Maher et al., Visualizing the origins of selfish de novo mutations in individual seminiferous tubules of human testes

Supplementary Appendix

Table of Contents

SI Text

Methods

Testis samples

Sections or blocks of formalin-fixed paraffin-embedded (FFPE) non-malignant testes removed from anonymized patients for reasons of coincidental pathology were acquired from the Department of Cellular Pathology/Oxford Centre for Histopathology Research (OCHRe), John Radcliffe Hospital, Oxford, UK, as previously described[1]. All patients had given informed written consent for research use and ethical approval was provided by the Oxfordshire Research Ethics Committee A (C03.076: Receptor tyrosine kinases and germ cell development: detection of mutations in normal testis, testicular tumors and sperm). The age of the donor at the time of surgery and clinical reason for orchidectomy was documented (Tables 1, S1 and S3). For the subset of testes that were processed for microdissection, a haematoxylin and eosin (H&E) stained section adjacent to those used for staining and microdissection was analyzed by a pathologist to determine any relevant pathology. Spermatogenesis of the immunopositive tubular cross-sections that were sequenced, as well as three neighboring tubular cross-sections with a normal MAGEA4 appearance, was scored, blinded to immunopositive status, using Johnsen's criteria[2] (Fig. 3 and Fig. S17) as presented in Table 1, Table S1 and Figs. S2-S5, S7-S16, S18.

Immunohistochemical screening

Staining of Testes 1-1, 2-1 and 3-1 has previously been reported[1]. For Testes 2-1 and 3-1, the regions previously analyzed included 4 sections on 1.0 polyethylene napthalate (PEN) membrane slides (Zeiss) that were used for DNA analysis in this study. For Testis 1-1 a further 56 sections of 5 µm were obtained from the same FFPE tissue block, 16 of which were placed on PEN membrane slides, which had been pre-treated under UV light for 30 minutes. For all other testes, initially, one to two 5 µm sections from each FFPE block (Table S3) were stained using a MAGEA4 antibody (clone 57B, a kind gift from Prof. Giulio C. Spagnoli) using methods previously described[1]. A further 20 (for Testis

7-1) or 30-35 (for Testes 8-E to 17-2E) consecutive 5 µm sections were obtained from each FFPE tissue block in which immunopositive tubules (i.e. tubular cross-sections with a darker appearance due to stronger staining and/or the presence of additional cells) were identified. Four (Testis 7-1) or twelve (Testes 8-E to 17-2E) sections were placed on UV-treated 1.0 PEN membrane slides and the remainder mounted on adhesive glass microscope slides. All slides were stored at 4°C until use. Immunohistochemistry was performed with antibodies to MAGEA4 (clone 57-B) (every 4-6 slides), FGFR3 (clone C-15, Santa Cruz Biotechnology) (single slide) and pAKT (clone 736E11, Cell Signaling Technology) (single slide) using the EnVision detection system (Dako) as previously described[12] or using the EXPOSE detection IHC kit (AbCam) following the manufacturer's instructions. Stained sections were digitally scanned at 20x magnification using either a dotSlide digital virtual microscope (Olympus) analyzed using the viewer software OlyVIA-Viewer (Olympus), or a NanoZoomer 2.0-HT slide scanner (Hamamatsu) analyzed using NDP.view2 (Hamamatsu). Details of the order of stained and microdissected (LCM) sections for each testis are presented in Figs. S2-S5, S7-S16, S18.

Laser capture microdissection and DNA extraction

In the proof-of-principle (PoP) study (Testes 1-1, 2-1, 3-1, 7-1) (Fig. S1), one to four neighboring cross-sections of tubules from 3 distinct regions (>2 mm apart) were isolated from a single methyl green-stained section into individual adhesive cap tubes (Zeiss) using a PALM laser capture MicroBeam microscope (Zeiss). For the main study (Testes 8-E to 17-2E) (Fig. S6) clusters of tubular cross-sections from the same region of two adjacent slides were pooled into a single adhesive cap tube. This was performed on six slides in total, generating three biological replicates for each cluster. DNA was extracted from each replicate independently using 10 µl Arcturus Picopure DNA extraction solution (Life Technologies) and whole genome amplified (WGA) using a GenomePlex single cell WGA kit (WGA4, Sigma Aldrich), omitting the initial fragmentation step (which was deemed unnecessary due to the fragmented nature of the formalin-fixed DNA). Negative (H_2O; PicoPure DNA extraction solution) and positive (gDNA) controls were included in each WGA procedure. Amplified

products were purified using a GenElute™ PCR CleanUp Kit (Sigma Aldrich). Further replicates were microdissected and extracted as above, but did not undergo WGA, to use for direct sequence validation. DNA was extracted from 3 x 5 µm whole sections using QIAamp DNA FFPE Tissue Kit (Qiagen) for use as constitutional DNA.

Targeted sequencing and variant calling

For the PoP study, 200 ng of HaloPlex Enrichment Control DNA (Agilent Technologies) or approximately 2 µg of WGA DNA were used as template DNA. For the main study approximately 1 µg of FFPE (constitutional) DNA or 2 µg of WGA DNA were used as template DNA. Targeted regions of the genome (Dataset S1) were captured and amplified with unique sample indices/barcodes (BC) using a HaloPlex custom design panel (Agilent Technologies) following the manufacturer's instructions. Equimolar ratios of the indexed WGA samples (and approximately half of the concentration of the control/constitutional samples) were pooled in groups of 13 to 19 samples and sequenced on one lane of the HiSeq 2000 platform (Illumina) using 2x100 paired-end reads (4 lanes in total were processed). 87.4% of target regions (range 53.2% - 95.6%) had a minimum median coverage of 20x in the WGA samples; 98.6% of target regions (range 98.0% - 99.1%) had a minimum median coverage of 20x in the whole section DNA samples (Dataset S1). 3' primer and adapter sequences were removed using Cutadapt[3]. 5' restriction enzyme footprints were removed by trimming the first 5 bases from all reads. For captured fragments shorter than 100 bases, 3' restriction enzyme footprints were also removed by trimming the last 5 bases using a custom script. Trimmed reads were aligned to hg19 genome build using NovoAlign version 3.01.00. For the PoP study (Testes 1-1, 2-1, 3-1, 7-1), single nucleotide variants (SNVs) and indels in the WGA tubular cross sections were identified by comparing variant calls to those in control DNA using VarScan version 2.3.6[4] (variant frequencies ≥ 0.15 in WGA tubular DNA, < 0.01 in control DNA; minimum coverage of 20 reads). Variants were annotated using ANNOVAR version 2014Nov12[5] and common SNPs (frequency ≥ 0.001 in ESP6500 (National Heart, Lung and Blood Institute [NHLBI] GO Exome

Sequencing Project)[6] or 1000 Genomes Project[7]) were removed. For the main study (Testes 8E to 16-D), SNVs and indels specific to the WGA cross-sections were identified by comparing variant frequencies with matched constitutional DNA using VarScan version 2.3.6 (variant frequencies ≥0.10 in WGA DNA; <0.01 in matched constitutional DNA; minimum coverage of 5 reads; minimum variant reads of 2). Variants were annotated using ANNOVAR version 2014Nov12. Subsequently, variants common to all three replicates were prioritized for validation. When triplicate variants were not identified or validated using the experimental outline (Fig. S6), candidate variants present in one or two replicates were prioritized using the experimental outline below, based on known disease association[8] or cancer association[9]. The number of the variants called for each tubule cluster are presented in Figs. S2-S5, S7-S16, S18.

Screening of two regions of *FGFR3* that were poorly covered by HaloPlex (Dataset S1) was performed by PCR using template-specific primers (Table S2) with common sequence (CS) tails on the same WGA samples processed by HaloPlex. The amplified regions were indexed and Ion PGM adapter sequences added by PCR using Access Array Barcode Library primers (Fluidigm). Indexed PCR products were pooled in equimolar ratios and sequenced to a minimum coverage of 20x using Ion PGM sequencing following the manufacturer's instructions (Life Technologies). To screen the ischemic testis (17-2E) for known PAE mutations, nine regions across *FGFR2* (2), *FGFR3* (2), *RET* (2), *PTPN11* (1) and *HRAS* (2) were amplified by PCR using template-specific primers (Table S2) with common sequence (CS) tails, indexed and sequenced on Ion PGM. Variants present at frequencies ≥ 0.15 in WGA tubular DNA were called using Ion Torrent variantCaller.

Variant validation

To validate candidate variants identified by the HaloPlex and PGM screens, non-WGA DNA from the same tubule on adjacent sections was amplified by PCR (Table S2) and dideoxy-sequenced. For some candidate variants that had the appearance of alignment errors, DNA from the same sample that

underwent HaloPlex capture was amplified by PCR and dideoxy-sequenced. Details of genetic analysis for each testis are presented in Figs. S2-S5, S7-S16, S18.

Accession codes

Genbank: *FGFR2* cDNA, NM_000141; *FGFR3* cDNA, NM_000142; *HRAS* cDNA, NM_005343; *KRAS* cDNA, NM_004985; *PTPN11* cDNA, NM_002834. cDNA numbering is given relative to the A (= 1) of the ATG initiation codon.

URLs

COSMIC: http://cancer.sanger.ac.uk/cosmic/

ESP6500: http://evs.gs.washington.edu/EVS/

OMIM: http://omim.org/

Supplementary References

1. Lim J*, et al.* (2012) Selfish spermatogonial selection: evidence from an immunohistochemical screen in testes of elderly men. *PLoS One* 7:e42382.
2. Johnsen SG (1970) Testicular biopsy score count - a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* 1:2-25.
3. Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal* 17:10-12.
4. Koboldt DC*, et al.* (2012) VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* 22:568-576.
5. Wang K, Li M, Hakonarson H (2010) ANNOVAR: Functional annotation of genetic variants from next-generation sequencing data. *Nucleic Acids Res.* 38:e164.
6. Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA. (Accessed at http://evs.gs.washington.edu/EVS/.)
7. Abecasis GR, *et al*. (2012) An integrated map of genetic variation from 1,092 human genomes. *Nature* 491:56-65.
8. Goriely A, Wilkie AOM (2012) Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. *Am. J. Hum. Genet.* 90:175-200.
9. Forbes SA*, et al.* (2015) COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res.* 43(D1):D805-D811.

Table S1. Detailed clinical and genetic data for testis samples included in Table 1.

Sample ID	Age (yrs)	Reason for orchidectomy	Pathology report for sections analyzed	Tubule cluster ID	Johnsen score for immune-positive tubule cluster [a]	Johnsen score in neigh-boring normal tubules [a]	Number of target genes	Constit-utional DNA sequenced	Variants identified in all replicates using HaloPlex	Variants identified in candidate screen	Germline Disorder ([d] = perinatal lethal)	OMIM reference number [e]	Reference for germline mutation	Somatic mutation (COSMIC ID) [f]	Somatic mutation (tissue distribution in COSMIC v67)
Testis 1-1	71	Prostate cancer	Normal	2	8	9	107	Y [c]	*FGFR2* c.1024T>A (p.C342S)	NA	Crouzon/Pfeiffer syndrome	123500 / 101600	Tartaglia et al., 1997 [18]	No	No
				6	6	9	107	Y [c]	*FGFR2* c.1024T>A (p.C342S)	NA					
Testis 2-1	75	Infected haematoma after excision of a benign epididymal cyst	Normal	7	9	9	107	N	-	*FGFR3* c.1118A>G (p.Y373C)	Thanatophoric dysplasia type I [d]	187600	Rousseau et al., 1996 [23]	COSM718; COSM1666838	urinary tract (n=396), skin (n=16), haematopoietic and lymphoid (n=8)
				11	4.33	10	107	N		*FGFR3* c.1118A>G (p.Y373C)					
				13	3	10	107	N	-	NA	NA	NA	NA	NA	NA
Testis 3-1	78	Inguinal hernia and transurethral resection of prostate	Edema, haemorrhage, mild chronic inflammation, focal atrophy and Leydig cell hyperplasia.	8	4	9	107	N	-	NA	NA	NA	NA	NA	NA
				12	4	9	107	N	-	*HRAS* c.37G>C (p.G13R)	None reported	NA	NA	COSM486; COSM99938	skin (n=28), thyroid (n=15), upper aerodigestive tract (n=8), urinary tract (n=8), prostate (n=3), salivary gland (n=3), soft issue (n=3), adrenal gland (n=2), lung (n=2)
				20	4	6	107	N	-	*HRAS* c.37G>C (p.G13R)					
Testis 7-1	70	Recurrent inguinal hernia repair. Wound sepsis. Necrotising infection.	Mild atrophy with seminiferous tubule basement membrane thickening, minimal focal chronic inflammation and minor Leydig cell hyperplasia.	1	5	4	107	N	-	NA	NA	NA	NA	NA	NA
				4	4	8	107	N	-	NA	NA	NA	NA	NA	NA
				7	5	9	107	N	-	NA	NA	NA	NA	NA	NA
Testis 8-E	39	Hernia	Mild edema, otherwise normal	A (x3)	9	10	135	Y	*FGFR2* c.758C>G (p.P253R)	NA	Apert syndrome	101200	Wilkie et al., 1995 [17]	COSM1152152; COSM915504; COSM49170; COSM915505	endometrium (n=5), Ovary (n=1), Lung (n=1)
				B (x3)	9.33	9	135	Y	*PTPN11* c.215C>T (p.A72V)	NA	None reported	NA	NA	COSM13015	haematopoietic and lymphoid tissue (n=42)
Testis 9-L2C	62	Prostate cancer	Edema, otherwise normal	A (x3)	5.33	9	135	Y	-	*PTPN11* c.181G>T (p.D61Y)	None reported	NA	NA	COSM13011	haematopoietic and lymphoid tissue (n=33), (large intestine (n=1), autonomic ganglia (n=1)
Testis 10-F	63	Inguinal hernia	Normal	B (x3)	9.33	9.67	135	Y	*FGFR2* c.1019A>G (p.Y340C)	NA	Pfeiffer syndrome [d]	101600	Khonsari et al., 2012 [20]	None reported	None reported
Testis 11-H	70	None provided	Normal	A (x3)	5.67	8	135	Y	-	*FGFR2* c.1019A>G (p.Y340C)	Pfeiffer syndrome [d]	101600	Khonsari et al., 2012 [20]	None reported	None reported

Testis 12-E	71	Inguinal hernia	Normal	A (x3)	5	8	135	Y	*KRAS* c.182A>G (p.Q61R)	NA	None reported	NA	NA	COSM1158660 ; COSM552	large intestine (n=27), lung (n=10), pancreas (n=9), thyroid (n=9), haematopoietic and lymphoid tissue (n=3), stomach (n=2), cervix (n=1), central nervous system (n=1), genital tract (n=1), prostate (n=1), skin (n=1), upper aerodigestive tract (n=1)
Testis 12-H			Focal Leydig cell hyperplasia, otherwise normal	A (x3)	6.5	9.67	135	Y	-	NA	NA	NA	NA	NA	NA
Testis 13-G	72	Cystic spermatic cord	Normal	A (x3)	4	10	135	Y	*PTPN11* c.215C>T (p.A72V)	NA	None reported	NA	NA	COSM13015	haematopoietic and lymphoid tissue (n=42)
				B (x3)	8	10	135	Y	*FGFR3* c.742C>T (p.R248C)	NA	Thanatophoric dysplasia type I [d]	187600	Tavormina et al., 1995 [22]	COSM714; COSM1133721	urinary_tract (n=165), skin (n=65), haematopoietic and lymphoid tissue (n=2), lung (n=1), upper aerodigestive tract (n=1)
Testis 14-D	80	Inguinal hernia	Normal	A (x3)	4.33	7	135	Y	-	*KRAS* c.182A>G (p.Q61R)	None reported	NA	NA	COSM1158660 ; COSM552	large intestine (n=27), lung (n=10), pancreas (n=9), thyroid (n=9), haematopoietic and lymphoid tissue (n=3), stomach (n=2), cervix (n=1), central nervous system (n=1), genital_tract (n=1), prostate (n=1), skin (n=1), upper aerodigestive tract (n=1)
Testis 15-B	80	Inguinal hernia	Areas of marked atrophy with completely atrophic seminiferous tubules, Leydig cell hyperplasia	A (x3)	8	10	135	Y	*KRAS* c.182A>G (p.Q61R)	NA	None reported	NA	NA	COSM1158660 ; COSM552	large intestine (n=27), lung (n=10), pancreas (n=9), thyroid (n=9), haematopoietic and lymphoid tissue (n=3), stomach (n=2), cervix (n=1), central nervous system (n=1), genital_tract (n=1), prostate (n=1), skin (n=1), upper aerodigestive tract (n=1)
Testis 16-D	87	Inguinal hernia	Normal	A (x3)	5	8.33	135	Y	-	*FGFR3* c.1948A>G (p.K650E)	Thanatophoric dysplasia type II [d]	187601	Tavormina et al., 1995 [22]	COSM719	urinary tract (n=23), skin (n=12), haematopoietic and lymphoid tissue (n=7), testis (n=2), central nervous system (n=1)
				B (x3)	3.67	7.33	135	Y	*FGFR2* c.870G>T (p.W290C)	NA	Pfeiffer syndrome	101600	Schaefer et al. 1998 [19]	COSM1346285	large intestine (n=1)
Testis 17-2E	90	Inguinal hernia	Poorly preserved tubular epithelium. Severe atrophy of seminiferous tubules. Vascular congestion.	A (x3)	3.33	8	5 [b]	N	-	*FGFR3* c.1948A>G (p.K650E)	Thanatophoric dysplasia type II [d]	187601	Tavormina et al., 1995 [22]	COSM719	urinary tract (n=23), skin (n=12), haematopoietic and lymphoid tissue (n=7), testis (n=2), central nervous system (n=1)

a = Johnsen scores[39] range between 1 (no seminiferous epithelium) and 10 (full spermatogenesis). b = targeted hotspots only (Table S2). c = DNA from normal tubule that underwent WGA. d = perinatal lethal. e = accessible at http://omim.org/; f = accessible at http://cancer.sanger.ac.uk/cosmic/. Reference numbering in this Table follows the main text

Table S2. Primer sequences used for mutation screening and dideoxy-sequencing

Primer sequences used for Ion PGM screen of low coverage regions of *FGFR3*

Gene	Exon	Genomic co-ordinates of targeted region (hg19)	Codons queried	Forward primer sequence 5' to 3' [CS1 tail underlined]	Reverse primer sequence 5' to 3' [CS2 tail underlined]	common tails used for secondary amplification	Target size (bp)	Tm	DMSO
FGFR3	7	chr4:1803532-1803684	p.R248; p.S249; p.P250;	ACACTGACGACATGGTTCTACAGTGGCCCCTGAGCGTCATCT	TACGGTAGCAGAGACTTGGTCTGCCACTGGATGTGGGGCTGT	CS1/2	153	60°C	0
FGFR3	15	chr4:1807829-1807954	p.K650	ACACTGACGACATGGTTCTACAGTGATGAAGATCGCAGACT TCGGGCTG	TACGGTAGCAGAGACTTGGTCTGCCAGGCGTCCTACTGGCATGA	CS1/2	126	60°C	5%

Primer sequences used for Ion PGM screen (37 PAE hotspots)

Gene	Exon	Genomic co-ordinates of targeted region (hg19)	Codons queried	Forward primer sequence 5' to 3' [CS1 tail underlined]	Reverse primer sequence 5' to 3' [CS2 tail underlined]	common tails used for secondary amplification	Target size (bp)	Tm	DMSO
FGFR2	8 (IIIa)	chr10:123279572-123279736	p.S252; p.P253	ACACTGACGACATGGTTCTACATTGGTCTCTCATTCTCCCATC CCCACTC	TACGGTAGCAGAGACTTGGTCTTGGGGCTGGGCATCACTGTAAACC	CS1/2	165	66°C	0
FGFR2	10 (IIIc)	chr10:123276833-123276963	p.Y328; p.N331; p.D336; p.A337; p.G338; p.Y340; p.T341; p.C342; p.A344; p.S347; p.S351	ACACTGACGACATGGTTCTACACACCACGGACAAAGAGATT GAGGTTC	TACGGTAGCAGAGACTTGGTCTCTGGCAGAACTGTCAACCATGCAG	CS1/2	131	66°C	0
FGFR3	10	chr4:1806034-1806163	p.E368; p.G370; p.S371; p.Y373; p.G375; p.G380; p.V381	ACACTGACGACATGGTTCTACACCTCAACGCCCATGTCTTTG C	TACGGTAGCAGAGACTTGGTCTCGTCACAGCCGCCACCACCA	CS1/2	130	60°C	0
FGFR3	15	chr4:1807829-1807954	p.D641; p. K650	ACACTGACGACATGGTTCTACAGTGATGAAGATCGCAGACT TCGGGCTG	TACGGTAGCAGAGACTTGGTCTGCCAGGCGTCCTACTGGCATGA	CS1/2	126	60°C	5%
HRAS	2	chr11:534203-534375	p.G12; p.G13; p.Q22	ACACTGACGACATGGTTCTACAGTGGGGCAGGAGACCCTGT AGGAGGAC	TACGGTAGCAGAGACTTGGTCTCAGGCTCACCTCTATAGTGGGGTC G	CS1/2	173	60°C	5%
HRAS	3	chr11:533818-533934	p.T58; p.A59; p.Q61; p.E62; p.E63-D69	ACACTGACGACATGGTTCTACAGGAAGCAGGTGGTCATTGA TGGGGAG	TACGGTAGCAGAGACTTGGTCTACAGGAAGCCCTCCCCGGTGCG	CS1/2	117	60°C	5%
PTPN11	8	chr12:112915408-112915583	p.F285; p.R289; p.N308; p.I309	ACACTGACGACATGGTTCTACACTGAAGCAGTCCAGGACTTA TGTGAC	TACGGTAGCAGAGACTTGGTCTATAGGCTAGAAATGTATGGTCAGA AAACACT	CS1/2	176	60°C	0
RET	11	chr10:43609885-43610015	p.C630; p.C634	ACACTGACGACATGGTTCTACAGAGCCTCTGGCGGTGCCAA GCCTCA	TACGGTAGCAGAGACTTGGTCTCAGAAGGCAGACAGCAGCACCGA GACGAT	CS1/2	131	60°C	5%
RET	16	chr10:43617335-43617490	p.M918	ACACTGACGACATGGTTCTACAACCCCTCCTTCCTAGAGAGT TAGAGT	TACGGTAGCAGAGACTTGGTCTCCCAAGAGAGCAACACCCACAC	CS1/2	156	60°C	0

Secondary amplification for addition of barcoded Ion PGM adapters

Forward primer	Reverse primer	Forward primer 5' to 3' [CS1 underlined]	Reverse primer 5' to 3' [CS2 underlined]	common tails used for secondary amplification	Target size (bp)	Tm	DMSO
A_BC_CS1	trP1_CS2	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**-[BC]-ACACTGACGACATGGTTCTACA	*CCTCTCTATGGGCAGTCGGTGAT*TACGGTAGCAGAGACTTGGTCT	NA	NA	60°C	0
trP1_CS1	A_BC_CS2	*CCTCTCTATGGGCAGTCGGTGAT*ACACTGACGACATGGTTCTACA	**CCATCTCATCCCTGCGTGTCTCCGACTCAG**-[BC]-TACGGTAGCAGAGACTTGGTCT	NA	NA	60°C	0

Primer sequences used for variant validation by dideoxy-sequencing

Gene		Genomic co-ordinates of targeted region (hg19)	Codons queried	Forward primer 5' to 3' [tail underlined]	Reverse primer 5' to 3' [tail underlined]	Primer tails used for dideoxy sequencing	Target size (bp)	Tm	DMSO
ALK		chr2:29416405-29416516	p.L1489	GTAAAACGACGGCCAGTGGTGGGTTTCTCTGTAAACCAG	AGCGGATAACAATTTCACACAGGAATTCTCTCAGTCCAACCCTCCT	M13F/R	112	60°C	0
ASXL1		chr20:31019124-31019241	p.L267	ACACTGACGACATGGTTCTACAGTCAAATGAAGCGCAACAGA	TACGGTAGCAGAGACTTGGTCTTGTGATGGTAAGGCATGGAA	CS1/2	118	60°C	5%
ASXL1		chr20:31023211-31023362	p.E914	ACACTGACGACATGGTTCTACATACCCATCCCATCGAATGATGA	TACGGTAGCAGAGACTTGGTCTACCCTCCTCAGCTGTCAAATCC	CS1/2	152	60°C	0
CHD7		chr8:61765888-61766001	p.E2222	ACACTGACGACATGGTTCTACAGAGAGCAAGCAGGAATGTGAG	TACGGTAGCAGAGACTTGGTCTCTCTTCGGAACCTTTCTCTGAA	CS1/2	114	60°C	0
DICER1		chr14:95592989-95593143	p.V255-p.L257	ACACTGACGACATGGTTCTACATTTTTAAATTGCATTCTCACTACTGC	TACGGTAGCAGAGACTTGGTCTTGCTTCTTCTAATTCCATCAGCA	CS1/2	155	60°C	5%
DICER1		chr14:95562561-95562741	p.V1525L	ACACTGACGACATGGTTCTACAGGGCTTCCACACAGTCCGCTAT	TACGGTAGCAGAGACTTGGTCTTGGGATGCAATGTGCTATCTGGA	CS1/2	181	60°C	5%
FGFR2		chr10:123279621-123279702	p.P253	ACACTGACGACATGGTTCTACATCTTCCCTCTCTCCACCAGAGC	TACGGTAGCAGAGACTTGGTCTCGACCACTGTGGAGGCATTT	CS1/2	82	60°C	0
FGFR2		chr10:123279527-123279658	p.W290	ACACTGACGACATGGTTCTACACCAAGCCGGACTGCC	TACGGTAGCAGAGACTTGGTCTCCGTATTTACTGCCGTTCTT	CS1/2	132	60°C	0
FGFR2		chr10:123276804-123276983	p.Y340; p.C342S	GTAAAACGACGGCCAGTTTCTAGGCCGCCGGTGTTA	AGCGGATAACAATTTCACACAGGAAAAACCCAGAGAGAAAGAACAGTAT	M13F/R	180	60°C	0
FGFR2		chr10:123274710-123274864	p.G384	ACACTGACGACATGGTTCTACATCTGTTCCTCCTCCTGTGATCT	TACGGTAGCAGAGACTTGGTCTGTGTTCTTCATTCGGCACAG	CS1/2	155	60°C	0
FGFR2		chr10:123247452-123247639	p.K641	ACACTGACGACATGGTTCTACATCTTGATTTCAGTGTATTCATCG	TACGGTAGCAGAGACTTGGTCTCATTCTGAGCCTCACCCC	CS1/2	188	60°C	0
FGFR3		chr4:1803532-1803684	p.R248	ACACTGACGACATGGTTCTACAGTGGCCCCTGAGCGTCATCT	TACGGTAGCAGAGACTTGGTCTGCCACTGGATGTGGGGCTGT	CS1/2	153	60°C	0
FGFR3		chr4:1807829-1807954	p.K650	ACACTGACGACATGGTTCTACAGTGATGAAGATCGCAGACTTCGGGCTG	TACGGTAGCAGAGACTTGGTCTGCCAGGCGTCCTACTGGCATGA	CS1/2	126	60°C	5%
FGFR4		chr5:176517856-176518024	p.T150-p.R154	ACACTGACGACATGGTTCTACACGCTGCTGCTCATCTGATCACT	TACGGTAGCAGAGACTTGGTCTAGCTGGACAGCGGAACTTGAC	CS1/2	169	60°C	0
FGFR4		chr5:176518607-176518720	p.H206	GTAAAACGACGGCCAGTATGAGGATCTAGCCTCCTGGTC	AGCGGATAACAATTTCACACAGGACTCTCCATCACGAGACTCCAG	M13F/R	114	60°C	5%
FGFR4		chr5:176518742-176518873	p.L242	ACACTGACGACATGGTTCTACACACATACACCTGCCTGGTAGAG	TACGGTAGCAGAGACTTGGTCTACCAAGAGGGCACAACTGAG	CS1/2	132	60°C	0
KRAS		chr12:25380250-25380371	p.Q61	ACACTGACGACATGGTTCTACACCAGACTGTGTTTCTCCCTTCTCA	TACGGTAGCAGAGACTTGGTCTGGTCCCTCATTGCACTGTACTCC	CS1/2	122	60°C	0
LRP5		chr11:68115485-68115603	p.C115	GTAAAACGACGGCCAGTAAGCAGACCTACCTGAACCAGA	AGCGGATAACAATTTCACACAGGAGAGTCCGTCCAGTACAGCTTCT	M13F/R	119	60°C	5%
MTOR		chr1:11319313-11319487	p.A12-p.T15	ACACTGACGACATGGTTCTACATCTAAAGAACCTCAGGGCAAGA	TACGGTAGCAGAGACTTGGTCTGTTCCATGGTGACATAGTGCTG	CS1/2	175	60°C	5%
PTPN11		chr12:112888088-112888207	p.D61	ACACTGACGACATGGTTCTACATCCCCTTGCCTCCCTTTCCAATGGA	TACGGTAGCAGAGACTTGGTCTCCAAAGTGGCAAATTTCTCCCCTCC	CS1/2	120	60°C	0
PTPN11		chr12:112888173-112888252	p.A72	ACACTGACGACATGGTTCTACATGACCTGTATGGAGGGGAGAAA	TACGGTAGCAGAGACTTGGTCTCTTTTAATTGCCCGTGATGTTCC	CS1/2	80	60°C	0

Table S3. Testis samples screened for immunopositive tubules with MAGEA4 antibody

Sample ID	Age (years)	Number of years since embedd-ing	Clinical reason for orchidectomy	Estimated size of sampled tissue (mm^2)	Presence of MAGEA4-immuno-positive tubules?	Presence of lumenal clusters of spermatogonia (see ref 34)	Sectioned further for microdissection?	Immunopositive tubules present in microdissection region	Sequenced?	Haloplex barcode
Testis 8-D	39	2	Inguinal hernia	296	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 8-E				301	Yes	No	Yes	Yes	Yes	BC24-BC26; BC46-48; BC27 [Whole section DNA]
Testis 8-F				151	Yes	No	No - prioritised block E	NA	NA	NA
Testis 9-L2C	62	<1	Prostate cancer	122	Yes	No	Yes	Yes	Yes	BC02-BC04; BC05 [Whole section DNA]
Testis 9-L4				192	Yes	No	No - prioritised block L2	NA	NA	NA
Testis 9-R1				380	Yes	No	No - prioritised block L2	NA	NA	NA
Testis 10-F	63	6	Inguinal hernia	189	Yes	No	Yes	Yes	Yes	BC10-BC12; BC13 [Whole section DNA]
Testis 11-H	70	6	None provided	233	Yes	No	Yes	Yes	Yes	BC14-BC16; BC17 [Whole section DNA]
Testis 12-G	72	1	Inguinal hernia	467	Yes	Yes	Yes	Yes	Yes	BC32-B34; BC40-42; BC35 [Whole section DNA]
Testis 12-H				368	Yes	No	Yes	Yes	No - prioritized block G	NA
Testis 13-D	71	3	Cystic spermatic cord	272	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 13-E				169	Yes	No	Yes	Yes	Yes	BC49-51
Testis 13-F				302	No	Yes	No - no obvious immunopositive tubules	NA	NA	NA
Testis 13-G				156	Yes	No	No - prioritised blocks E and G	NA	NA	NA
Testis 13-H				291	Yes	No	Yes	Yes	Yes	BC06-BC08; BC09 [Whole section DNA]
Testis 14-C	80	5	Inguinal hernia	158	Yes	No	No - prioritised block D	NA	NA	NA
Testis 14-D				206	Yes	Yes	Yes	Yes	Yes	BC20-BC22; BC23 [Whole section DNA]
Testis 15-A	80	1	Inguinal hernia	118	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 15-B				126	Yes	No	Yes	Yes	Yes	BC36-BC38; BC39 [Whole section DNA]
Testis 15-C				246	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 16-D	87	2	Inguinal hernia	408	Yes	Yes	Yes	Yes	Yes	BC28-BC30; BC43-45; BC31 [Whole section DNA]
Testis 16-E				341	Yes	Yes	Yes	Yes	No - prioritized block E	NA

Testis 16-F				432	Yes	Yes	Yes	Yes	No - prioritized block F	NA
Testis 18-F	57	3	Epidid-ymal cyst	137	No	Yes	No - no obvious immunopositive tubules	NA	NA	NA
Testis 18-G				67	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 19-F	62	1	Trauma	52	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 20-B	65	3	Inguinal hernia	229	No	Yes	No - no obvious immunopositive tubules	NA	NA	NA
Testis 20-C				352	Yes	No	Yes	No	NA	NA
Testis 20-D				170	No	Yes	No - no obvious immunopositive tubules	NA	NA	NA
Testis 20-E				171	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 20-F				328	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 21-D	79	5	Inguinal hernia	319	Yes	Yes	Yes	Yes	No - prioritized other samples due to age of block	NA
Testis 21-E				234	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 21-F				360	Yes	No	No - prioritised block D	NA	NA	NA
Testis 21-G				239	Yes	No	No - prioritised block D	NA	NA	NA
Testis 21-I				299	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 22-D	85	2	Inguinal hernia	312	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 22-E				375	Yes	No	Yes	Yes	No - prioritized other samples based on appearance	NA
Testis 22-F				91	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 22-G				17	No	No	No - no obvious immunopositive tubules	NA	NA	NA
Testis 23-E	96	3	Inguinal hernia	266	No	Yes	No - no obvious immunopositive tubules	NA	NA	NA
Testis 23-F				181	Yes	No	Yes	Yes	No - prioritized other samples based on appearance	NA

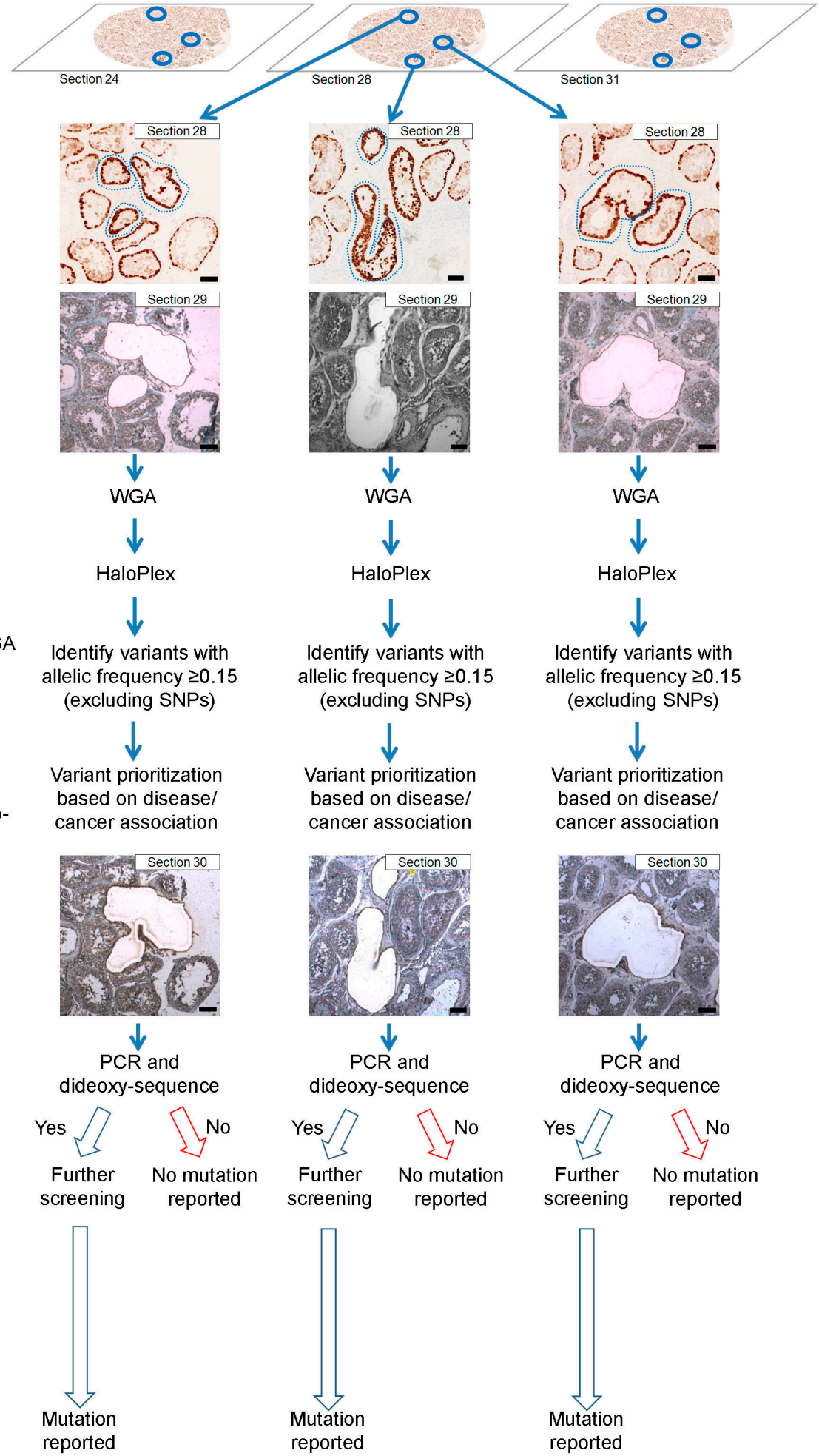


Fig. S1. Strategy to identify mutant clones in proof-of-principle study.

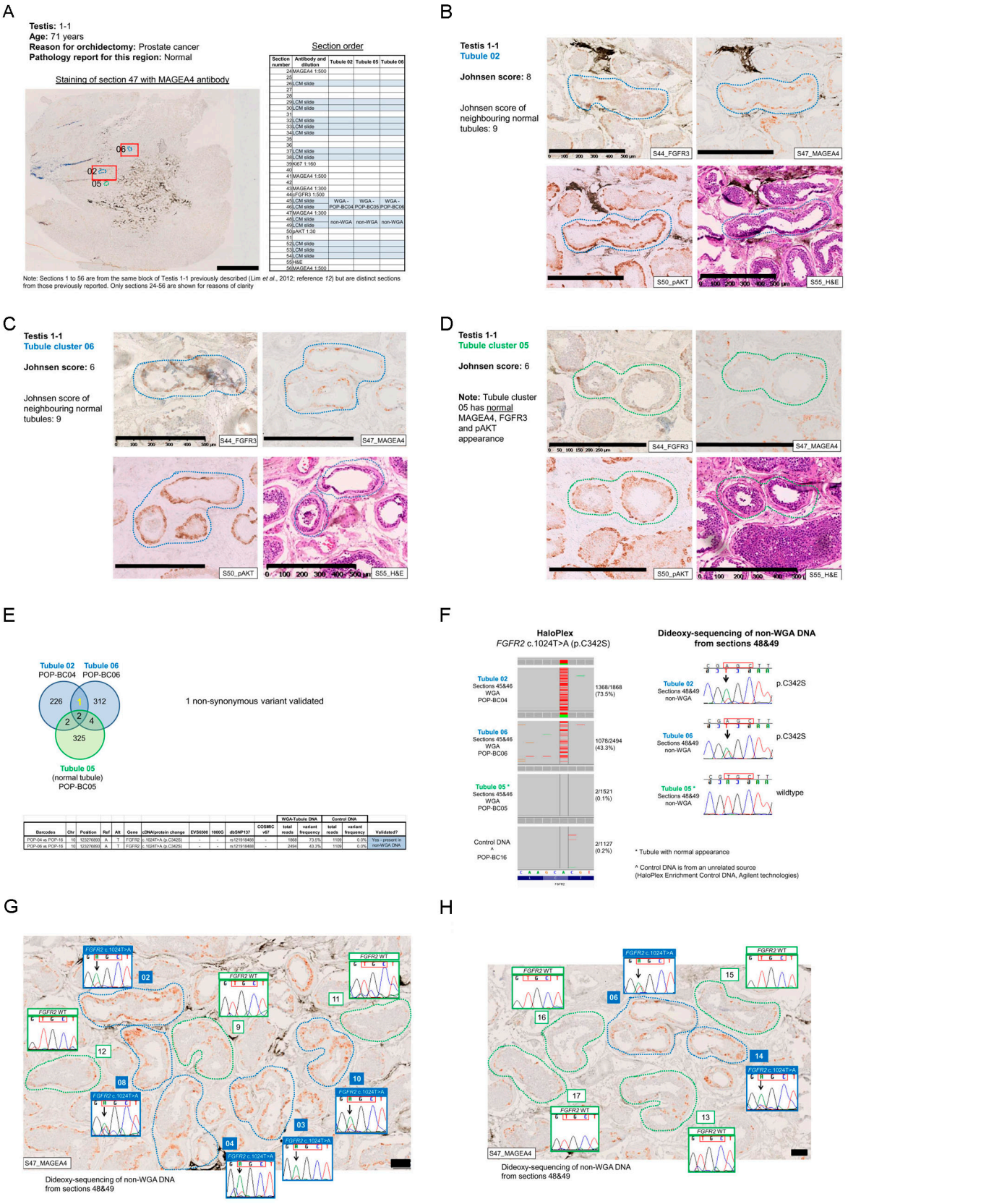


Fig. S2. Analysis of Testis 1-1. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubules (02, 05, 06) are indicated. Regions in red boxes are presented at higher magnification in (*G*) and (*H*). Scale bar: 5 mm. (*B-D*) Detailed view of the staining appearance (FGFR3, MAGEA4, pAKT and H&E) and Johnsen scoring of tubule 02 (*B*), tubule cluster 06 (*C*), and tubule cluster 05 (*D*). The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections; the green surround delineates the position of the normally-stained tubule cluster 05. Section numbers (e.g. S44) and staining performed on each section are indicated. Scale bars: 500 µm. (*E*) Number of protein-altering (non-synonymous, frameshift or splice site) variants identified in tubule clusters 02, 05 and 06, after excluding SNPs and variants in control genomic DNA, shown in Venn diagram. One variant was shared by both immunopositive tubule clusters (02 and 06) but not present in the tubule with normal appearance (05). (*F*) Identification of *FGFR2* c.1024T>A (p.C342S) in HaloPlex-screened WGA DNA from clusters 02 and 06 (viewed in Integrative Genomics Viewer (IGV)). POP-BC = HaloPlex barcode number used for proof-of-principle study. Mutations were confirmed in tubule clusters 02 and 06 by dideoxy-sequencing of non-WGA DNA extracted after microdissection of the equivalent tubule pooled from S48 and S49. (*G-H*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) appearance close to tubule 02 (*G*) and tubule cluster 6 (*H*) performed on non-WGA material extracted from S48 and S49. All immunopositive tubules (blue surround) in these regions are apparently homozygous for the *FGFR2* c.1024T>A mutation (wildtype somatic Sertoli cells within the tubule likely account for the minority reference "T" peak). All analyzed tubules with a normal MAGEA4 appearance (green surround) are mutation-negative. Scale bars: 100 µm.

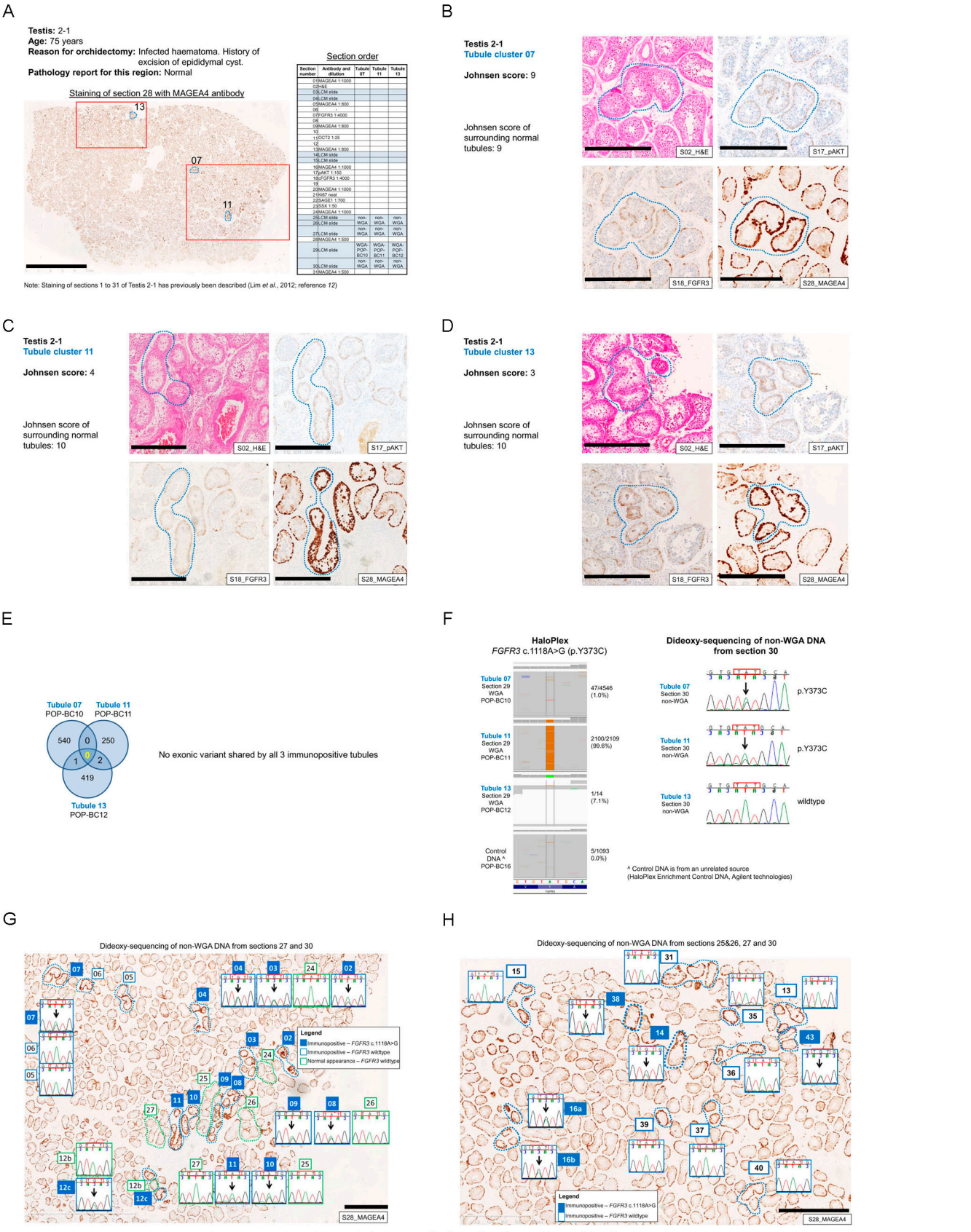


Fig. S3. Analysis of Testis 2-1. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubules (07, 11, 13) are indicated. Regions in red boxes are presented at higher magnificaiton in (*G*) and (*H*). Scale bar: 5 mm. (*B-D*) Detailed view of the staining appearance (H&E, pAKT, FGFR3 and MAGEA4) and Johnsen scoring of tubule clusters 07 (*B*), 11 (*C*), and 13 (*D*). The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S02) and staining performed on each section are indicated. Scale bars: 500 µm. (*E*) Number of protein-altering (non-synonymous, frameshift or splice site) variants identified in tubule clusters 07, 11 and 13, after excluding SNPs and variants in control genomic DNA, shown in Venn diagram. (*F*) Identification of *FGFR3* c.1118A>G (p.Y373C) in HaloPlex-screened WGA DNA from tubule cluster 11 (viewed in IGV). Dideoxy-sequencing non-WGA DNA of the equivalent tubule from S30 confirmed the apparently heterozygous mutation in tubule 11 and further identified the same mutation in tubule 07. The mutation was originally detected in the homozygous state in the WGA DNA from tubule cluster 11 but was not identified in the WGA DNA from tubule cluster 07, suggesting that allelic dropout occurred during WGA. Tubule cluster 13 sequence was poorly covered by the HaloPlex capture performed on WGA DNA but analysis of the non-WGA DNA confirmed that it did not harbor the mutation. (*G-H*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) appearance close to tubule clusters 07 and 11 (*G*) and tubule cluster 13 (*H*) performed on non-WGA material pooled from S25, S26, S27 and S30. In (*G*), although the majority of the MAGEA4-immunopositive tubules screened harbored the *FGFR3* c.1118A>G mutation (blue box with white font), some immunopositive tubules did not (white box with blue surround). All tubules with a normal appearance (white box with green surround) that were screened did not harbor the mutation. In (*H*), although tubule cluster 13 and some other neighboring immunopositive clusters did not harbor the *FGFR3* mutation (white box with blue surround), some immunopositive clusters harbored the *FGFR3* c.1118A>G mutation (blue box with white font). Part of the image shown in (*G*) is also reproduced in Fig. 1*A*. Scale bars: 1 mm.

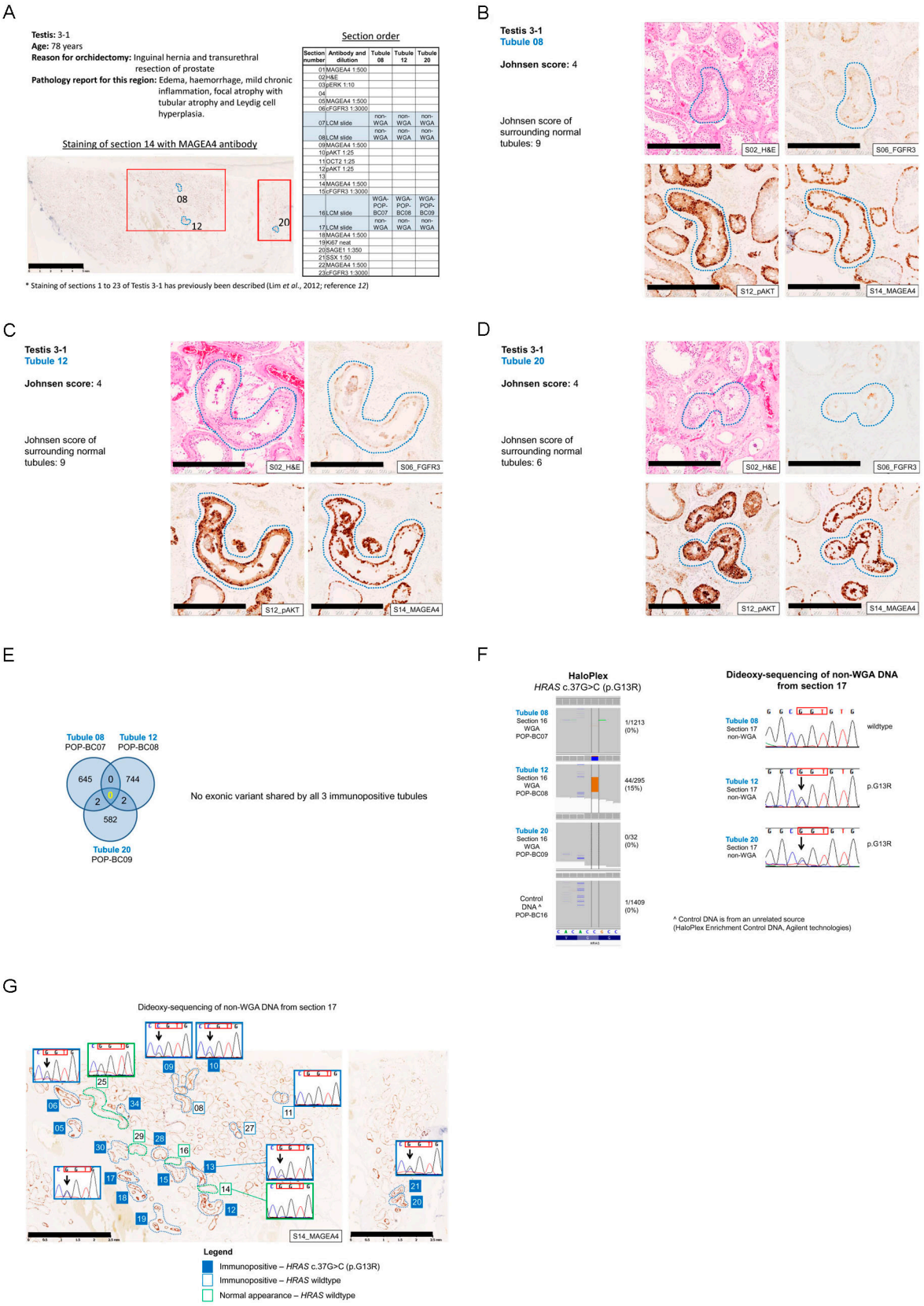
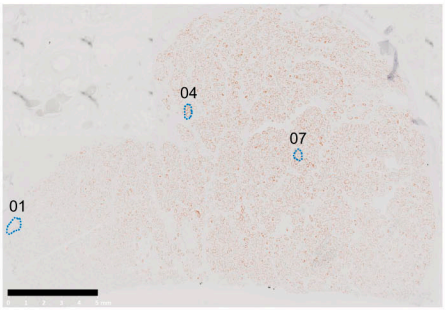


Fig. S4. Analysis of Testis 3-1. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubules (08, 12, 20) are indicated. Regions in red boxes are presented at higher magnification in (*G*). Scale bar: 5 mm. (**b-d**) Detailed view of the staining appearance (H&E, FGFR3, pAKT and MAGEA4) and Johnsen scoring of tubule 08 (*B*), tubule 12 (*C*), and tubule cluster 20 (*D*). The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S02) and staining performed on each section are indicated. Scale bars: 500 µm. (*E*) Number of protein-altering (non-synonymous, frameshift or splice site) variants identified in tubule clusters 08, 12 and 20, after excluding SNPs and variants in control genomic DNA, shown in Venn diagram. (*F*) Identification of *HRAS* c.37G>C (p.G13R) in HaloPlex-screened WGA DNA from tubule 12 (viewed in IGV). Dideoxy-sequencing non-WGA DNA of the equivalent tubule from S17 confirmed the presence of the heterozygous mutation in tubule 12 and further identified the same mutation in tubule 20. The mutation was not detected in the WGA DNA from tubule cluster 20, suggesting that allelic dropout occurred during WGA. Tubule cluster 08 did not harbor the mutation. (*G*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) appearance performed on non-WGA material from S17. Although the majority of the MAGEA4-immunopositive tubules screened harbored the *HRAS* c.37G>C mutation (blue box with white font), some immunopositive tubules did not (white box with blue surround). All tubules with a normal appearance (white box with green surround) that were screened did not harbor the mutation. For reasons of clarity, only a subset of the dideoxy-sequencing traces are shown. Note: regions 13 and 14 are presented at higher magnification in Fig. 1*B*. Scale bar: 2.5 mm.

A

Testis: 7-1
Age: 70 years
Reason for orchidectomy: Recurrent inguinal hernia repair. Wound sepsis. Necrotising infection.
Pathology report for this region: Mild atrophy with seminiferous tubule basement membrane thickening, minimal focal chronic inflammation and minor Leydig cell hyperplasia.

Staining of section 21 with MAGEA4 antibody



Section order

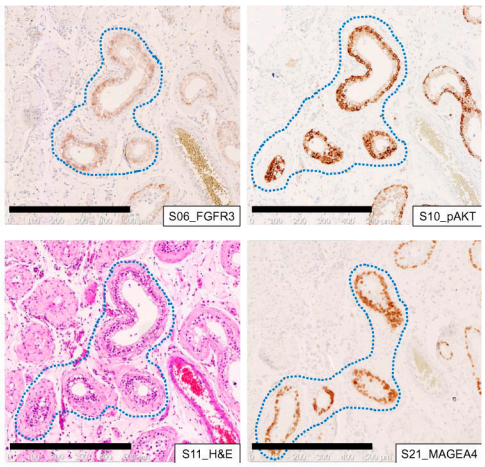
Section number	Antibody and dilution	Tubule 01	Tubule 04	Tubule 07
01	MAGEA4 1:500			
02				
03				
04				
05	MAGEA4 1:800			
06	cFGFR3 1:600			
07	LCM slide			
08	LCM slide			
09	MAGEA4 1:800			
10	pAKT 1:30			
11	H&E			
12	-			
13	MAGEA4 1:800			
14				
15	LCM slide			
16	LCM slide	WGA-POP-BC01	WGA-POP-BC02	WGA-POP-BC03
17	MAGEA4 1:800			
18	-			
19				
20				
21	MAGEA4 1:800			
22				

B

Testis 7-1
Tubule cluster 01

Johnsen score: 5

Johnsen score of surrounding normal tubules: 4

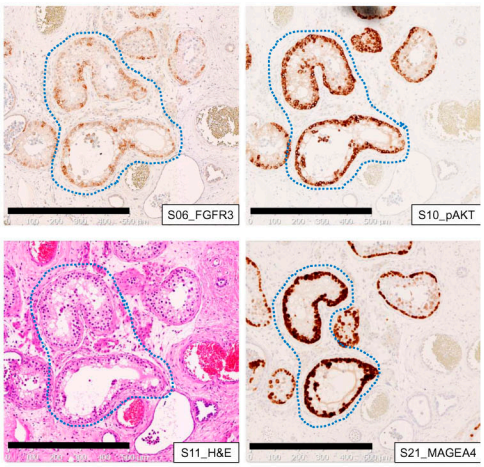


C

Testis 7-1
Tubule cluster 04

Johnsen score: 4

Johnsen score of surrounding normal tubules: 8

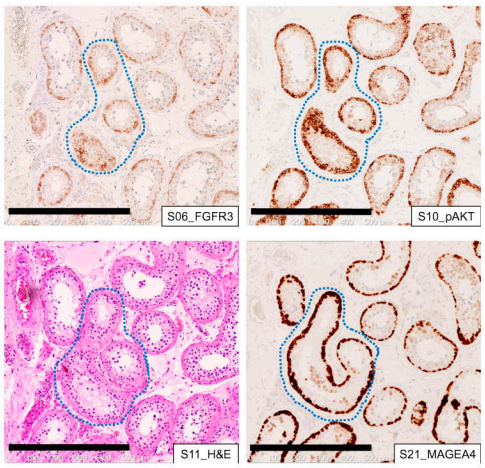


D

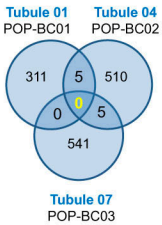
Testis 7-1
Tubule cluster 07

Johnsen score: 5

Johnsen score of surrounding normal tubules: 9



E



No exonic variant shared by all 3 immunopositive tubules

No candidate variant at hotspots in *FGFR2*, *FGFR3*, *HRAS*, *KRAS*, *PTPN11* or *RET*

Fig. S5. Analysis of Testis 7-1. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubules are indicated. Scale bar: 5 mm. (*B-D*) Detailed view of the staining appearance (FGFR3, pAKT, H&E and MAGEA4) and Johnsen scoring of tubule clusters 01 (*B*), 04 (*C*), and 07 (*D*). The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S06) and staining performed on each section are indicated. Scale bars: 500 µm. (*E*) Number of protein-altering (non-synonymous, frameshift or splice site) variants identified in tubule clusters 01, 04 and 07, after excluding SNPs and variants in control genomic DNA, shown in Venn diagram. No variants were shared by all three clusters and no candidate mutations from individual clusters were identified.

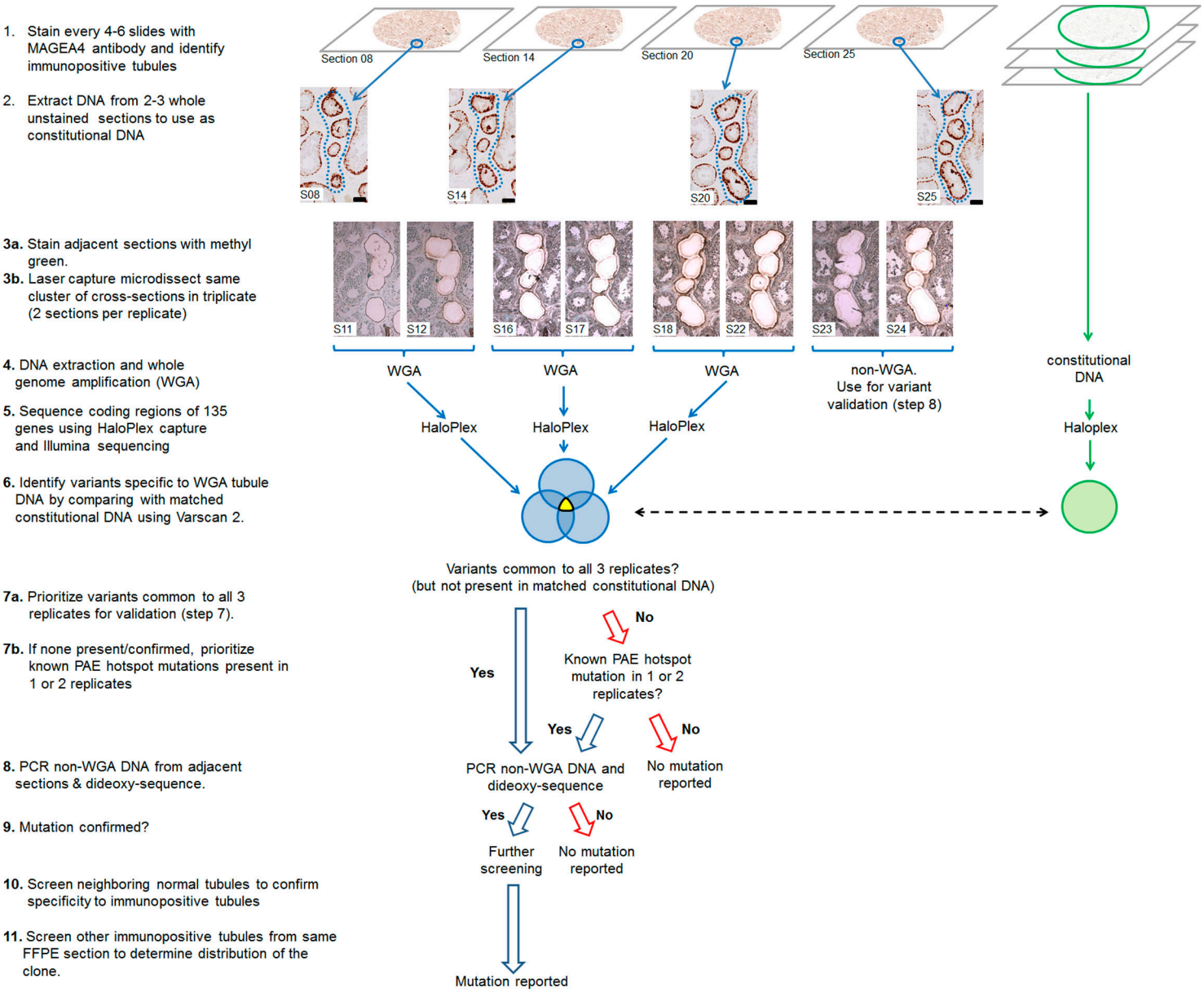


Fig. S6. Triplicate strategy to identify mutant clones in main study.

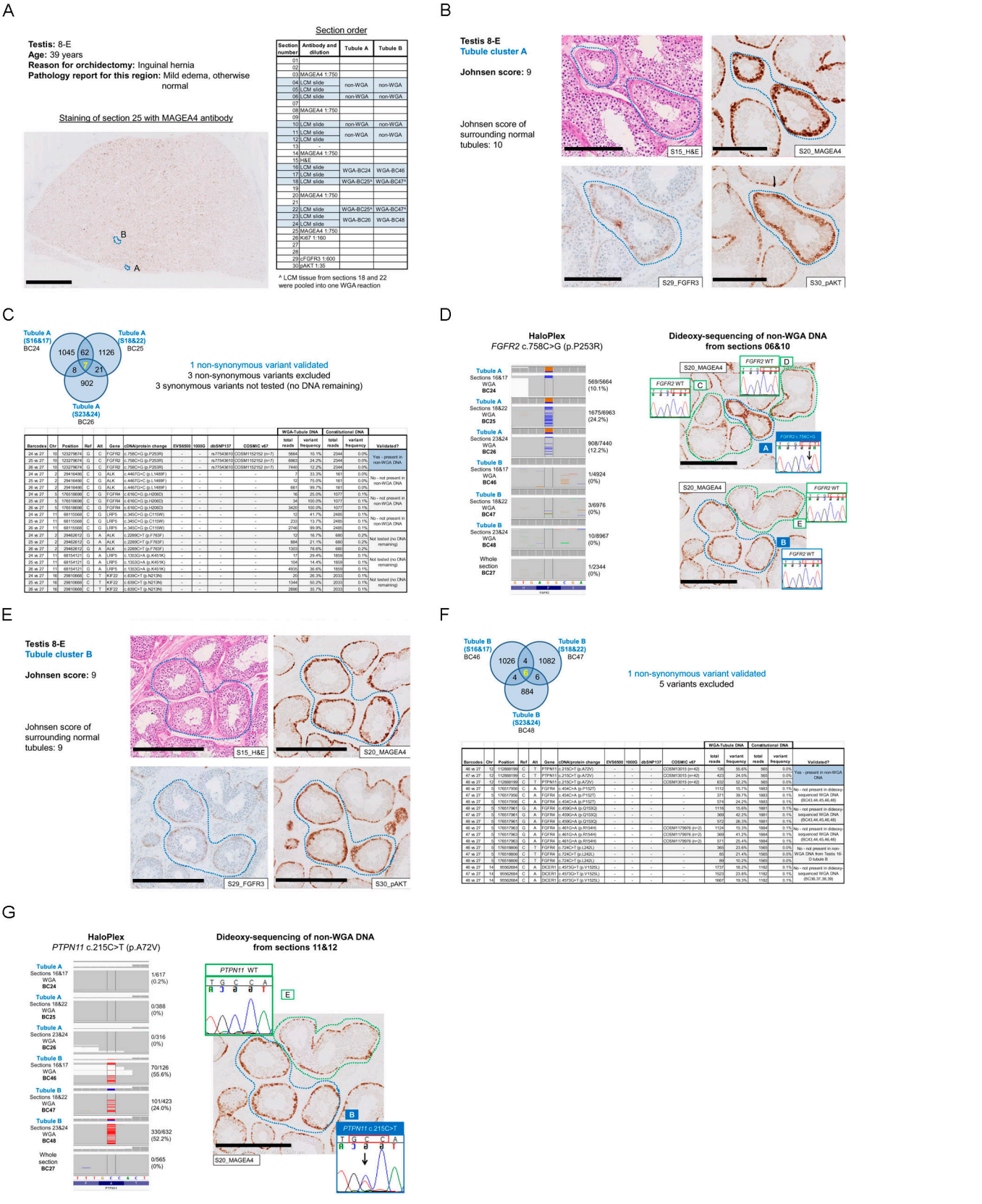
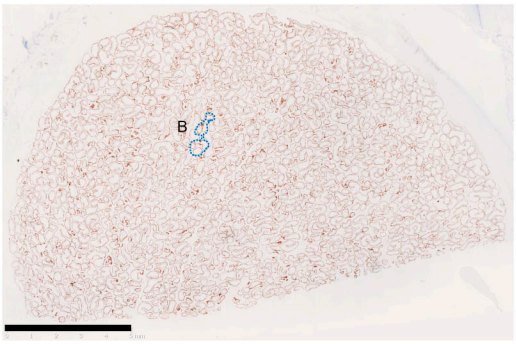


Fig. S7. Analysis of tubule clusters A and B in Testis 8-E. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubule clusters A and B are indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 250 µm. (*C*) Number of exonic single nucleotide variants (SNVs) and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Seven variants were common to each of the triplicates, only one of which was validated (blue box). Three variants were excluded as they were not detected in dideoxy-sequenced non-WGA DNA of the equivalent tubule from an adjacent section. Three synonymous variants were not tested due to lack of DNA. (*D*) Identification and validation of the heterozygous *FGFR2* c.758C>G (p.P253R) in tubule cluster A. The *FGFR2* c.758C>G mutation is present in the triplicate WGA DNA samples from tubule cluster A, but not in tubule cluster B or the whole section DNA (viewed in IGV). The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent tubule pooled from S06 and S10 (upper right; also illustrated in Fig. 1*C*). The mutation was not detected in the immunopositive tubule cluster B (lower right, blue surround) or tubules with normal MAGEA4-staining appearance (green surround). Scale bars: 500 µm. (*E*) Detailed view of the staining appearance (MAGEA4, FGFR3, pAKT and H&E) and Johnsen scoring of tubule cluster B. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Scale bars: 500 µm. (*F*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster B but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Six variants were common to each of the triplicates, only one of which was validated (blue box). Five variants were excluded as they were not detected in dideoxy-sequenced non-WGA DNA of the equivalent tubule from an adjacent section or were demonstrated to be alignment errors in other WGA samples. (*G*) Identification and validation of the heterozygous *PTPN11* c.215C>T (p.A72V) in tubule cluster B. The *PTPN11* c.215C>T mutation is present in the triplicate WGA DNA samples from tubule cluster B, but not in tubule cluster A or the whole section DNA. The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent immunopositive tubule (blue surround) pooled from S11 and S12. The mutation was not detected in neighboring tubules with normal MAGEA4-staining appearance (green surround). Scale bar: 500 µm.

A

Testis: 10-F
Age: 63 years
Reason for orchidectomy: Inguinal hernia
Pathology report for this region: Normal

Staining of section 12 with MAGEA4 antibody



Section order

Section number	Antibody and dilution	Tubule B
01	MAGEA4 1:750	
02	FGFR3 1:750	
03	NA	
04		
05	NA	
06	pAKT 1:45	
07	MAGEA4 1:750	
08	LCM slide	WGA-BC10
09	LCM slide	
10	LCM slide	WGA-BC11^
11	H&E	
12	MAGEA4 1:750	
13	pAKT 1:45	
14	LCM slide	WGA-BC11^
15	NA	
16	LCM slide	WGA-BC12^^
17	NA	
18	NA	
19	LCM slide	WGA-BC12^^
20		
21	MAGEA4 1:750	
22		
23		
24	NA	
25	NA	
26	NA	
27	NA	
28	Ki67 1:160	
29	MAGEA4 1:750	
30	LCM slide	
31	LCM slide	
32	NA	
33	NA	
34	NA	
35	NA	
36	MAGEA4 1:750	
37		
38	NA	
39	LCM slide	non-WGA
40	NA	
41	MAGEA4 1:750	

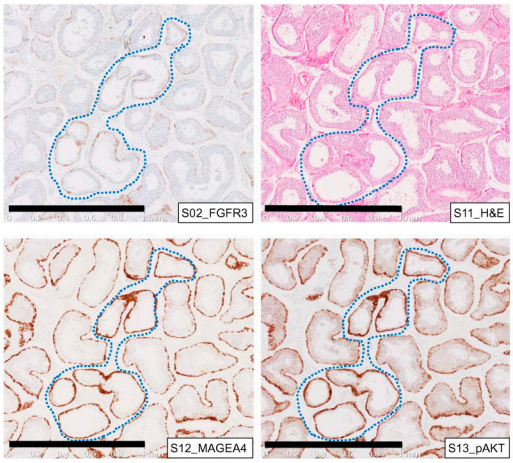
Tissue from sections 10 and 14 (^) were pooled into one WGA reaction.
Tissue from sections 16 and 19 (^^) were pooled into one WGA reaction.

B

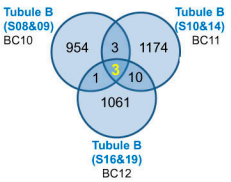
Testis 10-F
Tubule cluster B

Johnsen score: 9

Johnsen score of surrounding normal tubules: 10



C

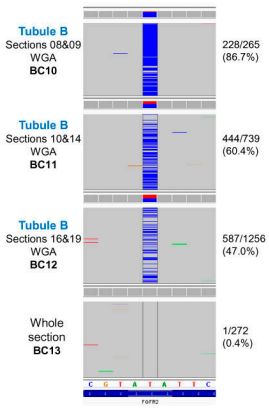


1 non-synonymous variant validated
2 variants excluded

											WGA-Tubule DNA		Constitutional DNA		
Barcodes	Chr	Position	Ref	Alt	Gene	cDNA/protein change	EVS6500	1000G	dbSNP137	COSMIC v67	total reads	variant frequency	total reads	variant frequency	Validated?
10 vs 13	10	123276898	T	C	FGFR2	c.1019A>G (p.Y340C)	-	-	-	-	265	86.7%	276	0.4%	Yes - present in non-WGA DNA
11 vs 13	10	123276898	T	C	FGFR2	c.1019A>G (p.Y340C)	-	-	-	-	739	60.4%	276	0.4%	
12 vs 13	10	123276898	T	C	FGFR2	c.1019A>G (p.Y340C)	-	-	-	-	1256	47.0%	276	0.4%	
10 vs 13	14	95593053	T	C	DICER1	c.767A>G (p.D256G)	-	-	-	-	3685	12.2%	874	0.2%	No - not present in dideoxy-sequenced WGA DNA (BC10,12)
11 vs 13	14	95593053	T	C	DICER1	c.767A>G (p.D256G)	-	-	-	-	5492	22.7%	874	0.2%	
12 vs 13	14	95593053	T	C	DICER1	c.767A>G (p.D256G)	-	-	-	-	6179	29.9%	874	0.2%	
10 vs 13	14	95593057	C	A	DICER1	c.763G>T (p.V255L)	-	-	-	-	3698	11.9%	874	0.2%	No - not present in dideoxy-sequenced WGA DNA (BC10,12)
11 vs 13	14	95593057	C	A	DICER1	c.763G>T (p.V255L)	-	-	-	-	5402	23.7%	874	0.2%	
12 vs 13	14	95593057	C	A	DICER1	c.763G>T (p.V255L)	-	-	-	-	6201	28.3%	874	0.2%	

D

HaloPlex
FGFR2 c.1019A>G (p.Y340C)



Dideoxy-sequencing of non-WGA DNA from section 39

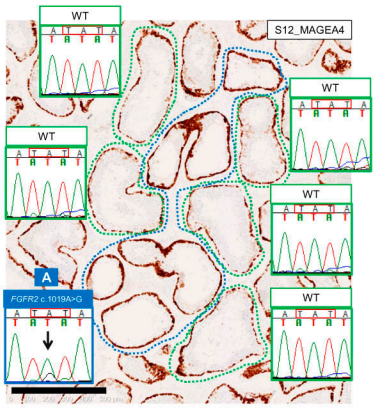


Fig. S8. Analysis of Testis 10-F. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster B is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (FGFR3, H&E, MAGEA4 and pAKT) and Johnsen scoring of tubule cluster B. The tubules surrounded by blue dotted lines are the immunopositive tubules that were micro-dissected and analyzed on adjacent sections. Section numbers (e.g. S02) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster B but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Three variants were common to each of the triplicates, only one of which was validated (blue box). Two variants were excluded as they were demonstrated to be alignment errors in other WGA samples. (*D*) Identification and validation of the heterozygous *FGFR2* c.1019A>G (p.Y340C) in tubule cluster B. The *FGFR2* c.1019A>G mutation is present in the triplicate WGA DNA samples from tubule cluster B, but not in the whole section DNA (viewed in IGV). The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent tubule from S39 (blue surround). The mutation was not detected in tubules with normal MAGEA4-staining appearance (green surround). Scale bar: 500 µm.

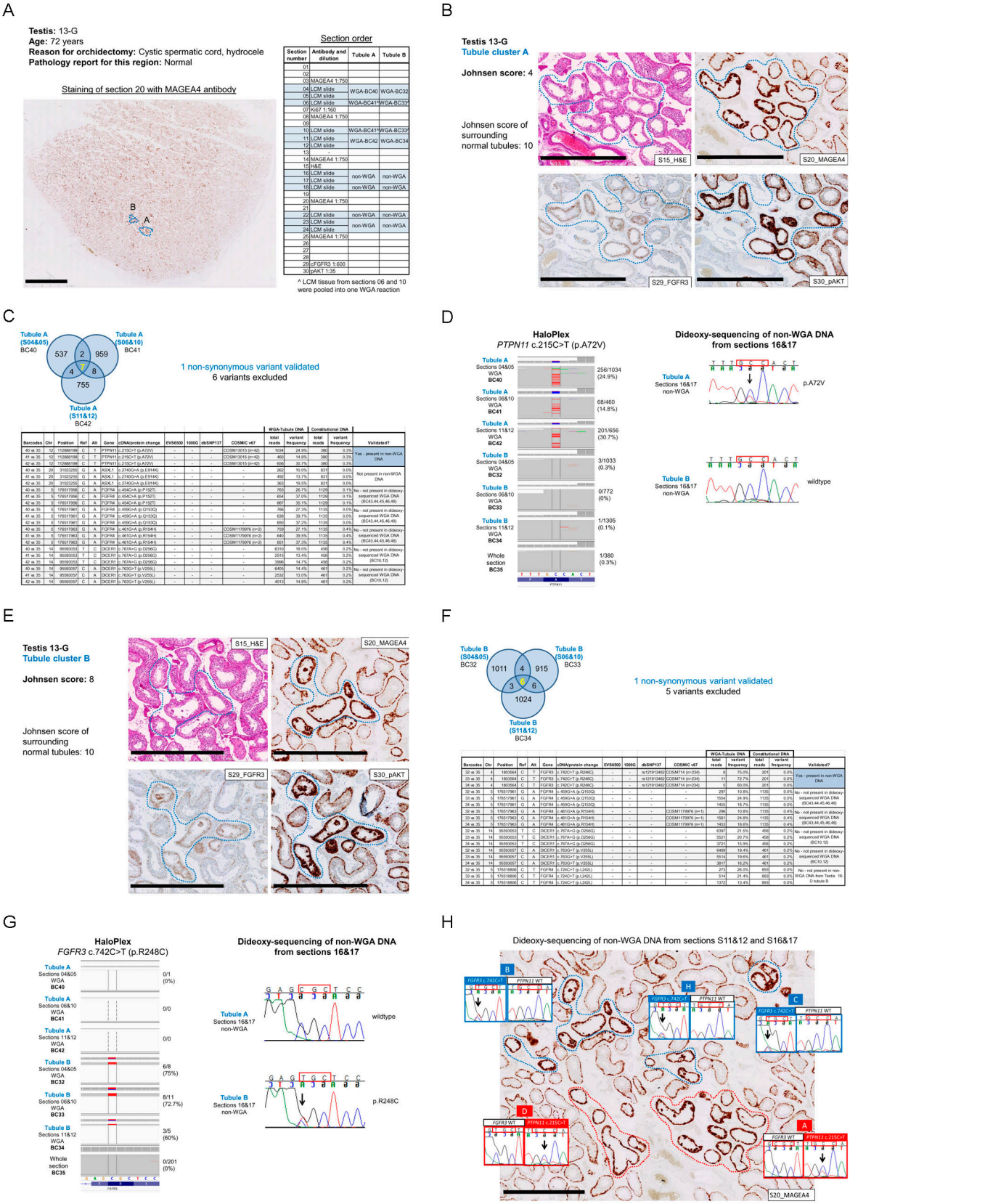


Fig. S9. Analysis of tubule clusters A and B in Testis 13-G. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubule clusters A and B are indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Seven variants were common to each of the triplicates, only one of which was validated (blue box). Six variants were excluded as they were not detected in dideoxy-sequenced non-WGA DNA of the equivalent tubule from an adjacent section or were demonstrated to be alignment errors in other WGA samples. (*D*) Identification and validation of the heterozygous *PTPN11* c.215C>T (p.A72V) in tubule cluster A. The *PTPN11* c.215C>T mutation is present in the triplicate WGA DNA samples from tubule cluster A, but not in tubule cluster B or the whole section DNA (viewed in IGV). The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent tubule pooled from S16 and S17 (upper right). The mutation was not detected in the immunopositive tubule cluster B (lower right). (*E*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster B. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Scale bars: 1 mm. (*F*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster B but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Six variants were common to each of the triplicates, only one of which was validated (blue box). Five variants were excluded as they were demonstrated to be alignment errors in other WGA samples. (*G*) Identification and validation of the heterozygous *FGFR3* c.742C>T (p.R248C) in tubule cluster B. The *FGFR3* c.742C>T mutation is present in the triplicate WGA DNA samples from tubule cluster B, but not in the whole section DNA. This region was not covered in the tubule cluster A triplicates. The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent immunopositive tubule pooled from sections S16 and S17 (lower right), but was not present in tubule cluster A (upper right). (*H*) Sequencing analysis of tubules with an immunopositive appearance in the vicinity of tubule clusters A and B performed on non-WGA material pooled from S11, S12, S16 and S17. Tubule clusters harboring the *PTPN11* c.215C>T (red surround) and *FGFR3* c.742C>T mutations (blue surround) are mutually exclusive suggesting independent mutational events in tubules that are in close proximity (also illustrated in Fig. 2*A*). Scale bar: 1 mm.

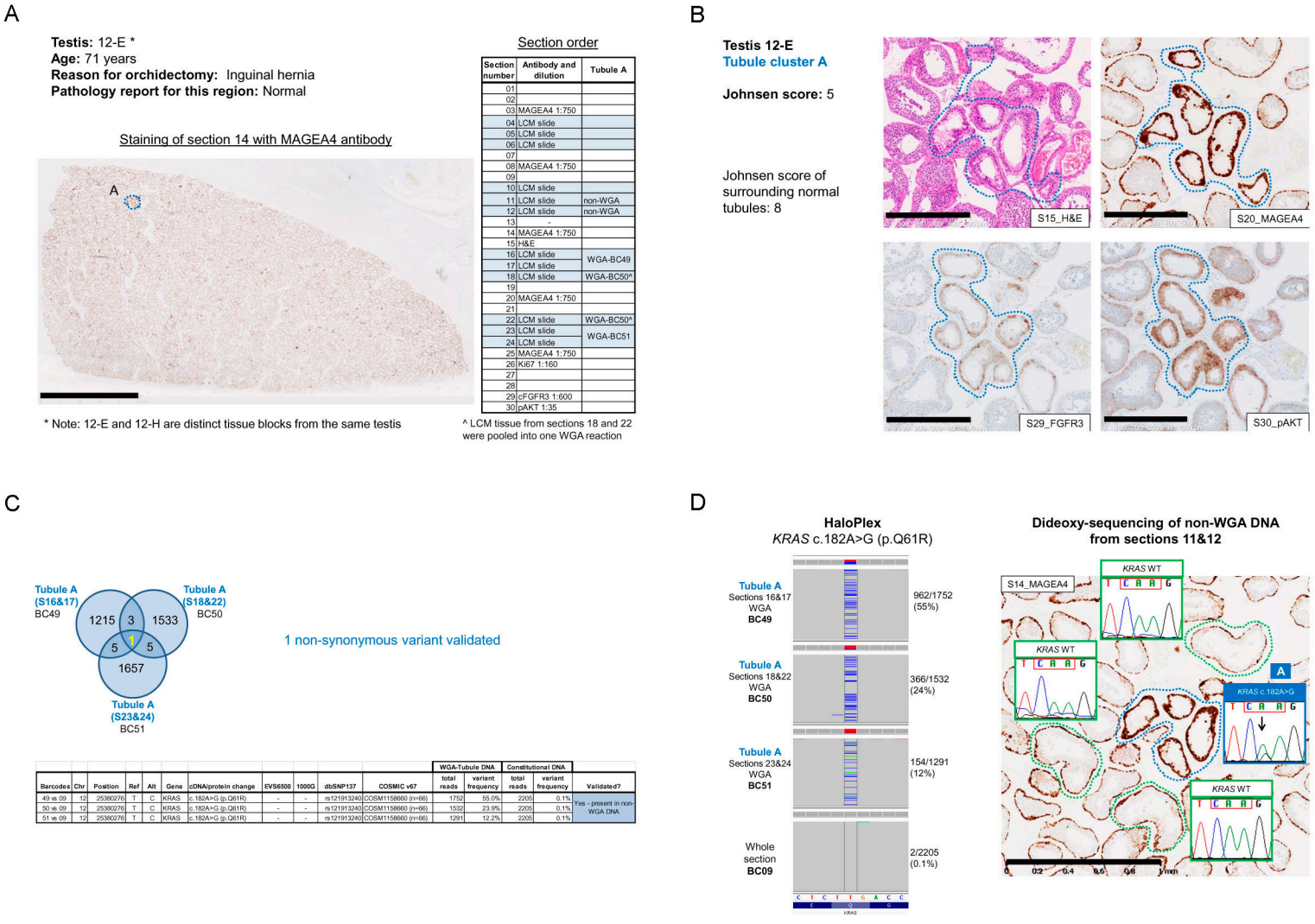


Fig. S10. Analysis of Testis 12-E. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 500 µm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Only one variant was common to each of the triplicates, and was subsequently validated (blue box). (*D*) Identification and validation of the heterozygous *KRAS* c.182A>G (p.Q61R) in tubule cluster A. The *KRAS* c.182A>G mutation is present in the triplicate WGA DNA samples from tubule cluster A, but not in the whole section DNA (viewed in IGV). The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent tubule from an adjacent section (blue surround). The mutation was not detected in tubules with normal MAGEA4-staining appearance (green surround). Scale bar: 1 mm.

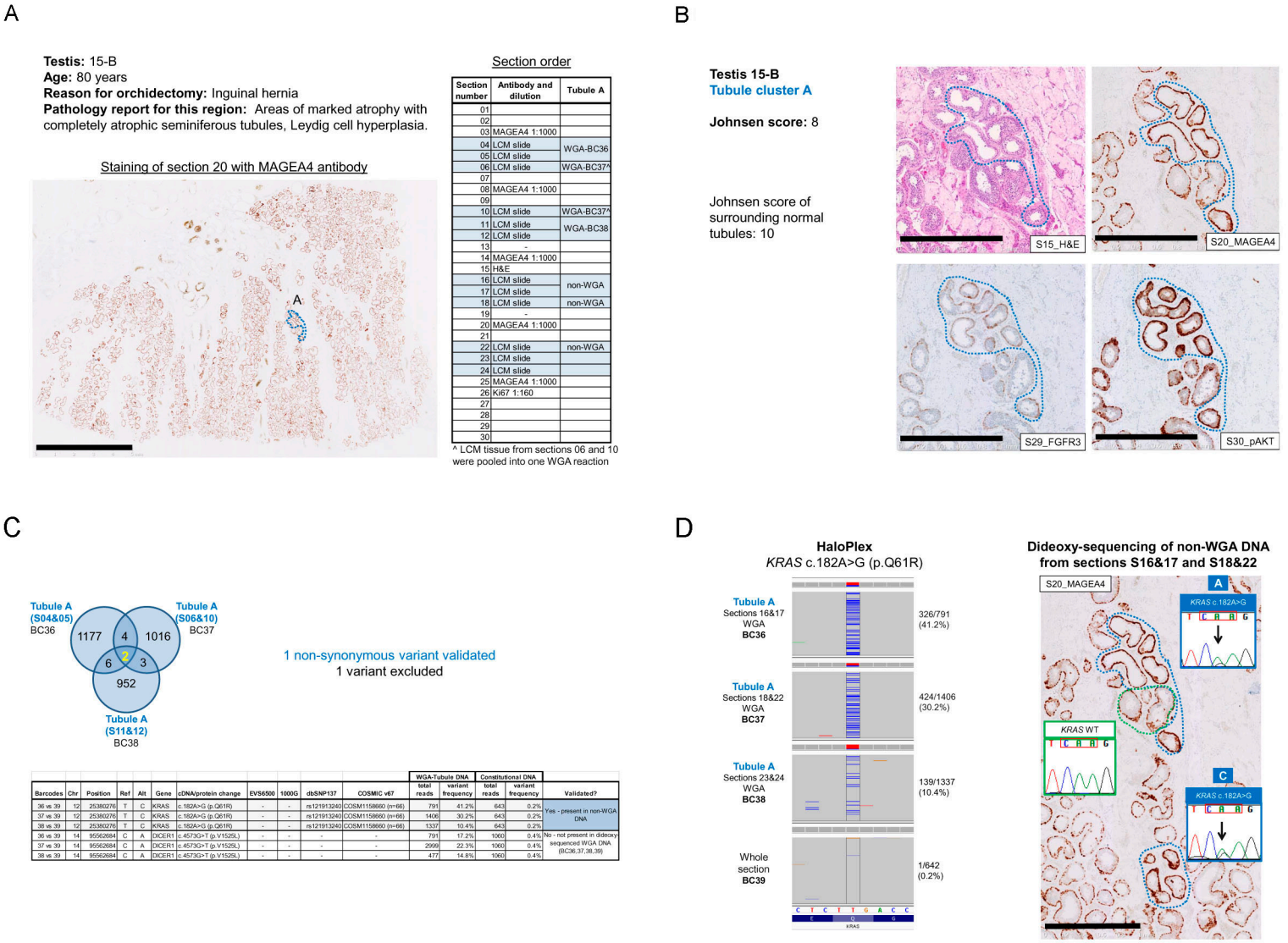


Fig S11. Analysis of Testis 15-B. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Two exonic variants were common to each of the triplicates; only one of which was subsequently validated (blue box). (*D*) Identification and validation of the heterozygous *KRAS* c.182A>G (p.Q61R) in tubule cluster A. The *KRAS* c.182A>G mutation is present in the triplicate WGA DNA samples from tubule cluster A, but not in the whole section DNA (viewed in IGV). The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent tubule pooled from S18 and S22. Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) MAGEA4 appearance close to tubule cluster A performed on non-WGA material pooled from S16, S17, S18 and S22. All immunopositive tubules (blue surround) in this region are heterozygous for the *KRAS* c.182A>G mutation. All analyzed tubules with a normal MAGEA4 appearance (green surround) are mutation-negative. Scale bar: 1 mm.

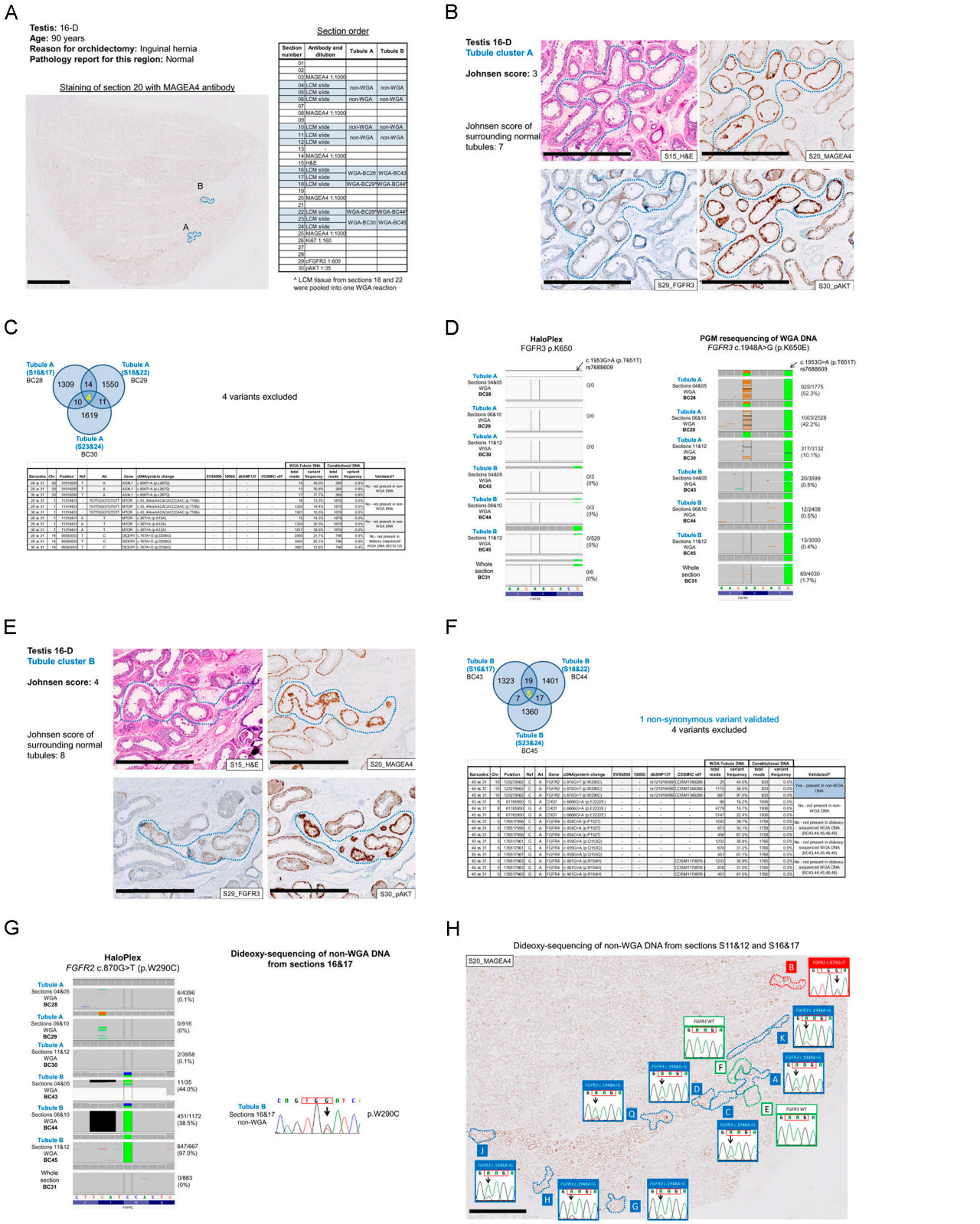


Fig. S12. Analysis of tubule clusters A and B in Testis 16-D. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The relative locations of the microdissected and HaloPlex-sequenced tubule clusters A and B are indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Four variants were common to each of the triplicates, all of which were excluded as they were not detected in dideoxy-sequenced non-WGA DNA of the equivalent tubule from an adjacent section or were demonstrated to be alignment errors in other WGA samples. (*D*) The mutation hotspot FGFR3 p.K650 was not covered by the HaloPlex experiment (left panel). PGM resequencing of PCR-amplified WGA DNA from the triplicate samples identified the *FGFR3* c.1948A>G (p.K650E) mutation in tubule cluster A (right panel). Note also that this individual is homozygous for the non-reference A allele of SNP rs7688609 at cDNA position 1953. HaloPlex and PGM sequencing reads were viewed in IGV. (*E*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster B. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Scale bars: 1 mm. (*F*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster B but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Five variants were common to each of the triplicates, only one of which was validated (blue box). Four variants were excluded as they were not detected in dideoxy-sequenced non-WGA DNA of the equivalent tubule from an adjacent section or were demonstrated to be alignment errors in other WGA samples. (*G*) Identification and validation of the heterozygous *FGFR2* c.870G>T (p.W290C) in tubule cluster B. The *FGFR2* c.870G>T mutation is present in the triplicate WGA DNA samples from tubule cluster B, but not in tubule cluster A or the whole section DNA. The mutation was validated by dideoxy-sequencing of non-WGA DNA of the equivalent immunopositive tubule pooled from nearby sections S16 and S17. (*H*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue or red surround) MAGEA4 appearance close to tubule clusters A and B performed on non-WGA pooled material from S11, S12, S16 and S17 (also shown in Fig. 2*B*). Whilst the *FGFR2* c.870G>T mutation (red surround) is limited in its distribution, the *FGFR3* c.1948A>G mutation (blue surround) extends over a large region of the tissue section (~4% of the total area of 440 mm2). Consistent with this, the *FGFR3* c.1948A>G mutation is detected at a low frequency (1.7%) in the whole section DNA (**d**, lower right). The *FGFR3* c.1948A>G mutation is not detected in neighboring tubules with normal MAGEA4-staining appearance (green surround). Scale bar: 2.5 mm.

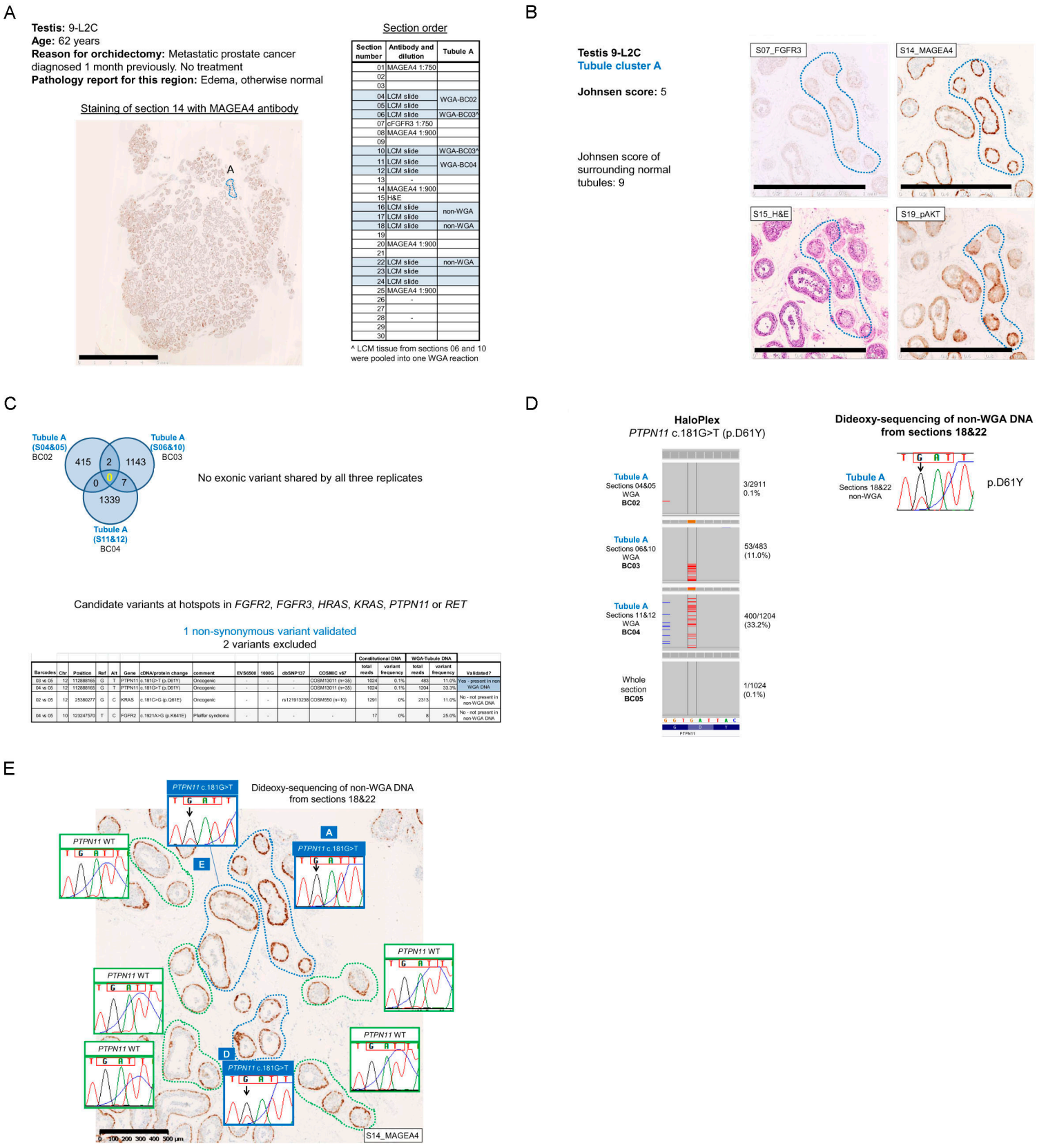
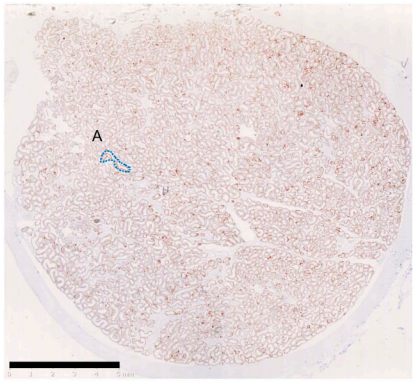


Fig. S13. Analysis of Testis 9-L2C. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (FGFR3, MAGEA4, H&E and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were micro-dissected and analyzed on adjacent sections. Section numbers (e.g. S07) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. No exonic variants were common to each of the triplicates. Three candidate variants at known mutational hotspots in *FGFR2*, *KRAS* and *PTPN11* present in one or two replicates were identified and subsequently screened using non-WGA DNA of the equivalent tubule from adjacent sections. One variant was validated (blue box). (*D*) A *PTPN11* c.181G>T (p.D61Y) mutation was identified by HaloPlex sequencing in two of the triplicate WGA DNA samples. This mutation was validated in the heterozygous state by dideoxy-sequencing of non-WGA DNA of the equivalent tubule cluster pooled from S18 and S22. (*E*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) MAGEA4 appearance close to tubule cluster A performed on non-WGA material pooled from S18 and S22. All immunopositive tubules (blue surround) in this region harbor the *PTPN11* c.181G>T mutation. All analyzed tubules with a normal MAGEA4 appearance (green surround) are mutation-negative. Scale bar: 500 µm.

A

Testis: 11-H
Age: 70
Reason for orchidectomy: None provided
Pathology report for this region: Normal

Staining of section 19 with anti-MAGEA4 antibody



Section order

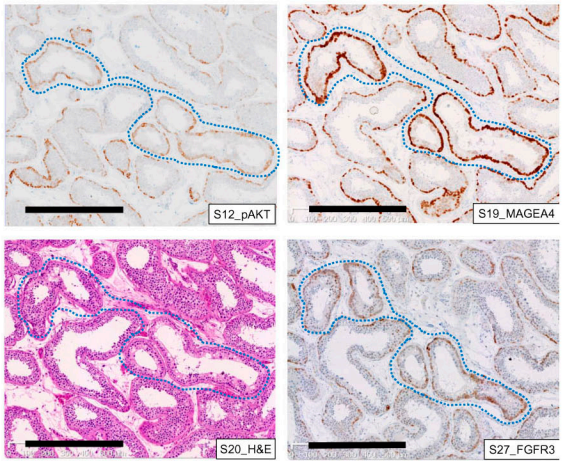
Section number	Antibody and dilution	Tubule A
01		
02	cFGFR3 1:750	
03	MAGEA4 1:750	
04	LCM slide	
05	LCM slide	
06	LCM slide	
07	MAGEA4 1:750	
08		
09	LCM slide	
10	LCM slide	
11	LCM slide	non-WGA
12	pAKT 1:45	
13	MAGEA4 1:750	
14	-	
15	LCM slide	non-WGA
16	LCM slide	WGA-BC14
17	LCM slide	
18		
19	MAGEA4 1:750	
20	H&E	
21	LCM slide	WGA-BC15
22	LCM slide	
23	LCM slide	WGA-BC16
24	LCM slide	
25	MAGEA4 1:750	
26	KI67 1:160	
27		
28		
29		
30	MAGEA4 1:750	

B

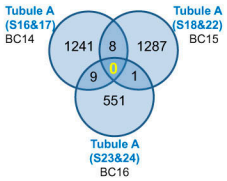
Testis 11-H
Tubule cluster A

Johnsen score: 6

Johnsen score of surrounding normal tubules: 8



C



No exonic variant shared by all three replicates

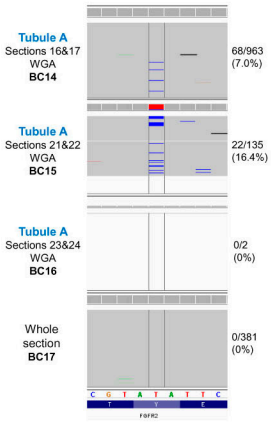
Candidate variants at hotspots in *FGFR2*, *FGFR3*, *HRAS*, *KRAS*, *PTPN11* or *RET*

1 non-synonymous variant validated
1 variant excluded

Barcodes	Chr	Position	Ref	Alt	Gene	cDNA/protein change	comment	EVS6500	1000G	dbSNP137	COSMIC v67	Constitutional DNA total reads	Constitutional DNA variant frequency	WGA-Tubule DNA total reads	WGA-Tubule DNA variant frequency	Validated?
15 vs 17	10	123276898	T	C	FGFR2	c.1019A>G (p.Y340C)	Pfeiffer syndrome	-	-	-	-	381	0%	135	16.4%	Yes - present in non WGA DNA
16 vs 17	12	25380277	G	C	KRAS	c.181C>G (p.Q61E)	Oncogenic	-	-	rs121913238	COSM550 (n=10)	849	0.1%	228	93.4%	No - not present in non-WGA DNA

D

Haloplex
FGFR2 c.1019A>G (p.Y340C)



Dideoxy-sequencing of non-WGA DNA from sections 11&15

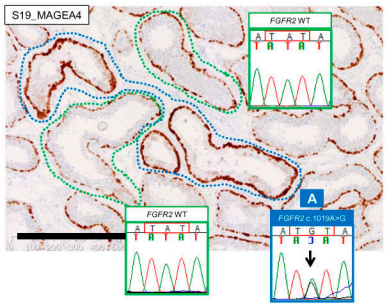


Fig. S14. Analysis of Testis 11-H. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (pAKT , MAGEA4, H&E and FGFR3) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S12) and staining performed on each section are indicated. Scale bars: 500 µm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. No exonic variants were common to each of the triplicates. Two candidate variants at known mutational hotspots in *FGFR2* and *KRAS* present in one or two replicates were identified and subsequently screened using non-WGA DNA of the equivalent tubule from an adjacent section. One variant was validated (blue box). (*D*) A *FGFR2* c.1019A>G (p.Y340C) mutation was identified by HaloPlex sequencing in one of the triplicate WGA DNA samples (viewed in IGV). The variant was present below the 10% calling threshold in BC14 and the region was poorly covered in BC16. The mutation was validated in the heterozygous state by dideoxy-sequencing of non-WGA DNA of the equivalent tubule cluster (blue surround) pooled from S11 and S15. All analyzed tubules with a normal MAGEA4 appearance (green surround) were mutation-negative. Scale bar: 500 µm.

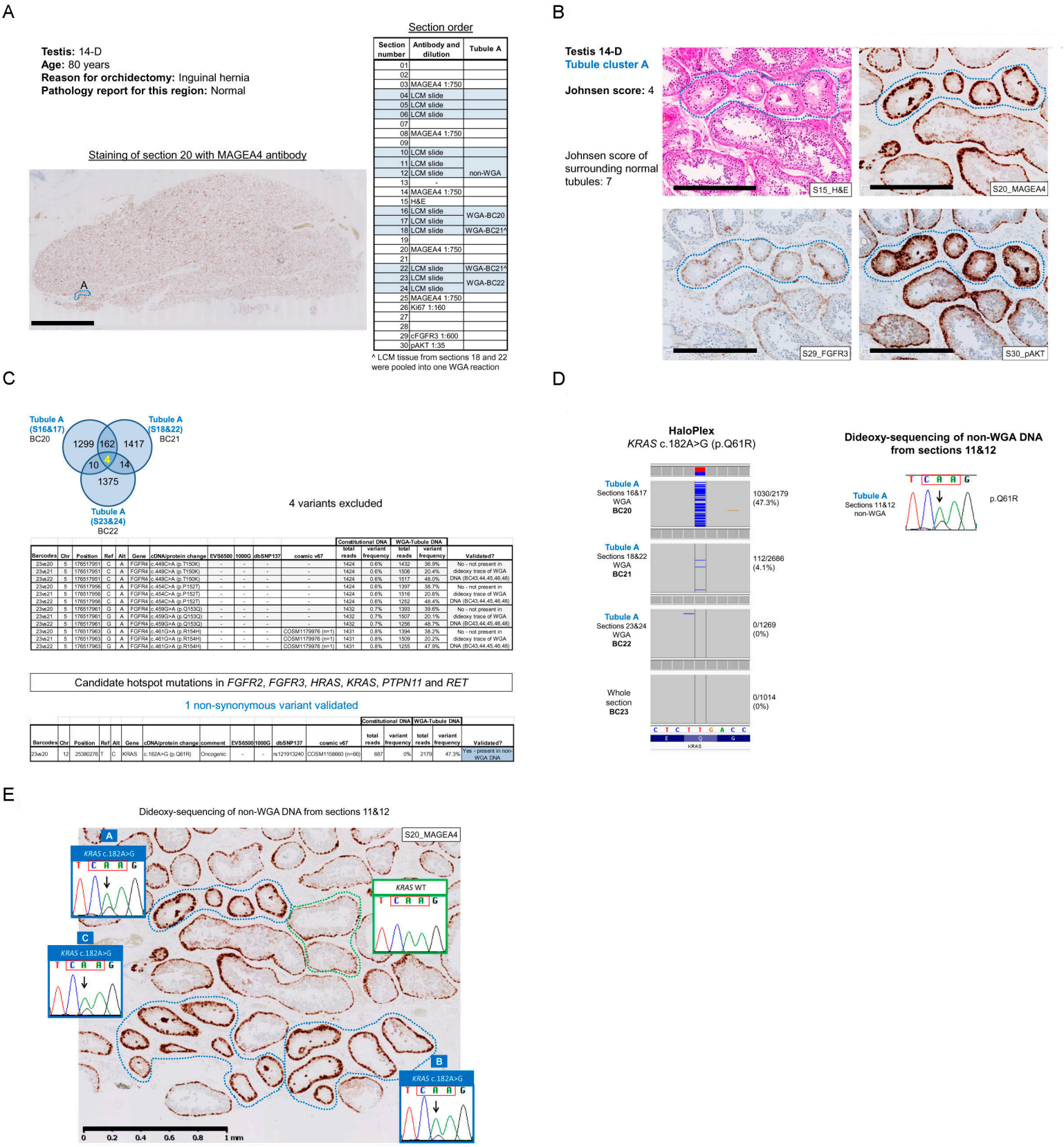
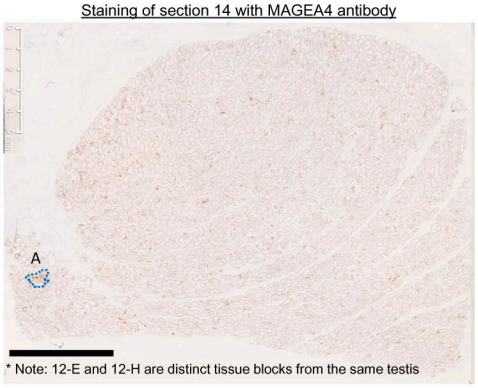


Fig. 15. Analysis of Testis 14-D. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 500 µm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. Four exonic variants were common to each of the triplicates but all four were demonstrated to be alignment errors in other WGA samples. A single candidate variant at a known mutational hotspot in *KRAS* present in one of the replicates was identified and subsequently validated (blue box). (*D*) A *KRAS* c.182A>G (p.Q61R) mutation was identified by HaloPlex sequencing in one of the triplicate WGA DNA samples (BC20) (viewed in IGV). The variant was present below the 10% calling threshold in BC21 and was not present in BC22. This mutation was validated in the heterozygous state by dideoxy-sequencing of non-WGA DNA of the equivalent tubule cluster pooled from S11 and S12. (*E*) Further dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) MAGEA4 appearance close to tubule cluster A performed on non-WGA material pooled from S11 and S12. All immunopositive tubules (blue surround) in this region harbor the *KRAS* c.182A>G mutation. All analyzed tubules with a normal MAGEA4 appearance (green surround) were mutation-negative. Scale bar: 1 mm.

A

Testis: 12-H *
Age: 71 years
Reason for orchidectomy: Inguinal hernia
Pathology report for this region: Focal Leydig cell hyperplasia

Staining of section 14 with MAGEA4 antibody



* Note: 12-E and 12-H are distinct tissue blocks from the same testis

Section order

Section number	Antibody and dilution	Tubule A
01		
02		
03	MAGEA4 1:500	
04	LCM slide	
05	LCM slide	
06	LCM slide	
07		
08	MAGEA4 1:750	
09		
10	LCM slide	WGA-BC06
11	LCM slide	
12	LCM slide	WGA-BC07^
13	-	
14	MAGEA4 1:750	
15	H&E	
16	LCM slide	WGA-BC07^
17	LCM slide	WGA-BC08
18	LCM slide	
19		
20	MAGEA4 1:750	
21		
22	LCM slide	non-WGA
23	LCM slide	non-WGA
24	LCM slide	
25	MAGEA4 1:750	
26	Ki67 1:160	
27		
28		
29	cFGFR3 1:750	
30	pAKT 1:30	

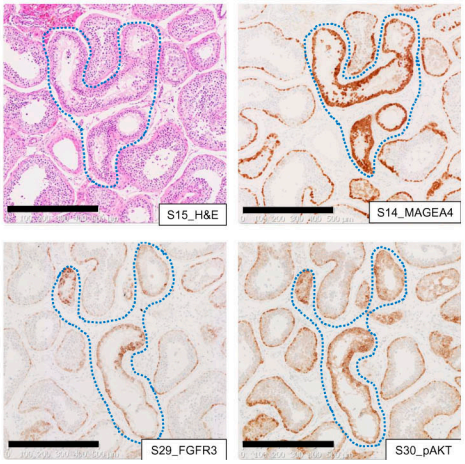
^ LCM tissue from sections 12 and 16 were pooled into one WGA reaction

B

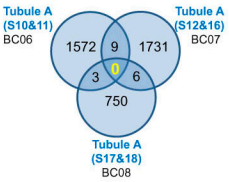
Testis 12-H
Tubule cluster A

Johnsen score: 7

Johnsen score of surrounding normal tubules: 10



C



No exonic variant shared by all three replicates

Candidate variants at hotspots in *FGFR2*, *FGFR3*, *HRAS*, *KRAS*, *PTPN11* or *RET*

1 variant excluded

												Constitutional DNA		WGA-Tubule DNA		
Barcodes	Chr	Position	Ref	Alt	Gene	cDNA/protein change	comment	EVS6500	1000G	dbSNP137	COSMIC v67	total reads	variant frequency	total reads	variant frequency	Validated?
08 vs 09	10	123274768	C	T	FGFR2	c.1150G>A (p.G384R)	Craniosynostosis	-	-	-	-	2458	0.0%	5809	67.9%	No - not present in non-WGA DNA

Fig. S16. Analysis of Testis 12-H. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. The location of the microdissected and HaloPlex-sequenced tubule cluster A is indicated. Scale bar: 5 mm. (*B*) Detailed view of the staining appearance (H&E, MAGEA4, FGFR3 and pAKT) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S15) and staining performed on each section are indicated. Scale bars: 500 µm. (*C*) Number of exonic SNVs and indels identified in the triplicate samples for tubule cluster A but not present in the matched whole section (constitutional) DNA shown in Venn diagram. No exonic variants were common to each of the triplicates. One candidate variant at a known mutational hotspot in *FGFR2* present in a single replicate was identified, but was excluded as it was not present in non-WGA DNA of the equivalent tubule pooled from S22 and S23.

A

Johnsen scoring system (main text ref [39])

10 = Full spermatogenesis
9 = Many late spermatids, disorganised epithelium
8 = Few late spermatids
7 = No late spermatids, many early spermatids
6 = No late spermatids, few early spermatids
5 = No spermatids, many spermatocytes
4 = No spermatids, few spermatocytes
3 = Spermatogonia only
2 = No germinal cells, Sertoli cells only
1 = No seminiferous epithelium

B

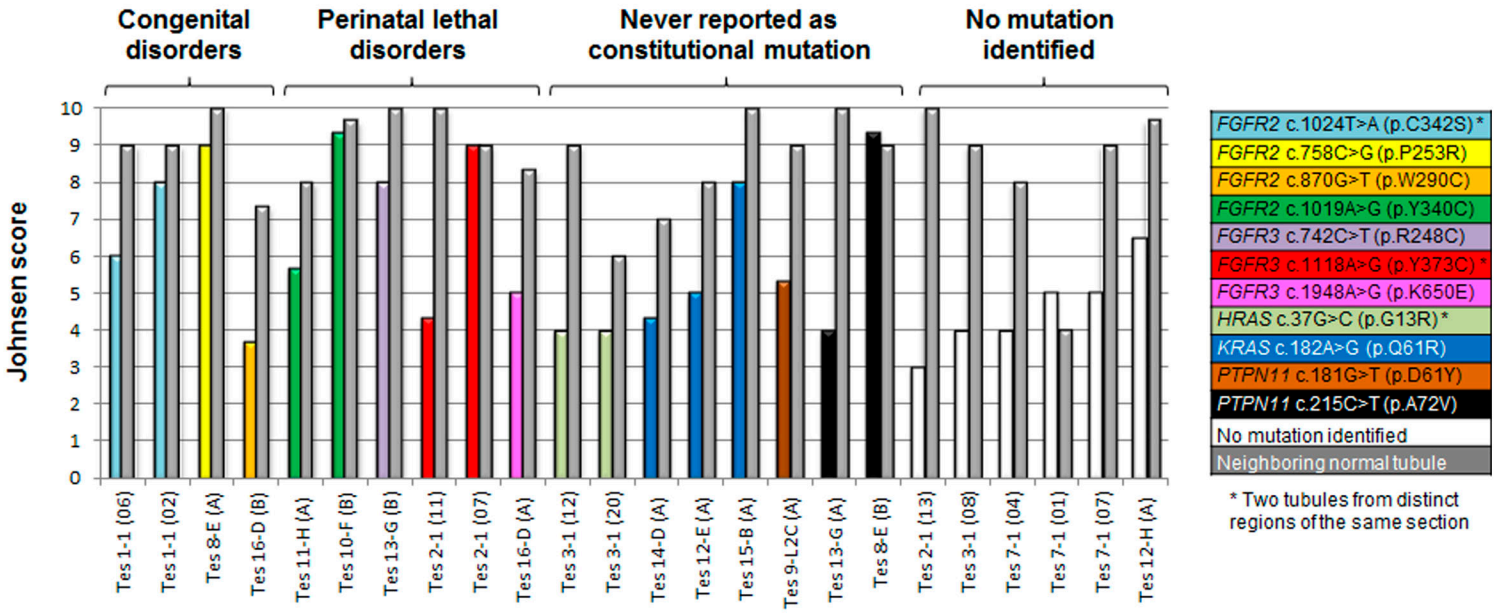


Fig. S17. Analysis of spermatogenesis in immunopositive and neighbouring normal tubules. (*A*) Details of the Johnsen scoring system for analysis of spermatogenesis. (*B*) Johnsen scores of immunopositive tubules (coloured bars as indicated) and neighbouring normal tubules (grey bars). Pairwise analysis of the data in (*B*) is presented in Fig. 3.

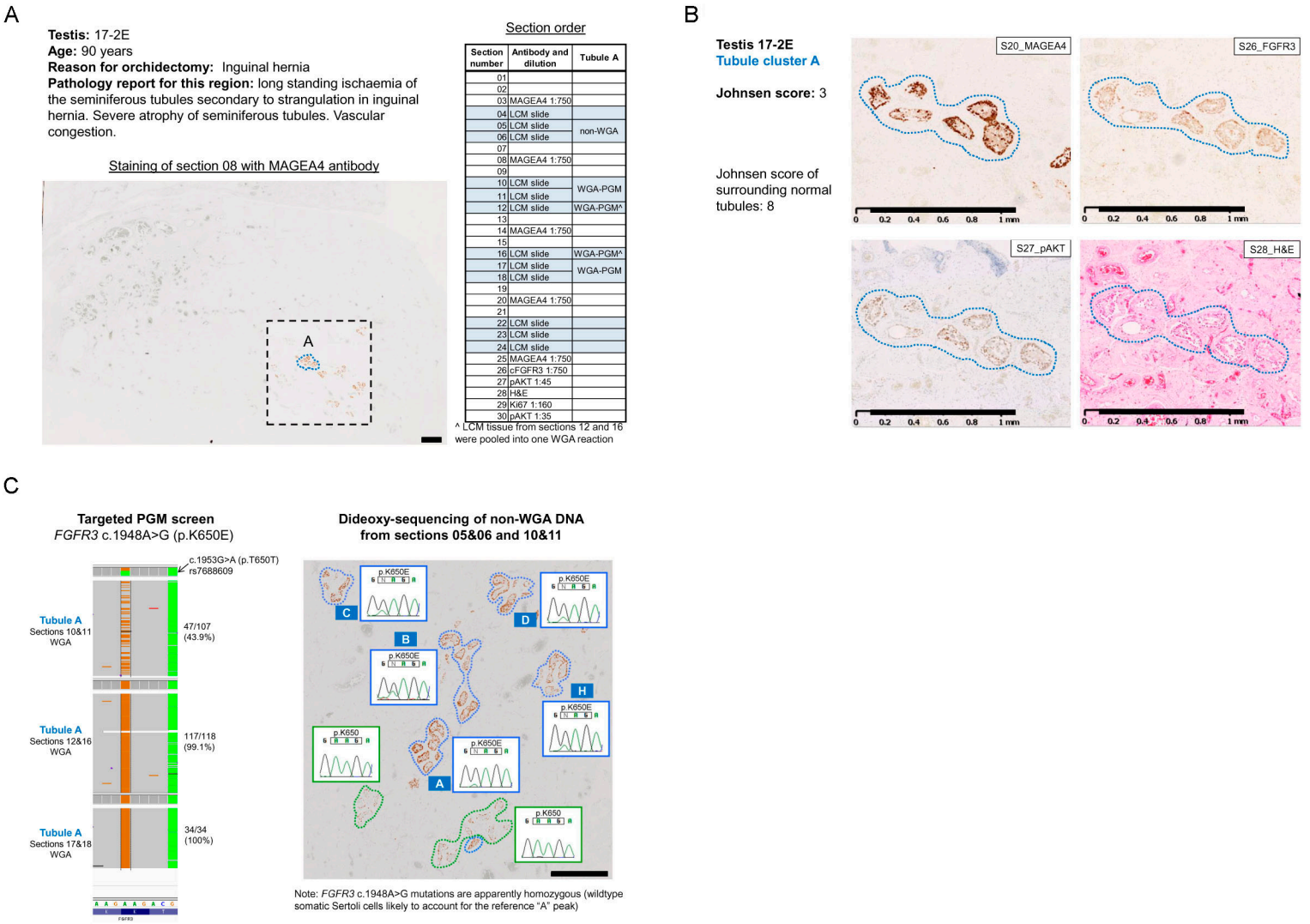


Fig. S18. Analysis of Testis 17-2E. (*A*) Low magnification view of the MAGEA4-stained sample, with pathology, serial section order and immunostaining details. Tubules with spermatogonia (identified by brown stain) are rare and are only present in the outlined box. Scale bar: 1 mm. (*B*) Detailed view of the staining appearance (MAGEA4, FGFR3, pAKT and H&E) and Johnsen scoring of tubule cluster A. The tubules surrounded by blue dotted lines are the immunopositive tubules that were microdissected and analyzed on adjacent sections. Section numbers (e.g. S20) and staining performed on each section are indicated. Scale bars: 1 mm. (*C*,left panel) Targeted PGM resequencing of PCR-amplified WGA DNA from the triplicate samples identified the *FGFR3* c.1948A>G (p.K650E) mutation in tubule cluster A from all triplicates. This individual is also homozygous for the non-reference A allele for SNP rs7688609 at cDNA position 1953. (**c**, right panel) Dideoxy-sequencing analysis of tubules with normal (green surround) or immunopositive (blue surround) MAGEA4 appearance close to tubule cluster A performed on non-WGA material pooled from S05, 06, S10 and S11. All of the strongly-immunopositive tubules (blue surround) in these regions are apparently homozygous for the *FGFR3* c.1948A>G mutation (wildtype somatic Sertoli cells within the tubule likely account for the minority reference "A" peak).The *FGFR3* c.1948A>G mutation was not detected in the few neighboring tubules with normal MAGEA4-staining appearance (green surround). Scale bar: 1 mm. Images in (*A*) and (*C*) are also shown in Fig. 4.