Loss of Kallmann syndrome-associated gene WDR11 disrupts primordial germ cell development by affecting canonical and non-canonical Hedgehog signalling

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Short title: WDR11 and primordial germ cell development

Key words: WDR11, Kallmann syndrome, primordial germ cell, Hedgehog signalling, primary cilia

ABSTRACT

Mutations of *WDR11* are associated with Kallmann syndrome (KS) and congenital hypogonadotropic hypogonadism (CHH), typically caused by defective functions of gonadotrophin-releasing hormone (GnRH) neurones in the brain. We previously reported that *Wdr11* knockout mice show profound infertility with significantly fewer germ cells present in the gonads. To understand the underlying mechanisms mediated by WDR11 in these processes, we investigated the effects of *Wdr11* deletion on primordial germ cell (PGC) development. Using live-tracking of PGCs and primary co-cultures of genital ridges (GR), we demonstrated that *Wdr11*-deficient embryos contained reduced numbers of PGCs which had delayed migration due to significantly decreased proliferation and motility. We found primary cilia-dependent canonical Hedgehog (Hh) signalling was required for proliferation of the somatic mesenchymal cells of GR, while primary cilia-independent non-canonical Hh signalling mediated by *Ptch2*/*Gas1* and downstream effectors *Src* and *Creb* was required for PGC proliferation and migration, which was disrupted by the loss of function mutations of WDR11. Therefore, canonical and non-canonical Hh signalling are differentially involved in the development of somatic and germ cell components of the gonads, and WDR11 is required for both of these pathways operating in parallel in GR and PGCs, respectively, during normal PGC development. Our study provides a mechanistic link between the development of GnRH neurones and germ cells mediated by WDR11, which may underlie some cases of KS/CHH and ciliopathies.

INTRODUCTION

Genetic defects affecting the development, integration and coordination of the hypothalamic-pituitary-gonadal (HPG) axis constitute most of the aetiologies in Kallmann syndrome (KS) and idiopathic congenital hypogonadotrophic hypogonadism (CHH), clinically defined by low plasma levels of sex steroids and gonadotropins with absent/delayed sexual maturation and infertility. KS patients also present with anosmia, a lack of the sense of smell (1). Current doctrine is that CHH/KS is a hypothalamic and/or pituitary disease caused by inappropriate development or failed reactivation of gonadotrophin-releasing hormone (GnRH) neurons at puberty. Therefore, infertility in patients with KS/CHH is routinely treated by GnRH or gonadotropin replacement therapy (2;3). The majority of male patients (75-95%) show normalised testosterone levels after treatment. However, only 5-20% of them achieve normal sperm concentrations and 20-40% show azoospermia, while the remainder exhibit severe oligospermia (1;4-6). These findings suggest that primary defects in the gonads may exist in these individuals.

Previously, we identified *WDR11* as a genetic locus for KS/CHH (7). Missense variants of *WDR11* have also been reported in septo-optic dysplasia, combined pituitary hormone deficiency and pituitary stalk interruption syndrome (8-10). *WDR11* belongs to a family of proteins with the evolutionarily conserved WD40-repeat (WDR) domains, forming β -propeller structures known to mediate protein-protein interactions (7;11). Our previous studies of a *Wdr11* knockout (KO) mouse model have indicated a critical role for *WDR11* in development (12). *Wdr11* is required for normal ciliogenesis as loss of *Wdr11* resulted in short and infrequent primary cilia. Since multiple developmental signalling pathways functionally rely on primary cilia, the majority of *Wdr11* KO embryos die in utero at mid-gestation (after E12.5) with severe developmental defects (12). Those rare individual

mice that survived through adulthood display features overlapping with KS/CHH such as delayed puberty and infertility, accompanied by reduced levels of GnRH and gonadotrophins. Migration of GnRH neurones is disrupted in these mice causing reduced total numbers of GnRH neurones reaching the hypothalamus. In addition, *Wdr11* KO mice are born with hypoplastic gonads containing fewer germ cells compared to the wild type (WT) littermates. *Wdr11*-deficient testes are smaller in size and contain fewer spermatocytes and spermatids with an increased frequency of morphologically abnormal sperm found in the seminiferous tubules (12). *Wdr11*-deficient ovaries are also smaller than WT and present with disproportionally higher numbers of oogonia or primordial follicles and reduced numbers of mature follicles (12). These data indicate that loss of *Wdr11* results in defective development of germ cells and gonads in both sexes.

Primordial germ cells (PGCs) are bipotential stem cells and the founders of gametes. They undergo distinctive developmental stages (specification, polarization, migration and invasion) before they become immobile and differentiate into either spermatozoa or oocytes in the gonads (13-15). In mouse, PGCs originate from the posterior primitive streak (E7.5) and move into the developing hindgut where they migrate along its anterior extension (E8-E9.5). Then they move out of the hindgut, travel through the mesentery of the dorsal body wall, and finally enter the bilateral genital ridges (GR) (E10.5) (13;14). Normal development and migration of PGCs are regulated by networks of signalling molecules and receptors expressed in the microenvironment of the germ cell niche, including chemokine SDF1 and its receptor CXCR4 (16;17). Interestingly, SDF1 and CXCR4 are also important in GnRH neuron migration, and decreased numbers of GnRH neurons are observed in *Cxcr4* knockout mice (18).

103 Hedgehog (Hh) is a major morphogen that binds to the cell surface receptors Ptch1 and
 104 Ptch2. Boc (bioregional Cdon binding protein), Cdon (cell-adhesion-molecule-
 105 related/downregulated by oncogenes, also called as Cdo) and Gas1 (growth arrest-
 106 specific gene 1) are membrane-associated co-receptors that interact with the primary
 107 Ptch receptors (19-22). They can bind Hh ligand independently of Ptch, facilitating
 108 ligand–receptor interactions at the cell surface (19). We recently reported that they are
 109 critically required for selective activation of Smo-downstream signalling (20). Gli
 110 transcription factors mediate canonical Hh signalling pathway via primary cilia-dependent
 111 mechanisms. Gli-independent non-canonical signalling also occurs, which does not
 112 require Smo localisation to the primary cilia (20;23;24). Studies in *Drosophila* and
 113 zebrafish have previously demonstrated that Hh signalling is involved in the development
 114 of PGCs, but not as a guidance cue or fate determinant (25-27). It was shown that Sonic
 115 Hedgehog (Shh) is not a chemo-attractant for PGCs in the mouse but non-canonical Hh
 116 signalling mediated by the Ptch2/Gas1 receptor complex, exclusively expressed on the
 117 surface of PGCs, is important in PGC motility (20). Notably, putative mutations of Hh
 118 signalling pathway genes including GLI, SMO and PTCH1 have been suggested in
 119 KS/CHH (28-30).

120

121 Here we investigate the potential involvement of the KS/CHH-associated gene WDR11
 122 in the establishment of germ cells in the gonads. Our data demonstrate that Wdr11 is
 123 essential for the proliferation and migration of PGCs as well as the growth of the
 124 surrounding soma, mediated by non-canonical and canonical Hh signalling, respectively.
 125 We propose that in addition to the hypothalamic GnRH deficiency, primary defects in the
 126 germ cells may underlie KS/CHH patients with WDR11 mutations. The mechanisms we
 127 revealed may apply to other ciliopathies where hypogonadism and infertility are part of
 128 the clinical features.

129

130 **RESULTS**

131

132 **WDR11 is expressed in the PGC developmental niche**

133 Initial analyses by RT-PCR showed that Wdr11 was expressed in the developing and
 134 adult urogenital organs of both sexes. Wdr11 mRNA was present in the regions through
 135 which PGCs migrate including the hindgut (HG) at E9.5 and the urogenital ridge area
 136 (UG) at E10.5 - 11.5. Wdr11 was also expressed in the post-pubertal testis, epididymis,
 137 ovary and kidney (Fig 1A). The spatio-temporal expression of Wdr11 in the developing
 138 urogenital system was further demonstrated by whole mount X-gal staining in Wdr11
 139 heterozygote embryos (Fig 1C) and by direct immunofluorescence staining for Wdr11
 140 (Fig 1D). Both methods indicated widespread expression in gonadal development
 141 including HG and mesonephric tubules. Co-immunostaining with SSEA1, a carbohydrate
 142 antigen specifically expressed by PGCs, confirmed the expression of Wdr11 in individual
 143 PGCs as well as in the surrounding somatic cells, showing diffuse peri-nuclear and
 144 cytoplasmic signal, which was absent in Wdr11 KO embryos (Fig 1D). Based on this
 145 broad expression of Wdr11 in both the mesenchymal and germ cell components of the
 146 gonads, we hypothesised that Wdr11 may have broad effects in reproductive system
 147 development including PGCs.

148

149 **Loss of Wdr11 affects PGC migration**

150 We have previously reported that the gonads of Wdr11-deficient mice were unusually
 151 small, which may be caused, at least in part, by a deficiency of germ cells (12). Wdr11
 152 deficient mice showed ovaries containing reduced numbers of oocytes and testes
 153 containing significantly fewer spermatocytes (12). Since defective development of PGCs
 154 during early embryogenesis can result in insufficient numbers of germ cells present in the

gonads at birth, leading to in/sub-fertility or premature ovarian failure, we investigated whether the absence of Wdr11 has any impact on PGC development.

First, we analysed the number and location of PGCs by anti-SSEA1 immunofluorescence and alkaline phosphatase staining at different developmental stages (E9.5 – E11.5). The results showed that Wdr11-null embryos were still populated with PGCs in their normal migratory path between the HG and GR, but many of them were inappropriately located for the stage of development (Fig 2A). Therefore, loss of Wdr11 did not completely prevent the specification of PGCs, but disrupted their migration. When we quantified the total numbers of PGCs by counting the SSEA1-positive cells, there was a significant reduction in Wdr11^{-/-} embryos compared to WT (Fig 2B). Our analyses confirmed an inappropriate accumulation of PGCs in the hindgut and mesentery, compared to WT at E10.5 (Fig 2C). This led to a significantly lower number of PGCs arriving in the GR at E10.5 in Wdr11^{-/-} embryos (Fig 2C), and concomitantly a significantly higher number of ectopic PGCs (Fig 2D). Mis-localised PGCs fail to develop normally owing to the lack of survival signals from their environment. These data suggest that Wdr11 KO caused impaired migration of PGCs, affecting germ cell establishment in the future gonads.

Defective motility of Wdr11-deficient PGCs

We hypothesised that Wdr11 KO would affect at least one of the following key processes in PGC development: motility, proliferation or survival. First, we examined the motile behaviours of PGCs and quantitatively analysed their intrinsic motility. For this purpose, we have established embryo slice cultures of explanted GR of mice expressing a Stella promoter-driven GFP transgene (*Stella*^{GFP}). Stella is the most specific marker for PGCs, being expressed soon after their specification at ~E7.5 and maintained until E13.5 in females and E15.5 in males (31). We performed a time-lapse live imaging and motion

analysis of PGCs in WT and *Wdr11* KO background using a *Stella^{GFP};Wdr11* hybrid strain at E10.5. The movements of PGCs in the time-lapse movies were manually tracked and analysed for directionality, targeting, distance and speed. When the migration over time (>10 hours) was compared in WT and *Wdr11*-null embryos, we found that PGCs in both genotypes were moving towards the GR area, showing no discernible differences in their targeting and directionality of migration (Fig 3A). To examine if *Wdr11* deficiency altered the intrinsic motility of PGCs, we performed quantitative motion analyses, which revealed that the velocity, accumulated distance and Euclidean distance of migration were significantly reduced in the *Wdr11* KO embryos (Fig 3B), consistent with the ectopic distribution of PGCs observed in our immunofluorescence experiments (Fig 2). Directionality values representing the degree to which the migratory path of a cell strayed from a straight line were not altered. Hence, the majority of the cells were still moving towards the GRs but the speed and distance of migration were reduced in *Wdr11* KO, resulting in a significantly reduced number of PGCs arriving at the GRs.

We observed some PGCs disintegrating progressively in our live-imaging, a hallmark of apoptotic cells (see supplementary movies 1 and 2). To ascertain whether the reduction in movement was due to reduced survival of PGCs in the mutants, we assessed the survival times by measuring the mean number of hours that the green fluorescence of individual cells could be observed in the movies and found no difference between the genotypes (Fig 3B). Combined, these data suggest that *Wdr11* is necessary for active PGC motility during migration towards the GRs but not the targeting and attraction of PGCs towards GRs, nor in maintaining survival.

Defective proliferation but normal apoptosis in *Wdr11* mutants

206 One possible explanation for the decreased number of PGCs is reduced proliferation. In
 207 mice, PGCs continue to proliferate during and after migration, rapidly expanding to a final
 208 population of ~25,000 cells per embryo at E13.5. Indeed, PGCs visibly divided during
 209 time-lapse imaging (see supplementary movies 1 and 2). To determine if loss of Wdr11
 210 affects PGC proliferation, we quantitatively assessed mitotically active cells by
 211 phosphorylated-histone H3 (PH3) staining of E10.5 GR (Fig 4A). The total number of
 212 PH3-positive and -negative PGCs and the mesenchymal somatic cells (GFP-negative,
 213 DAPI-positive) between the forelimb and hindlimb buds were manually counted and PH3-
 214 labelling index values generated. Wdr11^{-/-} embryos showed a lower PH3 labelling index
 215 compared to WT in both PGCs and mesenchymal cells (Fig 4B). Therefore, Wdr11 is
 216 required for the proliferation of both PGCs and mesenchymal cells in the migratory niche.
 217
 218 During PGC migration, there is an up-regulation of factors involved in apoptosis, and
 219 embryos with a defective apoptotic pathway exhibited ectopic PGCs that were not cleared
 220 effectively (32). To determine further whether loss of Wdr11 altered apoptosis, we carried
 221 out immunostaining for cleaved-Caspase 3 and manually counted Casp3-positive and -
 222 negative cells against total cell counts. The results indicated a significant increase of total
 223 apoptotic cells in Wdr11 KO embryos. However, upon careful examination, we found that
 224 this was due to the abnormally elevated numbers of ectopic PGCs present in these
 225 embryos, rather than enhanced apoptosis in general. This conclusion was based on the
 226 fact that the apoptotic index in the mesenchymal somatic cells was not different between
 227 the genotypes and the ectopic PGCs were equally positive for Casp3 in both WT and
 228 Wdr11 KO (Fig 4B). Therefore, loss of Wdr11 did not result in an overall increase in cell
 229 death, confirming our observation from the time-lapse imaging (Fig 3B).

230

231

232 **PGC developmental signalling in Wdr11 mutants**

233 It is possible that the defective establishment of PGCs in embryos lacking Wdr11 simply
234 reflects an overall retardation in development. To exclude such a notion, we validated
235 the developmental stages of the embryos used in our analyses by morphological
236 landmarks such as somite numbers, absence/presence of hind limbs and tail buds (E9.5
237 and E10.5, respectively) and the closure of lens vesicle (E11.5), which indicated that
238 mutant embryos did not have general developmental defects, at least during the period
239 we studied.

240

241 We next examined whether Wdr11 KO affected the expression of genes known to play
242 critical roles in the development of PGCs such as Blimp1, c-Kit, Steel (Kitl), Cxcr4 and
243 Sdf1 (Cxcl12). Our initial screening confirmed the expression of these genes in the WT
244 PGC migratory niche and adult urogenital organs (Fig 5A). Quantitative analyses by RT-
245 qPCR indicated that Wdr11^{-/-} embryos did not show significantly reduced mRNA levels
246 of these regulators, except for a significant decrease in c-Kit (Fig 5B). There was also a
247 numerical but non-significant reduction in Cxcr4. This is consistent with the reduced total
248 number of PGCs in the mutants, as both c-Kit and Cxcr4 are cell surface receptors
249 expressed by PGCs, while Cxcr4 is also more widely expressed (33;34). The expression
250 of the respective ligands for these receptors, Steel and Sdf1, which mediate the chemo-
251 attraction of PGCs towards the gonads, was not altered (Fig 5B). Therefore, the reduction
252 in c-Kit alone seemed unlikely to explain the decreased proliferation of PGCs and
253 mesenchymal somatic cells, nor the reduced PGC migration in Wdr11^{-/-} embryos.

254

255 **Wdr11 KO disrupts primary cilia and canonical Hh signalling**

256 Previously we reported that Wdr11 is required for ciliogenesis and cells lacking Wdr11
257 display short and infrequent primary cilia (12). Given the important role of primary cilia in

developmental signalling such as the Hh pathway, we hypothesised that the PGC deficiency in Wdr11 KO mice may be due to disruption in cilia-dependent signalling in the GR regions. Notably, the pluripotent PGCs are naturally un-ciliated but remain responsive to Hh signalling (20). Immunofluorescence staining of GR sections for ciliary marker Arl13b confirmed that the mesenchymal cells immediately surrounding the PGCs were ubiquitously ciliated in WT (Fig 6A and C). The mesenchymal cells in Wdr11 KO embryos, however, displayed significantly short and fewer cilia (Fig 6A and B). We have previously demonstrated that Hh signalling pathway genes are expressed in the PGC migratory niche in mice (20). Since Hh signalling is known to regulate proliferation of different cell types (35-37), we investigated if the reduced somatic cell proliferation in Wdr11 mutants was linked to an attenuation of Hh signalling caused by defective cilia. We first examined the expression of Ptch1 and Gli1/2/3 in the PGC migration routes in WT embryos, which demonstrated a significant induction of these genes from E9.5, reaching a maximum at E10.5 followed by a gradual decrease till E12.5 (Fig 7A). These results suggest active canonical Hh signalling in this site and period. Our analyses of Wdr11-null GR, however, showed significantly diminished expression of these genes even at the E10.5 peak (Fig 7B), suggesting a severe defect in canonical Hh signalling in the absence of Wdr11. Boc is an obligatory co-receptor for Ptch1 and mediates de-repression of Smo upon Hh ligand reception by Ptch1 (19;20;38). We have previously shown that Boc is broadly expressed in the PGC migratory niche on both somatic cells and PGCs (20). When we assessed Boc expression in Wdr11-null GR, significant reductions in both mRNA (Fig 7C) and protein levels (Fig 8B) were observed. Therefore, general depression of the canonical Hh signalling involving insufficient expression of Ptch1 and Boc may underlie the defective mesenchymal cell proliferation in Wdr11 KO.

284 **Wdr11 KO affects non-canonical Hh signalling in PGCs**

285 Loss of primary cilia cannot explain the reduced growth and migration of PGCs in Wdr11
 286 KO because PGCs are naturally unciliated and can receive Hh signalling through cilia-
 287 independent mechanisms (20). Thus, additional mechanisms must be involved. We
 288 have recently shown that post-specification migration of PGCs, as observed at E9.5 -
 289 E11.5, may be mediated by non-canonical Hh signalling. Locally secreted low
 290 concentration of Hh was required for maintenance of the intrinsic motility of PGCs (20).
 291 Desert hedgehog (Dhh) has also been associated with genitourinary tract development
 292 (39). Our qRT-PCR data showed Dhh was indeed expressed in the PGC niche at a
 293 slightly higher level than Shh, although there was no significant changes of Dhh during
 294 E9.5 – E12.5 (Fig 7D). Therefore, we investigated if the expression of Shh or Dhh was
 295 altered in Wdr11-null GRs. Interestingly, Wdr11 KO did not affect the expression of either
 296 Dhh or Shh (Fig 7E). These data demonstrate that the reduced proliferation and motility
 297 of PGCs in Wdr11 KO was not due to a reduced expression of Hh ligands themselves. If
 298 so, it may be the accessibility or reception of Hh ligand that is defective in Wdr11 KO
 299 embryos, preventing the activation of downstream effector pathways.

300
 301 It was reported that the Hh ligand is cooperatively received by Ptch2 and its co-receptor
 302 Gas1, exclusively expressed on PGCs (20). Upon stimulation with Hh ligand, the
 303 Ptch2/Gas1 hetero-complex mediated the rapid de-repression of Smo and induced non-
 304 canonical Hh signalling within minutes rather than days as required by the canonical Hh
 305 signalling associated with Gli transcription factors. This Ptch2/Gas1-dependent
 306 signalling did not require translocation of Smo to the primary cilium. Therefore, the
 307 unciliated PGCs could still respond to Hh ligands (20). To further define the role of Wdr11
 308 in this context, we investigated the status of these Hh receptors in WT and Wdr11-/-
 309 PGCs. Immunofluorescence analyses showed that Ptch2 and Gas1 expression was

310 virtually absent from Wdr11 KO PGCs (Fig 8A and B). Gas1 mRNA level was also
311 markedly reduced (Fig 7C), indicating that Wdr11-defective PGCs may be unable to
312 respond to Hh due to the lack of Hh receptors.

313

314 The nonreceptor tyrosine kinase Src is a regulator of cell motility and proliferation and
315 was shown to be activated by phosphorylation in migrating PGCs via Ptch2/Gas1-
316 dependent Hh signalling (20). Our immunofluorescence analyses of GR tissues using
317 phospho-Src antibody demonstrated that Wdr11-null PGCs exhibited significantly
318 reduced activation of Src (Fig 8 A and B). Therefore, defective expression of Ptch2/Gas1
319 on the Wdr11-null PGCs led to a failed induction of downstream signalling effectors
320 required for motility and proliferation such as p-Src. It has also been demonstrated that
321 Ptch2/Gas1-dependent Hh signalling can elicit a global induction of cAMP signalling and
322 phosphorylation of Creb in the cytoplasm, which is abolished in the absence of either
323 Ptch2 or Gas1 (20). Since activation of Creb has a pivotal role in cell proliferation and
324 motility (40) and agents that increase intracellular cAMP levels such as forskolin are
325 shown to enhance PGC proliferation (41), we sought to determine if Wdr11-deficient
326 PGCs failed to induce p-Creb in response to Hh. To this end, we generated primary
327 cultures of GR tissues and stimulated them with recombinant Shh protein (Shh-N) for 10
328 minutes, which was shown to induce p-Creb in PGCs but not in the somatic cells (20).
329 Primary GR cultures which were serum starved for 24 hours exhibited very little basal p-
330 Creb. Shh-N induced a significant upregulation of p-Creb in WT PGCs, which was
331 markedly attenuated in Wdr11 KO PGCs (Fig 8C and D). Combined, these data support
332 the notion that Ptch2/Gas1-dependent non-canonical Hh signalling involving Src and
333 Creb is disrupted in Wdr11-null PGCs.

334

335

Effects of Wdr11 mutations in mesenchymal cell proliferation

In an attempt to directly confirm the role of Wdr11 in mesenchymal cell proliferation and to predict the consequences of disease-associated mutations of Wdr11, we employed NIH3T3 cells as a model and engineered a targeted KO of Wdr11 by CRISPR/Cas9-mediated gene editing. In addition, we also introduced two clinically identified missense mutations of WDR11, namely MT and RC variants. The MT mutation (c.1610C>T; p.Pro537Leu) was originally found in two brothers with delayed puberty and childhood obesity (12). The RC mutation (c.1783T>A; p.Trp595Arg) was found in a 61-year old male patient with high grade clear-cell renal cell carcinoma (42). We also generated a targeted KO of IFT88, a gene critically required for ciliogenesis, disruption of which is known to cause defective cilia formation and function (43). The specific mutations and targeted KO were confirmed by both Sanger sequencing of genomic DNA and Western blotting of the endogenous proteins (Fig 9A). These genetic manipulations of NIH3T3 cells did not affect the gross cell morphology and the general cytoskeletal architecture (Fig 9B). However, when we examined the status of primary cilia, mutant cells showed a significant reduction in the cilia length compared to the WT (Fig 9C), except Wdr11-RC mutant which still maintained a comparable cilia length. Notably, ciliation frequency of Wdr11 mutants did not differ significantly from that of WT cells, while Ift88 KO caused a severe reduction in both cilia length and frequency (Fig 9C). We then asked whether these mutations altered cell proliferation by recording cell counts in normal growth medium over 3 days. Compared to the WT, cells with Wdr11 KO showed a severely attenuated growth, while those expressing Wdr11-MT and IFT88 KO showed a relatively moderate inhibition. Interestingly, the highest rate of proliferation was observed in Wdr11-RC mutant which had least affected cilia (Fig 10A), potentially implying pro-mitogenic effects associated with malignant cancers. The correlation between the proliferative capacity and the degree of cilia shortening suggests that cilia-dependent

canonical Hh signalling may regulate the proliferation of these cells. We speculate that a similar mechanism may underlie the reduced proliferation of somatic cells in the Wdr11-deficient GR exhibiting defective canonical Hh signalling (Fig 7).

Effects of Wdr11 mutations in proliferation and motility of PGCs

PGCs depend on the neighbouring somatic cells for survival and expansion (44-46). It is shown that isolated mouse PGCs can be cultured on feeder cell monolayer treated with Mitomycin-C (47). In such conditions, the feeder cells are not proliferating but physiologically alive, producing soluble factors and surface molecules necessary for stimulating PGC proliferation and motility while preventing apoptosis. To explore the consequences of Wdr11 mutations in the somatic cells which essentially govern the expansion and migration of PGCs, we established a PGC co-culture system where single-cell suspensions of GR tissues were seeded onto NIH3T3-CRISPR/Cas9 feeders expressing different mutations Wdr11 and lft88. Growth curves of PGCs, generated by counting GFP-positive cells in the co-cultures over 48 hours period, indicated that the mutant feeders expressing Wdr11-MT, Wdr11 KO or lft88 KO caused a significant reduction in PGC proliferation compared to the WT feeder (Fig 10B). On the contrary, Wdr11-RC feeder supported PGC proliferation almost as effectively as the WT feeder (Fig 10B). These results reinforce the notion that the property of somatic feeder cells can influence the growth of the PGCs.

Next, we investigated the impact of somatic mutations of Wdr11 on the motile capacity of PGCs. To this end, we analysed the random motility of isolated PGCs by time-lapse imaging of the co-cultures seeded on different feeders (Supplementary Movies 3-7). Analyses of the accumulated distance over 10 hours demonstrated that PGCs cultured on WT and Wdr11-RC mutant feeder maintained a similar level of intrinsic motility but

PGCs cultured on other mutant feeders had a significantly decreased motility (Fig 10C). This result also validates our slice culture experiment (Fig 3), confirming that the altered PGC migration in Wdr11-null embryo was not simply a consequence of morphological changes in the growing embryo, because even when the PGCs and their neighbouring somatic cells were dispersed and cultured as a monolayer in a dish, the effects of Wdr11 mutant feeders on the PGC motility were clearly demonstrable. Therefore, defective cilia on feeder cells caused by loss-of-function mutations of Wdr11 or lft88 may have significant impacts on PGC proliferation and migration.

Shh can rescue defective motility of PGCs

We previously reported that treatment of Hh agonists (Purmorphamine or recombinant Shh-N) increased the motility of PGCs and CRISPR/Cas9-mediated deletion of Wdr11 in NIH3T3 cells abolished the accumulation of Shh in the conditioned medium (20). If so, it is possible that the reduced motility of PGCs cultured on Wdr11 KO feeders might be due to an insufficient supply of Hh ligand or Hh signalling molecules by the feeders. Therefore, we investigated if an addition of exogenous Hh ligand could rescue the reduced PGC motility on Wdr11 KO feeders. The motility of PGCs cultured on Wdr11 KO feeder was markedly lower than those cultured on WT feeders (Fig 10D). However, after Shh-N treatment, it was increased to a level comparable to that of WT feeders. This finding is in line with the idea that Wdr11-null cells with defective cilia may be unable to produce Hh signalling molecules required for PGC motility, which can be partly rescued by addition of exogenous Shh-N (Fig 10D). Combined, these results suggest that the loss of Wdr11 which disrupts the function of primary cilia on the somatic cells affects the behaviour of PGCs, potentially due to a failed provision of Hh ligand necessary to induce the non-canonical signalling required for the migration and proliferation of PGCs.

DISCUSSION

PGCs migrate independently of one another mainly guided by their interaction with the environment. PGCs also express markers typical of embryonic stem cells including OCT4, NANOG and SOX2 (48). The fact that PGCs and their surrounding somatic cells originated from different niches and the unique property of PGCs naturally lacking primary cilia makes a fascinating model system where cilia-dependent and -independent Hh signalling pathways concurrently regulate cell behaviour in different context. We propose that canonical and non-canonical Hh signalling are differentially involved in the development of somatic and germ cell components of the gonads, and both pathways are each required, in parallel, for normal PGC development. How PGCs receive and interpret these different signals is not fully understood yet, but paracrine secretion or delivery of the Hh signalling component from the primary cilia may be involved. Although we did not specifically address this question in current paper, shedding of ciliary tips or release of ectosomes containing Hh signalling molecules, synchronised with cell cycle, has been reported (49-51). Using the PGC co-culture system, we demonstrated that primary cilia are not only important for the growth of soma itself but also critical for the proliferation and migration of PGCs growing on them. The fact that *Ift88* KO feeder also showed a significant impairment in supporting PGCs suggests that it is not just a specific effect by *Wdr11*, but may be applicable to other ciliopathy genes. We also established the genotype-phenotype correlations of clinically identified mutations of *Wdr11*, providing new insights for the pathogenesis of *Wdr11*-associated disorders such as CHH/KS (potential loss of function) and renal carcinomas (potential gain of function).

Defective migration and proliferation of PGCs in *Wdr11* mutants cannot simply be a secondary manifestation of primary cilia defects leading to a multitude of developmental

signalling failures, because we found that other key regulators of PGCs were not severely affected by *Wdr11* KO (Fig 5). We have recently shown that the local Hh signalling is required for the migration of post-specification PGCs. Treatment with recombinant Shh or a Smo agonist could enhance PGCs' intrinsic motility, while Hh antagonists such as cyclopamine and vismodegib inhibited it without affecting directionality (20). The unciliated PGCs rely on *Ptch2*/*Gas1*-dependent non-canonical Hh signalling pathway mediated by p-Src and p-Creb. Conversely, *Ptch1*/*Boc*-dependent canonical Hh signalling is likely responsible for the maintenance of the surrounding mesenchymal cell proliferation via Gli transcription factors. Our study provides further evidence for the requirement of these signalling pathways in PGC development, which is disrupted by *Wdr11* KO.

At least two lines of evidence suggest that the migrations of developing GnRH neurones and PGCs are linked or mediated by common signalling pathways. First, chemokine SDF-1 and its receptor CXCR4, which have an established role in directed migration of PGCs, can also regulate GnRH neuronal migration (18). SDF-1 is expressed in the nasal mesenchyme, whereas CXCR4 is localised in migrating GnRH neurons and olfactory/vomeroneasal nerve axons. *Cxcr4*-deficient mice contained significantly decreased numbers of GnRH neurones accompanied by defective migration. Secondly, Fibroblast Growth Factor (FGF) signalling pathway, a well-established KS/CHH-associated signalling pathway involving genes such as *FGFR1*, *FGF8* and Heparan sulfate 6-O-sulfotransferase 1 (52-54), is also important in development of PGCs (55). PGCs express two FGF receptors, *FGFR1-IIIc* and *FGFR2-IIIb*. FGF2, the ligand for *FGFR1-IIIc*, modulates PGC motility whereas FGF7, the ligand for *FGFR2-IIIb*, affects PGC proliferation (55). Importantly, FGFRs are also shown to localise to primary cilia, affecting the length and function of primary cilia (56). Loss of *FGFR1* or its FGF ligands

466 resulted in shorter cilia in zebrafish and *Xenopus* (57). Based on these findings we
 467 speculate that KS/CHH might be a ciliopathy. KS/CHH are traditionally considered as a
 468 'secondary' hypogonadism caused by defective development and function of GnRH
 469 neurones. Our data suggest that 'primary' hypogonadism (defects within the gonads) may
 470 also contribute. In a study, 26% of KS/CHH male patients did not respond to GnRH
 471 therapy (i.e. normalisation of testosterone levels, testes volume and spermatogenesis),
 472 suggesting that primary testicular defects may be involved (4). Nonetheless, these
 473 atypical responders were still considered as secondary (i.e. hypo-gonadotrophic)
 474 hypogonadism because GnRH administration could still increase LH/FSH levels. So far,
 475 up to 56 genes are reported to be associated with KS/CHH. Many of these genes are
 476 broadly expressed in the HPG axis; thus, the impact of the mutations may not be limited
 477 to hypothalamic GnRH neurons but produce more than one primary defect within the
 478 HPG axis. Those KS/CHH patients who do not respond to gonadotrophin therapies may
 479 suggest the possibility of primary hypogonadism, resulting from being born with
 480 significantly reduced numbers of germ cells. Investigation of the effects of other KS/CHH-
 481 associated genes in PGC migration may provide further evidence.

482

483 Here we report a previously undescribed role for *Wdr11* in development of the germ line
 484 with direct consequences in PGC development. Loss of *Wdr11* resulted in defective cilia
 485 and disrupted GnRH neuronal migration in mouse and zebrafish in vivo. ShRNA-
 486 mediated knockdown of WDR11 in human GnRH neuronal cells caused defective cilia,
 487 which was partially rescued by Hh agonist in vitro (12). Several members of the WDR
 488 protein family have been shown to play important roles in ciliogenesis. WDR11 is a multi-
 489 functional adaptor protein involved in cargo trafficking to the trans-Golgi network (58).
 490 Other studies have implicated WDR11 as a part of adaptor complexes regulating
 491 ciliogenesis (59) and autophagy (60). We speculate that WDR11 is involved in the

assembly and reabsorption of the primary cilium via endosome trafficking and targeted protein degradation at the ciliary base. The clinically identified mutations of WDR11 are mostly found on the surface of the protein, thus are likely to interfere with the interactions of other binding partners (7).

Hypogonadotrophic hypogonadism and infertility with or without anosmia are part of the clinical features of ciliopathies such as Bardet-Biedl syndrome (61), but in most cases, ciliopathy-related infertility was considered to be caused by the motile cilia defects affecting sperm flagella and oviduct epithelium. Our data show that non-motile primary cilium-dependent mechanisms also play an important role. Primary cilia-dependent Hh signalling is required for the proliferation and migration of PGCs that populate the foetal gonads, the lack of which could account for the germ cell insufficiency at birth, potentially leading to subfertility or infertility. Although several genes ensuring proper development of germ cells have been identified (14), the link with KS/CHH has not been established. KS/CHH patients show small testes and ovarian insufficiency, raising the possibility that normal gametogenesis might be still possible with the remaining germ cells, since loss of Wdr11 does not appear to affect the specification or fate determination of PGCs. Understanding the role of Wdr11 in later stages of reproductive development including gonadogenesis and steroidogenic cell differentiation will require further studies.

MATERIALS AND METHODS

Breeding of transgenic mice

Stella^{GFP} mice were originally obtained from Azim Surani (Gurdon Institute) (31) and maintained in a C57BL/6 background as described (20). The Wdr11 knockout mouse (International Gene Trap Consortium Ayu21-KBW205) was generated at the Institute of

Resource Development and Analysis, Kumamoto University in Japan (12). To establish the *Stella*^{GFP+/-};*Wdr11*^{+/-} hybrid line, the homozygote *Stella*^{GFP} mice were crossed with the heterozygote *Wdr11* mice. The noon copulation plug was counted as embryonic day 0.5 after timed mating. All experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986 in the Biological Research Facility at St. George's, University of London (PPL 70/8512) according to approved institutional guidelines and protocols.

Mouse genotyping

The genotypes of the parent mice and their litters were verified by performing PCR and qPCR analyses of the genomic DNA. The copy number of the GFP allele was determined quantitatively by qPCR to confirm the genotypes. To perform the relative quantification, the crossing point (Cp) value of the target gene (*GFP*) was normalised to the Cp of the reference gene (*β-tubulin*), based on which the Relative Copy Number Ratios (RCNR) were generated. Copy Number Variation (CNV) was calculated by $CNV = \frac{1}{baseline\ RCNR} \times Targeted\ gene\ RCNR$. The rounded CNV value of 1 was considered to indicate a heterozygote and 2 a homozygote. A CNV value of 0 indicated a WT mouse (i.e. no GFP). The presence of the *Stella*^{GFP} allele was further confirmed by test-breeding of randomly selected homozygous litters with WT mice, followed by PCR amplification of GFP. Primers used for genotyping analyses are shown in Supplementary Information.

qPCR and RT-PCR

For RT-PCR analyses, mouse tissues were harvested and homogenized before total RNA was extracted using an RNeasy Mini Kit according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized using oligo(dT) primers and the Precision nanoScript2 Reverse Transcription Kit (Primer Design). Quantitative real-

time PCR was performed using the Maxima® SYBR green qPCR master mix (Thermo Fisher Scientific) in a Light Cycler 2.0 instrument (Roche). The crossing point (Cp) values were obtained by LightCycler® Version 4.1 software (Roche). Cp values were analysed using the $2^{-\Delta\Delta CT}$ method normalised to *Gapdh*. All primers used are provided in Supplementary Information.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde prior to paraffin embedding. Sections cut at 6 µm thickness were de-paraffinised with HistoClear (National Diagnostics) and rehydrated in PBS. For β-galactosidase detection, whole-mount embryos were fixed with X-gal Fix buffer (0.2% glutaraldehyde, 2% paraformaldehyde, 5mM EGTA, 2mM MgCl₂ in PBS pH 7.4) for 1 hr at 4°C, washed in PBS and then incubated overnight at 37°C in X-gal solution (1mg/ml X-gal, 2mM MgCl₂, 5mM K₃Fe(CN)₆ in PBS at pH 7.4). After washing in PBS and paraffin-embedding, samples were sectioned at 12µm-thickness and counterstained with eosin. For alkaline phosphatase (ALP) staining, embryo sections were stained with BCIP-NBT (Roche) in ALP buffer at 4°C. Images of embryo sections were analysed by Zeiss Axioplan 2 Upright.

Immunofluorescence

Serial sections of dissected embryos at 5-7µm thickness were deparaffinized, rehydrated and washed in PBS. Following antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), sections were blocked with 10% goat serum in 0.5% Triton-X PBS for 1 hour at room temperature and then incubated overnight at 4 °C with primary antibodies diluted in 10% goat serum in 0.5% Tween in PBS. After washing, samples were incubated with fluorescence-labelled secondary antibodies at 1:500 dilution and counterstained with DAPI before mounting in Mowiol. For

immunofluorescence analyses of cultured cells, cells were plated on glass coverslips, fixed with 4% PFA, permeabilized with 0.2% Triton X-100 in PBS, and incubated in blocking buffer (2% heat-inactivated goat serum, 0.2% Triton X-100 in PBS) before probing with primary antibodies diluted in blocking buffer. After washing, secondary antibodies were added along with DAPI. Fluorescence microscopy was performed using a Zeiss Axiovert 200M Upright microscope and analysed by ImageJ software (<http://rsbweb.nih.gov/ij/>).

Imaging of primary cilia and F-actin

Cultured cells on glass cover slips were serum-starved for 18-24 hours before fixing to induce ciliogenesis. Embryo sections were prepared as above. Samples were analysed by immunofluorescence staining with anti-Arl13B antibody that visualises the cilia axoneme or anti-gamma-tubulin antibody that visualises the basal body. To generate ciliation frequency values, the total number of cilia and nuclei were counted from the maximum intensity projection images of each channel manually. The length of cilia was assessed in random fields of cells after Arl13b staining by measuring the maximum projection using ImageJ. To generate the 3D imaging of GR section, 3D volume rendering of the image stacks was performed in ImageJ software using the volume viewer plugin. For F-actin staining, Alexa Fluor 488-conjugated phalloidin (Invitrogen, LSA12379) was used.

Embryo slice culture and live imaging

Embryo slice organ culture and filming was performed as previously described (20). Briefly, transverse sections of E10.5 embryos were cultured in Hepes-buffered DMEM/F-12 medium with 0.04% lipid-free BSA and 100U/ml penicillin/streptomycin. A single optical section was captured every 15 min for approximately 10 hrs (total 40 frames). The

z-stack images were extracted as TIFF files and one stack per time interval was put together using ImageJ to create a movie. Motile behaviour of PGCs was evaluated based on accumulated distance (total cell path travelled), Euclidean distance (the shortest distance between cell start and end points), cell velocity and directionality (the ratio between Euclidean distance and accumulated distance indicating the straightness of the migration path), using the Chemotaxis and Migration Tool 2.0 plug-in software (Ibidi GmbH). Velocity measurements were generated for each time interval by using the formula $V = [\text{sqrt}(dx^2 + dy^2)](p)/0.25h$, where dx is the change in the x-axis, dy is the change in the y-axis, and p is the pixel size in μm . The velocities of all the tracked cells were averaged to obtain an overall mean velocity for each embryo slice/movie. Tracking was performed only on those PGCs that remained in focus and viable for the entire duration of filming. Ectopic PGCs localised in the mesentery and hindgut were not analysed as they tend to disintegrate during filming.

Genital ridge primary culture and live imaging

Dissected GR tissues of E10.5 embryos were digested in 0.25% trypsin, passed through a $0.4\mu\text{m}$ cell strainer and suspended in DMEM/L-15 medium supplemented with 20% knockout serum replacement (Invitrogen), 2mM L-glutamine, 0.1mM non-essential amino acids and 0.1mM 2-mercaptoethanol (Sigma-Aldrich), before being plated onto 0.1% gelatin-coated cover slips. Cells were incubated in 0.5% serum-containing media before treatment with 200 ng/mL recombinant Shh N-terminal peptide (R&D Systems, 1314-SH) diluted in dimethyl formamide (DMF).

For PGC co-cultures with feeder layers, single cell suspensions generated from dissected GR tissues were plated onto the NIH3T3 feeder layer pre-treated with Mitomycin-C ($5\mu\text{g/ml}$). Motile behaviors of PGCs were measured by time-lapse imaging of GFP-positive cells captured every 15 minutes for 10 hours. Live imaging was performed using

Nikon A1R laser scanning confocal microscope in a humidified 5% CO₂ chamber at 37.0±0.5°C. Random motility of PGC was analyzed using the Chemotaxis and Migration Tool 2.0 plug-in software (Ibidi GmbH).

NIH3T3 cell culture and CRISPR/Cas9

NIH 3T3 cells (American Type Culture Collection, Manassas, VA) were routinely cultured in DMEM with 2mM L-glutamine and 100µg/ml penicillin/streptomycin (Sigma-Aldrich), supplemented with 10% newborn calf serum (NCS). For growth curve analyses, NIH3T3 cells were plated at 2x10⁶ cells per 10 cm dish in the growth medium and total cell counts were assessed every 12 hours. NIH3T3 cells with targeted editing of Wdr11 and IFT88 were generated using CRISPR/Cas9 approach. Briefly, sgRNAs designed using the CRISPR Design Tool (<http://crispr.mit.edu>) were cloned into pSpCas9(BB)-2A-Puro (Addgene #48139) and transfected using Polyfect (Promega). To isolate single-cell clones, transfected cells were plated in 96-well plates. After selection in puromycin (Cambridge Bioscience), positive clones were confirmed by Sanger sequencing and western blot. The sequences of the sgRNA and primers used are provided in Supplementary Information.

Apoptosis and proliferation analyses

SSEA1-positive PGCs with co-localised staining of phosphohistone-H3 and cleaved caspase-3 were counted from every other sections of the entire length of the gonadal ridge of E10.5 embryos from each genotype. DAPI was used to determine the total number of cells. DAPI-positive cells negative for SSEA1 were counted as somatic cells. The PGC growth curves were generated by counting GFP-positive cells from 10 random fields of GR primary cultures plated on NIH3T3 feeder layer at 0, 9, 18, 24, 32 and 48 hours after plating. The percentage fold was calculated from the total cell count at 0 hours.

648 The images were captured using an Olympus IX70 inverted microscope (Hamamatsu
649 C4742–95, Hamamatsu, Japan).

650

651 **Western blot**

652 Total protein extracted in a lysis buffer (50mM HEPES, 150mM NaCl, 10% glycerol, 1%
653 Nonidet P-40, and 1mM EDTA) containing protease/phosphatase inhibitors (Sigma-
654 Aldrich) was separated by SDS-PAGE and transferred onto Hybond-ECL membrane
655 (Amersham) before being probed with primary antibodies diluted in blocking buffer (5%
656 skim milk in TBS with 0.05% Tween 20 (TBST)). After washing in TBST, membrane was
657 incubated with horseradish peroxidase-conjugated secondary antibodies before analyses
658 by enhanced chemiluminescence (GE Healthcare). B-Actin was used as loading control.

659

660 **Antibodies**

661 Primary antibodies used were against GFP (Rabbit IgG, 1:200, Abcam, ab290), SSEA1
662 (Mouse IgG, 1:200, Developmental Studies Hybridoma Bank, MC-480), Stella (Rabbit
663 IgG, 1:200, Abcam, ab19878), phospho-histone H3 (Rabbit IgG, 1:500, Millipore, 06-570),
664 cleaved-caspase 3 (Rabbit IgG, 1:200, Cell Signalling, 9661), Arl13B (Rabbit IgG, 1:1000,
665 Proteintech, 17711-1-AP), gamma-tubulin (mouse IgG, 1:1000, Sigma T6557), phospho-
666 Src (Rabbit IgG, 1:200, Invitrogen, 44-660G), phospho-Creb (Rabbit IgG, 1:200, Cell
667 Signaling, 9198), Wdr11 (rabbit IgG, 1:100, Abcam, ab175256; goat IgG, 1:100, Santa
668 Cruz, sc-163523), IFT88 (rabbit IgG, 1:500, Proteintech, 13967-1-AP) and b-actin (rabbit
669 IgG, 1:500, CST, 4967L). Secondary antibodies, all of which were from Invitrogen
670 Thermo Fisher Scientific and used at 1:5000 dilution, include Alexa Fluor 488 (Goat anti-
671 rabbit, A-11008), Alexa Fluor 555 (Goat anti-mouse, A-21422), Alexa Fluor 568 (Goat
672 anti-rabbit, A-11011), Alexa Fluor 555 (Goat anti-rabbit, A-27039) and Alexa Fluor 488
673 (Donkey anti-goat, A-11055).

674

675 **Statistical analyses**

676 Statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA, USA). The
 677 numbers of independent replicated experiments (n) are indicated in the relevant figure
 678 legends where possible. In some experiments where percentage values are indicated,
 679 the values were calculated from the average raw data value of the sample divided by the
 680 average raw data value of the control. Significance was tested using an unpaired
 681 student's t-test. In experiments where the number of repeated measures was unequal
 682 per group, a one-way analysis of variance (ANOVA) was used with Welch's test.

683

684 **Acknowledgements**

685 YJK, JYL and S-HK were supported by Medical Research Council (MRC) grant
 686 MR/L020378/1 awarded to S-HK and LCL. JYL and YJK were also supported by St
 687 George's Research Bridging Fund Scheme and Global Educational Trust awarded to S-
 688 HK. We thank Chris Wylie for his generous help and advice with the embryo slice culture.
 689 We appreciate Gregory Perry for technical assistance with confocal microscopy live
 690 imaging.

691

692 **Author contributions**

693 Conceptualization: S-HK, LCL, H-GK; Funding acquisition: S-HK, LCL; Supervision: S-
 694 HK, NAB, DB; Methodology (creation of models and design of methods): YJK, JYL, S-
 695 HK, D-WK, PA; Investigation (performing the experiments): YJK, JYL; Original draft
 696 writing: S-HK; Manuscript review and editing: YJK, JYL, H-GK, D-WK, DCB, NAB, LCL
 697 and S-HK.

698

699 **Competing interests**

700 The authors declare no competing interests.

701

702 **FIGURE LEGENDS**

703

704 **Figure 1. Wdr11 is expressed in embryonic urogenital tissues and PGCs.**

705 **(A)** Expression of Wdr11 and Gapdh in the hindgut (HG) at E9.5, the urogenital ridge
706 area (UG) at E10.5 - 11.5 and the post-pubertal reproductive organs (testis, epididymis,
707 ovary) and kidney in 8-week-old mice was assessed by RT-PCR. Representative results
708 are shown from 3 independent biological samples.

709 **(B)** Genotyping analyses of Wdr11 knockout mice by PCR. The WT and gene-trap allele
710 are indicated (left panel). The presence of the GFP-specific allele in the *Stella^{GFP};Wdr11*
711 hybrid line was confirmed by PCR (right panel) as well as by a test breeding (see
712 Materials and Methods).

713 **(C)** Images of transverse sections of whole-mount X-gal-stained paraffin-embedded
714 E10.5 embryos with eosin counterstaining. WT (+/+) and heterozygous (+/-) embryos are
715 shown (scale bar, 100µm). n, neural tube; ao, aorta; nc, notochord; mt, mesonephric
716 tubules; hg, hindgut.

717 **(D)** Immunofluorescence staining of WDR11 (green) and DAPI (blue) on the transverse
718 sections of Wdr11 WT and KO embryos at E10.5. Scale bar represents 100µm.

719 **(E)** Transverse sections of E10.5 *Wdr11* WT and KO embryos stained with anti-WDR11
720 (green), anti-SSEA1 (red) and DAPI (blue), confirming expression of Wdr11 in PGCs
721 (SSEA1-positive) and the surrounding somatic cells in the GR area. A zoomed-in image
722 of the dotted area is shown. Scale bar represents 20µm.

723

724 **Figure 2. Wdr11 KO causes a reduction in total PGCs with increased ectopic PGCs.**

725 **(A)** Immunofluorescence (left) and alkaline phosphatase (right) staining analyses of
 726 PGCs in their migratory route from hindgut towards the GRs (yellow outlines) at different
 727 developmental stages as indicated. Compared to the WT, *Wdr11*^{-/-} embryos contain
 728 fewer PGCs in the GRs, and more ectopically located PGCs (red arrowheads). n, neural
 729 tube; ao, aorta; hg, hindgut; me, mesentery. Scale bar, (E9.5) 20µm; 100µm (E10.5,
 730 E11.5).

731 **(B)** *Wdr11* KO embryos show a reduction in total PGC numbers. Total PGCs were
 732 counted from every other slide of the serial sections of E9.5, E10.5 and E11.5 embryos
 733 (n=5 per genotype). Total count per embryo is shown as mean ± SD.

734 **(C)** *Wdr11* KO embryos contain an increased number of ectopic PGCs. Total number of
 735 PGC population in each location at E10.5 are shown for each genotype (n=5). hg, hindgut;
 736 mes, mesentery; gr, genital ridge.

737 **(D)** The proportion of ectopic PGCs present in WT and KO embryos (n=5 per genotype)
 738 at E10.5 is shown as a percentage value of total PGCs. Error bars represent SD.
 739 Unpaired Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

740

741 **Figure 3. Loss of *Wdr11* disrupts PGC migration.**

742 **(A)** Time-lapse images of embryo slice cultures from *Stella*^{GFP};*Wdr11* hybrid mice.
 743 Representative images of a plane of the z-stack at 0, 5 and 10 hours are shown from
 744 movies of E10.5 *Stella*^{GFP+/+;}*Wdr11*^{+/+} and *Stella*^{GFP+/+;}*Wdr1*^{-/-} embryos in biologically
 745 independent experiments (Supplementary Movies 1 and 2). n, neural tube; a, aorta; hg;
 746 hindgut. Scale bar represents 100µm. The PGCs (GFP-positive) migrating towards the
 747 GRs (indicated in red) were tracked using ImageJ. Trajectory plots of migration (right
 748 panel) were generated by placing the starting points of individual PGC tracks onto the
 749 same point. Origins of all tracks were centred at the 0,0 XY coordinate with distance in

micrometers on x- and y-axes. The direction of migration in relevance to the embryo orientation is shown with dotted lines. Dor, dorsal; lat, lateral; vent, ventral.

(B) Comparison of the velocity (total accumulated distance over time period), accumulated distance (total path travelled) and Euclidean distance (the shortest distance between the start and end points) indicates a significant reduction in PGC migration in *Wdr11*-null embryos compared to WT. There was no significant difference between the two genotypes in the directionality of migration (the straightness of the migration path, represented by the ratio between Euclidean distance and accumulated distance) or cell survival (assessed by the number of hours that the GFP fluorescence from a cell was detected during the imaging shown in A). Error bars represents SEM. Data were from independent slice cultures (WT, n=9; KO, n=6) where 7-10 PGCs were tracked from each embryo slice. Statistical analysis by unpaired Student's t-test (* $P < 0.05$; **** $P < 0.0001$; directionality, $P = 0.061$; survival, $P = 0.64$).

Figure 4. *Wdr11* KO affects PGC proliferation but not directly their survival.

(A) Representative images of immunofluorescence staining of SSEA1 (red) and phospho-histone H3 (PH3, green) on *Wdr11* WT and KO embryos at E10.5. Yellow line indicates the GRs. Zoomed-in images of the dotted area are shown on the right. ao, aorta; hg, hindgut; me, mesentery. Scale bar, 100 μ m (left); 20 μ m(right).

(B) Analyses of cell proliferation based on the PH3-positive cell counts. The percentage values are obtained by manually counting the total positive cells against the total cell counts labelled with DAPI from every other section of the PGC migratory route (top panel). The percentage of PH3-positive cells in the somatic cell population (middle panel) and the PGC population (bottom panel) are compared between WT and KO embryos. Error bars represent SEM. Statistical analysis by unpaired Student's t-test (n=5, number of embryos for each genotype; * $P < 0.05$; ** $P < 0.01$).

776 **(C)** Representative images of immunofluorescence staining of SSEA1 (red) and
 777 Caspase-3 (green) on E10.5 Wdr11 WT and KO embryos. Yellow line indicates the GRs.
 778 Scale bar, 100µm.

779 **(D)** Analyses of apoptosis based on Caspase-3 immunofluorescence. CASP3-positive
 780 and total cells were counted from every other section of the PGC migratory route (top
 781 left). The percentages of CASP3-positive somatic cells (top right), PGCs (bottom left)
 782 and ectopic PGCs (bottom right) are compared between WT and KO embryos. Error
 783 bars represent SEM. Statistical analysis by unpaired Student's t-test (n=5, number of
 784 embryos for each genotype; ***P<0.001).).

785

786 **Figure 5. Wdr11 KO did not affect the expression of key genes required for the PGC**
 787 **migration and urogenital development.**

788 **(A)** RT-PCR analysis of Blimp1, c-Kit, Steel, Cxcr4 and Sdf1 mRNA in the PGC migratory
 789 niche of E9.5, E10.5 and E11.5 embryos (HG, hindgut; UG, urogenital ridge) and the
 790 reproductive organs and kidney in adults. NC, no template control.

791 **(B)** Comparison of gene expression in WT and Wdr11 KO by qRT-PCR. Loss of Wdr11
 792 did not affect expression of these genes in the PGC migratory niche area, except c-kit
 793 showed a decrease. Values are shown as means ± SD. Statistical analysis by unpaired
 794 Student's t-test (n=5 embryos for each genotype; **P < 0.01).

795

796 **Figure 6. Primary cilia are disrupted in the Wdr11 KO PGC migratory niche**

797 **(A)** Immunofluorescence analyses of primary cilia by anti-ARL13B (green) and anti-
 798 SSEA1 (red) staining on GR tissue sections of Wdr11 WT and KO embryos at E10.5.
 799 Zoomed-in images of the dotted area are shown on the right. Scale bar, 10 µm (left panels)
 800 and 50 µm (right panels). Representative images are shown from 5 independent
 801 biological samples.

802 **(B)** Comparison of the ciliation frequency and cilium length observed in GR sections.
 803 Ciliation frequency values are generated from the total number of cilia and nuclei counted
 804 from the maximum intensity projection images of each channel manually. The length of
 805 cilia was assessed by measuring the maximum projection of Arl13b signal using ImageJ.
 806 WT (n=119) and KO (n=103). Unpaired t-test. ***P < 0.001.

807 **(C)** 3D reconstruction of GR region in E10.5 WT embryo stained with anti-Arl13B and
 808 anti-SSEA1. ImageJ software using the volume viewer plugin was used to build the image
 809 stacks. Scale bar, 10 μ m.

810

811 **Figure 7. Expression of Hh pathway genes in the PGC migratory niche is disrupted**
 812 **by Wdr11 KO**

813 **(A)** Expression of Ptch1, Gli1, Gli2 and Gli3 in the PGC migratory niche was assessed
 814 by RT-qPCR of WT mouse embryos at E9.5, E10.5, E11.5 and E12.5. Data were
 815 normalised to Gapdh. Means $\pm$ SD are shown. Statistical analysis by multiple t-test
 816 (number of embryos for each stage, n=5). *P < 0.01, **P < 0.001; ***P < 0.0001; ****P <
 817 0.000001.

818 **(B)** Expression levels of Ptch1, Gli1, Gli2 and Gli3 are significantly reduced in the GR
 819 area of Wdr11-deficient embryos compared to WT litter mates at E10.5. Means $\pm$ SD are
 820 shown. Statistical analysis by multiple t-test (number of embryos for each genotype, n=5);
 821 **P = 0.003730; ***P = 0.000045; ****P < 0.000001).

822 **(C)** Expression of Boc and Gas1 are significantly reduced in the GR area of Wdr11-
 823 deficient embryos compared to WT litter mates at E10.5. Means $\pm$ SD are shown.
 824 Statistical analysis by multiple t-test (n=5, number of embryos for each genotype; **P =
 825 0.003730; ***P = 0.000045; ****P < 0.000001).

826 **(D)** Dhh mRNA levels did not show statistically significant difference in GRs of E9.5 – E
 827 12.5 embryos (Welch's ANOVA test).

828 **(E)** mRNA levels of Dhh and Shh in the GRs at E10.5 was not altered by loss of Wdr11
829 ($P = 0.76$; $P = 0.29$ respectively).

830

831 **Figure 8. Loss of Hh receptors and p-Src in Wdr11-deficient PGCs.**

832 **(A)** Immunofluorescence analyses of Ptch2, Gas1, Boc and p-Src on the GR sections of
833 WT and Wdr11 KO embryos at E10.5. PGCs are labelled by SSEA1 staining. The
834 merged images are shown without DAPI signal to improve the clarity. Scale bar, 10 μ m.

835 **(B)** The relative fluorescence intensity values of Ptch2, Gas1, Boc and p-Src, that were
836 normalised with the fluorescence intensity values of SSEA1 in each cell. Data are
837 obtained from WT (n=8), KO (n=10) for Ptch2; WT (n=10), KO (n=12) for Gas1; WT (n=9),
838 KO (n=11) for Boc; WT (n=11), KO (n=12) for p-Src. Error bars represent means $\pm$ SD.
839 Statistical analysis by unpaired t-test with Welch's correction. **** $P < 0.0001$.

840 **(C)** PGCs in the GR primary cultures generated from E10.5 embryos of WT and Wdr11
841 KO were analysed after immunofluorescence co-staining of p-Creb and SSEA1. Cells
842 plated on 0.1% gelatin-coated cover slips were treated with solvent dimethyl formamide
843 (DMF) or recombinant Shh protein (Shh-N) for 10 minutes. Representative images are
844 shown. Scale bar, 10 μ m.

845 **(D)** The relative fluorescence intensity values of p-Creb normalized with the intensity
846 values of SSEA1 observed in each PGC were compared in each of the genotype group,
847 with or without Shh-N treatment for 10 minutes. Data are obtained from DMF (n=8) and
848 Shh-N (n=9) for WT; DMF (n=9) and Shh-N (n=9) for Wdr11 KO. Error bars represent
849 means $\pm$ SD. Statistical analysis by unpaired t-test with Welch's correction. **** $P <$
850 0.0001.

851

852 **Figure 9. Characterisation of NIH3T3 cells after CRISPR/Cas9-mediated**
853 **mutagenesis.**

854 **(A)** Western blot analyses of NIH3T3 cells with targeted gene editing confirmed the
855 absence of endogenous proteins after KO. The missense variants Wdr11-RC and
856 Wdr11-MT still produced the full-length proteins although at reduced levels. All variants
857 were also confirmed by direct Sanger sequencing.

858 **(B)** The phase contrast microscope imaging (scale bar 100µm) and F-actin phalloidin
859 staining (scale bar 100µm) showed that the gross cell morphology and cytoskeletal
860 organisation of different NIH3T3-CRISPR/Cas9 cells were not altered significantly. The
861 primary cilium (scale bar 10µm) was visualised with Arl13B labelling for axoneme (green)
862 and gamma-tubulin labelling for basal body (red). Representative images are shown.

863 **(C)** NIH3T3 CRISPR/Cas9 cells plated onto the glass cover slips coated with 0.001%
864 poly-L-lysine in PBS were incubated in serum-free medium for 24 hours to induce primary
865 cilia formation. The length of the cilia axoneme was measured from WT (n=186), Wdr11-
866 RC (n=98), Wdr11-MT (n=52), Wdr11 KO (n=101) and lft88 KO (n=68). The ciliation
867 frequency was assessed by counting the total number of nuclei and cilia in the random
868 fields of cells from WT (n=11), Wdr11-RC (n=9), Wdr11-MT (n=10), Wdr11 KO (n=9) and
869 lft88 KO (n=16). Error bars represent means $\pm$ SD after unpaired t-test with Welch's
870 correction. *P < 0.01, ****P < 0.0001, ns (non-significant).

871

872 **Figure 10. Effects of Wdr11 mutations on primary PGC co-cultures.**

873 **(A)** The mitotic effects of various mutations introduced in NIH3T3 cells are shown as
874 growth curves. Cells were plated at 2×10^6 cells per 10 cm dish in the normal growth
875 medium and counted at the time points indicated.

876 **(B)** Proliferation of PGCs cultured on different NIH3T3 CRISPR/Cas9 feeder cell layers.
877 The growth curves were generated by counting GFP-positive cells from 10 random fields
878 at the time points indicated. The percentage was calculated from the total cell count at 0
879 hours.

880 **(C)** Intrinsic random motility of PGCs cultured on different NIH3T3 CRISPR/Cas9 feeder
 881 cell layers assessed by live time-lapse imaging (see Supplementary Movies 3 - 7). The
 882 average of accumulated moving distance of 20 GFP-positive cells in random fields of
 883 view tracked for 16 hours in 3 biologically independent experiments are shown. Error bars
 884 represent means $\pm$ SD after unpaired t-test with Welch's correction. *P < 0.01, ****P <
 885 0.0001.

886 **(D)** Effects of Shh-N on the motility of PGCs cultured on WT and Wdr11 KO feeder cell
 887 layers (see Supplementary Movies 8 - 11). The average of accumulated moving distance
 888 of 20 GFP-positive cells in random fields of view tracked for 16 hours in 3 biologically
 889 independent experiments are shown. Error bars represent means $\pm$ SD after unpaired t-
 890 test with Welch's correction. PGCs on WT feeder were increased by $30.2 \pm 0.6\%$ upon
 891 Shh-N treatment, while those on Wdr11 KO feeder were increased by $22.9 \pm 0.7\%$.

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Lee et al. Figure 1.

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Lee et al. Figure 2.

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Lee et al. Figure 10.

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SUPPLEMENTARY TABLES

Supplementary Table 1. List of primers used for mouse genotyping

Gene	Genebank	Tm (°C)	Primer sequences (5' to 3')	Product size
WT allele	NM_172255.3	58	F: ATGGCCTGGGATTTGATGACC R: AGAGTGGTCTGAGAGGAAAGG	233bp
Trapped (KO) allele		58	F: ATGGCCTGGGATTTGATGACC R: TGTGAGCGAGTAACAACCCG	753bp
<i>GFP</i> allele	M73708	60	F: CGACGGCAACTACAAGAC F: TAGTTGTACTCCAGCTTGTGC	307bp
<i>GFP</i> (qPCR)	M73708	60	F: CTTTCCCAAGAGAAGGGTCC R: TGCAGAGACATCTGAATGGC	107bp
<i>β-Tubulin</i>	M28739	60	F: GCCAGAGTGGTGCAGGAAATA R: TCACCACGTCCAGGACAGAGT	81bp

Supplementary Table 2. List of primers used for RT-PCR analysis

Gene	Genebank	Tm (°C)	Primer sequences (5' to 3')	Product size
<i>Dhh</i>	NM_007857.5	56	F: GGGACCTCGTACCCAACTAC R: CTTTGCAACGCTCTGTCATC	139bp
<i>Shh</i>	NM_009170.3	60	F: CAGCGACTTCCTCACCTTCCT R: AGCGTCTCGATCACGTAGAAG	129bp
<i>Gapdh</i>	NM_001289726.1	60	F: TGTGTCCGTCGTGGATCTGA R: CCTGCTTCACCACCTTCTTGA	77bp
<i>Gli1</i>	NM_010296.2	57	F: CTATCCTCAGCCTCCCCATG R: CCTCCCACAACAATTCCTGC	146bp
<i>Gli2</i>	NM_001081125.1	57	F: CAGTCCTGAGCTATCCCCAG R: GAGGCTGCATGAGACCAAAG	117bp
<i>Gli3</i>	NM_000168.5	57	F: CTGCAGTGAGAGTGGACAGG R: GTATCCAGTTGTGGGCTGCT	162bp
<i>Ptch1</i>	NM_000264.3	55	F: TGTTCCAGTTAATGACTCCC R: ACACTCTGATGAACCACCTC	145bp
<i>Blimp1</i>	NM_007548.4	55	F: CACACAGGAGAGAAGCCACA R: TTGTGACACTGGGCACACTT	209bp
<i>c-Kit</i>	NM_001122733.1	55	F: TGTAAGGCCTCCAACGATGT R: ACCACAAAGCCAATGAGCAG	122bp
<i>Steel</i>	NM_013598.3	55	F: AGGTCCCGAGAAAGATTCCA R: TTGTAGGCCCGAGTCTTCAG	136bp
<i>Cxcr4</i>	NM_009911.3	55	F: AACCTCTACAGCAGCGTTCT R: GATCCAGACGCCACATAGA	132bp
<i>Sdf-1</i>	NM_001012477.2	55	F: GCCAACGTCAAGCATCTGAA R: TTCGGGTCAATGCACACTTG	101bp
<i>Wdr11</i>	NM_172255.3	60	F: CATTGACCAACCACAGCAC R: GACCACGGACGCTAAACATT	131bp
<i>Gapdh</i>	NM_001289726.1	60	F: TGTGTCCGTCGTGGATCTGA R: CCTGCTTCACCACCTTCTTGA	170bp

Supplementary Table 3. Guide RNAs used for CRISPR/Cas9

Target gene	Targeting sequences (200bp) before PAM
Wdr11(RC)	F: CACCAAGATAAGCCCCTGGAATTA R: AAACCTAATTCAGGGGCTTATCTT
Wdr11(MT/KO)	F: CACCGGATGAACCTTATGAAAGTAG R: AAACCTACTTTTCATAAGGTTTCATCC
Ift88 (KO)	F: CACCGGATGAACCTTATGAAAGTAG R: AAACCTACTTTTCATAAGGTTTCATCC

Supplementary Table 4. ssODN for generating point mutations

Target gene	ssODN sequences (90bp)
Wdr11(RC)	GAAACTTTTATGACATTTCTCTAAGAAGAGTGCAAGTT CTAATATCCCTTAATTCAGGGGCTTATCTTTGAAAA CAACTGCCAAATACTG
Wdr11(MT)	GAATGGACAAGCTTGACCAGTTTCCTTTCTTTGCTG CTTCGACCCTAAACAACATGGGGTTAGTAAGAAATG AACTTCAGCTGGTTGAT

Supplementary Table 5. Primers used for validation of gene targeting after CRISPR/Cas9

Target gene	Primer sequences (5' to 3')
Wdr11 (RC)	F: ATGCTGTCTGAGTCCTACCCTC R: CCACACAGATCAGTACCCAAGA
Wdr11 (MT/KO)	F: TAGGGGTATTGAATGGACAAGC R: AAACCATGTTGTTTGGGGTCGAAG
Ift88 (KO)	F: TAAGAGTGAACGACTGAGTGCC R: TAGACAGTGCAAACCCAATGAC

SUPPLEMENTARY MOVIE LEGENDS

Supplementary Movie 1. PGC migration in E10.5 WT embryo.

A time-lapse movie of an embryo slice culture from *Stella*^{GFP+/+}; *Wdr11*^{+/+} mouse as described in the Materials and Methods. PGCs are labelled as green fluorescence.

Supplementary Movie 2. PGC migration in E10.5 Wdr11 KO embryo.

A time-lapse movie of an embryo slice culture from *Stella*^{GFP+/+}; *Wdr11*^{-/-} mouse as described in the Materials and Methods. PGCs are labelled as green fluorescence.

Supplementary Movie 3. WT genital ridge co-culture on WT feeder.

A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on WT NIH3T3 cell feeder.

Supplementary Movie 4. WT genital ridge co-culture on Wdr11-RC feeder.

A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder edited for Wdr11-RC mutation.

Supplementary Movie 5. WT genital ridge co-culture on Wdr11-MT feeder.

A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder edited for Wdr11-MT mutation.

Supplementary Movie 6. WT genital ridge co-culture on Wdr11 KO feeder.

1155 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder
1156 with Wdr11 KO.

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1158 **Supplementary Movie 7. WT genital ridge co-culture on IFT88 KO feeder.**

1159 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder
1160 with IFT88 KO.

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1162 **Supplementary Movie 8. WT genital ridge co-culture on WT feeder treated with
1163 DMF.**

1164 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on WT NIH3T3 cell
1165 feeder after treatment with DMF.

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1167 **Supplementary Movie 9. WT genital ridge co-culture on WT feeder treated with
1168 Shh-N.**

1169 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on WT NIH3T3 cell
1170 feeder after treatment with Shh-N.

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1172 **Supplementary Movie 10. WT genital ridge co-culture on Wdr11 KO feeder treated
1173 with DMF.**

1174 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder
1175 with Wdr11 KO after treatment with DMF.

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1177 **Supplementary Movie 11. WT genital ridge co-culture on Wdr11 KO feeder treated
1178 with Shh-N.**

1179 A time-lapse movie of E10.5 *Stella*^{GFP+/+} mouse GR cells cultured on NIH3T3 cell feeder
1180 with Wdr11 KO after treatment with Shh-N.

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