010445.1	*Hmx1*	H6 homeobox 1	0.04	0.00	-20.5
NM_009330.1	*Hnf1b*	HNF1 homeobox B	0.00	0.00	-6.3
NM_026489.1	*Hormad1*	HORMA domain containing 1	0.02	0.00	-7.7
AK015939.1	*Hormad2*	HORMA domain containing 2	0.04	0.00	-2.6

AK016553.1	*Hsf2bp*	heat shock transcription factor 2 binding protein	0.00	0.00	-3.1
CN837332.1	*Hsf5*	heat shock transcription factor family member 5	0.01	0.00	-3.3
U23921.1	*Hspa4l*	heat shock protein 4 like	0.00	0.00	-2.5
NM_016865.2	*Htatip2*	HIV-1 tat interactive protein 2, homolog (human)	0.04	0.00	-2.1
NM_153072.1	*Hus1b*	Hus1 homolog b (S. pombe)	0.00	0.00	-3.1
NM_027407.2	*Ica1l*	islet cell autoantigen 1-like	0.01	0.00	-3.5
NM_008331.1	*Ifit1*	interferon-induced protein with tetratricopeptide repeats 1	0.01	0.00	-6.3
NM_010501.1	*Ifit3*	interferon-induced protein with tetratricopeptide repeats 3 (Ifit3)	0.03	0.00	-5.2
NM_015777.1	*Igbp1b*	immunoglobulin (CD79A) binding protein 1b	0.00	0.00	-3.5
NM_009951.2	*Igf2bp1*	insulin-like growth factor 2 mRNA binding protein 1	0.00	0.00	-2.5
NM_018738.2	*Igtp*	interferon gamma induced GTPase	0.03	0.00	-5.0
AK052469.1	*Igtp*	interferon gamma induced GTPase	0.00	0.00	-5.3
NM_021792.3	*Iigp1*	interferon inducible GTPase 1 (Iigp1)	0.03	0.00	-7.7
NM_021792.3	*Iigp1*	interferon inducible GTPase 1	0.02	0.00	-8.1
NM_134109.1	*Ildr1*	immunoglobulin-like domain containing receptor 1	0.01	0.00	-2.4
NM_016851.1	*Irf6*	interferon regulatory factor 6	0.00	0.00	-3.3
NM_016850.1	*Irf7*	interferon regulatory factor 7	0.00	0.00	-3.7
NM_021459.2	*Isl1*	ISL1 transcription factor, LIM/homeodomain	0.00	0.00	-2.5
NM_027721.1	*Katnal2*	katanin p60 subunit A-like 2	0.04	0.00	-5.0
NM_010598.2	*Kcnab2*	potassium voltage-gated channel, shaker-related subfamily, beta member 2 (Kcnab2)	0.00	0.00	-3.2
NM_010601.2	*Kcnh3*	potassium voltage-gated channel, subfamily H (eag-related), member 3	0.03	0.00	-2.0
NM_008430.1	*Kcnk1*	potassium channel, subfamily K, member 1	0.00	0.00	-2.5
BM198622.2	*Kdm5b*	lysine (K)-specific demethylase 5B	0.02	0.00	-4.0
NM_010616.1	*Kif12*	kinesin family member 12	0.01	0.00	-2.1
D12645.1	*Kif3a*	kinesin family member 3A	0.01	0.00	-2.2
BC057064.1	*Klhdc10*	kelch domain containing 10	0.00	0.00	-2.8
NM_172565.1	*Klhl11*	kelch-like 11 (Drosophila)	0.00	0.00	-2.7
NM_172565.1	*Klhl11*	kelch-like 11 (Drosophila)	0.01	0.00	-2.8

AK009417.1	*Klrg2*	killer cell lectin-like receptor subfamily G, member 2	0.02	0.00	-8.4
NM_008468.1	*Kpna6*	karyopherin (importin) alpha 6	0.00	0.00	-2.2
NM_016958.1	*Krt14*	keratin 14	0.00	0.00	-4.6
NM_010663.1	*Krt17*	keratin 17	0.01	0.00	-4.4
AK009986.1	*Krt24*	keratin 24	0.01	0.00	-3.2
NM_031170.1	*Krt8*	keratin 8	0.00	0.00	-3.4
NM_027221.1	*Krtcap3*	keratinocyte associated protein 3	0.01	0.00	-2.1
NM_028622.1	*Lce1c*	late cornified envelope 1C	0.05	0.00	-2.2
NM_013580.2	*Ldhc*	lactate dehydrogenase C	0.00	0.00	-3.5
NM_010710.2	*Lhx2*	LIM homeobox protein 2	0.00	0.00	-2.9
NM_008500.1	*Lhx6*	LIM homeobox protein 6	0.00	0.00	-4.1
NM_010713.1	*Lhx8*	LIM homeobox protein 8	0.00	0.00	-4.6
D49658.1	*Lhx8*	LIM homeobox protein 8	0.01	0.00	-7.4
NM_145833.1	*Lin28a*	lin-28 homolog A (C. elegans)	0.01	0.00	-2.4
NM_057173.1	*Lmo1*	LIM domain only 1	0.00	0.00	-3.7
CD541541.1	*LOC673430*	zinc finger protein 160-like	0.03	0.00	-2.6
AK016522.1	*Lonrf3*	LON peptidase N-terminal domain and ring finger 3	0.01	0.00	-4.3
NM_022983.2	*Lpar3*	lysophosphatidic acid receptor 3	0.01	0.00	-2.6
NM_029627.1	*Ly6k*	lymphocyte antigen 6 complex, locus K	0.00	0.00	-2.2
NM_175296.3	*Mael*	maelstrom homolog (Drosophila)	0.01	0.00	-4.1
BM225062.2	*Magea10*	melanoma antigen family A, 10	0.02	0.00	-3.2
NM_020018.1	*Magea5*	melanoma antigen, family A, 5 (Magea5)	0.03	0.00	-7.0
BC031147.1	*Map3k15*	mitogen-activated protein kinase kinase kinase 15	0.00	0.00	-3.1
NM_008279.1	*Map4k1*	mitogen-activated protein kinase kinase kinase kinase 1	0.00	0.00	-2.3
NM_025979.2	*Mastl*	microtubule associated serine/threonine kinase-like	0.00	0.00	-2.3
AY512920.1	*Mdn1*	midasin homolog (yeast)	0.01	0.00	-3.2
NM_008578.1	*Mef2b*	myocyte enhancer factor 2B (Mef2b)	0.00	0.00	-3.2
NM_008588.1	*Mesp1*	mesoderm posterior 1	0.01	0.00	-4.8
AK052278.1	*Mgat4a*	mannoside acetylglucosaminyltransferase 4, isoenzyme A	0.01	0.00	-2.8
NM_177282.2	*Mical2*	microtubule associated monoxygenase, calponin and LIM domain containing 2	0.02	0.00	-2.8
NM_027696.1	*Mier1*	mesoderm induction early response 1 homolog (Xenopus laevis	0.01	0.00	-3.9

U81317.1	*Mobp*	myelin-associated oligodendrocytic basic protein	0.04	0.00	-3.2
NM_010816.1	*Morc1*	microrchidia 1	0.01	0.00	-2.6
AK084586.1	*Mphosph9*	M-phase phosphoprotein 9	0.02	0.00	-2.3
NM_031870.1	*Msh4*	mutS homolog 4 (E. coli)	0.01	0.00	-2.4
AK030316.1	*Mtap7d2*	MAP7 domain containing 2	0.01	0.00	-4.6
M74753.1	*Myh3*	myosin, heavy polypeptide 3, skeletal muscle, embryonic	0.02	0.00	-2.5
AK039333.1	*Naa30*	N(alpha)-acetyltransferase 30, NatC catalytic subunit	0.02	0.00	-2.9
NM_031389.1	*Nalp4c*	leucine rich repeat and PYD containing 4C (Nalp4c)	0.00	0.00	-2.7
BC056473.1	*Nanos1*	nanos homolog 1 (Drosophila)	0.01	0.00	-3.8
NM_145602.1	*Ndrg4*	N-myc downstream regulated gene 4	0.00	0.00	-3.3
BE915912.1	*Necab3*	N-terminal EF-hand calcium binding protein 3	0.00	0.00	-2.8
NM_011848.1	*Nek3*	NIMA (never in mitosis gene a)-related expressed kinase 3	0.00	0.00	-2.4
AK083512.1	*Neto2*	neuropilin (NRP) and tolloid (TLL)-like 2	0.01	0.00	-4.1
BE292026.1	*Nfib*	nuclear factor I/B	0.01	0.00	-2.6
NM_008690.2	*Nfkbie*	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	0.02	0.00	-2.2
NM_025998.1	*Nkain1*	Na+/K+ transporting ATPase interacting 1	0.00	0.00	-2.6
AF202039.1	*Nkx2-4*	NK2 transcription factor related, locus 4 (Drosophila)	0.01	0.00	-5.1
NM_008708.1	*Nmt2*	N-myristoyltransferase 2	0.00	0.00	-2.8
NM_021315.1	*Noc3l*	nucleolar complex associated 3 homolog (S. cerevisiae)	0.00	0.00	-2.4
CF613235.1	*Npw*	neuropeptide W	0.00	0.00	-3.1
BM120306.2	*Nr3c2*	nuclear receptor subfamily 3, group C, member 2	0.01	0.00	-2.1
NM_020610.1	*Nrip3*	nuclear receptor interacting protein 3	0.00	0.00	-3.8
BU756239.1	*Nup62cl*	nucleoporin 62 C-terminal like	0.00	0.00	-2.3
NM_170591.1	*Nupl1*	nucleoporin like 1	0.05	0.00	-2.6
NM_031259.1	*Nxf2*	nuclear RNA export factor 2	0.01	0.00	-5.5
NM_145227.1	*Oas2*	2'-5' oligoadenylate synthetase 2	0.00	0.00	-3.3
NM_011854.1	*Oasl2*	2'-5' oligoadenylate synthetase-like 2	0.04	0.00	-3.1
NM_207201.1	*Olfr8*	olfactory receptor 8	0.03	0.00	-2.0
NM_011015.1	*Orc1*	origin recognition complex, subunit 1	0.02	0.00	-2.9
NM_152818.2	*Osbp2*	oxysterol binding protein 2	0.00	0.00	-2.0
CA570565.1	*Osbpl9*	oxysterol binding protein-like 9	0.02	0.00	-2.0

AK015275.1	*Otud4*	OTU domain containing 4	0.00	0.00	-2.3
NM_011023.2	*Otx1*	orthodenticle homolog 1 (Drosophila)	0.03	0.00	-9.4
NM_019935.2	*Ovol1*	OVO homolog-like 1 (Drosophila)	0.04	0.00	-2.4
NM_026924.2	*Ovol2*	ovo-like 2 (Drosophila)	0.02	0.00	-2.1
NM_011060.1	*Padi3*	peptidyl arginine deiminase, type III	0.00	0.00	-4.5
NM_011035.1	*Pak1*	p21 protein (Cdc42/Rac)-activated kinase 1	0.04	0.00	-2.9
BC052527.1	*Pank4*	pantothenate kinase 4	0.02	0.00	-3.3
AK005563.1	*Parp14*	poly (ADP-ribose) polymerase family, member 14	0.00	0.00	-2.4
NM_011040.2	*Pax8*	paired box gene 8	0.02	0.00	-2.4
NM_029508.1	*Pcgf5*	polycomb group ring finger 5	0.01	0.00	-3.6
NM_008806.1	*Pde6b*	phosphodiesterase 6B, cGMP, rod receptor, beta polypeptide	0.00	0.00	-2.9
BF534000.1	*Pdxk*	pyridoxal (pyridoxine, vitamin B6) kinase	0.00	0.00	-2.4
NM_008821.1	*Pet2*	plasmacytoma expressed transcript 2	0.01	0.00	-2.1
AK017146.1	*Pgap1*	post-GPI attachment to proteins 1	0.00	0.00	-3.2
BB469950.2	*Phb*	prohibitin	0.01	0.00	-2.6
NM_199299.1	*Phf15*	PHD finger protein 15	0.01	0.00	-2.7
AK004823.1	*Phf15*	PHD finger protein 15	0.00	0.00	-4.3
BI695483.1	*Phf17*	PHD finger protein 17	0.00	0.00	-2.7
NM_009434.2	*Phlda2*	pleckstrin homology-like domain, family A, member 2	0.00	0.00	-10.4
NM_178621.2	*Phyhipl*	phytanoyl-CoA hydroxylase interacting protein-like	0.01	0.00	-4.8
BF450484.1	*Pign*	phosphatidylinositol glycan anchor biosynthesis, class N	0.04	0.00	-2.3
NM_029094.1	*Pik3cb*	phosphatidylinositol 3-kinase, catalytic, beta polypeptide	0.00	0.00	-2.0
NM_021308.1	*Piwil2*	piwi-like homolog 2 (Drosophila)	0.03	0.00	-5.9
NM_001024145.1	*Pla2g4f*	phospholipase A2, group IVF	0.00	0.00	-3.1
NM_172285.1	*Plcg2*	phospholipase C, gamma 2	0.01	0.00	-2.1
NM_183191.1	*Plch1*	phospholipase C, eta 1	0.01	0.00	-4.5
BG298178.1	*Pnldc1*	poly(A)-specific ribonuclease (PARN)-like domain containing 1	0.04	0.00	-2.4
BB698563.1	*Pnma5*	paraneoplastic antigen family 5	0.01	0.00	-2.4
NM_011138.1	*Pou2f2*	POU domain, class 2, transcription factor 2	0.00	0.00	-3.0
NM_008900.1	*Pou3f3*	POU domain, class 3, transcription factor 3	0.00	0.00	-6.1
NM_175363.2	*Pphln1*	periphilin 1	0.00	0.00	-2.7

NM_029948.1	*Pramef12*	PRAME family member 12	0.01	0.00	-7.3
NM_031377.1	*Pramel1*	preferentially expressed antigen in melanoma-like 1	0.05	0.00	-2.1
NM_031390.1	*Pramel3*	preferentially expressed antigen in melanoma-like 3 (Pramel3)	0.02	0.00	-4.8
BF152648.1	*Prr19*	proline rich 19	0.01	0.00	-4.8
AK006612.1	*Prss44*	protease, serine, 44	0.03	0.00	-3.3
NM_146227.2	*Prss50*	protease, serine, 50	0.04	0.00	-2.5
NM_133351.1	*Prss8*	protease, serine, 8 (prostasin)	0.00	0.00	-4.5
NM_011178.2	*Prtn3*	proteinase 3	0.03	0.00	-2.5
AK010717.1	*Psma8*	proteasome (prosome, macropain) subunit, alpha type, 8	0.03	0.00	-2.4
NM_013585.1	*Psmb9*	proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2)	0.04	0.00	-3.1
NM_016899.2	*Rab25*	RAB25, member RAS oncogene family	0.01	0.00	-2.1
NM_011234.2	*Rad51*	RAD51 homolog (S. cerevisiae)	0.04	0.00	-2.3
AK076551.1	*Rad51c*	RAD51 homolog c (S. cerevisiae)	0.03	0.00	-2.4
W11780.1	*Rap1gap*	Rap1 GTPase-activating protein	0.00	0.00	-3.0
NM_053268.1	*Rasa2*	RAS p21 protein activator 2	0.05	0.00	-2.1
BF319710.1	*Rasal2*	RAS protein activator like 2	0.01	0.00	-3.4
AK015898.1	*Rasd2*	RASD family, member 2	0.00	0.00	-4.1
BC042449.1	*Rasgrf1*	RAS protein-specific guanine nucleotide-releasing factor 1	0.01	0.00	-2.4
NM_019547.1	*Rbm38*	RNA binding motif protein 38	0.00	0.00	-2.8
NM_029660.1	*Rbmxl2*	RNA binding motif protein, X-linked-like 2	0.02	0.00	-7.3
NM_011253.1	*Rbmy1a1*	RNA binding motif protein, Y chromosome, family 1, member A1	0.01	0.00	-4.8
NM_020002.2	*Rec8*	REC8 homolog (yeast)	0.01	0.00	-2.4
NM_058214.1	*Recql4*	RecQ protein-like 4	0.03	0.00	-2.3
NM_011261.1	*Reln*	reelin	0.02	0.00	-2.2
NM_026097.1	*Rffl*	ring finger and FYVE like domain containing protein	0.00	0.00	-2.3
AK007253.1	*Rhox13*	reproductive homeobox 13	0.00	0.00	-6.2
NM_027897.2	*Rhpn2*	rhophilin, Rho GTPase binding protein 2 (Rhpn2)	0.01	0.00	-3.0
NM_025660.1	*Ribc1*	RIB43A domain with coiled-coils 1	0.01	0.00	-3.0

AY497009.1	*Rictor*	RPTOR independent companion of MTOR, complex 2	0.00	0.00	-2.4
NM_023663.3	*Ripk4*	receptor-interacting serine-threonine kinase 4	0.01	0.00	-3.1
NM_023270.3	*Rnf128*	ring finger protein 128	0.03	0.00	-2.4
NM_013894.1	*Rnf17*	ring finger protein 17	0.01	0.00	-6.1
BB014105.2	*Rnf17*	ring finger protein 17	0.01	0.00	-9.6
NM_026594.1	*Rpl39l*	ribosomal protein L39-like	0.04	0.00	-2.5
NM_027491.1	*Rragd*	Ras-related GTP binding D	0.00	0.00	-2.5
BC023785.1	*Rreb1*	ras responsive element binding protein 1	0.00	0.00	-2.1
NM_153457.6	*Rtn1*	reticulon 1 (Rtn1), transcript variant 1	0.00	0.00	-2.0
NM_198620.1	*Rundc3b*	RUN domain containing 3B	0.04	0.00	-3.4
NM_025393.1	*S100a14*	S100 calcium binding protein A14	0.01	0.00	-2.5
BC062937.1	*Sall1*	sal-like 1 (Drosophila)	0.00	0.00	-3.0
AK017633.1	*Sall4*	sal-like 4 (Drosophila)	0.01	0.00	-3.1
NM_026283.1	*Samd8*	sterile alpha motif domain containing 8	0.03	0.00	-2.2
BG833093.1	*Satb1*	special AT-rich sequence binding protein 1	0.03	0.00	-2.7
BG087102.2	*Scml1*	Vsex comb on midleg-like 1 (Drosophila)	0.02	0.00	-3.5
NM_133194.2	*Scml2*	sex comb on midleg-like 2 (Drosophila)	0.04	0.00	-5.4
NM_019460.1	*Sfmbt1*	Scm-like with four mbt domains 1	0.02	0.00	-2.0
AK076687.1	*Sfmbt2*	Scm-like with four mbt domains 2	0.01	0.00	-2.9
AV304616.2	*Shh*	sonic hedgehog	0.00	0.00	-3.2
NM_009170.2	*Shh*	sonic hedgehog	0.01	0.00	-4.9
NM_181590.2	*Shq1*	SHQ1 homolog (S. cerevisiae)	0.00	0.00	-2.5
AK012978.1	*Shq1*	SHQ1 homolog (S. cerevisiae)	0.00	0.00	-2.5
NM_009173.1	*Siah1b*	seven in absentia 1B	0.03	0.00	-3.0
NM_011380.1	*Six2*	sine oculis-related homeobox 2 homolog (Drosophila)	0.01	0.00	-2.9
NM_029084.1	*Slamf8*	SLAM family member 8	0.00	0.00	-2.8
NM_178386.2	*Slc25a31*	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31	0.01	0.00	-3.8
BY761173.1	*Slc39a9*	solute carrier family 39 (zinc transporter), member 9	0.00	0.00	-2.1
NM_007514.1	*Slc7a2*	solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	0.00	0.00	-3.0

NM_080470.1	*Smc1b*	structural maintenance of chromosomes 1B	0.00	0.00	-4.0
NM_024230.1	*Smtnl1*	smoothelin-like 1	0.00	0.00	-2.7
NM_013914.2	*Snai3*	snail homolog 3 (Drosophila)	0.00	0.00	-6.1
NM_001001714.1	*Sohlh1*	spermatogenesis and oogenesis specific basic helix-loop-helix 1	0.00	0.00	-7.0
NM_028937.1	*Sohlh2*	spermatogenesis and oogenesis specific basic helix-loop-helix 2	0.01	0.00	-3.6
BC052024.1	*Sox3*	SRY-box containing gene 3	0.00	0.00	-13.3
NM_030220.2	*Sp2*	Sp2 transcription factor	0.00	0.00	-4.1
NM_022435.2	*Sp5*	trans-acting transcription factor 5	0.02	0.00	-4.5
NM_029299.1	*Spata19*	spermatogenesis associated 19	0.02	0.00	-2.7
NM_021343.1	*Spata5*	spermatogenesis associated 5	0.03	0.00	-3.3
NM_030061.1	*Spink12*	serine peptidase inhibitor, Kazal type 11	0.01	0.00	-3.4
NM_016907.2	*Spint1*	serine protease inhibitor, Kunitz type 1	0.02	0.00	-2.6
NM_138673.1	*Stab2*	stabilin 2	0.05	0.00	-2.7
U06924.1	*Stat1*	signal transducer and activator of transcription 1	0.00	0.00	-3.7
NM_027399.1	*Steap1*	six transmembrane epithelial antigen of the prostate 1	0.01	0.00	-2.8
NM_009292.1	*Stra8*	stimulated by retinoic acid gene 8	0.03	0.00	-26.7
NM_013873.3	*Sult4a1*	sulfotransferase family 4A, member 1	0.00	0.00	-3.0
BC032960.1	*Suv39h2*	suppressor of variegation 3-9 homolog 2 (Drosophila)	0.02	0.00	-2.6
NM_011516.1	*Sycp1*	synaptonemal complex protein 1	0.00	0.00	-5.8
AK014411.1	*Sycp2*	synaptonemal complex protein 2	0.01	0.00	-8.9
NM_011517.1	*Sycp3*	synaptonemal complex protein 3	0.03	0.00	-3.3
NM_021482.1	*Syngr4*	synaptogyrin 4	0.00	0.00	-4.5
AK129267.1	*Synpo*	synaptopodin	0.03	0.00	-2.7
AB026802.1	*Syt9*	synaptotagmin IX	0.03	0.00	-2.9
NM_028958.2	*Taf7l*	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.03	0.00	-6.7
NM_001001176.1	*Taf9b*	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor	0.01	0.00	-2.6

NM_013683.1	*Tap1*	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	0.01	0.00	-4.0
NM_019636.1	*Tbc1d1*	TBC1 domain family, member 1	0.02	0.00	-2.9
NM_178254.2	*Tcfl5*	transcription factor-like 5 (basic helix-loop-helix)	0.03	0.00	-4.6
NM_011560.2	*Tcte3*	t-complex-associated testis expressed 3	0.00	0.00	-2.2
NM_001002238.1	*Tdrd1*	tudor domain containing 1	0.02	0.00	-5.5
AK012596.1	*Tdrkh*	tudor and KH domain containing protein	0.03	0.00	-3.0
NM_019981.1	*Tex101*	testis expressed gene 101	0.00	0.00	-3.9
NM_031384.1	*Tex11*	testis expressed gene 11	0.02	0.00	-15.4
NM_025687.1	*Tex12*	testis expressed gene 12	0.00	0.00	-5.9
NM_031386.1	*Tex14*	testis expressed gene 14	0.02	0.00	-3.3
NM_031374.1	*Tex15*	testis expressed gene 15	0.02	0.00	-3.7
NM_031382.1	*Tex16*	testis expressed gene 16	0.01	0.00	-3.2
NM_031385.1	*Tex18*	testis expressed gene 18	0.02	0.00	-5.4
NM_028602.2	*Tex19.1*	testis expressed gene 19.1	0.00	0.00	-3.0
NM_009359.1	*Tex9*	testis expressed gene 9	0.01	0.00	-2.1
NM_011575.1	*Tff3*	trefoil factor 3, intestinal	0.04	0.00	-2.0
NM_011579.2	*Tgtp1*	T-cell specific GTPase 1	0.03	0.00	-8.4
NM_012033.2	*Tinag*	tubulointerstitial nephritis antigen	0.01	0.00	-3.1
NM_028927.1	*Tktl2*	transketolase-like 2	0.00	0.00	-7.5
NM_009389.1	*Tle3*	transducin-like enhancer of split 3, homolog of Drosophila E(spl)	0.01	0.00	-2.5
NM_025781.1	*Tmem170*	transmembrane protein 170	0.03	0.00	-2.7
BY731679.1	*Tmem170b*	transmembrane protein 170B	0.00	0.00	-4.3
NM_177013.2	*Tmem229a*	transmembrane protein 229A	0.00	0.00	-4.0
NM_001008973.1	*Tmem232*	transmembrane protein 232	0.00	0.00	-2.0
BB365838.2	*Tnn*	tenascin N	0.03	0.00	-2.5
NM_198601.1	*Trim52*	tripartite motif-containing 52	0.00	0.00	-4.0
AV117999.1	*Trim71*	tripartite motif-containing 71	0.05	0.00	-2.1
BC049168.1	*Trnp1*	TMF1-regulated nuclear protein 1	0.00	0.00	-3.6
BC050805.1	*Ttc25*	tetratricopeptide repeat domain 25	0.01	0.00	-2.7
AK007856.1	*Ttc39b*	tetratricopeptide repeat domain 39B	0.00	0.00	-2.2
NM_009445.1	*Ttk*	Ttk protein kinase	0.00	0.00	-2.0
NM_009446.1	*Tuba3*	tubulin, alpha 3 (Tuba3)	0.00	0.00	-4.5
BB268360.2	*Uba3*	ubiquitin-like modifier activating enzyme 3	0.04	0.00	-2.1
NM_172712.2	*Uba6*	ubiquitin-like modifier activating enzyme 6	0.02	0.00	-2.6

AK078845.1	*Ube2e3*	ubiquitin-conjugating enzyme E2E 3, UBC4/5 homolog (yeast)	0.00	0.00	-7.6
NM_011670.1	*Uchl1*	ubiquitin carboxy-terminal hydrolase L1	0.00	0.00	-2.4
NM_010633.3	*Uhmk1*	U2AF homology motif (UHM) kinase 1	0.00	0.00	-2.2
NM_009477.1	*Upp1*	uridine phosphorylase 1 (Upp1)	0.00	0.00	-3.4
AK006739.1	*Usp12*	ubiquitin specific peptidase 12	0.03	0.00	-3.8
NM_011909.1	*Usp18*	ubiquitin specific peptidase 18	0.00	0.00	-5.1
NM_176972.2	*Usp37*	ubiquitin specific peptidase 37	0.00	0.00	-2.2
NM_152825.1	*Usp45*	ubiquitin specific petidase 45	0.03	0.00	-2.2
NM_009482.1	*Utf1*	undifferentiated embryonic cell transcription factor 1	0.02	0.00	-7.3
BC048955.1	*Utp20*	UTP20, small subunit (SSU) processome component, homolog (yeast)	0.02	0.00	-2.0
NM_173028.2	*Vps13a*	vacuolar protein sorting 13A (yeast)	0.02	0.00	-4.7
NM_172840.2	*Vwa2*	von Willebrand factor A domain containing 2	0.00	0.00	-3.0
NM_027963.1	*Wdr16*	WD repeat domain 16	0.00	0.00	-2.1
NM_080848.1	*Wdr5*	WD repeat domain 5	0.00	0.00	-2.1
AK129287.1	*Whsc1*	Wolf-Hirschhorn syndrome candidate 1 (human)	0.01	0.00	-2.2
AK010881.1	*Wipi2*	WD repeat domain, phosphoinositide interacting 2	0.04	0.00	-2.5
NM_011719.2	*Wnt9b*	wingless-type MMTV integration site 9B	0.02	0.00	-4.9
NM_011726.1	*Xlr3a*	X-linked lymphocyte-regulated 3a (Xlr3a)	0.00	0.00	-2.5
NM_011726.1	*Xlr3a*	X-linked lymphocyte-regulated 3A	0.00	0.00	-8.8
NM_031493.1	*Xlr5c*	X-linked lymphocyte-regulated 5C	0.02	0.00	-6.2
NM_026858.2	*Xrcc6bp1*	XRCC6 binding protein 1	0.00	0.00	-2.1
NM_021394.1	*Zbp1*	Z-DNA binding protein 1	0.00	0.00	-9.7
AA882005.1	*Zbtb16*	zinc finger and BTB domain containing 16	0.04	0.00	-3.1
BC051084.1	*Zbtb42*	zinc finger and BTB domain containing 42	0.00	0.00	-3.8
BU756529.1	*Zcchc2*	zinc finger, CCHC domain containing 2	0.01	0.00	-2.1
AK016727.1	*Zfp597*	zinc finger protein 597	0.03	0.00	-2.7
NM_028913.1	*Zfp819*	zinc finger protein 819	0.00	0.00	-3.7
BM240956.2	*Zfp850*	zinc finger protein 850	0.04	0.00	-2.1
NM_009571.1	*Zfy2*	zinc finger protein 2, Y linked (Zfy2)	0.00	0.00	-5.8
AF401983.1	*Zim2*	zinc finger, imprinted 2	0.00	0.00	-4.2

NM_015785.1	*Zpbp*	zona pellucida binding protein	0.04	0.00	-2.3
NM_178375.2	*Zswim3*	zinc finger, SWIM domain containing 3	0.01	0.00	-2.8
BE946949.1	*Zyg11b*	zyg-ll homolog B (C. elegans)	0.00	0.00	-2.5
CA479032.1	*1190003J15Rik*	RIKEN cDNA 1190003J15 gene	0.01	0.00	-2.8
AK005608.1	*1700001L05Rik*	RIKEN cDNA 1700001L05 gene	0.00	0.00	-3.1
NM_029372.1	*1700011F14Rik*	RIKEN cDNA 1700011F14 gene	0.00	0.00	-2.8
AK005953.1	*1700013H16Rik*	RIKEN cDNA 1700013H16 gene	0.00	0.00	-9.2
NM_198637.1	*1700016K19Rik*	RIKEN cDNA 1700016K19 gene	0.00	0.00	-3.3
NM_025493.2	*1700018B24Rik*	enhancer of rudimentary homolog pseudogene	0.00	0.00	-5.4
AK006179.1	*1700020N01Rik*	RIKEN cDNA 1700020N01 gene	0.00	0.00	-2.5
AK006257.1	*1700023A16Rik*	RIKEN cDNA 1700023A16 gene	0.00	0.00	-3.2
CN843262.1	*1700024P04Rik*	RIKEN cDNA 1700024P04 gene	0.02	0.00	-2.4
NM_025503.1	*1700029P11Rik*	RIKEN cDNA 1700029P11 gene	0.01	0.00	-7.0
AK006959.1	*1700080O16Rik*	RIKEN cDNA 1700080O16 gene	0.02	0.00	-3.0
BY707078.1	*1700086P04Rik*	RIKEN cDNA 1700086P04 gene	0.00	0.00	-6.2
AK007177.1	*1700112H15Rik*	RIKEN cDNA 1700112H15 gene	0.00	0.00	-2.3
AK007250.1	*1700123I01Rik*	RIKEN cDNA 1700123I01 gene	0.00	0.00	-4.4
AK019107.1	*2410006F04Rik*	RIKEN cDNA 2410006F04 gene	0.01	0.00	-3.8
NM_025572.1	*2610528J11Rik*	RIKEN cDNA 2610528J11 gene	0.05	0.00	-2.5
NM_027512.1	*3830417A13Rik*	RIKEN cDNA 3830417A13 gene	0.05	0.00	-4.0
NM_025723.1	*4921515J06Rik*	RIKEN cDNA 4921515J06 gene	0.00	0.00	-10.8
AK015678.1	*4930502E18Rik*	RIKEN cDNA 4930502E18 gene	0.00	0.00	-2.1
NM_026262.1	*4930524B15Rik*	RIKEN cDNA 4930524B15 gene	0.05	0.00	-3.8
BC024760.1	*4930529M08Rik*	RIKEN cDNA 4930529M08 gene	0.02	0.00	-2.5
NM_198607.1	*4930572J05Rik*	RIKEN cDNA 4930572J05 gene	0.01	0.00	-2.5
AK016695.1	*4933406J08Rik*	RIKEN cDNA 4933406J08 gene	0.00	0.00	-3.6

AK048125.1	*4933411K20Rik*	RIKEN cDNA 4933411K20 gene	0.00	0.00	-4.1
AV281902.2	*4933427D06Rik*	RIKEN cDNA 4933427D06 gene	0.00	0.00	-7.1
NM_175017.2	*4933427D06Rik*	RIKEN cDNA 4933427D06 gene	0.01	0.00	-9.7
AV347067.2	*6430590A07Rik*	RIKEN cDNA 6430590A07 gene	0.00	0.00	-2.3
AK033245.1	*8030474K03Rik*	RIKEN cDNA 8030474K03 gene	0.00	0.00	-4.0
AK020619.1	*9530062K07Rik*	RIKEN cDNA 9530062K07 gene	0.04	0.00	-4.6
BC042745.1	*A330069E16Rik*	RIKEN cDNA A330069E16 gene	0.02	0.00	-2.3
NM_178796.3	*A530064D06Rik*	RIKEN cDNA A530064D06 gene	0.04	0.00	-3.3
NM_001004174.1	*AA467197*	expressed sequence AA467197	0.03	0.00	-3.0
BC020182.1			0.00	0.00	-2.0
NM_134063.2			0.00	0.00	-2.0
BC036332.1			0.03	0.00	-2.1
AK015922.1			0.05	0.00	-2.1
BB554612.2			0.02	0.00	-2.1
AK045740.1			0.01	0.00	-2.1
BY703358.1			0.01	0.00	-2.1
CO806983.1			0.02	0.00	-2.2
BY742236.1			0.01	0.00	-2.2
AI323998.1			0.00	0.00	-2.2
NM_201364.1			0.05	0.00	-2.2
BB559411.2			0.04	0.00	-2.2
CA464433.1			0.04	0.00	-2.2
BB268253.1			0.03	0.00	-2.3
AK082203.1			0.01	0.00	-2.3
BY368931.1			0.00	0.00	-2.3
BY710919.1			0.01	0.00	-2.3
AK079871.1			0.03	0.00	-2.3
BQ550570.1			0.04	0.00	-2.6
BE957117.1			0.00	0.00	-2.6
AI507010.1			0.00	0.00	-2.6
AK076976.1			0.00	0.00	-2.7
BB729271.1			0.02	0.00	-2.7
NM_023781.3			0.01	0.00	-2.7
AV254043.2			0.02	0.00	-2.7
BB520952.2			0.02	0.00	-2.8
NM_170757.1			0.02	0.00	-2.8
BG082668.2			0.00	0.00	-2.9
BB858483.1			0.00	0.00	-3.0
AK015184.1			0.04	0.00	-3.1

AK015606.1			0.00	0.00	-3.1
BB048290.1			0.02	0.00	-3.1
BE944454.1			0.02	0.00	-3.3
AI451225.1			0.04	0.00	-3.4
AK015130.1			0.00	0.00	-3.4
NM_009109.1			0.01	0.00	-3.4
BQ174097.1			0.00	0.00	-3.6
AI118499.1			0.03	0.00	-3.7
BX630487.1			0.00	0.00	-3.7
BB185781.1			0.00	0.00	-4.5
BG075960.2			0.04	0.00	-4.6
BF018919.1			0.02	0.00	-4.7
AW061221.1			0.00	0.00	-4.8
NM_023386.3			0.00	0.00	-4.9
BQ257921.1			0.03	0.00	-5.7
BG083749.2			0.00	0.00	-6.3
BY706965.1			0.00	0.00	-6.4
AI591895.1			0.02	0.00	-14.4

Supplemental Table 2a

Annotation Cluster 1	**Enrichment Score**	**2.09**
Term	**p-value**	**Genes**
GO0006935~chemotaxis	0.002	ROBO1, S100A9, CCL9, CCL17, CCL6
GO0042330~taxis	0.002	ROBO1, S100A9, CCL9, CCL17, CCL6
GO0007610~behavior	0.010	ROBO1, S100A9, CCL9, ZIC1, APBB1, CCL17, CCL6
GO0007626~locomotory behavior	0.024	ROBO1, S100A9, CCL9, CCL17, CCL6
GO0006955~immune response	0.063	THEMIS, CCL9, CFI, LTB, CCL17, CCL6
Annotation Cluster 2	**Enrichment Score**	**1.99**
Term	**p-value**	**Genes**
GO0016477~cell migration	0.005	VAV3, TNS1, MDGA1, S100A9, ESR2, APBB1
GO0006928~cell motion	0.006	VAV3, TNS1, ROBO1, MDGA1, S100A9, ESR2, APBB1
GO0048870~cell motility	0.009	VAV3, TNS1, MDGA1, S100A9, ESR2, APBB1
GO0051674~localization of cell	0.009	VAV3, TNS1, MDGA1, S100A9, ESR2, APBB1
GO0001764~neuron migration	0.041	MDGA1, ESR2, APBB1
Annotation Cluster 3	**Enrichment Score**	**0.94**
Term	**p-value**	**Genes**
GO0030029~actin filament-based process	0.046	ACTA1, S100A9, CNN1, APBB1
GO0030036~actin cytoskeleton organization	0.174	ACTA1, S100A9, CNN1
GO0007010~cytoskeleton organization	0.186	ACTA1, S100A9, CNN1, TACC2

Supplemental Table 2b

Annotation Cluster 1	**Enrichment Score**	**12.61**
GO Term	**p-value**	**Genes**
0048232~male gamete generation	0.000	RAD51C, RNF17, SOX3, ADAD1, MAEL, GGT1, ZBTB16, SOHLH1, SOHLH2, TAF7L, STRA8, DDX25, OVOL1, PIWIL2, DAZL, SPATA5, TDRD1, CCNB1IP1, MSH4, TLE3, MORC1, HMGA1, REC8, CLGN, TEX15, SYCP3, TCFL5, D1PAS1, SPATA19, DMC1
0007283~spermato-genesis	0.000	RAD51C, RNF17, SOX3, ADAD1, MAEL, GGT1, ZBTB16, SOHLH1, SOHLH2, TAF7L, STRA8, DDX25, OVOL1, PIWIL2, DAZL, SPATA5, TDRD1, CCNB1IP1, MSH4, TLE3, MORC1, HMGA1, REC8, CLGN, TEX15, SYCP3, TCFL5, D1PAS1, SPATA19, DMC1
0019953~sexual reproduction	0.000	RAD51C, RNF17, SOX3, ADAD1, FIGLA, MAEL, ASZ1, GGT1, ZBTB16, SYCP2, SOHLH1, SOHLH2, TAF7L, ZPBP, STRA8, DDX25, OVOL1, PIWIL2, DAZL, DND1, SPATA5, TDRD1, CCNB1IP1, MSH4, TLE3, MORC1, HMGA1, REC8, TEX15, CLGN, TCFL5, SYCP3, D1PAS1, SPATA19, DMC1, TEX11
0007276~gamete generation	0.000	RAD51C, RNF17, SOX3, ADAD1, FIGLA, MAEL, ASZ1, GGT1, ZBTB16, SOHLH1, SOHLH2, TAF7L, STRA8, DDX25, OVOL1, PIWIL2, DAZL, DND1, SPATA5, TDRD1, CCNB1IP1, MSH4, TLE3, MORC1, HMGA1, REC8, CLGN, TEX15, TCFL5, SYCP3, D1PAS1, SPATA19, DMC1
0032504~multicellular organism reproduction	0.000	RAD51C, RNF17, SOX3, ADAD1, FIGLA, MAEL, ASZ1, GGT1, ZBTB16, SOHLH1, SOHLH2, TAF7L, STRA8, DDX25, OVOL1, PIWIL2, DAZL, CHRNA7, DND1, SPATA5, TDRD1, CCNB1IP1, FOXL2, MSH4, TLE3, MORC1, HMGA1, REC8, CLGN, TEX15, TCFL5, SYCP3, D1PAS1, SPATA19, DMC1
0048609~reproductive process in a multicellular organism	0.000	RAD51C, RNF17, SOX3, ADAD1, FIGLA, MAEL, ASZ1, GGT1, ZBTB16, SOHLH1, SOHLH2, TAF7L, STRA8, DDX25, OVOL1, PIWIL2, DAZL, CHRNA7, DND1, SPATA5, TDRD1, CCNB1IP1, FOXL2, MSH4, TLE3, MORC1, HMGA1, REC8, CLGN, TEX15, TCFL5, SYCP3, D1PAS1, SPATA19, DMC1
Annotation Cluster 2	**Enrichment Score**	**10.53**
GO Term	**p-value**	**Genes**
0007127~meiosis I	0.000	CCNB1IP1, RAD51C, MSH4, MAEL, CPEB1, SYCP1, RAD51, REC8, TEX15, STRA8, SYCP3, OVOL1, PIWIL2, DMC1, TEX11

0007126~meiosis	0.000	CCNB1IP1, RAD51C, MSH4, MAEL, CPEB1, SYCP2, SYCP1, RAD51, SUV39H2, REC8, CLGN, TEX15, STRA8, SYCP3, OVOL1, PIWIL2, DMC1, TDRD1, TEX11, SMC1B
0051327~M phase of meiotic cell cycle	0.000	CCNB1IP1, RAD51C, MSH4, MAEL, CPEB1, SYCP2, SYCP1, RAD51, SUV39H2, REC8, CLGN, TEX15, STRA8, SYCP3, OVOL1, PIWIL2, DMC1, TDRD1, TEX11, SMC1B
0051321~meiotic cell cycle	0.000	CCNB1IP1, RAD51C, MSH4, MAEL, CPEB1, SYCP2, SYCP1, RAD51, SUV39H2, REC8, CLGN, TEX15, STRA8, SYCP3, OVOL1, PIWIL2, DMC1, TDRD1, TEX11, SMC1B
0000279~M phase	0.000	RAD51C, NEK3, MAEL, CPEB1, SYCP2, SYCP1, STRA8, OVOL1, PIWIL2, TDRD1, HELLS, CCNB1IP1, MSH4, RAD51, SUV39H2, FSD1, REC8, CLGN, TEX15, SYCP3, HORMAD1, HORMAD2, DMC1, SMC1B, TEX11
0022403~cell cycle phase	0.000	RAD51C, NEK3, MAEL, CPEB1, SYCP2, SYCP1, STRA8, OVOL1, PIWIL2, TDRD1, HELLS, CCNB1IP1, MSH4, RAD51, SUV39H2, FSD1, REC8, CLGN, TEX15, SYCP3, HORMAD1, HORMAD2, DMC1, SMC1B, TEX11
0022402~cell cycle process	0.000	RAD51C, NEK3, MAEL, CPEB1, SYCP2, SYCP1, UHMK1, CDKN2A, STRA8, OVOL1, PIWIL2, TDRD1, HELLS, CCNB1IP1, MSH4, RAD51, SUV39H2, FSD1, REC8, CLGN, TEX15, SYCP3, HORMAD1, HORMAD2, DMC1, TEX11, SMC1B
0007049~cell cycle	0.000	RAD51C, NEK3, MAEL, CPEB1, SYCP2, SYCP1, UHMK1, CDKN2A, STRA8, FANCI, OVOL1, PIWIL2, TDRD1, HELLS, CCNB1IP1, TRNP1, MSH4, SUV39H2, RAD51, FSD1, REC8, TEX15, CLGN, SYCP3, UBA3, HORMAD1, RBM38, HORMAD2, DMC1, TEX11, SMC1B
Annotation Cluster 3	**Enrichment Score**	**2.73**
GO Term	p-value	Genes
0007062~sister chromatid cohesion	0.001	RAD51C, SYCP3, STRA8, SMC1B
0045132~meiotic chromosome segregation	0.001	RAD51C, SYCP3, STRA8, TEX11
0007066~female meiosis sister chromatid cohesion	0.002	RAD51C, SYCP3, STRA8
0007143~female meiosis	0.003	RAD51C, SYCP3, STRA8, SYCP2
0051177~meiotic sister chromatid cohesion	0.004	RAD51C, SYCP3, STRA8

Annotation Cluster 4	**Enrichment Score**	**2.33**
GO Term	**p-value**	**Genes**
0010557~positive regulation of macromolecule biosynthetic process	0.001	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, LIN28A, ISL1, HMGA1, SHH, SALL4, OVOL2, KRT17, IRF6, PAX8, POU2F2, POU3F3, PIWIL2, DAZL, EAF2, NFIB
0031328~positive regulation of cellular biosynthetic process	0.002	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, LIN28A, ISL1, HMGA1, SHH, SALL4, OVOL2, KRT17, IRF6, PAX8, POU2F2, POU3F3, PIWIL2, DAZL, EAF2, NFIB
0009891~positive regulation of biosynthetic process	0.002	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, LIN28A, ISL1, HMGA1, SHH, SALL4, OVOL2, KRT17, IRF6, PAX8, POU2F2, POU3F3, PIWIL2, DAZL, EAF2, NFIB
0010604~positive regulation of macromolecule metabolic process	0.002	FGF8, HNF1B, HTATIP2, GLIS1, EHF, LIN28A, SHH, OVOL2, POU2F2, PAX8, POU3F3, PIWIL2, DAZL, UTF1, FOXL2, SIX2, GRHL3, RICTOR, ISL1, HMGA1, SALL4, KRT17, IRF6, EAF2, NFIB
0010628~positive regulation of gene expression	0.005	FOXL2, UTF1, HNF1B, FGF8, HTATIP2, GLIS1, SIX2, EHF, GRHL3, ISL1, HMGA1, SHH, SALL4, OVOL2, IRF6, PAX8, POU2F2, POU3F3, EAF2, NFIB
0045941~positive regulation of transcription	0.008	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, ISL1, HMGA1, SHH, SALL4, OVOL2, IRF6, PAX8, POU2F2, POU3F3, EAF2, NFIB
0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.017	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, ISL1, HMGA1, SHH, SALL4, OVOL2, IRF6, PAX8, POU2F2, POU3F3, EAF2, NFIB
0051173~positive regulation of nitrogen compound metabolic process	0.022	FOXL2, UTF1, HNF1B, HTATIP2, GLIS1, SIX2, EHF, GRHL3, ISL1, HMGA1, SHH, SALL4, OVOL2, IRF6, PAX8, POU2F2, POU3F3, EAF2, NFIB
Annotation Cluster 5	**Enrichment Score**	**2.21**
GO Term	**p-value**	**Genes**
0022602~ovulation cycle process	0.003	FOXL2, STRA8, MSH4, CHRNA7, DMC1, SOHLH1
0042698~ovulation cycle	0.003	FOXL2, STRA8, MSH4, CHRNA7, DMC1, SOHLH1
0001541~ovarian follicle development	0.004	FOXL2, STRA8, MSH4, DMC1, SOHLH1
0048511~rhythmic process	0.049	FOXL2, STRA8, MSH4, CHRNA7, DMC1, SOHLH1

Annotation Cluster 6	**Enrichment Score**	**2.13**
GO Term	**p-value**	**Genes**
0001541~ovarian follicle development	0.004	FOXL2, STRA8, MSH4, DMC1, SOHLH1
0008585~female gonad development	0.004	FOXL2, STRA8, MSH4, LHX8, DMC1, SOHLH1
0046545~development of primary female sexual characteristics	0.006	FOXL2, STRA8, MSH4, LHX8, DMC1, SOHLH1
0008406~gonad development	0.009	FOXL2, STRA8, MSH4, LHX8, DMC1, SOHLH1, TEX11
0046660~female sex differentiation	0.010	FOXL2, STRA8, MSH4, LHX8, DMC1, SOHLH1
0048608~reproductive structure development	0.017	FOXL2, STRA8, MSH4, LHX8, DMC1, SOHLH1, SHH, TEX11
Annotation Cluster 7	**Enrichment Score**	**1.99**
GO Term	**p-value**	**Genes**
0021795~cerebral cortex cell migration	0.004	DAB1, POU3F3, LHX6, RELN
0022029~telencephalon cell migration	0.005	DAB1, POU3F3, LHX6, RELN
0021885~forebrain cell migration	0.006	DAB1, POU3F3, LHX6, RELN
0021799~cerebral cortex radially oriented cell migration	0.011	DAB1, POU3F3, LHX6
0021543~pallium development	0.022	FGF8, DAB1, POU3F3, LHX6, RELN
0021987~cerebral cortex development	0.039	DAB1, POU3F3, LHX6, RELN
Annotation Cluster 8	**Enrichment Score**	**1.96**
GO Term	**p-value**	**Genes**
0044265~cellular macromolecule catabolic process	0.003	FOXL2, UCHL1, UBA6, HERC3, RFFL, OAS2, DNAHC12, LIN28A, PSMA8, PSMB9, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, STRA8, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
0009057~macromolecule catabolic process	0.007	FOXL2, UCHL1, UBA6, HERC3, RFFL, OAS2, DNAHC12, LIN28A, PSMA8, PSMB9, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, STRA8, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45

0051603~proteolysis involved in cellular protein catabolic process	0.013	UCHL1, UBA6, HERC3, RFFL, DNAHC12, PSMA8, PSMB9, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
0044257~cellular protein catabolic process	0.014	UCHL1, UBA6, HERC3, RFFL, DNAHC12, PSMA8, PSMB9, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
0043632~modification-dependent macromolecule catabolic process	0.016	UCHL1, UBA6, HERC3, RFFL, DNAHC12, PSMA8, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
0019941~modification-dependent protein catabolic process	0.016	UCHL1, UBA6, HERC3, RFFL, DNAHC12, PSMA8, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
0030163~protein catabolic process	0.019	UCHL1, UBA6, HERC3, RFFL, DNAHC12, PSMA8, PSMB9, ATE1, ZYG11B, ASB9, UBE2E3, ARIH1, USP18, SIAH1B, RNF128, UBA3, USP12, USP37, FBXO15, USP45
Annotation Cluster 9	**Enrichment Score**	**1.91**
GO Term	**p-value**	**Genes**
0010605~negative regulation of macromolecule metabolic process	0.002	SATB1, GTPBP4, SPINK12, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, LIN28A, SHH, ZBTB42, SALL4, CDKN2A, OVOL2, STRA8, RNF128, OVOL1, PIWIL2, POU3F3, TDRD1, HELLS
0010629~negative regulation of gene expression	0.010	SATB1, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, LIN28A, SHH, ZBTB42, SALL4, OVOL2, OVOL1, POU3F3, PIWIL2, TDRD1, HELLS
0045892~negative regulation of transcription, DNA-dependent	0.010	SATB1, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, OVOL1, POU3F3, HELLS
0051253~negative regulation of RNA metabolic process	0.011	SATB1, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, OVOL1, POU3F3, HELLS
0010558~negative regulation of macromolecule biosynthetic process	0.012	SATB1, GTPBP4, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, STRA8, RNF128, OVOL1, POU3F3, HELLS
0031327~negative regulation of cellular biosynthetic process	0.015	SATB1, GTPBP4, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, STRA8, RNF128, OVOL1, POU3F3, HELLS

0045934~negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.016	SATB1, GTPBP4, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, STRA8, OVOL1, POU3F3, HELLS
0009890~negative regulation of biosynthetic process	0.016	SATB1, GTPBP4, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, STRA8, RNF128, OVOL1, POU3F3, HELLS
0051172~negative regulation of nitrogen compound metabolic process	0.017	SATB1, GTPBP4, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, STRA8, OVOL1, POU3F3, HELLS
0000122~negative regulation of transcription from RNA polymerase II promoter	0.020	SATB1, HMX1, SALL4, GABPA, GLIS1, OVOL1, MAEL, POU3F3, CBX2, SHH, ZBTB42
0016481~negative regulation of transcription	0.041	SATB1, HMX1, GLIS1, GABPA, MAEL, CBX2, ZBTB16, SHH, ZBTB42, SALL4, OVOL2, OVOL1, POU3F3, HELLS
Annotation Cluster 10	**Enrichment Score**	**1.90**
GO Term	**p-value**	**Genes**
0030539~male genitalia development	0.003	FGF8, TEX15, SYCP2, SHH
0046546~development of primary male sexual characteristics	0.022	FGF8, TEX15, SYCP2, SHH, TEX11
0046661~male sex differentiation	0.028	FGF8, TEX15, SYCP2, SHH, TEX11
Annotation Cluster 11	**Enrichment Score**	**1.86**
GO Term	**p-value**	**Genes**
0034587~piRNA metabolic process	0.004	MAEL, PIWIL2, TDRD1
0043046~DNA methylation during gametogenesis	0.011	MAEL, PIWIL2, TDRD1
0006306~DNA methylation	0.011	MAEL, PIWIL2, TDRD1, HELLS
0006305~DNA alkylation	0.011	MAEL, PIWIL2, TDRD1, HELLS
0006304~DNA modification	0.012	MAEL, PIWIL2, TDRD1, HELLS
0016458~gene silencing	0.028	MAEL, PIWIL2, LIN28A, TDRD1, HELLS

0040029~regulation of gene expression, epigenetic	0.054	MAEL, PIWIL2, LIN28A, TDRD1, HELLS
Annotation Cluster 12	**Enrichment Score**	**1.74**
GO Term	**p-value**	**Genes**
0001701~in utero embryonic development	0.009	CCNB1IP1, HNF1B, KIF3A, GABPA, SPINT1, CX43, CDH1, ASCL2, FOXF1A, SALL4, OVOL2, COL4A3BP, KRT8
0043009~chordate embryonic development	0.025	CCNB1IP1, FGF8, HNF1B, KIF3A, GABPA, CX43, SPINT1, SIX2, CDH1, SHH, ASCL2, FOXF1A, SALL4, OVOL2, COL4A3BP, KRT8
0009792~embryonic development ending in birth or egg hatching	0.027	CCNB1IP1, FGF8, HNF1B, KIF3A, GABPA, CX43, SPINT1, SIX2, CDH1, SHH, ASCL2, FOXF1A, SALL4, OVOL2, COL4A3BP, KRT8
Annotation Cluster 13	**Enrichment Score**	**1.69**
GO Term	**p-value**	**Genes**
0051674~localization of cell	0.013	ASZ1, CX43, ISL1, SHH, TAF7L, DAB1, OVOL2, POU3F3, CHRNA7, TNN, LHX6, RELN, MESP1
0048870~cell motility	0.013	ASZ1, CX43, ISL1, SHH, TAF7L, DAB1, OVOL2, POU3F3, CHRNA7, TNN, LHX6, RELN, MESP1
0016477~cell migration	0.025	DAB1, OVOL2, ASZ1, CX43, POU3F3, LHX6, RELN, TNN, ISL1, MESP1, SHH
0006928~cell motion	0.037	ASZ1, CX43, ISL1, SHH, TAF7L, DAB1, OVOL2, LHX2, POU3F3, RELN, CHRNA7, TNN, LHX6, MESP1
Annotation Cluster 14	**Enrichment Score**	**1.51**
GO Term	**p-value**	**Genes**
0043414~biopolymer methylation	0.015	SATB1, MAEL, PIWIL2, TDRD1, HELLS, SUV39H2
0032259~methylation	0.022	SATB1, MAEL, PIWIL2, TDRD1, HELLS, SUV39H2
0006730~one-carbon metabolic process	0.090	SATB1, MAEL, PIWIL2, TDRD1, HELLS, SUV39H2
Annotation Cluster 15	**Enrichment Score**	**1.36**
GO Term	**p-value**	**Genes**
0045727~positive regulation of translation	0.003	KRT17, PIWIL2, DAZL, LIN28A
0032270~positive regulation of cellular protein metabolic process	0.146	KRT17, PIWIL2, DAZL, RICTOR, LIN28A

0051247~positive regulation of protein metabolic process	0.181	KRT17, PIWIL2, DAZL, RICTOR, LIN28A
Annotation Cluster 16	**Enrichment Score**	**1.25**
GO Term	**p-value**	**Genes**
0045109~inGO Termediate filament organization	0.028	KRT17, KRT14, SHH
0045104~inGO Termediate filament cytoskeleton organization	0.073	KRT17, KRT14, SHH
0045103~inGO Termediate filament-based process	0.091	KRT17, KRT14, SHH

Supplemental Table 3

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	Ccl9, CDKN2A, CRKL, CXCL9, DAB1, DAZ2, DDX4, DNMT3B, ERK, FAM3B, GAD1, GAS2, CX43, Histone h3, HMGA1, HOXA11, IFIT1B, Iigp1/Iigp1b, IL12 (complex), Interferon alpha, IRF7, KPNA2, LTB, MHC CLASS I (family), Ms4a4b (includes others), PIWIL2, PSMB9, RELN, RNA polymerase II, STAT1, TAP1, Tgtp1, UPP1, USP18, ZBP1	33	26	Infectious Disease, Gene Expression, Antigen Presentation
2	ABCB7, ACTB, B2M, CASP9, Ccl6, CCL21, CD274, CXCL9, EAF2, EBI3, ERO1L, FGL2, GBP4, Gbp4, Gbp8, GBP2 (includes EG:14469), IFIT1B, IFNB1, IFNG, IGF2BP1, Igtp, Iigp1/Iigp1b, IL5, IL27, IL7R, ISG15, NR3C1, OAS2, Oasl2, PCP4, SERINC3, SOCS1, STAT2, Tgtp1, TXN (includes EG:116484)	18	18	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Tissue Morphology
3	ACTA1, BAZ1A, BCL2L1, BIRC5, CCND1, CHRNA7, CRABP1, CXCL3, CXCL10, CYP2F1, DKK2, ESR2, FABP4, GGT1, GNAQ, GTF2A1, HELLS, HMGCR, IKBKB, JAK2, LEP, Mapk, MAT2A, MYH3, Myhs, MYOD1, NFKBIE, RBBP4, SOCS3, STAT1, SUV39H2, TNF, TNN, TNNC2, YY1	15	16	Gene Expression, Cancer, Cardiac Necrosis/Cell Death
4	ARG2, ARL5B, CBX2, CDK2, DMRT1, DMRTC2, DUSP4, E2F1, E2F4, FGFR2, FOXL2, FSHB, ISL1, LHB, MPHOSPH9, NR1H3, NR5A1, OSBP2, PIN1, PPM1H, SMAD3, SOHLH1, STAR, STRA8, UTF1, ZFP36	13	13	Embryonic Development, Organ Development, Organismal Development
5	AK4, ARIH1, CCL17, CDK2, CEBPZ, CLGN, E330020D12Rik, ERK1/2, F2RL1, FBXW12, FIGLA, FOS, GLI2, HDAC2, Histone h3, Histone h4, IL13, IL1B, Jnk, JUN, KITLG, LDHC, MAPK14, P38 MAPK, RASA2, RB1, RNA polymerase II, SOCS3, SRF, Taf7l, TCOF1, TDRD1, TLR4, TNFSF11, UBA3	12	14	Gene Expression, Tissue Morphology, Cellular Development

6	ADAD1, BCL2, BCL2L1, CD8A, CHCHD2, CSF1, CSF1R, DNAH12, Fcer1, FDFT1, HMGCR, IFIT3, IgG1, Igm, IL4, IL21, IL27, ITGAV, ITGB3, KCNAB2, LCK, MT1E, NFATC1, PHB, PLCG2, POU2F2, RASGRF1, RHOA, RORA, S100A9, SATB1, SPATA19, VAV3, YBX2, ZBTB16	12	14	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cellular Growth and Proliferation
7	Akt, Alp, BCL2, BCL2L1, BRCA1, BRCA2, CASP3, CCND1, CDKN1A, CDKN2A, CNR1, CREBBP, DDX25, EP300, GATA1, HDAC1, HNF1A, HOXA10, HTT, KIF12, KRT14, MDM2, MEIS2, NFKBIA, PLCB1, RAD51, RAP1GAP, RB1, RNASE4, RUNX2, SLC7A2, STAT1, TP53, TTK, WDR5	11	13	Gene Expression, Cancer, Cellular Development
8	Actg2, ALDOB, CDH1, CNN1, DYNC2LI1, FHL1, FOXA1, FOXA2, GATA6, GLI1, GLI2, GRHL1, HDAC1, HDAC2, HHIP, HLTF, HNF1B, HNF4A, IKZF3, KRT8, KRT17, LRP5, Mi2, MYBPC1, MYOCD, NR1H4, NR5A2, ONECUT1, PAX2, RBBP4, RHPN2, SALL1, SALL4, SHH, SLC2A2	11	13	Gene Expression, Embryonic Development, Organismal Development
9	Akr1b7, Alp, APBB1, APP, AQP7, ATF2, BLNK, CAV1, CCND1, CCND2, CCND3, CEBPA, CEBPB, CTNNB1, DKK1, DTX2, EGFR, FABP4, FGF8, FKBP6, Histone h3, MAP4K1, MESP1, MSX1, MYC, MYOG, NDRG4, PHLDA2, PPARD, PPARG, PRKG2, PRSS8, RUNX2, Tex19.1, WNT3A	11	13	Cellular Development, Gene Expression, Cell Cycle
10	ARC, CAMK4, CEBPB, CREB1, Creb, Crem, DLG4, DUSP14, EGR4, FMR1, GABPA, IRS2, NXF1, NXF2/NXF2B, PAK1, PDXK, PIK3CB, PPARA, PPARD, PPARGC1A, SIK1	8	9	Neurological Disease, Organismal Injury and Abnormalities, Tissue Morphology

Supplemental Table 4

ACCN	Gene Symbol	Gene Name	T-Test p-value	RP p-value	FC KO|WT
BE945380.1	*Ajap1*	adherens junction associated protein 1	0.39	0.11	1.3
NM_010288.2	*Gja1*	gap junction protein, alpha 1	0.00	0.00	**-3.4**
NM_016975.2	*Gja3*	gap junction protein, alpha 3	0.35	0.21	1.2
NM_008120.2	*Gja4*	gap junction protein, alpha 4	0.47	0.51	1.1
BB627115.1	*Gja5*	gap junction protein, alpha 5	0.50	0.21	1.2
BY760342.1	*Gja6*	gap junction protein, alpha 6	0.34	0.07	-1.7
CF739442.1	*Gja8*	gap junction protein, alpha 8	0.66	0.56	1.1
NM_008123.2	*Gja8*	gap junction protein, alpha 8	0.77	0.59	1.0
NM_008124.2	*Gjb1*	gap junction protein, beta 1	0.88	0.09	-1.1
NM_008125.2	*Gjb2*	gap junction protein, beta 2	0.27	0.17	-1.2
NM_008126.1	*Gjb3*	gap junction protein, beta 3	0.66	0.03	-1.3
NM_008127.3	*Gjb4*	gap junction protein, beta 4	0.53	0.56	1.1
NM_010291.2	*Gjb5*	gap junction protein, beta 5	0.95	0.24	1.0
NM_008128.3	*Gjb6*	gap junction protein, beta 6	0.53	0.53	1.1
NM_008122.1	*Gjc1*	gap junction protein, gamma 1	0.02	0.02	**-1.6**
NM_080454.2	*Gjc2*	gap junction protein, gamma 2	0.43	0.53	1.1
NM_010290.2	*Gjd2*	gap junction protein, delta 2	0.39	0.05	1.3
NM_178596.1	*Gjd3*	gap junction protein, delta 3	0.05	0.04	**-1.4**
NM_153086.3	*Gjd4*	gap junction protein, delta 4	0.79	0.62	1.0
NM_198652.1	*Hjurp*	Holliday junction recognition protein	0.36	0.21	1.2
AI020871.1	*Jam2*	junction adhesion molecule 2	0.31	0.01	1.5
AK039971.1	*Jam3*	junction adhesion molecule 3	0.62	0.15	1.1
NM_023277.1	*Jam3*	junction adhesion molecule 3	0.94	0.36	1.0
BU840990.1	*Jmy*	junction-mediating and regulatory protein	0.05	0.41	1.1

NM_021310.2	*Jmy*	junction-mediating and regulatory protein	0.22	0.06	-1.3
NM_028001.1	*Jsrp1*	junctional sarcoplasmic reticulum protein 1	0.90	0.41	-1.0
NM_010593.1	*Jup*	junction plakoglobin	0.13	0.44	1.1
NM_028751.2	*Tjap1*	tight junction associated protein 1	0.02	0.21	-1.2
NM_009386.1	*Tjp1*	tight junction protein 1	0.74	0.38	-1.0
NM_011597.1	*Tjp2*	tight junction protein 2	0.99	0.34	-1.0
NM_013769.1	*Tjp3*	tight junction protein 3	0.10	0.01	-1.8
NM_172538.2	*Vezt*	vezatin, adherens junctions transmembrane protein	0.71	0.13	1.1
AK041354.1	*Vezt*	vezatin, adherens junctions transmembrane protein	0.80	0.59	1.0