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**Title:** The Sertoli cell expressed gene *secernin-1* (*Scrn1*) is dispensable for male fertility in the mouse

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**Keywords:** Sertoli cell, spermatogenesis, sex development

**Funding:** National Health and Medical Research Council Australia: APP1120356

**Running title:** Scrn1 is not required for male fertility

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/dvdy.299](https://doi.org/10.1002/dvdy.299)

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**Abstract**

Background: Male infertility is a prevalent clinical presentation for which there is likely a strong genetic component due to the thousands of genes required for spermatogenesis. Within this study we investigated the role of the gene *Scrn1* in male fertility. *Scrn1* is preferentially expressed in XY gonads during the period of sex determination and in adult Sertoli cells based on single cell RNA sequencing. We investigated the expression of *Scrn1* in juvenile and adult tissues and generated a knockout mouse model to test its role in male fertility.

Results: *Scrn1* was expressed at all ages examined in the post-natal testis, however its expression peaked at postnatal day 7-14 and SCR1 protein was clearly localized to Sertoli cells. *Scrn1* deletion was achieved via removal of exon 3, and its loss had no effect on male fertility or sex determination. Knockout mice were capable of siring litters of equal size to wild type counterparts and generated equal numbers of sperm with comparable motility and morphology characteristics.

Conclusions: *Scrn1* was found to be dispensable for male fertility, but this study identifies SCR1 as a novel marker of the Sertoli cell cytoplasm.

## Introduction

Male infertility is a common condition affecting up to 7% of men in Western nations that places significant emotional and financial burden on couples attempting pregnancy.<sup>1</sup> The causes of infertility can originate from genetic, environmental, or a combination of these factors. However, genetic changes are likely the predominant cause of male infertility, in line with the expression of ~20,000 genes during spermatogenesis in mammals.<sup>2,3</sup> While the number of validated genetic causes of male infertility is growing, the majority of genes expressed during spermatogenesis remain to be investigated.

An analysis of testis single cell expression data identified *Scrn1* expression is highly enriched within Sertoli cells.<sup>4</sup> A literature search revealed that *Scrn1* is a relatively uncharacterized gene and is preferably expressed in male somatic cells of embryonic gonads at embryonic days 11.5, 12.5 and 13.5, coincident with the timing of fetal gonad differentiation, then up-regulated in adult Sertoli cells.<sup>5</sup> As such *Scrn1* has been suggested to play a role in Sertoli cell maturation or function. Sertoli cells comprise the somatic cell compartment of the seminiferous epithelium. They provide nutritional and structural support to developing germ cells and are critical for male fertility.<sup>6</sup> The number of Sertoli cells present within the testis is set in the pre-pubertal period and ultimately defines the upward limit of sperm production.<sup>7</sup> Defects in Sertoli cell function can result in a variety of infertility phenotypes, including sex reversal, loss of blood-testis barrier integrity, premature release of germ cells at an immature stage or abnormal sperm retention (spermiation failure).<sup>6,7</sup>

Although the role of *Scrn1* in male fertility has not yet been investigated, in humans and mice the gene encodes a 46 kDa protein with a potential pro-tumorigenic role in exocytosis in a variety of cancers, particularly those affecting the colon.<sup>8,9</sup> Alternative *Scrn1* transcripts (Figure 1A) are predicted to encode for proteins of 18.6, 8.3 and 6 kDa. *Scrn1* belongs to the secernin family, which includes *Scrn1*, *Scrn2* and *Scrn3*, neither of which have been studied in any detail. Secernins are members of the peptidase 69 family and are proposed to have roles in exocytosis in a number of cell types (InterPro IPR005322). All three *Scrn* genes contain a dipeptidase domain (PF03577). Within the study we sought to test the role of SCR1 in male fertility broadly through the generation of a *Scrn1* knockout mouse. We assessed knockout males at 3 and 6 months of age for any fertility defects. Knockout animals were overtly healthy and male fertility was unaffected. As such, SCR1 is not an absolute requirement for male sex determination or fertility.

## Results

### Scrn1 expression in the testis and across major organs

As sourced from *Ensembl*, the *Scrn1* gene encodes 6 isoforms (Figure 1A), 2 of which do not generate a protein product. The *Scrn1* gene has two paralogs, *Scrn2* and *Scrn3* (Figure 1B), which all contain a mapped PF03577 peptidase domain (coded by exons 2-4). As shown by a comparison of the full length SCRIN proteins and their peptidase domains (Figure 1C), there is ~50% conservation between each of the paralogues.

*Scrn1* expression in mouse testis was assessed by qPCR and in whole tissues by western blotting (Figure 2). The qPCR primers specifically detect the long transcript as they align to exons 5 (forward) and 6 (reverse). *Scrn1* testis mRNA was detected in the testes from day 0, peaked in expression at day 7, and persisted throughout the establishment of the first wave of spermatogenesis and into the adult testis (Figure 2A). This data is consistent with RNAseq data recently published,<sup>4</sup> where the highest ranking for *Scrn1* expression was in the Sertoli cell cluster (Figure 2B). The mRNA data was similar to SCRIN1 protein expression data but peaked at day 14-18. We identified SCRIN1, at the predicted size of 46 kDa (Figure 2C), at all ages examined (postnatal days 0 to 50). Protein concentration peaked at days 14-18 consistent with the first wave of meiosis and Sertoli cell maturation.<sup>2</sup> SCRIN1 was also clearly expressed in the brain, epididymis, lung and testis, and was enriched in the brain. The predicted 18.6 kDa protein was detected with an independent antibody (Thermo Fisher PA5-20992) within the liver but not in the testis (data not shown). It is unclear if the 8.3 and 6 kDa proteins are detected with either antibody used, but they were not identified in any tissue surveyed.

An analysis of *Scrn2* and *Scrn3* RNA expression using the single cell sequencing library mentioned above revealed that both genes are preferentially expressed in the germ cell compartment, and notably within spermatogonia and pre-prophase I spermatocytes (Figure 2B).

### Scrn1 testis localization

Within the testis, SCRIN1 localized solely to the Sertoli cell cytoplasm in both juvenile (postnatal day 14) and adult mouse testes (Figure 3). This clear Sertoli cell cytoplasm localization was emphasized by co-

staining with the well-established nuclear Sertoli cell marker, Wilms tumour 1 (WT1) protein (Figure 3, right panels).<sup>10</sup> Background staining was detected in blood vessels and interstitial fluid in both negative controls (Figure 3C and 3C inset [IgG control]). No expression was seen in *Scrn1*<sup>-/-</sup> testis tissue, thus confirming the specificity of the antibody in this context (Figure 4B, right vs left panel). SCR1 was expressed in Sertoli cells throughout the entirety of the spermatogenic cycle (data not shown).

#### *Scrn1* expression is not essential for male fertility or sex determination

We generated a *Scrn1* knockout mouse model to explore its role in male fertility and sex determination (Figure 4A). Here, exon 3 of *Scrn1* was removed, causing a shift in the canonical open reading frame and generating a premature stop codon in exon 4. Successful knockout of *Scrn1* was identified by a significant reduction in *Scrn1* transcript. Success deletion of the *Scrn1* gene was confirmed using immunohistochemical and western blotting methods (Figure 4B-C). No effect on *Scrn2* or *Scrn3* transcripts levels was observed, suggesting they do not compensation for the loss of *Scrn1* (Figure 4B).

We acknowledge that our approach targeted the *Scrn1*-201 and 203 transcripts but did not modify two much smaller coding isoforms *Scrn1*-205 and 206. However, these isoforms were not detected within the testis and do not contain encode the peptidase domain. Thus, they are likely not relevant in this context.

We first investigated whether there was a difference in the phenotypic sex ratios of *Scrn1*<sup>+/-</sup> and *Scrn1*<sup>-/-</sup> offspring. No differences were found compared to wildtype offspring (Figure 5A;  $P = 0.7$ ). To test fertility, wildtype and *Scrn1* knockout mice were aged to 10-14 weeks of age and bred with wildtype females or knockout females. Comparison of litter sizes across genotypes revealed no effect of loss of *Scrn1* on male fertility (Figure 5B). We next assessed *Scrn1* wildtype and knockout male mouse fertility at 3 or 6 months of age. At both ages, *Scrn1* knockout mice had comparable body weights to wildtype mice (Figure 5C) and produced testes of the same mass (Figure 5D). In accordance with this data there was no significance difference in seminal vesicle weights at 6 months of age suggesting that testosterone signalling was normal (Figure 5E). Similarly, the daily sperm production and average number of sperm resident within the epididymis was identical between genotypes (Figure 6A, B). Furthermore, all major sperm motility and morphology parameters (Figure 6C, D), as well as histology of the testis and epididymis were comparable between genotypes (Figures 7, 8).

Finally, and as a first step towards exploring the potential for an age-dependent compensation for a more subtle sex determination phenotype, we investigated testis morphology at embryonic day 13.5, the time point at which *Scrn1* is normally up-regulated during the sex determination process<sup>5</sup> (Figure 9). We saw no notable differences in testis morphology (Figure 9 top panels) and did not identify any overt differences in SOX9 staining as a marker of Sertoli cells (Figure 9 bottom panels). Our data indicated that SCR1 is not required for male sex determination or fertility.

## Discussion

To the best of our knowledge, this is the first paper to describe the expression of *Scrn1* in adult mice and comprehensively assess its role in male fertility. SCR1 was clearly localized to the Sertoli cells of the testis and provides an excellent marker of the Sertoli cell cytoplasm in juvenile and adult testes (Figure 3). Global depletion of SCR1 in the mouse model, however, did not result in any discernible effects on male fertility. While clearly not essential for fertility, the possibility existed that SCR1 function may be compensated for by paralogs during sex development or its role may only come critical under exposure to environmental stress. In opposition to the concept of compensation by SCR2 or SCR3, loss of *Scrn1* did not alter the expression of *Scrn2* and *Scrn3* in adult testes (Figure 4B). Further, while *Scrn1* is enriched within Sertoli cells, *Scrn2* is expressed in both Sertoli cells and early germ cells (spermatogonia and early meiotic cells), and *Scrn3* is expressed predominantly in early germ cell populations.

While previous reports have suggested *Scrn1* has a potential role in sex determination,<sup>5</sup> our data does not lend support to this hypothesis. The loss of *Scrn1* did not alter the sex ratio across wild type, heterozygous or knockout *Scrn1* genotypes (Figure 5B). Male reproductive organs were also indistinguishable in appearance, weight and function (Figure 5D) between wild type and knockout genotypes. Although sex determination was not rigorously investigated, embryonic testis histology at day e13.5 and SOX9 expression (Sertoli cell marker) was comparable between wildtype and *Scrn1* knockout mice.

While not of direct relevance to the focus of the current study, evidence is mounting to suggest that aberrant SCR1 expression plays a role in the establishment of many cancers, particularly colon cancer.<sup>8,11,12</sup> Here, SCR1 may be involved in exocytosis of factors that promote tumour progression.<sup>8,9</sup>

Whether it plays a role in remodeling of the endoplasmic reticulum in other tumors, or in germ cells, as has been recently shown in neurons,<sup>13</sup> is yet to be investigated. We did not find any published evidence in published articles or screens to suggest that SCR1 plays a role in the establishment of testicular cancers, however.<sup>23</sup>

## **Experimental Procedures**

### Ethics approval

All animal experiments were approved by the Monash University Animal Experimentation Ethics Committee (number BSCI/2017/31) and followed animal ethics guidelines stated by the Australian National Health and Medical Research Council (NHMRC).

### SCR1 expression

SCR1 localization was determined in testis sections from juvenile (day 14) and adult (> day 50) mice, using standard conditions previously reported.<sup>14</sup> The SCR1 primary antibody LS-C338451 (LifeSpan BioSciences, Seattle, USA) was diluted to 6.6 µg/ml in Dako Antibody Diluent (Agilent, Santa Clara, USA) and applied overnight at 4°C. After washing slides (3 x 5 min) in PBS-Tween-20 (0.05%) (PBST), EnVision anti-mouse polymer (Agilent) was applied for 30 min, followed by DAB liquid chromogen (Agilent) for 4 min. Slides were again washed for 3 x 5 min in PBST, stained with Mayer's haematoxylin (Amber Scientific, Midvale, Australia) and developed with Scott's Tap Water Substitute (Amber Scientific) for one minute each. After washing in tap water for 5 min, slides were mounted with DPX (Sigma Aldrich, Castle Hill, Australia). SCR1 localisation was also conducted using immunofluorescence with AlexaFluor-555 secondary 1/500, co-stained with 0.25 mg/ml Wilms tumour protein 1 (WT1, a known Sertoli cell nuclear marker<sup>10</sup>) primary antibody Ab89901 (Abcam, Melbourne, Australia) and 1/500 secondary AlexaFluor 488 (Thermo Fisher Scientific, Scoresby, Australia), using the same conditions as above.

To define the expression of SCR1 across tissues, protein was extracted from wild type adult male organs, including: brain, epididymis, heart, kidney, liver, lung, seminal vesicles, spleen and testis. Additional testes samples were collected at key time points during the establishment of the first wave of spermatogenesis. Protein was also extracted from the testis of 10-week-old knockout and wild type

male siblings to assess the success of gene knockout of *Scrn1* and SCR1 antibody specificity. Each tissue was snap frozen on ice prior to storage at  $-80^{\circ}\text{C}$  and then thawed and sonicated in RIPA buffer (containing  $5\ \mu\text{l/ml}$  protease inhibitor cocktail III [539134, Calbiochem, Merck, MA, USA]) on ice to extract protein. The supernatant was collected after centrifugation at  $13000 \times g$  for 10 min and protein concentration was determined using the DC protein assay kit (BioRad, Gladesville, Australia). Proteins ( $10\ \mu\text{g/sample}$  for testis age series and  $20\ \mu\text{g/sample}$  for tissue survey) were size separated on 10% SDS-PAGE gels, transferred to a PVDF membrane and probed as previously described.<sup>15</sup> The SCR1 antibody (LS-C338451) was used at a concentration of  $1.98\ \mu\text{g/ml}$  and was then detected with Dako goat anti-rabbit IgG-horse-radish peroxidase (Agilent; 1/10,000) and the Clarity Western ECL substrate kit (BioRad). A beta-actin antibody (Sigma Aldrich, A2066) was used as a loading control for all western blotting experiments at a concentration of  $0.67\ \mu\text{g/ml}$ . Densitometry analysis was performed using ImageJ, by comparing the SCR1 band intensity in each sample to the intensity of the loading control, Beta actin.

RNA was isolated from testes (postnatal day 0-50) of wild type mice at key points during the establishment of the first wave of spermatogenesis to ascertain cell types in which *Scrn1* is expressed. Tissue samples were homogenised in TRIzol Reagent (Thermo Fisher Scientific) under RNase-free conditions. RNA was converted to cDNA using SuperScriptIII (Thermo Fisher Scientific) and used for quantitative PCR (QuantStudio3; Thermo Fisher Scientific). The qPCR was run with SYBR Green mastermix (Thermo Fisher Scientific) with primers listed in Table 1. Expression of *Scrn1* was presented relative to housekeeping gene *Ppia* and then normalized to the day 0 sample. This data was also cross-referenced against single cell RNA sequencing data obtained by our laboratory.<sup>4</sup> Please see this reference for further explanation of expression calculations.

#### Mouse knockout production

*Scrn1* knockout mice were generated through the Australian Phenomics Network (APN) at Monash University, using CRISPR guide sequences (CCCAAGTTCTGTGATTACCC and GTGAATCACC GAAGTTATAG) to target excision of exon 3 of *Scrn1*, leading to a premature stop codon in exon 4 and a truncated protein. Specific changes in gene sequence were validated with Sanger sequencing. Mice heterozygous for the *Scrn1* deletion were supplied by APN, which were mated to generate knockout

males. All genotyping was performed externally via Transnetyx, using primers listed in Table 1. Mice were generated on the C57BL/6J background.

### Fertility analysis

Knockout males and wild type male littermates were assessed for fertility defects using the strategy outlined previously.<sup>16,17</sup> Briefly, mice were aged to 10-14 weeks prior to assessment of fertility via mating with adult wild type females aged to 6-10 weeks. The presence of copulatory plugs was used as an indicator of successful mating and reproductive behaviour. Genetic combinations assessed included wildtype male x wildtype female, knockout male x wildtype female, and knockout male x knockout female breeding pairs. Litter sizes were recorded for each mating and phenotypic sex ratio was monitored in order to assess the possibility that SCR<sup>N1</sup> played a role in sex determination.

Mice were culled at 3 or 6 months of age then body and testis weights were recorded, and mice were assessed for any overt phenotypic abnormalities. One testis per mouse was snap-frozen on dry ice and the other was fixed in Bouin's fixative (Amber Scientific) for 5 h at room temperature. One epididymis per mouse was fixed in 4% paraformaldehyde, while the cauda of the other was used to harvest sperm. Fixed tissues were alcohol processed, embedded in paraffin wax and sectioned using standard methods. Daily sperm production was assessed on testis tissue using the Triton-X-100 solubilization method,<sup>18</sup> with addition of a sonication step. A minimum of  $n = 3$  tissues from separate mice per genotype were analysed per experiment.

For the analysis of sperm motility, computer assisted semen analysis (CASA) was used. Caudal epididymal sperm were collected by retrograde perfusion via the vas deferens as described previously<sup>19</sup>. Epididymides were dipped in pre-warmed mineral oil (Sigma), tied to plastic tubing with silk thread at the vas deferens, and nicked. Air was blown through the tubing with a syringe and sperm were collected in 10  $\mu$ l glass microcapillaries (Drummond Scientific, PA, USA). Sperm were allowed to disperse in modified Tyrode's 6 medium (MT6) for 15 min at 37°C and then loaded into 80  $\mu$ m deep CASA slides.<sup>20</sup> Motility was assessed with a MouseTraxx CASA system (Hamilton Thorne, USA), using criteria previously established.<sup>21</sup> A minimum of 1000 sperm were assessed per animal and five biological replicates were used per genotype. In order to assess sperm morphology, residual sperm were pelleted at 500  $\times$  g for 5 min and re-suspended in phosphate buffer saline (PBS). Sperm were spread across

glass slides and air-dried overnight. Each slide was immersed in 4% paraformaldehyde for 10 min at room temperature to fix cells and then washed in PBS for 5 min.

Sperm slides and dewaxed epididymal sections from wild type and *Scrn1* knockout mice were stained with haematoxylin and eosin. Dewaxed testis sections were stained using periodic acid-Schiff's and haematoxylin reagents. All slides were dehydrated and mounted under a glass coverslip with DPX (Sigma Aldrich, USA). Sperm morphology and tissue histology was assessed via light microscopy with reference to a gold standard manual<sup>22</sup>. All microscopy was performed on an Olympus BX-53 microscope (Olympus America, Center Valley, PA, USA) equipped with an Olympus 392 DP80 camera.

#### Investigation of embryonic testis morphology

Embryos were dissected from pregnant (wildtype x wildtype and heterozygous x knockout mates) females at embryonic day 13.5, corresponding to the middle period of sex determination. Males were identified via gonad morphology and the presence of a coelomic vessel. Embryos from het x KO mates were genotyped via limb tissue. All embryos were fixed in Bouin's solution for 24 hours at room temperature then processed into paraffin blocks via standard protocols. Serial sectioning of embryos was undertaken to isolate testis sections, which were stained with haematoxylin and eosin to investigate testis histology. Sections were also immunochemically stained with a SOX9 antibody (1/1000; Merck Millipore AB5535) to mark Sertoli cells and inspect the testis cords (using methods detailed above).

#### Statistical analysis

Statistical significance was assessed using t-tests between wildtype and knockout genotypes for 2 variables or ANOVA and post-hoc Dunnett's test compared to the wildtype for 3 or more variables. We utilised GraphPad Prism 7 with an  $\alpha$  of 0.05.

#### SCRN1 paralog sequence comparison

SCRN1 (ENSMUST00000019268.10), SCRN2 (ENSMUST00000021249.10) and SCRN3 (ENSMUST00000090811.10) protein sequences were extracted from *Ensembl* and used for comparison via NCBI's protein BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A peptidase domain (PF03577) in SCRN1 encoded by amino acids 89-231 was also used for BLAST analysis to determine domain identity across paralogs.

## Acknowledgements

We acknowledge the Australian Phenomics Network for design of the knockout strategy and generation of the mice, and the Monash Histology Platform Clayton Node. We thank Amy Luan for extracting the expression data for Figure 2B.

## Funding

MKO'B, KIA and DFC are the recipients of an NHMRC project grant APP1120356 supporting this study.

## Author contributions

BJH, HO, AEO'C, DJM and MKO'B designed and performed mouse experiments

BJH and MKO'B wrote the paper

BJH, LN, KIA, DFC and MKO'B provided feedback on the paper and were involved in the study intellectually

## Conflicts

KO is a consultant for medical electronic systems

## References

1. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nature Reviews Urology*. 2018/06/01 2018;15(6):369-384. <https://doi.org/10.1038/s41585-018-0003-3>.
2. Yu Z, Guo R, Ge Y, et al. Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. *Biol Reprod*. Jul 2003;69(1):37-47. <https://doi.org/10.1095/biolreprod.102.012609>.
3. Soumillon M, Necsulea A, Weier M, et al. Cellular source and mechanisms of high transcriptome complexity in the mammalian testis. *Cell Rep*. Jun 27 2013;3(6):2179-90. <https://doi.org/10.1016/j.celrep.2013.05.031>.
4. Jung M, Wells D, Rusch J, et al. Unified single-cell analysis of testis gene regulation and pathology in five mouse strains. *Elife*. Jun 25 2019;8. <https://doi.org/10.7554/eLife.43966>.

5. Bouma GJ, Hudson QJ, Washburn LL, Eicher EM. New candidate genes identified for controlling mouse gonadal sex determination and the early stages of granulosa and Sertoli cell differentiation. *Biol Reprod*. Feb 2010;82(2):380-9. <https://doi.org/10.1095/biolreprod.109.079822>.
6. Griswold M. Sertoli Cell Biology (2<sup>nd</sup> ed.). Elsevier, USA. ISBN: 978-0-12-417047-6. 2015
7. Sharpe RM, McKinnell C, Kivlin C, Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*. 2003; 125:769-784
8. Lin S, Jiang T, Yu Y, et al. Secernin-1 Contributes to Colon Cancer Progression through Enhancing Matrix Metalloproteinase-2/9 Exocytosis. *Disease Markers*. 2015;2015:12. <https://doi.org/10.1155/2015/230703>.
9. Way G, Morrice N, Smythe C, O'Sullivan AJ, Malhotra V. Purification and Identification of Secernin, a Novel Cytosolic Protein that Regulates Exocytosis in Mast Cells. *Molecular Biology of the Cell*. 2002;13(9):3344-3354. <https://doi.org/10.1091/mbc.e01-10-0094>.
10. Wang XN, Li ZS, Ren Y, et al. The Wilms tumor gene, Wt1, is critical for mouse spermatogenesis via regulation of sertoli cell polarity and is associated with non-obstructive azoospermia in humans. *PLoS Genet*. 2013;9(8):e1003645. <https://doi.org/10.1371/journal.pgen.1003645>.
11. Song P, Zhu H, Zhang D, et al. A genetic variant of miR-148a binding site in the SCR1 3'-UTR is associated with susceptibility and prognosis of gastric cancer [Article]. *Scientific Reports*. 11/17/online 2014;4:7080. <https://doi.org/10.1038/srep07080>
12. Miyoshi N, Ishii H, Mimori K, Sekimoto M, Doki Y, Mori M. SCR1 is a novel marker for prognosis in colorectal cancer. *Journal of Surgical Oncology*. 2010;101(2):156-159. <https://doi.org/10.1002/jso.21459>.
13. Lindhout FW, Cao Y, Kevenaar JT, et al. VAP-SCR1 interaction regulates dynamic endoplasmic reticulum remodeling and presynaptic function. *EMBO J*. Oct 15 2019;38(20):e101345. <https://doi.org/10.15252/embj.2018101345>.
14. Hu J, Merriner DJ, O'Connor AE, et al. Epididymal cysteine-rich secretory proteins are required for epididymal sperm maturation and optimal sperm function. *MHR: Basic science of reproductive medicine*. 2018;24(3):111-122. <https://doi.org/10.1093/molehr/gay001>.
15. Gaikwad AS, Loh KL, O'Connor AE, Reid HH, O'Bryan MK. Expression and purification of recombinant mouse CRISP4 using a baculovirus system. *Protein Expression and Purification*. 2020; 105543
16. Borg CL, Wolski KM, Gibbs GM, O'Bryan MK. Phenotyping male infertility in the mouse: how to get the most out of a 'non-performer'. *Hum Reprod Update*. Mar-Apr 2010;16(2):205-24. <https://doi.org/10.1093/humupd/dmp032>.

17. Houston BJ, Conrad DF, O'Bryan MK. A framework for high-resolution phenotyping of candidate male infertility mutants: from human to mouse. *Human Genetics*. 2020  
<https://doi.org/10.1007/s00439-020-02159-x>
18. Cotton L, Gibbs GM, Sanchez-Partida LG, Morrison JR, de Kretser DM, O'Bryan MK. FGFR-1 signaling is involved in spermiogenesis and sperm capacitation. *Journal of Cell Science*. 2006;119(1):75-84.  
<https://doi.org/10.1242/jcs.02704>
19. Baker MA, Hetherington L, Weinberg A, Velkov T. Phosphopeptide analysis of rodent epididymal spermatozoa. *J Vis Exp*. Dec 30 2014;(94). <https://doi.org/10.3791/51546>.
20. D. FA, S. KN, Ryuzo Y. Alteration of sperm thiol-disulfide status and capacitation in the guinea pig. *Gamete Research*. 1986;13(2):93-102. <https://doi.org/10.1002/mrd.1120130202>.
21. Gibbs GM, Orta G, Reddy T, et al. Cysteine-rich secretory protein 4 is an inhibitor of transient receptor potential M8 with a role in establishing sperm function. *Proc Natl Acad Sci U S A*. Apr 26 2011;108(17):7034-9. <https://doi.org/10.1073/pnas.1015935108>.
22. Russell, LD, Ettlín, RA, Hikim, APS. and Clegg, ED. *Histological and Histopathological Evaluation of the Testis*. 1990. Cache River Press, Clearwater, Florida.
23. Chang, YC, Wang, X, Xu, Y et al. Comprehensive characterization of cancer - testis genes in testicular germ cell tumor. *Cancer Medicine*. 2019; 8(7):3511-3519.

### Figure legends

**Figure 1. *Scrn1* mouse transcripts.** (A) Mouse *Scrn1* transcript variants, sourced from *Ensembl*: gene ENSMUSG00000019124.10. (B) SCR1, SCR2 and SCR3 protein length and domains. Exon length is represented as coding length relative to whole transcript, with *Scrn1* and *Scrn3* possessing large 3' untranslated regions. PF03577 = peptidase domain. (C) SCR1, SCR2, SCR3 sequence comparison at the whole protein and PF03577 domain level.

**Figure 2. *Scrn1*/SCR1 expression analysis within the mouse testis and across major mouse organs.** (A) *Scrn1* transcript level across the developing testis postnatal day 0-50 assessed by quantitative PCR, relative to *Ppia* and normalized to day 0 ( $n = 1$ ). (B) *Scrn1*, *Scrn2* and *Scrn3* expression across major cell types in the mouse testis as determined by single cell RNA sequencing (extracted from data generated by Jung et al., 2019). X-axis left to right: undifferentiated spermatogonia, differentiated spermatogonia, leptotene/zygotene spermatocytes, pachytene spermatocytes, round

spermatids, elongating spermatids, Sertoli cells and Leydig cells. (C) SCR1 protein level in the developing testis postnatal day 0-50 as assessed by western blotting and band intensity analysis compared to housekeeping gene beta actin ( $n = 1$ ). (D) SCR1 protein level in major body organs assessed by western blotting and band intensity analysis compared to housekeeping gene beta actin ( $n = 1$ ).

**Figure 3. SCR1 Sertoli cell localization.** SCR1 protein localization was investigated in (A, B) juvenile (postnatal day 14) and (C, D) adult testis (> day 50) using immunohistochemistry (DAB staining). An IgG control in inset in C. Sertoli cell localization of SCR1 (red) was confirmed by co-staining juvenile (E) and adult testis (F) sections with the known Sertoli cell marker, Wilms tumor 1 (WT1) protein (green) and DAPI nuclear stain (blue). Scale bars = 50  $\mu$ m.

**Figure 4. *Scrn1* knockout confirmation.** (A) The *Scrn1* gene was knocked out in mice, using CRISPR to remove exon 3. (B) *Scrn1* and paralog (*Scrn2*, 3) transcript levels were assessed via qPCR in *Scrn1* KO testes. Statistical analysis was undertaken using unpaired t-tests, NS = not significant. SCR1 protein loss was also confirmed in the testis in comparison to tissue from wildtype mice using (C) immunohistochemical DAB staining and (D) western blotting. The intensity of bands in (D) was quantified and significance was assessed with an unpaired t-test. Scale bars = 50  $\mu$ m. \*\*\*  $P < 0.001$  compared to wildtype. VII-VIII denotes tubule stage.

**Figure 5. Fertility of *Scrn1* knockout mice.** (A) Male sex ratio of offspring per genotype was recorded for wildtype (WT), heterozygous (het) and knockout (KO) mice generated from WT x WT, het x het and KO x KO mates. The numbers in the columns refer to the number of mice used per genotype for sex assessment. Chi-squared analysis revealed no significant changes in male:female ratio. (B) Average litter size from breeding trials conducted with wildtype and *Scrn1* knockout mice. A one-way ANOVA determined there was no significant difference in average litter size with either knockout combination. (C) Body mass and (D) testis mass are shown for each male WT and KO mouse. (E) Seminal vesicle weight measured for WT and KO males at 6 months of age.

**Figure 6. Sperm production rate and activity in *Scrn1* knockout mice.** (A) Daily sperm production, assessed by solubilization of the testis. (B). Epididymal sperm content, assessed by homogenization of the epididymis. (C) Objective sperm motility was assessed using computer assisted semen analysis. Circles represent wildtype sperm, while squares represent *Scrn1* knockout sperm. Sperm motility

represents all moving spermatozoa, while progressive motility denotes only the percentage of sperm moving in a forward motion (D) Sperm morphology was assessed on haematoxylin and eosin stained sperm cells. Scale bars = 20  $\mu$ m.

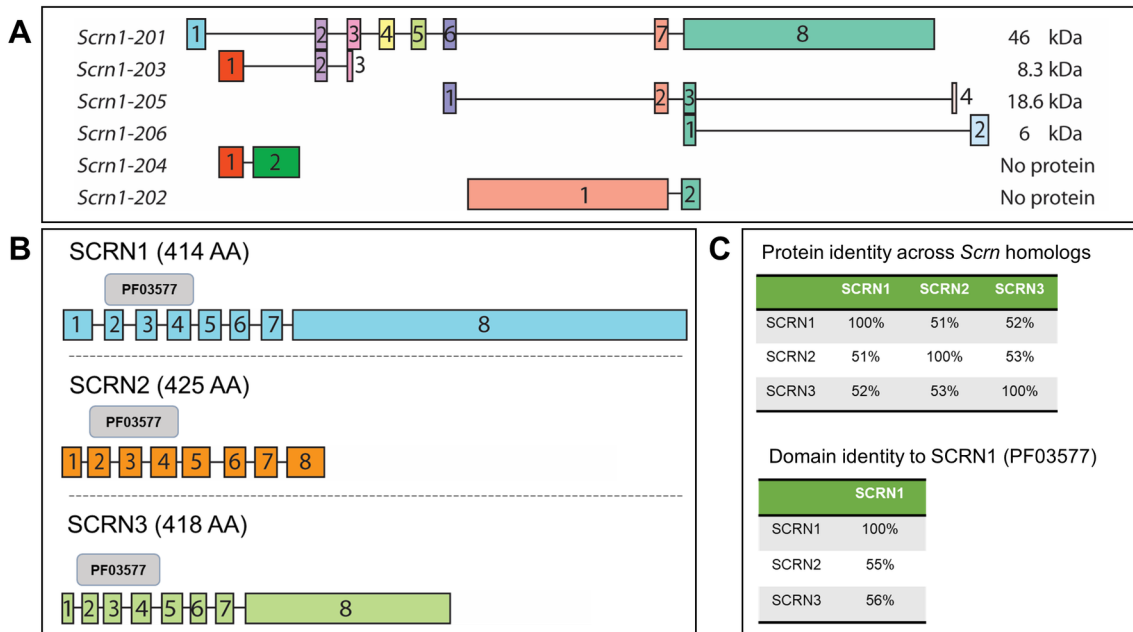
**Figure 7. Adult testis histology of *Scrn1* knockout mice.** Testis sections were stained with periodic acid-Schiff reagents and normality of spermatogenesis was assessed. VII-VIII denotes tubule stage. Scale bar = 50  $\mu$ m in all panels.

**Figure 8. Epididymal histology of *Scrn1* knockout mice.** Epididymal sections were stained with haematoxylin and eosin and assessed for morphology and presence of sperm. Cauda epididymis sections are shown. Scale bar = 50  $\mu$ m in all panels.

**Figure 9. Embryonic testis histology of *Scrn1* knockout mice.** Testis sections were stained with haematoxylin and eosin (top panels) and a SOX9 antibody (bottom panels) to mark Sertoli cells. Scale bar = 50  $\mu$ m in all panels.

**Table 1.** Genotyping and quantitative PCR primers used to target *Scrn1* in this study.

**Figure 1**



**Figure 2**

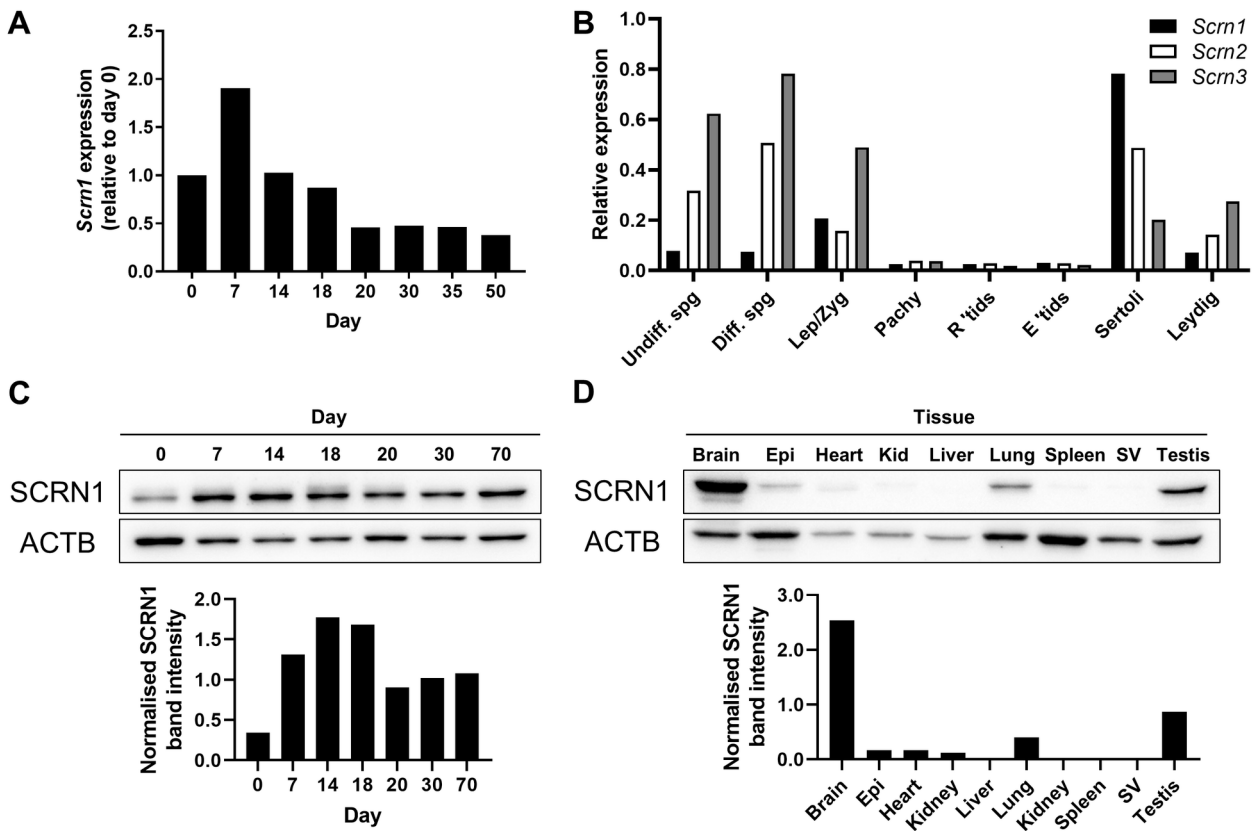
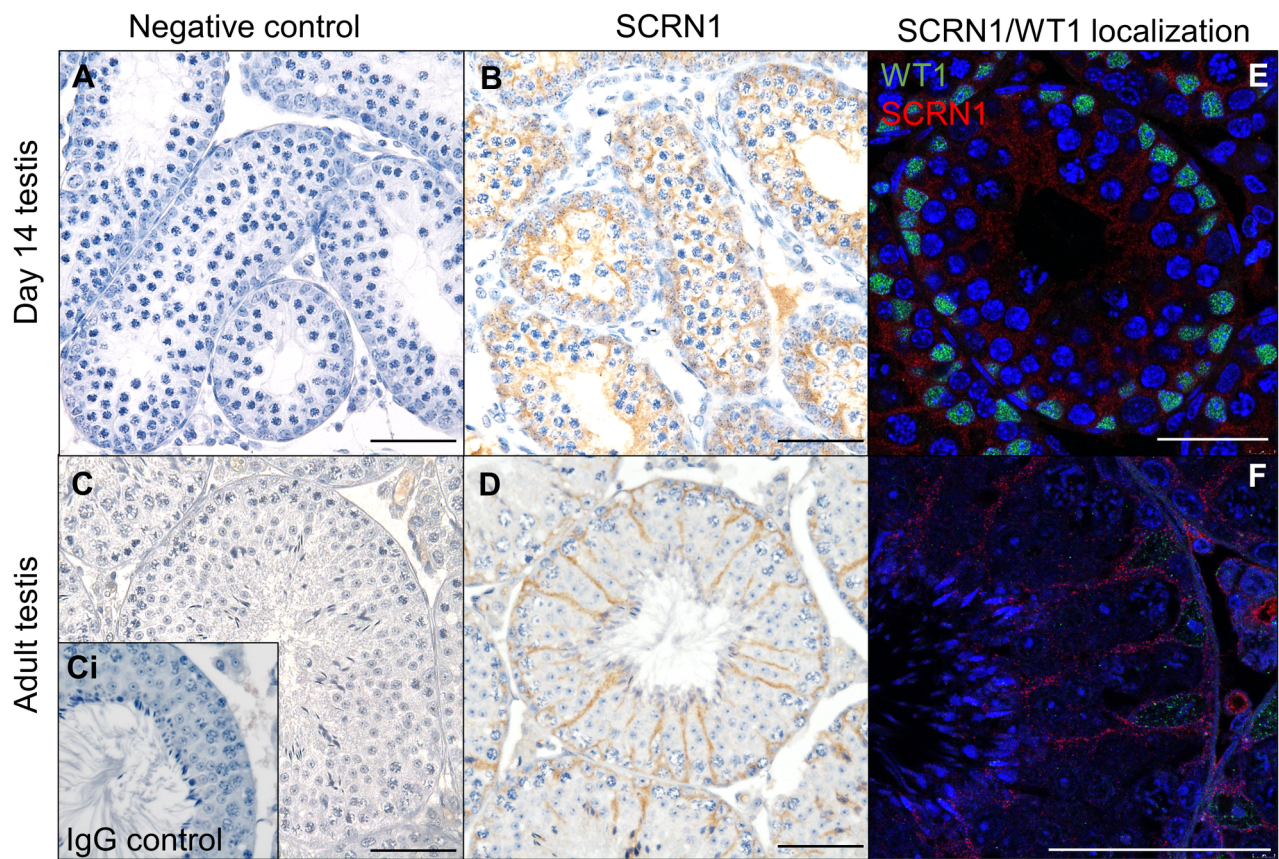
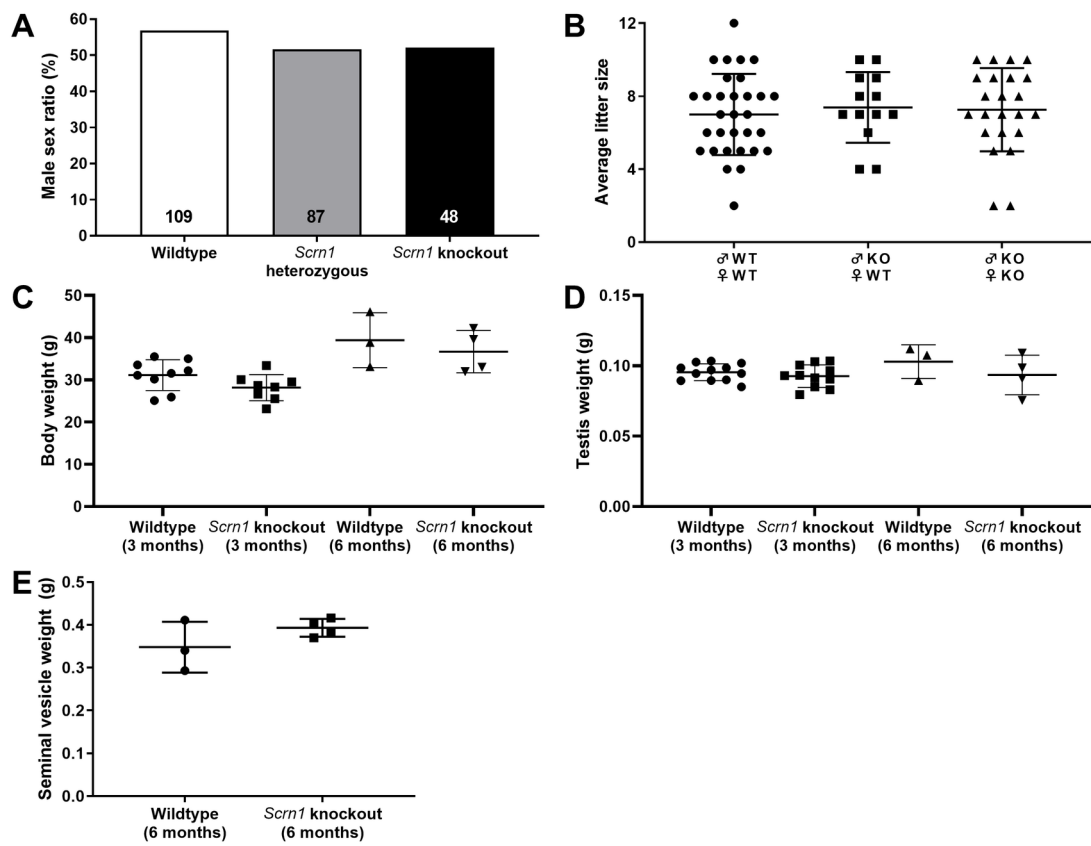


Figure 3

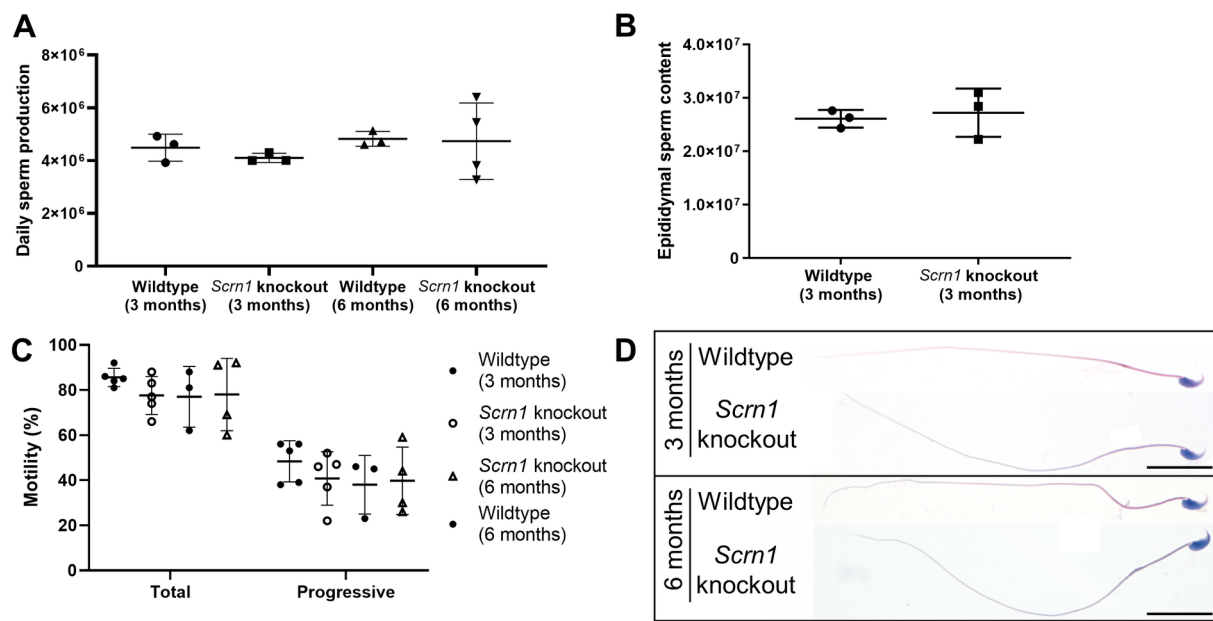


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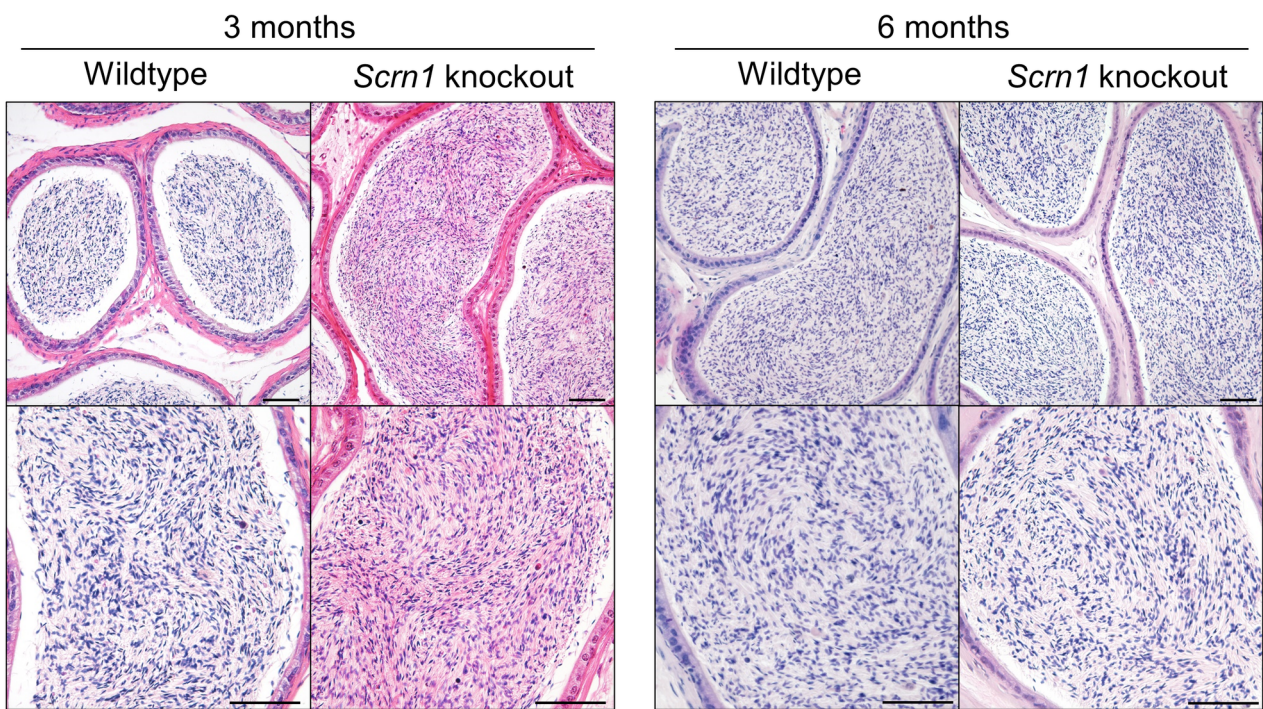
**Figure 5**



**Figure 6**

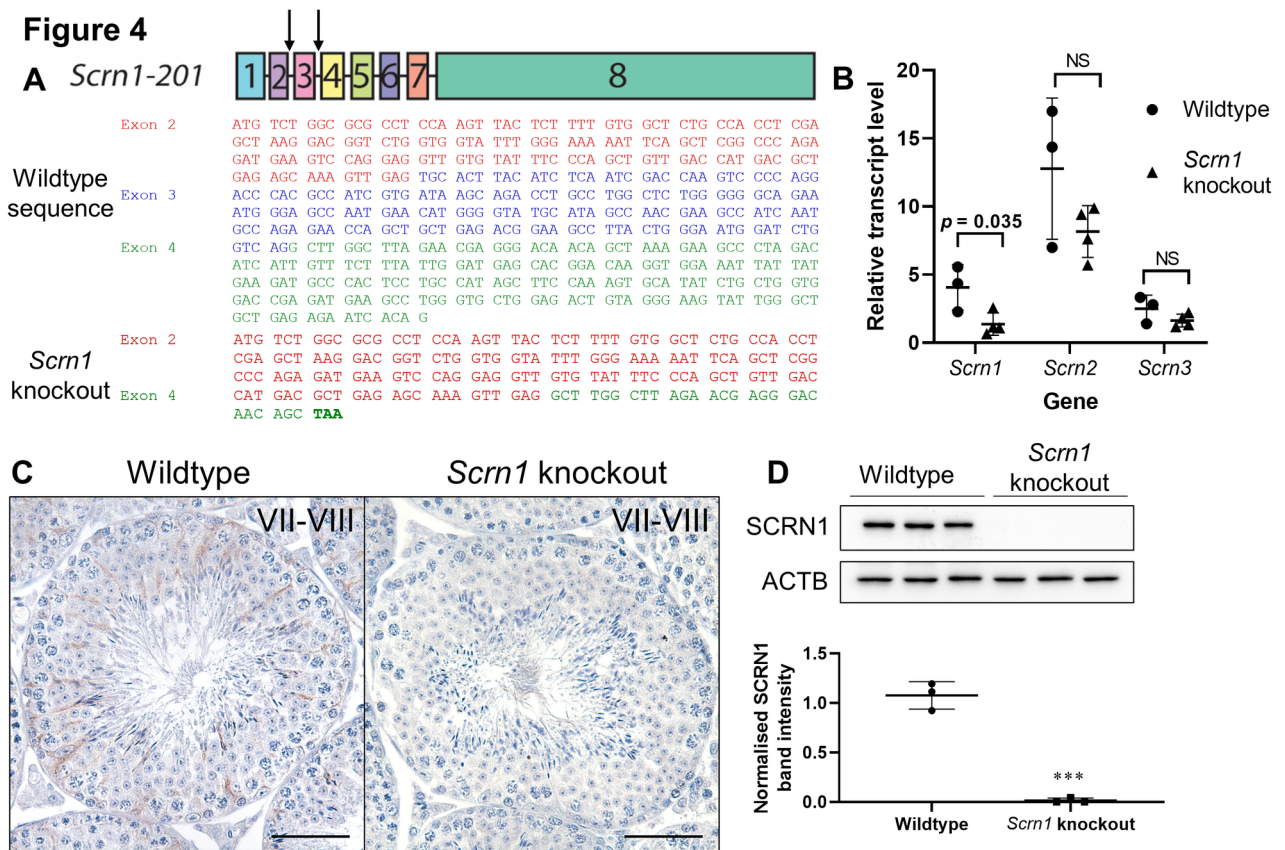


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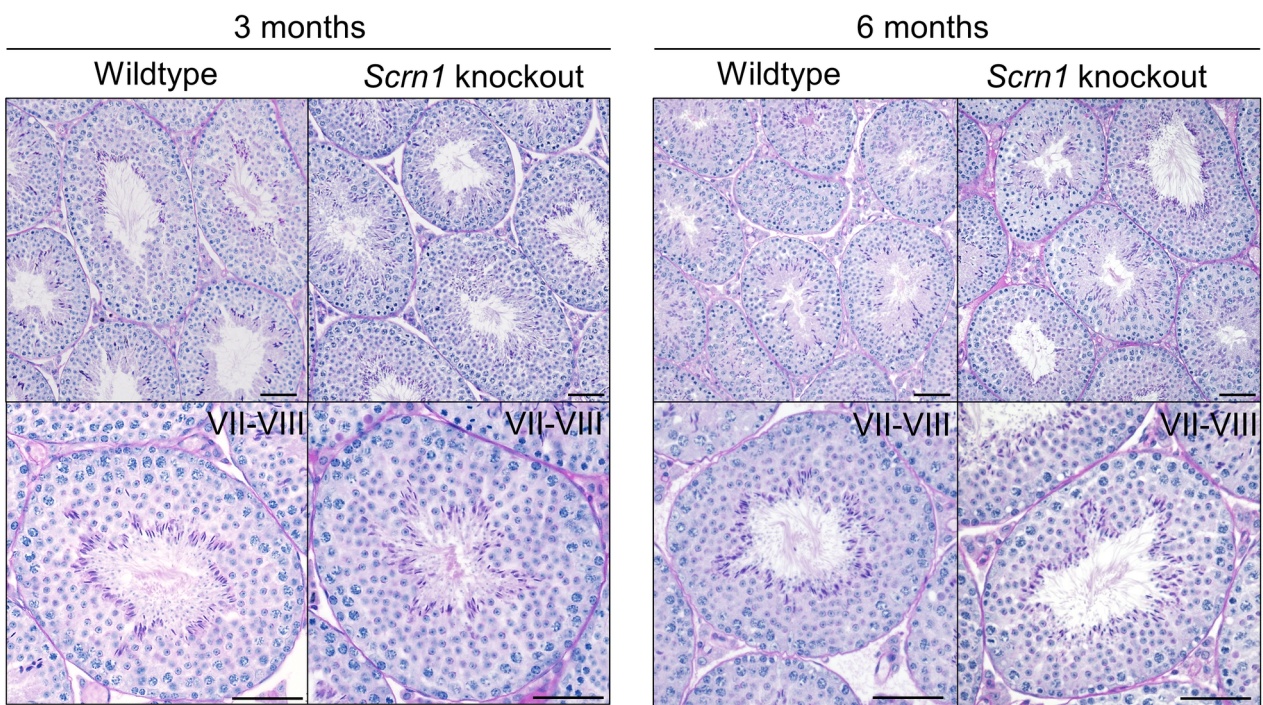


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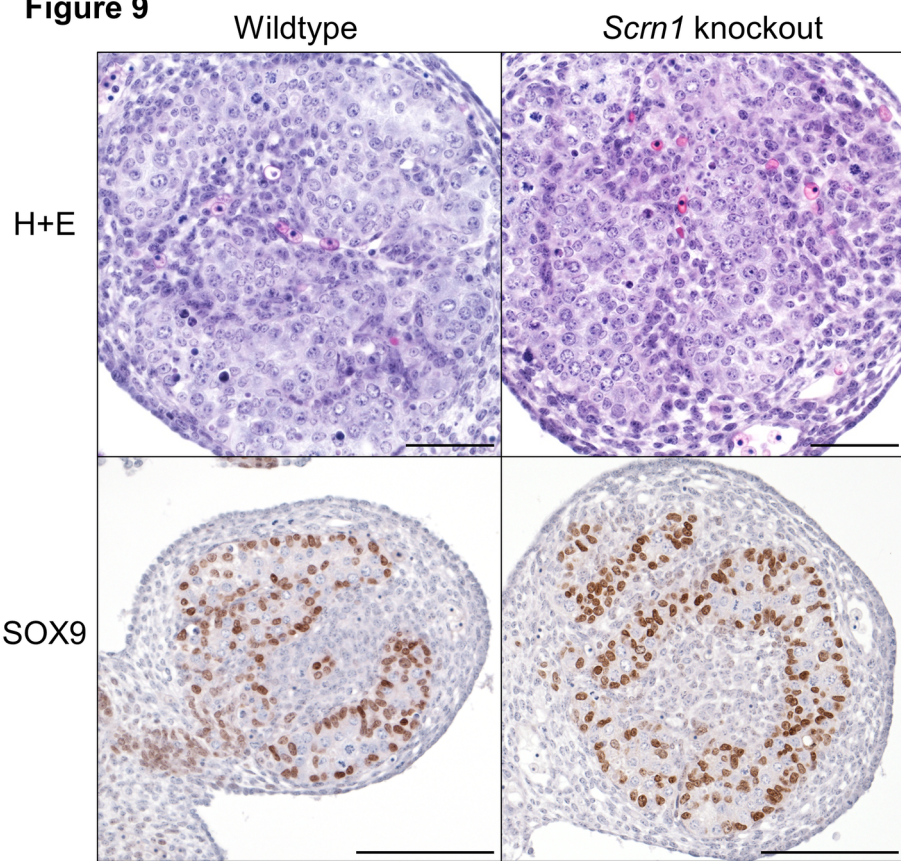
**Figure 4**



**Figure 7**



**Figure 9**



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