Defects in efferent duct multiciliogenesis underlie male infertility in GEMC1-, MCIDAS- or CCNO-deficient mice

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Summary statement

We demonstrate that male mice lacking GEMC1, MCIDAS or CCNO are infertile due to defects in the multiciliated cells of the efferent ducts of the epididymis.

Abstract

GEMC1 and MCIDAS are Geminin family proteins that transcriptionally activate E2F4/5-target genes during multiciliogenesis, including *FoxJ1* and *Ccno*. Male mice lacking *Gemc1, Mcidas* or *Ccno* were found to be infertile, but the origin of this defect has remained unclear. Here we show that all three genes are necessary for the generation of functional multiciliated cells in the efferent ducts that are required for spermatozoa to enter the epididymis. In mice mutant for *Gemc1, Mcidas* or *Ccno,* we observed a similar spectrum of phenotypes, including thinning of the seminiferous tubule epithelia, dilation of the rete testes, sperm agglutinations in the efferent ducts and lack of spermatozoa in the epididymis (azoospermia). These data suggest that defective efferent duct development is the dominant cause of male infertility in these mouse models and this likely extends to patients with the ciliopathy Reduced Generation of Multiple Motile Cilia with mutations in *MCIDAS* and *CCNO*.

Introduction

Spermatogenesis is a highly regulated developmental process that generates haploid sperm. To become capable of fertilization, spermatazoa must detach from the seminiferous epithelium, enter the tubule lumen and travel through the epididymis that promotes maturation and motility. The efferent ducts (ED) connect the rete testes to the epididymis and are important for sperm concentration, reabsorbing most of the luminal seminiferous fluid (Clulow et al., 1998). The ED epithelia contains poorly characterized multiciliated cells (MCCs) that mobilize luminal fluids through the action of hundreds of motile cilia on the apical surface of these cells (Joseph et al., 2011). The luminal turbulence generated by the MCCs of the ED has been proposed to prevent the agglutination of spermatozoa and promote fluid reabsorption by non-MCCs in the ED (Yuan et al., 2019).

Differentiation of airway MCCs is initiated in part through the action of the *Mir449/34* family of miRNAs that downregulate numerous genes, including *Cp110*, an inhibitor of cilia assembly, as well as *Notch* and *Dll1*, that together inhibit MCC differentiation (Kyrousi et al., 2015; Lafkas et al., 2015; Marcet et al., 2011; Song et al., 2014; Tsao et al., 2009; Zhou et al., 2015). This is followed by the activation of a transcriptional program by the Geminin family members GEMC1 (*GMNC*) and MCIDAS (*MCIDAS*) that interact with E2F4/5-DP1 and are required for the generation of MCCs in fish, frogs and mammals (Arbi et al., 2016; Balestrini et al., 2010; Boon et al., 2014; Chong et al., 2018; Danielian et al., 2007; Kyrousi et al., 2015; Lu et al., 2019; Ma et al., 2014; Stubbs et al., 2012; Terre et al., 2016; Zhou et al., 2015). In addition, the transcription factors MYB (Pan et al., 2014; Tan et al., 2013), FOXJ1 (You et al., 2004; Yu et al., 2008), RFX2/3 (Chung et al., 2012; Didon et al., 2013; El Zein et al., 2009) and TAp73 (transcriptionally active isoform encoded by the *Trp73* gene) (Marshall et al., 2016; Nemajerova et al., 2016), as well as the atypical cyclin, CCNO (Funk et al., 2015; Nunez-Olle et al., 2017; Wallmeier et al., 2014), are necessary to promote deuterosome-mediated centriole amplification and the generation of multiple motile cilia. Mutations in *MCIDAS* or *CCNO* underlie Reduced Generation of Multiple Motile Cilia (RGMC), a rare

ciliopathy characterized by hydrocephalus, mucus accumulation in the respiratory system and reduced fertility, all presumably due to defects in MCC differentiation (Amirav et al., 2016; Boon et al., 2014; Funk et al., 2015; Wallmeier et al., 2014).

Male and female infertility occurs in a number of mice mutant for genes involved in MCC development, including *Ccno*, the *miR-34b/c and miR-449a/b/c (miR-dKO)* loci, *TAp73*, *E2f4 and 5, Mcidas* and *Gemc1*. Where this has been addressed in females, it appears to be due to the loss of MCCs in the oviducts (Chen et al., 1998; Lu et al., 2019; Marshall et al., 2016; Nunez-Olle et al., 2017; Terre et al., 2016; Wu et al., 2014). However, in males, the origin of the defect has not been clearly established in all cases. Both *miR-34/449* and *TAp73* are expressed in the testes, and *miR-dKO* mice are impaired in meiosis and spermiogenesis and exhibit a “nearly empty” seminiferous tubule phenotype (Comazzetto et al., 2014; Holembowski et al., 2014; Wu et al., 2014; Yuan et al., 2015). *TAp73* deficient mice showed a similar seminiferous tubule phenotype (Tomasini et al., 2008), as well as degeneration of the Sertoli cells (SCs) that support spermatid development and ensure integrity of the blood-testes barrier (Holembowski et al., 2014). However, a conditional knockout of 3 out of 4 *E2f4* and *E2f5* alleles in the EDs, but not spermatogonia or spermatocytes, as well as the *FoxJ1*-Cre (MCC specific) mediated *miR-dKO* deletion, phenocopied the seminiferous tubule phenotype(Danielian et al., 2016; Yuan et al., 2019). This indicates that ED defects, particularly in the MCC population, are likely to be sufficient to cause testicular atrophy and male infertility

Here, we show that male mice lacking *Gemc1, Mcidas* or *Ccno* exhibited a testes phenotype similar to *miR-dKO*, *TAp73* deletion or loss of multiple *E2f4* and *E2f5* alleles (Comazzetto et al., 2014; Danielian et al., 2016; Holembowski et al., 2014; Inoue et al., 2014; Terre et al., 2016; Wu et al., 2014; Yuan et al., 2015). We found that *Gemc1*, *Mcidas*, or *Ccno* deficient mice exhibited luminal dilation of the seminiferous tubules, atrophy of the germinal epithelium, rete testes dilation, SC degeneration and spermatozoa failed to enter the epididymis, instead accumulating in the EDs. In each case,

defects in MCC maturation were clearly evident. Moreover, we show that similar to *FoxJ1*, *Trp73* expression is high in the EDs and is dependent on GEMC1 but not MCIDAS or CCNO, further establishing distinct temporal roles of these factors. Our results demonstrate that GEMC1, MCIDAS and CCNO are required for ED MCC differentiation and this further underscores that these defects are likely to be the primary cause of male infertility in several mouse lines with MCC defects, and potentially in human RGMC patients with mutations in *MCIDAS* or *CCNO*.

Results and Discussion

GEMC1 loss impairs the late stages of spermatogenesis

We analyzed adult testes of *Gemc1*$^{-/-}$ mice over the first three months and found no consistent changes in size and weight compared with wild type (*Wt*) or *Gemc1*$^{+/-}$ littermates when normalized to body size (Fig. 1A). Histological evaluation during the first semi-synchronous wave of spermatogenesis revealed no overt differences between *Wt, Gemc1*$^{+/-}$ or *Gemc1*$^{-/-}$ testes during the first 20 days post partum (p0-p20) (Fig. 1B, 1C). However, by p27-p35, the thinning of the seminiferous germinal epithelia became obvious, corresponding to the first appearance of elongating spermatids (ES) (Fig. 1B, 1D). Despite the reduction in cellularity, numbers of mitotic cells, dead cells, meiotic progression and levels of hormonal gene expression were normal in *Gemc1*$^{-/-}$ mice (Fig. S1A-E).

Consistent with histological observations, *Gemc1* mRNA expression peaked around p27, although peak levels were considerably lower than in the trachea that contains a large number of MCCs (Figs. 1E, 1F). As the peak of *Gemc1* expression and appearance of seminiferous tubule dilation correlated with late stages of spermatogenesis (Fig. 1E), we isolated and quantified enriched populations of testicular cell types (leptotene-zygotene (LZ), pachytene-diplotene (PD), round spermatids (RS) and elongating spermatids (ES)) by fluorescence activated cell sorting (FACS) (Fig. S1F). *Gemc1* mRNA was enriched in RS and ES populations compared to the germ cell pellet (Fig.

1G) and a significant reduction in RS and ES populations was observed in testes from *Gemc1*$^{-/-}$ mice (Fig. 1H), compared to similar numbers of prophase cells (LZ and PD). This suggested that GEMC1 may support late stages of spermatogenesis through the control of transcription, but the prominent role of GEMC1 in MCC differentiation and the phenotypic similarity to *miR-dKO* mice prompted us to consider that these effects may be secondary to defects in MCC function in the EDs(Comazzetto et al., 2014; Yuan et al., 2019).

Hypocellularity and dilation of the seminiferous tubules and rete testes

In conditional *E2f4*$^{-/-}$ *E2f5*$^{+/-}$ or *miR-dKO* mice generated with Cre transgenes active in the EDs of the epididymis and not the testes, defects in MCC formation in the EDs and signs of fluid backpressure, namely seminiferous tubule and rete testes dilation, have been described (Danielian et al., 2016; Yuan et al., 2019). We examined the transcriptional activation of GEMC1 target genes in the testes and in contrast to tissues containing MCCs, we did not observe any significant alterations in *Ccno, Mcidas*, *FoxJ1, Trp73 and Cdc20b* in the absence of GEMC1 (Fig. 2A) This was further confirmed at the protein level for TP73 (Fig. 2B). As male mice mutant for *Mcidas* and *Ccno* that, like GEMC1, play key roles in MCC development, were also infertile (Lu et al., 2019; Nunez-Olle et al., 2017), we histologically examined the testes of these animals in parallel to *Gemc1*$^{-/-}$. In each case they exhibited a similar seminiferous tubule phenotype characterized by reduced cellularity and luminal dilation (Fig. 2C, D). Moreover, extensive rete testes dilation was apparent (Fig. 2E), indicative of fluid backpressure.

Previous work reported seminiferous tubule dilation accompanied by extensive SC degeneration and spermatid detachment due to loss of *TAp73,* that is also required for MCC generation (Holembowski et al., 2014; Inoue et al., 2014; Marshall et al., 2016; Nemajerova et al., 2016; Tomasini et al., 2008). Immunostaining of Vimentin-containing intermediate filaments, one of the main components of the SC cytoskeleton (Aumuller et al., 1988), revealed marked structural abnormalities in the SCs of *Gemc1, Mcidas* and *Ccno*

deficient testes (Fig. 2F, G). This was characterized by abnormally shorter and thinner cytoplasmic projections and the appearance of SC-only seminiferous tubules (Fig. 2H) and was accompanied by extensive spermatid detachment in *Gemc1*$^{-/-}$ mice (Fig. 2I). In contrast, the SCs of *Wt* mice extended long cytoplasmic arms in contact with germ cell populations (Fig. 2F, G, I). Thus, *Gemc1, Mcidas* and *Ccno* mutants phenocopied the loss of *TAp73, E2f4/5* and *miR-dKO*, exhibiting dilated seminiferous tubules and SC degeneration with the loss of GEMC1 having little impact on the expression of known MCC transcriptional targets in the testes.

Defective movement of spermatazoa to the epididymis in *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice

Spermatazoa enter the caput epididymis but remain immature until reaching the cauda epididymis, where they acquire motility and fertilization competency (Fig. 3A). *Gemc1*$^{-/-}$ and *Ccno*$^{-/-}$ epididymes appeared paler, smaller and thinner than *Wt* (Fig. 3B). Histological analysis of *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ revealed no detectable sperm in the caput, corpus or cauda epididymes in any case, in contrast to *Wt* where sperm were abundant in all sections (Fig. 3C). Consistent with this, the lumen of *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ epididymes was often filled with amorphous PAS-positive material, a phenotype typically observed in the absence of spermatozoa (Abe and Takano, 1988). *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ males thus have an apparently complete block in transit of spermatozoa to the epididymis.

As the EDs are critical for sperm transit and contain MCCs, we examined *Gemc1, Mcidas* and *Ccno* expression in the testes, cauda epididymis and EDs. The expression of *Gemc1* was considerably higher in the EDs than testes (Fig. 3D) and similar to its levels in the trachea that contains abundant MCCs. A similar pattern was observed for its target gene *Mcidas* (Fig. 3E), as well as *Trp73* (Fig. 3F). In contrast, *Ccno* was marginally upregulated in the ED compared to the testes and further increased in the cauda epididymis (Fig. 3G), suggesting it may play roles independently of

GEMC1 and MCIDAS that both appear restricted to MCCs in other tissues (Kyrousi et al., 2015).

We next examined the expression levels of key MCC regulators in *Gemc1*$^{-/-}$ EDs. While *Ccno* levels were moderately increased, other known target genes including *Mcidas, Cdc20B,* and *FoxJ1*, as well as *Trp73*, were strongly downregulated (Fig. 3H), consistent with what has been observed in the trachea (Terre et al., 2016). Similar to what has been reported for *miR-dKO,* or *TAp73* and *E2f4/5* deficient mice, PAS staining of histological sections revealed sperm agglutinations in the EDs of *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice, where they are normally not detected in *Wt* animals due to the rapid transit through this region (Fig. 3I). Therefore, regardless of gene expression patterns in the testes, spermatazoa were unable to enter the epididymis of *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ males, corresponding with defects in ED formation or function. In the case of GEMC1, this appeared to reflect its transcriptional role, similar to other MCC containing tissues.

Distinct temporal roles of GEMC1, MCIDAS and CCNO in the efferent ducts

Staining of motile cilia with anti-Ac-tub antibodies revealed that columnar MCCs were absent in *Gemc1*$^{-/-}$ EDs (Fig. 4A). A less severe phenotype was observed in both *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice, as some cells with MCC morphology were apparent, but few cilia visible (Fig. 4A). In all 3 mutants, Ac-tub staining was observed in spermatazoa agglutinations in the central cavity of the ED.

The ED epithelium of *Gemc1*$^{-/-}$ was thinner than *Wt, Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ deficient mice (Fig. 4A), so we examined the expression of the FOXJ1 and TP73 transcription factors that are critical for MCC formation in other tissues (Marshall et al., 2016; Nemajerova et al., 2016; You et al., 2004). Cells lining the EDs were strongly immunopositive for FOXJ1 in *Wt* and *Ccno*$^{-/-}$ but staining was completely absent in *Gemc1*$^{-/-}$ (Fig. S2A). Similarly, TP73 positive cells were absent in *Gemc1*$^{-/-}$, but readily identifiable in both *Mcidas*$^{-/-}$

and *Ccno*$^{-/-}$ mice. We, and others, have previously shown that the transient transfection of GEMC1 can activate early MCC factors, including both *MCIDAS* and *FOXJ1,* in AD293 cells (Arbi et al., 2016; Lu et al., 2019; Terre et al., 2016). Transient overexpression of *GEMC1* led to a strong increase in *Trp73* mRNA and protein levels, in contrast to *CCNO* overexpression that did not influence its levels (Fig. 4C), demonstrating that GEMC1 can activate TP73 expression.

Collectively, our experiments demonstrated that male mice lacking *Gemc1*, *Mcidas* or *Ccno* were infertile and exhibited a testes phenotype closely resembling that of *TAp73* deficient mice or ED-specific *miR-dKO* or *E2f4*$^{-/-}$*E2f5*$^{+/-}$ deletions (Comazzetto et al., 2014; Holembowski et al., 2014; Yuan et al., 2019). The impairment of the MCC transcriptional program and agglutination of spermatozoa in the EDs appears to be sufficient to generate backpressure that prevents spermatozoa from entering the epididymis(Yuan et al., 2019). We propose that impaired MCC function is likely the dominant cause of infertility in *Gemc1*$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice, and potentially in male RGMC patients that harbor mutations in *MCIDAS* or *CCNO* (Fig. 4D). The seminiferous tubule and rete testes dilation, degeneration of the SC support structures and failure of spermatazoa to enter the epididymis is most likely a secondary effect of backpressure caused by loss of MCC function that results in the agglutination of spermatozoa in the EDs. We cannot however rule out a role for these factors in other cell types of the ED associated with reabsorption of seminal vesicle fluids, as has been proposed for both *ESR1* and *E2f4/5* mutants(Danielian et al., 2016; Joseph et al., 2011). However, we observed normal staining of the E2F4/5-dependent AQP1 marker of secretory cells in CCNO and GEMC1 mutants, suggesting that E2F4/5 mediated transcription is not severely affected in non-MCC cell types of the ED in these mice (Fig. 4E and S2B)(Danielian et al., 2016).

The roles of GEMC1 and MCIDAS in multiciliogenesis are primarily related to transcription and our data lends further support to them acting in a stepwise manner, with GEMC1 playing a key role in specification and MCIDAS required for ciliogenesis (Fig.4B). However, the function of CCNO

remains enigmatic. Recent work demonstrated that the mitotic oscillator and fine-tuning of CDK activity is required for stepwise deuterosome-mediated centriole amplification in MCCs (Al Jord et al., 2017; Vladar et al., 2018). Given that CCNO interacts with CDK1 and CDK2 (Roig et al., 2009) and CCNO deficient mice exhibit abnormal deuterosomes (Funk et al., 2015), it seems plausible that one of its primary functions is CDK regulation during deuterosome formation. Future work will be needed to understand precisely how GEMC1, MCIDAS and CCNO regulate different aspects of the transcriptional response and deuterosome formation, as well as their potential roles in other tissues.

Materials and Methods

Histopathology and immunohistochemistry of murine tissues

Gemc1$^{-/-}$, *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice on a mixed C57BL/6-129SvEv background were described previously (Lu et al., 2019; Nunez-Olle et al., 2017; Terre et al., 2016). Animals were maintained in accordance with the European Community (86/609/EEC) guidelines in the Specific-Pathogen Free (SPF) facilities of the Barcelona Science Park (PCB) and in SPF facilities following guidelines of the Biological Resource Center (BRC) of the Agency for Science, Technology and Research (A*STAR) of Singapore. Protocols were approved by the Animal Care and Use Committee of the PCB (IACUC; CEEA-PCB) in accordance with applicable legislation (Law 5/1995/GC; Order 214/1997/GC; Law 1201/2005/SG) and ethical guidelines of the Singapore National Advisory Committee on Laboratory Animal Research (NACLAR). All efforts were made to minimize use and suffering. Sample sizes were not defined to detect pre-determined effect size. Animals were not randomized, were identified by genotyping for analysis and ages, were all males and ages are indicated in the figure legends.

Testes and epididymis were harvested and fixed in 4% PFA or Bouin's solution (Electron Microscopy Sciences) overnight at 4ºC and embedded in paraffin using standard procedures. Sections were cut at 3-5 μm thickness. dewaxed and stained with hematoxylin and eosin (H&E) and Periodic acid/Schiff reagent (PAS, Sigma-Aldrich) using a CoverStainer (Dako - Agilent) following manufacturer procedures. For immunohistochemistry, epitope retrieval was performed using Citrate pH6 buffer in autoclave at 121º for 12min. Washings were performed using the Wash Solution AR (AR10211-2, Dako, Agilent). Quenching of endogenous peroxidase was performed by 10 min of incubation with Peroxidase-Blocking Solution at RT (S2023, Dako, Agilent). Non-specific unions were blocked using 5 % of goat normal serum (16210064, Life technology) mixed with 2.5 % BSA diluted in wash buffer for 60 min at RT. Ac-tubulin was also blocked with Vector® M.O.M.™ Blocking Reagent (ref: MK-2213 – Vector) following manufacturer procedures.

Immunohistochemistry was performed using an Autostainer Plus (Dako - Agilent) for p73 antibody (90 min at room temperature) and manually for Ac-tubulin staining (over night at 4ºC). The secondary antibodys used were a BrightVision Poly-HRP-Anti Rabbit IgG Biotin-free, ready to use for 45' at RT (DPVR-110HRP, Immunologic) and a Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (ref: P0447 – Dako) diluted at 1:100 for 30 min at RT. Antigen–antibody complexes were reveled with 3-3′-diaminobenzidine (K346811, Dako), with the same time exposure (10 min). Sections were counterstained with hematoxylin (CS700, Dako, Agilent) and mounted with Mounting Medium, Toluene-Free (CS705, Dako, Agilent) using a Dako CoverStainer. Specificity of staining was confirmed by omission of the primary antibody or staining with rabbit IgG, polyclonal - Isotype control (ref: ab27478, Abcam) or a mouse IgG - Isotype Control (ref: ab37355, Abcam). Brightfield images were acquired with a NanoZoomer-2.0 HT C9600 digital scanner (Hamamatsu) equipped with a 20X objective. All images were visualized with the NDP.view2 U123888-01 software (Hamamatsu, Photonics, France). All images were visualized with a gamma correction set at 1.8 in the image control panel of the NDP.view 2 U12388-01 software (Hamamatsu, Photonics, France).

Sections were cut at 10 μm thickness and stained with hematoxylin and eosin (H&E) and Periodic acid/Schiff reagent (PAS, Sigma-Aldrich). For colorimetric visualization, sections were incubated with primary antibody overnight at RT after quenching endogenous peroxidase using 0.6% H_2O_2 (vol/vol) in methanol. Slides were washed and incubated with biotinylated secondary antibody and avidin-biotin complex (Vectastain Elite kit, Vector Labs). Immunoreactive signals were visualized with the VIP substrate kit (Vector Labs) using the manufacturer's protocol. Sections were counterstained with 0.1% methyl green (wt/vol), dehydrated, and mounted in DPX (Fluka). For TUNEL staining, testes sections were labeled with In Situ Cell Death Detection Kit (TUNEL) according to the manufacturer's instructions (Roche, 11684795910).

Antibodies

Primary antibodies used in the publication:

Antigen	Species	Vendor	Clone	Reference no.	Dilution
Ac-tub	mouse	Sigma	6-11B-1	T6793	1:1000
Vimentin	rabbit	Abcam	EPR3776	ab92547	1:2000
Vinculin	rabbit	Abcam	EPR8185	ab129002	1:2000
TP73	rabbit	Abcam	EP436Y	ab40658	1:50
Myc	rabbit	Abcam		ab9132	1:1000
FLAG	mouse	Sigma	M2	F3165	1:1000
AQP1	rabbit	Merck		AB2219	1:1000
SCP3	mouse	abcam	Cor 10G11/7	ab97672	1:500
SCP1	rabbit	abcam		ab15087	1:500
γH2AX	mouse	Millipore	JBW301	05-636	1:500
pH3Ser10	Rabbit	Millipore		05-598	1:500
AQP1	rabbit	EMD Millipore		AB2219	1:1000
FOXJ1	mouse	eBioscience	2A5	14-9965-83	1:500

All antibodies have been previously validated and were subjected to additional controls. The Ac-tub antibody has been used widely in the field and recognizes multiciliated cells that are absent in *Gemc1* deficient mice(Terre et al., 2016). The AQP1 and Vimentin antibodies have been validated in knockouts and extensively in publications (see Merck and Abcam product page) and the TP73 Ab was validated in knockout animals (Marshall et al., 2016) and signal is in the expected cell types and correlates with the mRNA level in our experiments. The Vinculin antibody recognizes a band of the correct size and was used as a loading control. Myc and FLAG epitope antibodies have been validated extensively and recognize proteins of the correct size only when tagged cDNAs are expressed. SCP1/3, γH2AX, FOXJ1 and pH3S10 antibodies have been validated (see product pages or

CiteAb.com references) and we have previously reported their use for meiotic staging or testes characterization (Marjanovic et al., 2015).

Microscopy

Histological sections from testes, epididymis and efferent ducts were imaged with the digital slide scanner Nanozoomer 2.0HT (Hamamatsu) and analyzed using the NDP view 2 free software (Hamamatsu). Image analysis and quantification of IHC were performed with the TMARKER free software (GitHub). For quantitation of % of lumen space, tubule area and lumen area were analyzed for ten tubules of PAS-stained sections (two sections per animal). The length of cytoplasmic SC arms was determined in μm using Vimentin staining. Vimentin marks intermediate filaments that are seen as cytoplasmic arms surrounding the nucleus and extending from the basal region towards the tubular lumen. SC arm's length was calculated in ten tubules of Vimentin-stained sections (2 sections per animal). Vimentin was also used to detect SC-only tubules, characterized by intense staining of the entire seminiferous tubule combined with the lack or dramatic decrease of germ cells.

Quantitative real-time PCR (qRT-PCR)

Testes, epididymis, efferent ducts or pituitary gland were carefully dissected and collected on ice, washed in PBS and frozen. Testes were disrupted in Tri-Reagent (Sigma) by zirconium beads in a mechanical tissue disruptor (Precellys 24, Bertin technologies). Total RNA was isolated according to manufacturer recommendations (PureLink RNA mini kit, Ambion) and 1ug of RNA was treated with DNase I prior to cDNA synthesis (Thermo Fisher). cDNA was generated using 0.5-1 μg of total RNA and a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real time-PCR (RT-QPCR) was performed using the comparative CT method and a Step-One-Plus real-time PCR Applied Biosystems Instrument. Amplification was performed using

Power SYBR Green PCR Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems). All assays were performed in duplicate. For TaqMan assays, *ActB* (mouse and human) probe was used as an endogenous control for normalization and a specific Taqman probe was used for mouse or human *Gemc1*(*Mm02581229_m1), Mcidas (Mm01308202_m1), Ccno(Mm01297259_m1), FoxJ1 (Mm01267279_m1)* and *Trp73 (Mm00660220_m1) and TP73 (Hs01056231_m1).* Primers used for SYBR Green assays (Sigma) are listed below.

Oligo	Sequence (5'-3')
*mCdc20b*_FW	TATCTTAGGAGATTCAGCGG
*mCdc20b*_RV	ATGTATACAGAGGTTCCGAG
*mFSH*_FW	CAGTAGAGAAGGAAGAGTGCCG
*mFSH*_RV	CGGTCTCGTATACCAGCTCC
*mLH*_FW	GCCGGCCTGTCAACGCAACT
*mLH*_RV	TGGGGTCTACACCCGGTGGG
*mActB*_FW	GGCTGTATTCCCCTCCATCG
*mActB*_RV	CCAGTTGGTAACAATGCCATGT

Germ cell isolation for fluorescence-activated cell sorting (FACS)

Testes from adult male mice were isolated, decapsulated and processed together in a 15 ml falcon tube. Testes were first digested in EKRB medium (120 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_2, 1.2 mM MgSO4, 1.3 mM CaCl2, 11 mM Glucose, non-essential aminoacid (Invitrogen), penicillin-streptomycin (Invitrogen)) with collagenase (0.5 mg/ml, Sigma T6763) in shaking water bath at 32 ºC for 10 min. Seminiferous tubules were let to sediment by gravity for 1-3 min and washed twice with EKRB. The tubules were further dissociated in EKRB with trypsin (1 mg/ml, Sigma T6763) and DNase (5 μg/ml, Sigma D4263) in shaking at 32 ºC for 15 min. Cells were resuspended thoroughly with a pipette until obtaining a single cell suspension and 1 ml of FBS was added to neutralize the Trypsin. Cell suspension was filtered with a 70 μm cell strainer and total cell number was counted using the Neubauer chamber. 1 million cells/ml were resuspended in EKRB

supplemented with 10% FBS and Hoechst 33342 (10 μg/ml) (Life Technologies) in shaking at 32 ºC for 30-60 min. Propidium iodide (PI, 30 μg/ml) (Life Technologies) was added for the discrimination of dead cells. Flow cytometric experiments were carried out using a FacsAria I SORP cell sorter (Beckton Dickinson, San Jose, California), using a 70-micron nozzle at 60PSI. Excitation of the sample was done using a blue (488nm) laser for forward scatter (FSC) parameter; green-orange laser (561nm) was used for the excitation of PI and side scatter (SSC) signal, and a UV laser (350nm) was used for Hoechst 33342 excitation. Cells were gated according to their scatter (FSC vs SSC) parameters; fluorescence of Hoechst 33342 was measured on live (not stained with PI), non-aggregated cells. Red emission (660/40 nm) vs. blue emission (395/25 nm) from the UV laser was used on a dot plot in order to discriminate populations. Results were analyzed using the FlowJo software.

Immunofluorescence

Slides containing meiotic squashes were washed several times in PBS followed with washing in PBS-T (0.4% Triton X-100 in PBS) and blocked with 5% goat serum and 1% BSA in PBS-T for 1 h. Slides were incubated overnight at 4ºC with primary antibody, washed several times in PBS-T and stained with the Alexa Fluor-conjugated complementary antibody (Life Technologies) for 1 h at RT. After final washing, DNA was counterstained with DAPI. Slides were mounted using Vectashield Antifade reagent (Vector Laboratories) and imaged using a Leica TCS SP5 confocal microscope equipped with 63x NA 1.40 oil immersion objective and HyD detectors. For paraffin sections, heat-induced eptiope retrieval was performed in a Rodent Decloaker (Biocare Medical, Concord, CA). Tissue sections were blocked with 10% normal sera in PBS-0.1% Triton X-100 for 1 hour at room temperature. After washes with PBS, tissue sections were sequentially probed with primary antibodies and fluorescently labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA).

Cell culture, transfection and Western Blotting

AD293 cells (Stratagene) were cultured in DMEM (Gibco) with 10% FBS (Hyclone) and routinely tested for mycoplasma and found negative. For transient transfections, AD293 cells were seeded in 10 cm plates at 70% confluence and 10μg of plasmid were transfected the day after with Polyethylenimine (Polysciences). The medium was changed 12 h post-transfection and cells were collected 48 h after with RIPA buffer (50mM TrisHCl pH 8, 150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium Deoxycholate). For tissue samples, testes were disrupted in RIPA buffer using zirconium beads in a mechanical tissue disruptor (Precellys 24, Bertin technologies). Samples were incubated 20 min on ice and sonicated using a Bioruptor XL sonication device (Diagenode) for 15 min with 15 s intervals and centrifuged at 4 ºC for 20 min at 1300 rpm. Antibodies to the following epitope tags were used: Flag-tag (Sigma, F3165), Myc-tag (Abcam, ab9132), p73 EP436Y (Abcam, ab50658), and Vinculin (Sigma, V9264).

DNA constructs

The expression construct for FLAG tagged human *GEMC1* (pcDNA5-FRT/TO-flag-hGemc1) was previously described in (Terre et al., 2016), Human *CCNO* cDNA was obtained from Expressed Sequence Tag EST IMAGE: 6421733 (gi22331975) as a template and amplified using 5'-GCGAATTCCATGGTGACCCCCTGTCCCACCAGCC-3' and 5'-GCTCTAGATTATTTCGAGCTCGGGGGCAGG-3' primers. hCCNO cDNA was cloned into the pBluescript (I) SK(+) vector (Addgene) at the EcoRI and XbaI restriction sites and then into the mammalian expression vector pCDNA3.1 (Invitrogen) modified with a n N-terminal myc-tag to produce proteins N-terminally fused to myc under the control of the constitutive CMV promoter (pcDNA3.1-myc-hCcno). The pCMX-flag plasmid was used as an empty-vector control (a gift from Ron Evans, the Salk Institute for Biological Studies).

Meiotic squashes

Spermatocyte squashes were performed as previously described(Page et al., 1998; Sitaram et al., 2014). In brief, tubules from adult mice were placed in fixative solution (2% formaldehyde, 0.05% Triton-X 100 in PBS) for 10 min. To release spermatocytes from tubules, a small aliquot of the tubules was minced with tweezers on a glass slide pre-treated with poly-L lysine. Cells were covered with a coverslip and gently squashed by applying pressure with the thumb. The slide was snap-frozen in liquid nitrogen, the coverslip was removed and the slide was processed for immunofluorescence.

Histopathology and immunohistochemistry

Testes and epididymis were harvested and fixed in 4% PFA or Bouin's solution (Electron Microscopy Sciences) overnight at 4ºC and embedded in paraffin using standard procedures. Sections were cut at 10 μm thickness and stained with hematoxylin and eosin (H&E) and Periodic acid/Schiff reagent (PAS, Sigma-Aldrich). For colorimetric visualization, sections were incubated with primary antibody overnight at RT after quenching endogenous peroxidase using 0.6% H_2O_2 (vol/vol) in methanol. Slides were washed and incubated with biotinylated secondary antibody and avidin-biotin complex (Vectastain Elite kit, Vector Labs). Immunoreactive signals were visualized with the VIP substrate kit (Vector Labs) using the manufacturer's protocol. Sections were counterstained with 0.1% methyl green (wt/vol), dehydrated, and mounted in DPX (Fluka). For TUNEL staining, testes sections were labeled with In Situ Cell Death Detection Kit (TUNEL) according to the manufacturer's instructions (Roche, 11684795910).

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Competing interests

The authors declare no competing interests

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Figure legends

Figure 1. *Gemc1* loss impairs late stages of spermatogenesis. (**A**) Example of testes from littermate mice of the indicated genotypes at 3 months (top panel). Ruler=mm. Testes weight relative to the whole body at the indicated ages is plotted (n=8 for 1 month and n=6 animals/genotype for 2-3 month). (**B**) Schematic of semi-synchronous stages of spermatogenesis in mice, "p" = postnatal days (adapted from (Comazzetto et al., 2014)). (**C**) PAS staining of developing testes from *Wt, Gemc1*$^{+/-}$ and *Gemc1*$^{-/-}$ littermates at p0, p7, p9, p14 and p20. Scale bar=100μm. (**D**) PAS staining of p27 and p35 testes, note thinner seminiferous tubule epithelia in *Gemc1*$^{-/-}$. Scale bars=100μm (p27) and 50μm (p35). (**E**) RT-PCR analysis of *Gemc1* expression in testes at the indicated postnatal days (n=2 for p7, p18, p20, p30 and n=3 animals for p12, p22, p27, p35) and plotted relative to the trachea. Normalization control = *Actb.* (**F**) RT-PCR analysis of *Gemc1* expression in 1-2 mo. old testes (n=6) compared to trachea (n=4). Normalization control = *Actb.* (**G**) Ratio of *Gemc1* expression in isolated RS/ES populations compared to germ cell pellets (RT-PCR, n=8 animals). Normalization control = *Actb.* (**H**) Comparative abundance of each spermatogenic cell type of control and *Gemc1*$^{-/-}$ mice by fluorescence activated cell sorting (FACS) (*Wt* n=10, *Gemc1*$^{-/-}$ n=5 animals*)*. *p=0.023 and **p=0.0023, unpaired t-test, two-tailed.

Figure 2. Seminiferous tubule and rete testes dilation and Sertoli cell degeneration in testes of *Gemc1*, *Mcidas* or *Ccno* mutant mice (**A**) RT-PCR analysis of *Ccno*, *Mcidas*, *FoxJ1, Trp73* and *Cdc20b* in the p27 testes of *Wt* (n=4) and *Gemc1*$^{-/-}$ mice (n=3). For *Trp73* n=5 for both genotypes.

Normalization control = *Actb.* (**B**) A representative western blot of TP73 levels in testes lysates from p27 *Wt* or *Gemc1*$^{-/-}$ (n=2). Vinculin used as a loading control. (**C**) Quantification of empty lumen space (n=4 testes/genotype, top right, ***p<0.0001, unpaired t-test, two-tailed). (**D**) H&E staining of testes sections from p35-p37 *Gemc1*$^{-/-}$. *Mcidas*$^{-/-}$ and *Ccno*$^{-/-}$ mice revealed thinning of the spermatogenic cell layer. Scale bar=100μm. (**E**) Examples of rete testes dilation in the indicated genotypes at p35. Scale bar=200μm. (**F**) Vimentin staining of SC intermediate filaments in the testes of the indicated genotype. Scale bars=100μm. (**G**) Quantifications of the length of vimentin positive SC arms (n=4, 2, 2, 2 animals/genotype). Genotype key in panel H. (**H**) SC only tubules (n=4 testes/genotype). Results from p30-37 testes are shown. (**I**) Ac-tubulin staining of p35 seminiferous tubules of *Wt* and *Gemc1*$^{-/-}$ mice. Detached spermatids in *Gemc1*$^{-/-}$ indicated by black arrowheads. Scale bars=50μm.

Figure 3. *Gemc1*, *Mcidas* or *Ccno* deficiency causes sperm agglutination in the ED (**A**) Schematic of a vertical section of the testis, rete testis, efferent ducts and epididymis (caput, corpus, cauda). (**B**) Gross morphology of the p35-p37 epididymes of the indicated genotypes. Ruler=mm. (**C**) PAS staining of the three major regions of the mouse epididymis (caput, corpus and cauda) from adult mice of the indicated genotype. Scale bar=100μm and applies to all panels in each row. (**D-G**) RT-PCR analysis of the expression levels of the indicated gene in different tissues (TR=trachea, TS=testes, ED=efferent ducts, CA=cauda epididymis) normalized to the trachea (n=4 p35 animals). *Actb* was used as a normalization control. (**H**) RT-PCR analysis of the expression levels of the indicated gene in the efferent ducts of p35 *Wt* (n=3) or *Gemc1*$^{-/-}$ animals (n=2). *Actb* was used as a normalization control. (**I**) PAS staining of the p35-p37 efferent ducts of mice of the indicated genotype. Black arrowheads indicate the aberrant accumulation of spermatazoa in the *Gemc1*$^{-/-}$ and *Ccno*$^{-/-}$ mice compared to *Wt.* Scale bar=100μm (top panels) and 50μm (bottom panels).

Figure 4. GEMC1, MCIDAS and CCNO are required for ED MCC development (**A**) Representative Ac-tubulin staining of p35-p37 EDs of the indicated genotypes. Scale bars=100μm. (**B**) ED sections of the indicated genotypes stained with an antibody against TP73. Scale bars=100μm (main panels) and 50μm (bottom insets). (**C**) Overexpression of FLAG-GEMC1, but not Myc-CCNO, in AD293 cells by transient transfection induces TP73 expression. RT-PCR was used to measure relative mRNA levels (n=4) and a representative western blot of 3 independent experiments is shown. *ACTB* was used as a RT-PCR normalization control and Vinculin as a loading control for westerns. (**D**) Mice with mutations in *Mir449/34*, *Gemc1, Mcidas, E2f4/5, Trp73* and *Ccno* exhibit defects in MCC development and a similar phenotypic spectrum that includes dilation of the seminiferous tubules and rete testes, SC degeneration, and lack of spermatozoa in the epididymis (azoospermia). We propose that the failure of the EDs and resulting agglutination of spermatozoa contributes directly to fluid backpressure, preventing spermatozoa from entering the epididymis. This potentially occurs in human RGMC patients with *MCIDAS* or *CCNO* mutations. (**E**) IF co-immunostaining of AQP1 and TP73 in EDs of p30-35 animals of the indicated genotypes. Scale bars=200μm