FGF independent MEK1/2 signalling is essential for male germline development in mice

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Abstract

Germline development provides the founding cells for spermatogenesis and oogenesis in males and females, respectively. Disrupted germline differentiation or compromised testis development can lead to subfertility or infertility and are strongly associated with testis cancer in humans. In mice, SRY and SOX9 induce expression of a range of genes, including *Fgf9,* that promote Sertoli cell differentiation and testis development. FGF9 is also thought to promote male germline differentiation but the pathway through which it signals is unknown. As FGFs signal through Mitogen-Activated Protein Kinases (MAPKs) in other tissues, we explored whether FGF9 regulates male germline development through MAPK by inhibiting either FGF or MEK1/2 signalling in fetal testis cultures from embryonic day (E)12.5, immediately after gonadal sex determination and testis cord formation, but prior to male germline commitment. Inhibition of MEK1/2 disrupted mitotic arrest, dysregulated a broad range of male germline development genes and prevented the upregulation of key male germline markers DPPA4 and DNMT3L. In contrast, when FGF signalling was inhibited, the male germline specific transcriptional program and the expression of male germline markers DPPA4 and DNMT3L were unaffected, and germ cells entered mitotic arrest normally. While male germline development was not disrupted by FGF inhibition, some genes were commonly altered after 24h of FGF or MEK1/2 inhibition including genes involved in maintenance, germline stem cells, Nodal signalling, proliferation, and germline cancer. Together, these data demonstrate a novel and essential role for MEK1/2 signalling in male germline differentiation, but a surprisingly limited role for FGF signalling. Our data strongly indicate that additional ligands act through MEK1/2 to promote male germline differentiation and highlight a need for further mechanistic understanding of male germline development.

Introduction

Development of functional sperm and oocytes is essential for fertility and the transmission of genetic and epigenetic information to offspring. A critical part of germline development involves the commitment of germ cells to male or female development, which occurs in response to somatic cell signalling (Adams and McLaren, 2002). Germ cells within a developing testis commit to male development at around embryonic day (E)12.5 and enter mitotic arrest between E13.5 and E15.5 (Adams and McLaren, 2002, Western et al., 2008). In contrast, germ cells in a developing ovary commit to female development and enter meiosis by E15.5 (Western et al., 2008, Adams and McLaren, 2002, Miles et al., 2010).

Male germline differentiation depends on development of an appropriate testicular environment. In XY mice, the Y-chromosome located male sex determining gene, *Sry* (Sex region, Y-chromosome) and the related transcriptional factor SOX9 (SRY box gene 9) drive commitment of pre-supporting cells to Sertoli cell development at E11.5 (Koopman et al., 1991, Kent et al., 1996). Sertoli cells proliferate and form testis cords that enclose germ cells, a process supported by FGF9 (fibroblast growth factor 9), which further induces and maintains *Sox9* expression (Kim et al., 2006, Colvin et al., 2001). *Fgf9* expression peaks at E11.5 in the somatic cells of the developing testis, before declining to low levels by E13.5 (Bowles et al., 2010, Colvin et al., 2001, Kim et al., 2006, Yildirim et al., 2020, Jameson et al., 2012b). Loss of function mutations in *Fgf9* or its receptor, *Fgfr2*, disrupt testis development in XY mice leading to the development of ovaries or ovotestes (Jameson et al., 2012a, Yildirim et al., 2020, Bowles et al., 2010, Colvin et al., 2001, Kim et al., 2006, Bagheri-Fam et al., 2008, Bagheri-Fam et al., 2017).

While these studies highlight an essential role for FGF signalling in testis determination, loss of FGF9 also led to reduced germ cell numbers and indirect sex-reversal of the germline (DiNapoli et al., 2006). Further work indicated that FGF9 promotes male germline development by repressing expression of the retinoic acid responsive gene, *Stra8*, preventing germ cell entry into meiosis and ensuring expression of male germline genes (Bowles et al., 2010). Homozygous deletion of *Cyp26b1* resulted in higher *Stra8* expression, consistent with a failure of CYP26B1 to degrade retinoic acid (Bowles et al., 2010). In addition, experiments

using urogenital ridges cultured with FGF9 or heterozygous global deletion of *Fgf9* on a *Cyp26b1* null background indicated that FGF9 antagonises *Stra8* transcription independently of CYP26B1, consistent with FGF9 acting as a meiosis inhibitory substance (Bowles et al., 2010, Barrios et al., 2010). Moreover, addition of FGF9 or an FGFR inhibitor to cultured germ cells indicated that FGF9 acted on germ cells to increase expression of the male germline markers *Nanos2* and *Dnmt3l* and the pluripotency markers *Sox2* and *Oct4* in XY germ cells (Bowles et al., 2010). It was proposed that FGF9 acts not only as a meiosis inhibitory factor, but actively promotes male germ cell fate (Bowles et al., 2010). However, male germ cells developed in *Wnt4/Fgf9* double null mice (Jameson et al., 2012a), indicating that *Fgf9* is not essential in this double null background and the mechanism through which FGF9 might promote male germ cell development and mitotic arrest in wild type mice remains unknown.

FGF ligands are also required for the establishment of undifferentiated, functionally pluripotent embryonic germ cells (EGCs) from primordial germ cells (PGCs). EGC derivation is dependent on exposure of PGCs to FGF2, or other FGF family ligands including FGF9, an induction process that primes PGCs to form EGCs in culture (Durcova-Hills et al., 2006). Consistent with this, exposure of isolated E11.5 or E12.5 XY germ cells to high levels of FGF9 maintained proliferation, but low levels of FGF9 promoted male germ cell differentiation (Ulu et al., 2017). Moreover, FGF9 has been implicated in initiating Nodal signalling and transiently maintaining pluripotent gene expression in E12.5 and E13.5 male germ cells (Miles et al., 2013, Spiller et al., 2012). *Fgf9* is downregulated by E13.5 and the potential for germ cells to make EGCs is lost after E12.5, consistent with the germ cell entry into mitotic arrest and the repression of pluripotency between E13.5 and E15.5 (Durcova-Hills et al., 2008, Western et al., 2010, Bowles et al., 2010).

The intracellular signalling mechanism(s) through which FGF9 achieves its influence on somatic and germ cells remain unclear. FGF ligands mediate their actions by binding to FGF receptors and commonly activating intracellular responses via MAP kinase signalling, including through MEK1/2 and phosphorylation of its target ERK1/2 (Dorey and Amaya, 2010). Phosphorylation of ERK1/2 is low at E11.5 in both XX and XY germ cells, but gradually increases between E12.5 and E14.5 (Sorrenti et al., 2020), highlighting a potential role for MEK1/2 signalling during fetal germ cell development. Moreover, inhibiting ERK1/2 signalling

maintains stem cells in ground state pluripotency (Ying et al., 2008), demonstrating a role for ERK1/2 in priming differentiation of pluripotent cells, an activity facilitated by the E26 transformation-specific (ETS) factors, ETV4 and ETV5 (Kalkan et al., 2019, Mulas et al., 2019).

We hypothesised that FGF9 signals through MEK1/2 to promote male germline development soon after supporting cells commit to Sertoli cell development and testis differentiation. We used a model in which we inhibited FGF receptor or MEK1/2 at E12.5, after commitment to testis development at E11.5 but before germ cells irreversibly committed to male development. Our data demonstrate that germ cells are not irreversibly committed to male germline fate at E12.5 but require FGF-independent MEK1/2 signalling to successfully mediate male germline differentiation.

Results

Inhibition of FGF or MEK1/2 signalling disrupts testis development

To determine the role of FGF and potential downstream target signalling pathways required for male germ cell development, E12.5 XY gonad-mesonephros samples were cultured with vehicle control (DMSO) or a range of small molecule inhibitors of FGF receptors (FGFR1-3, MEK1/2, p38MAPK and PI3K (Table 1)). To ensure the most specific inhibition approach possible, we used inhibitors with IC50 values in the low nM range that have been extensively validated and are either currently in, or have undergone clinical trials in human patients demonstrating their high specificity, potency and cell tolerance (Table 1). Initially gonad-mesonephros samples were cultured for 72h with DMSO or 500nM of pan-FGFR (FGFR1-3) inhibitor BGJ398/Infigratinib (FGFRi; IC50 ~1.0nM), MEK1/2 inhibitor PD0325901/Mirdametinib (MEKi; IC50 0.33nM), p38MAPK inhibitor PH-797804 (p38i; IC50 26nM) or PI3K inhibitor GSK1059615 (PI3Ki; IC50 5nM). A starting concentration of 500nM was based on the cell free assay IC50s of <5nM for all bar one of these drugs (Table 1), and our observations that drugs with similar IC50s have observable effects in gonad cultures in the 100-1000nM range. To initially screen for effects of each drug on testis development, we observed whole cultured testes under bright field and fluorescence conditions, with germ cell specific expression of *Oct4-GFP* providing an initial readout of structure based on germ cell organisation within testis cords (Fig 1A). DMSO treated controls developed well-defined cords

that contained germ cells. In contrast, FGFRi and MEKi treatment resulted in poorly defined gonadal structure with testis cord structure disrupted and GFP positive germ cells scattered throughout the testis. p38i and PI3Ki treated gonads were morphologically similar to the DMSO controls, with GFP positive germ cells contained within well-defined testis cords (Fig 1A). DMSO was used at a dilution of at least 1 in 5,000 in all experiments, a concentration that does not affect gonad or germline development (Miles et al., 2013, Gustin et al., 2016b).

We previously demonstrated that FGF9 induces robust proliferation of XX somatic cells to rates similar to that observed in XY gonads (Gustin et al., 2016b). To confirm the ability of FGFRi and MEKi to block FGF9 activity, XX E12.5 gonads were cultured for 48h in media containing DMSO, 50ng/mL FGF9, 500nM FGFRi, 500nM MEKi, FGF9+FGFRi or FGF9+MEKi, with 5-ethynyl-2'-deoxyuridine (EdU) added for the final two hours of culture to mark cells in S-phase (Fig 1B-C). Using flow cytometry, mouse vasa homologue (MVH, also known as DDX4) positive germ cells were separated from somatic cells and proliferation assessed using EdU incorporation and propidium iodide to measure DNA content (S1) (Miles et al., 2013, Gustin et al., 2016b, Gustin et al., 2016a). As expected, addition of exogenous FGF9 substantially increased the proliferation of gonadal somatic cells compared to XX controls ($P<0.05$, $P<0.0001$; Fig 1B-C). Critically, both FGFRi and MEKi completely neutralised FGF9, with somatic cell proliferation decreased to XX control levels in FGF9+FGFRi or FGF9+MEKi treated gonads (Fig 1B-C). In contrast, neither p38i nor PI3Ki counteracted FGF9 induced somatic cell proliferation in XX gonads, indicating that neither p38MAPK nor PI3K regulate primary pathways through which FGF drives somatic cell proliferation in developing gonads (S2A).

To assess the effects of each treatment on testis development, we used EdU and flow cytometry to measure Sertoli and germ cell proliferation in E12.5 gonad-mesonephric samples cultured for 72h with each inhibitor (Fig 1D-E). Sertoli and germ cells were identified by staining for SOX9 or MVH, respectively (S1). As expected, Sertoli cells were highly proliferative in DMSO controls (Fig 1D). However, FGFR or MEK1/2 inhibition significantly reduced Sertoli cell proliferation compared to XY DMSO controls ($P<0.05$ or $P<0.001$, respectively, Fig 1D). The ability of germ cells to enter mitotic arrest is a key milestone in male germline differentiation (Western et al., 2008). As expected, germ cell proliferation was very low in DMSO controls (Fig 1E), confirming that the majority of germ cells had entered mitotic

arrest. In contrast, germ cell proliferation was substantially higher in MEKi treated gonads ($P<0.05$), demonstrating that germ cells had not entered mitotic arrest (Fig 1E). Surprisingly, the percentage of germ cells incorporating EdU in FGFRi-treated testes was the same as that observed in DMSO controls, demonstrating that the germ cells had normally entered mitotic arrest (Fig 1E). The effect of FGFR inhibition using BGJ398 was confirmed using another potent FGFR inhibitor (AZD4547; Table 1) which confirmed that while inhibiting FGF reduced Sertoli cell proliferation, it did not disrupt mitotic arrest in germ cells (S2B-C). Neither p38i (PH-797804) nor PI3Ki (GSK1059615) affected Sertoli cell proliferation or germ cell mitotic arrest (Fig 1D-E), an outcome confirmed using independent, specific p38 and PI3K inhibitors, ralimetanib dimesylate (LY2228820) and PF-04691502 (Table 1; S2D-E).

FGF and MEK1/2 signalling is required for Sertoli cell proliferation, but only MEK1/2 is required for germ cell mitotic arrest

To determine the minimal dose of FGFRi and MEKi to effectively disrupt testis and germline development, E12.5 XY gonad-mesonephros samples were cultured for 72h with each inhibitor at 0 (DMSO diluted ≥1/5,000), 125, 250, 500 and 1000nM and Sertoli and germ cell proliferation determined using flow cytometry (S3). Compared to DMSO control, Sertoli cell proliferation was reduced by all doses of FGFRi ($P<0.0001$), with the effect plateauing at 125nM or higher (Fig 2Ai). As with 500nM FGFRi (Fig 1E), germ cell mitotic arrest remained unaffected even with an FGFRi dose of 1000nM (~750-1000x the drug IC50; Fig 2Bi). MEKi also dose-dependently reduced Sertoli cell proliferation, though a dose of ≥250nM (slightly higher than for FGFRi) was required to mediate statistically significant reductions ($P<0.0001$, Fig 2Aii). In contrast, even when used at 125nM, MEKi potently disrupted germ cell mitotic arrest, with increasingly high proportions of EdU positive proliferative germ cells as MEKi concentration increased ($P<0.0001$, Fig 2Bii). Consistent with these outcomes, Sertoli cell proliferation was reduced but mitotic arrest was not disrupted even when an excessively high dose of 2500nM FGFRi was used (S2F-G). Similarly, the VEGF/FGFR inhibitor SU5402, a drug used in a previous study of germ cell development (Bowles et al., 2010), reduced Sertoli cell proliferation to a similar extent as FGFRi, but did not inhibit germ cell mitotic arrest (Table 1; S2F-G). Together these data demonstrate that regardless of the inhibitor used, FGFR inhibition consistently reduced Sertoli cell proliferation but did not disrupt germ cell mitotic arrest.

As FGF9 expression peaks at E11.5 (Jameson et al., 2012b), a potential explanation for the inability of FGFRi to disrupt germ cell mitotic arrest may have been that FGF9 was inhibited too late in the E12.5 cultures. To address this possibility, XY E11.5 gonad-mesonephros samples were cultured for 96h with DMSO or 500nM FGFRi or MEKi and Sertoli and germ cell proliferation assessed using flow cytometry (Fig 2C-D). As in E12.5 cultures, FGFRi or MEKi resulted in significantly reduced Sertoli cell proliferation compared to control ($P<0.01$ and $P<0.001$ respectively, Fig 2C). Germ cell proliferation was moderately higher in FGFRi treated gonads compared to DMSO control ($p<0.05$, Fig 2D). However, MEKi resulted in much more robust maintenance of germ cell proliferation ($P<0.0001$, Fig 2D), again demonstrating the potent ability of MEKi, but a substantially lower impact of FGFRi to disrupt mitotic arrest.

To determine if MEK1/2 inhibition affected Sertoli cell proliferation and germ cell mitotic arrest after E13.5, XY E13.5 gonad-mesonephros samples were cultured for 48h with either DMSO or 500nM MEKi. MEKi treatment from E13.5 resulted in significantly reduced Sertoli cell ($P<0.01$, Fig 2E) and increased germ cell proliferation ($P<0.01$, Fig 2F), although the MEKi effect on germ cell mitotic arrest was diminished compared to that in E12.5 and E11.5 cultures (EdU 6% for E13.5 vs 15% and 25% for E12.5 and E11.5, Fig 2Bii,D,F), demonstrating that the ability of MEKi to disrupt germ cell mitotic arrest rapidly decreased in E11.5, E12.5 and E13.5 gonads, despite similar impacts on Sertoli cell proliferation at all ages.

FGF and MEK1/2 signalling is required to ensure all germ cells localise inside testis cords

As gonad wholemount images indicated that testis cords did not form properly following FGFRi or MEKi (Fig 1A), we used IF to investigate MVH positive germ cells relative to SOX9-expressing Sertoli cells, laminin-delineated testis cords and SMA-expressing peritubular myoid cells. In DMSO controls the majority of Sertoli cells were organised in a single layer at the outer edge of the testis cords, delineated by SMA-expressing peritubular myoid cells or laminin, with germ cells very rarely found outside the cords (Fig 3). In FGFRi and MEKi treated samples some Sertoli cells localised to the outer edge of testis cords, but gaps were evident between the Sertoli cells and many Sertoli cells remained dispersed throughout the interior of the testis cords (Fig 3A). Furthermore, germ cells were occasionally present in the gaps

between Sertoli cells at the testis cord basement membrane (Fig 3A) and were mis-localised outside of the testis cords in FGFRi treated cultures, although this was more common in MEKi treated gonads (Fig 3B).

MEK1/2 signalling is required for male germline differentiation

As MEKi prevented germ cell mitotic arrest, the expression of male germline differentiation markers was assessed using IF and flow cytometry in E12.5 XY gonad-mesonephros samples cultured for 72h with DMSO, 125nM or 500nM FGFRi or MEKi. DPPA4 is expressed in all XX and XY germ cells at E12.5, but is upregulated in XY germ cells and repressed in XX germ cells as they differentiate (Gustin et al., 2016b). As expected, DPPA4 was not detected in germ cells of XX gonads but was detected in all germ cells of XY E12.5 gonads cultured for 72h with DMSO, and fluorescence intensity appeared more intense in XY E12.5+72h germ cells than in E12.5 XY germ cells (Fig 4A, S4A). While DPPA4 germ cell levels were similar in XY E12.5+72h DMSO and FGFRi cultures, DPPA4 intensity appeared lower in MEKi treated samples and was comparable to E12.5 XY germ cells (Fig 4A, S4A). Confirming this, flow cytometry revealed that the relative DPPA4 germ cell intensity was 2x higher in XY E12.5+72h DMSO and FGFRi cultures than in E12.5 XY germ cells ($P<0.0001$, Fig 4B), but was comparable to E12.5 XY germ cells in MEKi-treated gonads (Fig 4B).

As the germ cell proliferation and DPPA4 data indicated that male germ cells failed to properly differentiate in MEKi-treated XY gonads, we examined the well-characterised male germline markers DNMT3L and PIWIL2. IF and flow cytometry revealed that the majority of germ cells were DNMT3L positive in the DMSO control and FGFRi treated gonads (Fig 4C-E, S4B). In contrast, very few germ cells were DNMT3L positive in MEKi-treated gonads, and the intensity of DNMT3L staining intensity was significantly lower than in the DMSO- or FGFRi-treated samples ($P<0.01$, $P<0.0001$; Fig 4C-E, S4B). Similarly, PIWIL2 was expressed at relatively uniform levels in germ cells of DMSO- and FGFRi-treated samples but was variable in MEKi-treated samples, with some germ cells staining strongly for PIWIL2 and others negative (Fig 4F, S4C). This was not possible to confirm using flow cytometry because a reliable PIWIL2 flow assay could not be developed.

MEK1/2 inhibition induced *Stra8*, but failed to promote widespread meiosis in XY germ cells

Since MEKi inhibited mitotic arrest and male germline differentiation, the expression of female germline markers was investigated to determine if FGFRi or MEKi induced female development in XY germ cells. As expected, the pre-meiotic marker STRA8 was detected in almost all germ cells of XX E12.5+72h DMSO-treated gonads but was not detected in germ cells of XY DMSO controls (Fig 5A, S5A). While some germ cells appeared very weakly positive for STRA8 in FGFRi treated samples, STRA8 positive germ cells were commonly found in MEKi-treated gonads (Fig 5A, S5A), particularly in germ cells closer to the mesonephric-gonadal boundary than in more distally located germ cells (S5A). Moreover, STRA8 staining was localised in the germ cell nucleus in XX controls but was detected in the germ cell cytoplasm and nucleus in MEKi treatments (Fig 5A, S5A). Flow cytometry demonstrated that 74% of germ cells were STRA8 positive in XX DMSO controls, but only 5% and 7% were STRA8 positive in XY control and FGFRi treated samples (Fig 5B). The proportion of STRA8 positive germ cells in MEKi-treated gonads was 38%, significantly higher than XY controls ($P<0.0001$), but lower than XX controls ($P<0.0001$, Fig 5B).

To determine whether germ cells in FGFRi- or MEKi-treated XY gonads had entered meiosis, gonad sections were triple stained using antibodies specific for SYCP3, phospho-γH2AX (p-γH2AX) and MVH (Fig 5C, S5B). SYCP3 and p-γH2AX were detected in most germ cells in 72h XX DMSO controls but not in XY DMSO- or FGFRi-treated gonads (Fig 5C, S5B). A small number of germ cells were positive for SYCP3 in MEKi-treated gonads and a subset also stained for p-γH2AX (Fig 5C, S5B). In addition, cells positive for p-γH2AX were detected in XY control, FGFRi and MEKi treated gonads (S5B), however, most did not express MVH and were likely to be apoptotic somatic cells in which p-γH2AX also marks double strand DNA breaks.

To test the possibility that meiotic entry of germ cells in FGFRi- or MEKi-treated gonads was delayed, we cultured E12.5 XX and XY gonad-mesonephros for 96h with DMSO or 500nM FGFRi or MEKi. IF staining revealed that most XX DMSO germ cells were p-γH2AX positive, indicating they had entered meiosis (Fig 5D, S5C). p-γH2AX positive germ cells were rarely detected in XY DMSO- or FGFRi-treated gonads but were more common in MEKi-treated samples (Fig 5D, S5C). Quantification using flow cytometry revealed that 88% of all germ cells

were positive for p-γH2AX in XX DMSO samples while only 2%, 4% and 19% were p-γH2AX positive in XY control, FGFRi and MEKi treated samples, respectively (Fig 5E).

Cell cycle analysis of germ cells from the same gonads using EdU incorporation (S-phase) and propidium iodide staining to quantify DNA synthesis and content demonstrated that the majority of germ cells were in G2/M in XX DMSO controls, while the majority of germ cells in XY DMSO- and FGFRi treated gonads were in G0/G1 in E12.5+96h cultures (Fig 5F). Significantly more germ cells were in G2/M in MEKi than in XY control or FGFRi cultures ($P<0.0001$), but this proportion remained substantially lower than in XX controls ($P<0.0001$, Fig 5F). Therefore, while MEKi treatment resulted in a significantly greater percentage of p-γH2AX expressing germ cells that were in G2/M, this proportion was substantially lower than in XX control gonads indicating that germ cell meiosis was not properly induced within the normal temporal window following MEK inhibition.

The majority of transcriptional divergence occurs after E12.5 in XY and XX germ cells

We next used RNA sequencing to gain greater insight into genome-wide transcriptional changes in FACS isolated *Oct4*-GFP positive germ cells of E12.5 XX and XY gonads cultured for 24 and 72h with DMSO, FGFRi and MEKi (Fig 6A). In addition, germ cells were collected from XX and XY E12.5 gonads to provide time 0 controls. Differential gene expression analysis identified 183 and 234 genes that were expressed higher in XY and XX germ cells at E12.5, respectively (time 0; FDR<0.05; absolute Fold-Change (FC) $\geq$ 1.5, absolute Log-FC $\geq$ 0.585; Fig 6B, Table S1.1-1.2). Included in the differentially expressed genes (DEGs) that were higher in E12.5 XY germ cells were a range of Nodal signalling associated genes, including *Nodal, Tdgf1 (Cripto), Lefty1*, *Lefty2, Pitx2* and *Otx2*, which are known to be high in XY germ cells at this time point (Spiller et al., 2012, Miles et al., 2013, Wu et al., 2015, Mayère et al., 2021) (Table S1.1). E12.5 XX germ cells expressed higher levels of a range of BMP target genes, including *Msx1, Msx2, Id1, Id2, Id3, Stra8* and *Gata2,* consistent with recent observations that BMP2 promotes female germline development (Nagaoka et al., 2020), (Mayère et al., 2021) (Table S1.2). However, despite these sex-specific transcriptional differences in E12.5 germ cells, our functional data strongly indicated that these differences were insufficient to ensure male germline commitment as MEK1/2 inhibition at E12.5 substantially disrupted male germline differentiation (Figs 1-4).

To identify male and female transcriptional changes that occurred as a normal part of sex-specific germline differentiation, XX and XY germ cells of DMSO control samples were compared after 24 and 72h of culture. Multidimensional scaling (MDS) revealed that although time 0, XY and XX E12.5 samples were different, they diverged substantially more after 24 and 72h of gonad culture (Fig 6C). Differential gene expression analysis revealed that 211 and 3437 genes were increased, and 217 and 2160 genes were decreased in XY germ cells after 24 and 72h compared to E12.5 (time 0) XY germ cells, respectively (Fig 6B, Table S1.3-1.4). Similarly, 340 and 3214 genes were increased, and 157 and 2592 genes were decreased in XX germ cells compared to E12.5 XX germ cells after 24 and 72h, respectively (Fig 6B, Table S1.5-1.6). Together, these data indicated that while male and female germline differentiation progressed in the first 24h, the greatest transcriptional change occurred between 24 and 72h.

To identify genes specifically associated with male and female germline differentiation we compared XY with XX germ cells from DMSO controls. This revealed 589 and 3548 genes higher in XY than XX germ cells after 24 and 72h, (XY germline genes; Fig 6B, Table S1.7-1.8), including male germline genes *Dnmt3l, Dppa4, Nanos2, Piwil1, Piwil2, Piwil4, Tdrd1, Tdrd5* and *Tdrd9*. By comparison, 978 and 2898 genes were higher in XX than XY germ cells after 24 and 72h (XX germline genes; Fig 6B, Table S1.9-1.10), including female germline and meiosis markers *Atr, Atm, Chek1, Ccnb3, H2ax, Irx3, Irx5, Msx1, Id1, Id2, Id3, Sycp2, Sycp3* and *Stra8*, confirming that E12.5 germ cells diverged in the expected sex-specific manner over time.

FGF and MEK1/2 commonly regulate a subset of genes involved in germ cell tumours, stem cell biology and proliferation

To determine the initial impacts of MEKi and FGFRi on germline development we compared outcomes in XY germ cells cultured with DMSO or 500nM FGFRi or MEKi for 24h. MDS indicated that germ cells from XX E12.5+24h controls were transcriptionally distinct from all XY groups. XY E12.5+24h DMSO, FGFRi or MEKi cultures were also transcriptionally distinct (Fig 6D) and comparison of XY germ cells from MEKi and FGFRi cultures with DMSO controls revealed 43 and 25 DEGs, respectively (FDR<0.05, |FC|≥1.5; Fig 6E, Table S2.1-2.2). These included 23 and 19 genes that were lower than expected (i.e. for which transcription was not properly activated) and 20 and six genes that were higher than expected (i.e. not properly

repressed, or unexpectedly derepressed) in FGFRi or MEKi treatments. Nine genes were commonly dysregulated by MEKi and FGFRi implying that they depend on FGF signalling via MEK1/2 (Fig 6Fi, Table S2.3). Importantly, the direction of change (up- or downregulated) was concordant (roast test *P*=0.00025) for each of the nine common DEGs, with eight expressed lower and one higher than expected (Fig 6Fii). The simplest interpretation of these data is that the DEGs that were lower than expected were FGF responsive genes that depended on MEK1/2 signalling for their upregulation, and the DEGs that were higher than expected depended on FGF-MEK1/2 for their repression.

Further examination of the genes that were lower than expected revealed that six of the FGFRi-MEKi DEGs were associated with stem cell differentiation, cell self-renewal, cancer, cell proliferation and survival and germ cell tumours, including *Etv1* and *Etv4* (Akagi et al., 2015, Gashaw et al., 2005, Oh et al., 2012, Cai et al., 2007), *Pdk1* (Anwar et al., 2021), *Ret* (Miles et al., 2012, Naughton et al., 2006), *Tdgf1* (*Cripto*) (Spiller et al., 2016) and *Calcoco2* (Cui et al., 2021). Few genes commonly associated with sex-specific germline differentiation were represented in the FGFRi and MEKi DEG lists after 24h of culture. However, although unaffected by FGFRi, *Nanos2,* which regulates male germline differentiation (Suzuki and Saga, 2008), was 9.12-fold lower than control in 24h MEKi samples (Table S2.2), consistent with a requirement for MEK1/2 signalling for its upregulation.

Male specific germline differentiation depends on MEK1/2, but FGF signalling is dispensable

To determine the impacts of FGFRi and MEKi on later stages of male germline differentiation we analysed samples after 72h of culture. Surprisingly, germ cells from 72h XY DMSO and FGFRi cultures were transcriptionally similar (Fig 6G). Although 43 genes were differentially expressed after 24h of FGFR inhibition (FDR<0.05, |FC|≥1.5; Fig 6Ei; Table S2.1), only one gene was differentially expressed after 72h, and was higher than expected (FDR<0.05, |FC|≥1.5; Fig 6Hi; Table S2.4). In contrast, MEKi samples were transcriptionally distinct from XX and XY DMSO controls at 72h (Fig 6G), with 817 genes lower and 586 higher than controls (FDR<0.05, |FC|≥1.5; Fig 6Hii, Table S2.5). Of the 817 genes that were lower, 686 were male germline genes (i.e. normally upregulated in the germ cells of XY vs XX E12.5+72h DMSO cultures defined in Fig 6B) and were therefore defined as MEK1/2-dependent male germline genes (Fig 6Ii, Table S2.6). Genes lower than expected included key male germline markers

Dppa4, *Nanos2*, *Dnd1*, *Nxf2* and *Brdt* (Maldonado-Saldivia et al., 2007, Suzuki and Saga, 2008). Interestingly, the transcriptional level of *Dppa4* and the germline teratoma gene *Dnd1* (Youngren et al., 2005, Cook et al., 2009) resembled that of undifferentiated XY germ cells at E12.5 (Fig 6J), consistent with our observations of DPPA4 protein expression in MEKi treated samples (Fig 4A-B). In addition, many DNA methylation and piRNA pathway associated genes, including *Dnmt3a*, *Dnmt3l*, *Tdrd1*, *Tdrd5*, *Tdrd9*, *Spocd1*, *Piwil1*, *Piwil4* and *Henmt1* (Sakai et al., 2004, Aravin et al., 2008, Shoji et al., 2009), were not properly upregulated in MEKi treated samples, with levels remaining substantially lower than in XY DMSO-treated controls (Fig 6J). Consistent with persistent germ cell proliferation observed after MEK inhibition (Fig 2Cii), genes that regulate the G1-S transition, DNA synthesis and germ cell proliferation, including *Mki67, Pcna*, *Ccne1*, *Ccne2* and *Ccnd1*, remained high in germ cells after 72h of MEKi treatment (Fig 6J).

MEK1/2 signalling inhibition did not effectively promote female germline differentiation

Of the 586 genes that were higher than control in XY germ cells after MEKi treatment (i.e. genes derepressed or not properly repressed via MEK1/2 signalling), 382 were genes normally expressed at higher levels in differentiating XX than XY germ cells (Fig 6B), suggesting feminisation of the germline (Fig 6Iii; Table S2.7). However, of these 382 genes, 218 were not informative as they were expressed at similar levels in E12.5 germ cells, consistent with MEKi blocking germline differentiation rather than increasing feminisation (S6). These included genes such as the cell cycle regulators, *Ccne1, Pcna* and *Ki67* which mark proliferating germ cells and germ cells transitioning G1-S into G2 to enter meiosis (Miles et al., 2010, Western et al., 2008) (Fig 6J). As MEKi blocked mitotic arrest but most germ cells did not enter meiosis (Fig 5E, F), the simplest explanation is that these genes remained high due to continued germ cell proliferation, rather than female differentiation.

MEKi upregulated *Stra8*, *Meioc*, *Irx3* and *Irx5* (Soh et al., 2017, Bowles et al., 2006, Miles et al., 2010, Fu et al., 2018) and some other genes that increase during female germline development (genes in red box in S6). However, many genes marking meiosis initiation or progression including *Msx1, Sycp2, Sycp3*, *Ccnb3* (Cyclin B3)*, Atr and Atm* (Miles et al., 2010) (Le Bouffant et al., 2011) were not increased by MEKi (Fig 6J). Moreover, *Ccnb1 and Msx1,* which are repressed in differentiating XX germ cells (Miles et al., 2010), remained high in MEKi

cultures (Fig 6J). Other well-known meiosis markers including *Check1* and *H2ax* were transcribed higher in both MEKi and E12.5 XY controls than DMSO controls, so were not informative (Fig 6J). Together, despite higher levels of some female germline markers including *Stra8 and Meioc,* the low expression of many key meiosis markers such as *Atr*, *Atm* and *Msx1* (Fig 6J) was consistent with the limited entry of germ cells into meiosis based on cell cycle and IF analyses of p-γH2AX in MEKi treated samples (Fig 5D-F).

MEK1/2 inhibition retained germ cells in a relatively undifferentiated state

MEK1/2 inhibition maintained XY germline proliferation and a range of genes including *Dppa4, Dnd1, Ccne1, Ccne2, Ki67, Pcna, H2ax, Chek1* and *Ccnb1* remained at similar levels in E12.5+72h MEKi samples as in E12.5 XY germ cells. *Nodal* and associated genes including *Lefty1, Lefty2* and *Cripto* (*Tdgf1*) are transcribed at high levels in E12.5 XY germ cells and Nodal signalling is important to maintain pluripotency and prevent premature male germ cell differentiation (Spiller et al., 2012, Miles et al., 2013). *Lefty1* and *Lefty2* were maintained at higher levels in germ cells of 72h MEKi treated samples than controls, although they were not expressed as highly as in E12.5 XY germ cells (Fig 6J). Although *Nodal* and *Tdgf1* were not affected by MEKi at 72h, the Nodal signalling target *Pitx2* was also upregulated by MEKi (Fig 6J).

Key regulators of pluripotency *Sox2* and *Nanog* are expressed in E12.5 in XX and XY germ cells but repressed after E13.5 (Western et al., 2010, Maldonado-Saldivia et al., 2007, Bullejos and Koopman, 2004). MEKi maintained *Nanog*, but not *Sox2* transcription in germ cells. *Oct4* transcription remained unchanged, but as it is normally transcriptionally maintained between E12.5 and E15.5 in XY germ cells (Western et al., 2010), this was not informative. As expected, the majority of XY germ cells were positive for OCT4 and SOX2 protein at E13.5, but were negative in XX DMSO samples cultured for 72h (S7B-C). Although OCT4 and SOX2 staining intensity was significantly lower than in E13.5 XY gonads, many germ cells remained positive in XY gonads treated with DMSO, FGFRi or MEKi for 72h and the proportion of germ cells positive for OCT4 or SOX2 and the staining intensity of these proteins remained unaffected by FGFRi and MEKi (S7B-E). In addition, MEKi altered *Dmrt1, Etv3, Sox3* and *Rhox10,* which have been associated with germ cell or other tumours (Kanetsky et al., 2011, Taguchi et al., 2021), or spermatogonial stem cell function (Raverot et al., 2005, Song et al., 2016). *Dmrt1*

and *Sox3* were higher and *Rhox10* was lower in XY 72h MEKi germ cells compared to 72h XY and XX DMSO controls (Fig 6J). Rather than being down-regulated as normally occurs in differentiating male germ cells, *Etv3* was maintained at levels higher than or similar to E12.5 germ cells (Fig 6J).

Discussion

We have identified an essential role for MEK1/2 signalling in male germ cell differentiation. Our data demonstrate that MEK1/2 signalling is required for XY germ cells to enter mitotic arrest, upregulate a wide range of genes that mark male germline differentiation, and repress a range of female germline genes. However, although FGF signalling has been implicated in directly promoting male germline differentiation our data indicates that FGF signalling is dispensable for germ cell mitotic arrest and male germline differentiation. Instead, our data support a role for FGF-MEK1/2 signalling in regulating genes associated with Nodal signalling and stem cell characteristics in E12.5-E13.5 XY germ cells. This is consistent with the proposal that NODAL maintains pluripotency in XY germ cells, germ cell tumours and in pluripotent stem cells (Spiller et al., 2012, Vallier et al., 2009, Mesnard et al., 2006), and with the well-defined role for FGF in the derivation of pluripotent EG cells (Durcova-Hills et al., 2006).

Inhibition of MEK1/2 disrupted germ cell mitotic arrest, maintained expression of genes associated with germ cell proliferation and prevented appropriate upregulation of 686 male germline development genes. A prominent germline signature of MEK1/2 inhibition was a failure of germ cells to properly activate DNA methylation, including deficient upregulation of *de novo* DNA methylation genes and a range of genes involved in piRNA biology that are required for silencing repetitive elements. This implies that the DNA methylation pathway either responds directly to MEK1/2 signalling or is not properly activated due to the poor differentiation of XY germ cells in the absence of MEK1/2 signalling. Either way, germline DNA methylation is likely to remain low if MEK1/2 signalling is compromised in the germline, potentially allowing de-repression of repetitive elements.

DNA methylation is low and transcription of repetitive elements is increased in GCNIS and human embryonal carcinoma cells, undifferentiated cells of human germline tumours

(Oosterhuis and Looijenga, 2005). While speculative, activity of these elements could induce germline mutations through their random integration into the genome, or cause genomic instability characteristic of germline tumours. In addition, several genes associated with germline tumours, including members of the Nodal signalling pathway and genes associated with germline tumours in human GWAS and other studies, including *DMRT1*, were expressed in germ cells of MEK1/2 inhibited samples. While we did not observe increased OCT4 or SOX2 protein levels, *Nanog* transcription remained high, germ cell proliferation was sustained, and germline differentiation was inhibited in the absence of MEK1/2 signalling. Combined with low DNA methylation, these factors could render germ cells more susceptible to germline tumours, a possibility that requires further investigation.

A previous report indicated that FGF9 is required for transiently maintaining pluripotency in male germ cells and for male germ cell differentiation (Bowles et al., 2010). In this study, we observed a requirement for FGF signalling to regulate 43 genes and MEK1/2 to regulate 24 genes in E12.5 germ cells after 24h of culture. Nine genes were commonly dysregulated by FGFRi and MEKi, suggesting that these genes are regulated by FGF-MEK-ERK signalling. Consistent with a role for FGF-MEK-ERK in regulating stem cell characteristics in E12.5 germ cells, six of these genes have been associated with germ cell tumours, stem cells, cell self-renewal, cancer, cell proliferation and cell survival.

In contrast, although inhibition of FGFR for 24h from E12.5 resulted in dysregulation of 43 genes, only one gene was dysregulated after 72h of FGFRi treatment, while 1403 genes were dysregulated by MEKi. Moreover, despite inhibition of FGF signalling at E12.5, germ cells progressed normally into mitotic arrest. Together, these data indicate that although some aspects of male germ cell development were disrupted by FGFR inhibition at E12.5-E13.5, the germ cells were able to enter the male fate normally. Our observations support a role for FGF signalling in priming stemness in male germ cells but indicate that FGF signalling is dispensable for male germline differentiation. This is consistent with previous observations that FGF9 can be used for deriving EG cells (Durcova-Hills et al., 2006). While it remains possible that a low level of residual FGF activity could explain the ability of E12.5 germ cells to enter male differentiation in our study, our conclusion is consistent with the observations in the Jameson study in which male germline development occurred in fetal gonads lacking *Wnt4/Fgf9*

(Jameson et al., 2012a). Together these findings demonstrate that germ cells require MEK-ERK signalling to properly establish male identity and that this is likely to occur through a ligand that is independent of FGF9-FGFR signalling (Fig 7).

While our data strongly indicate that FGF signalling is dispensable for male germline differentiation at E12.5, FGFR inhibition at E11.5 partially disrupted germ cell mitotic arrest. There are a number of explanations for this. One possibility is that FGFR inhibition was too late to disrupt male germline development. However, this seems unlikely as FGF-MEK signalling is very rapid and MEK inhibition profoundly disrupted male germline differentiation at E12.5, whereas FGFR inhibition did not. A second possibility is that FGFR inhibition was insufficient, leaving residual FGF activity that promoted male germline development. However, FGFRi reduced Sertoli cell proliferation to a similar extent in E11.5 and E12.5 XY gonads and completely abrogated FGF9 induced somatic cell proliferation in XX gonads. Moreover, two independent, highly potent and specific FGFR inhibitors provided similar outcomes. Together these data suggest we achieved a complete block of FGF signalling. Indeed, even when E12.5 gonads were treated with 2500nM FGFRi, a dose ~2000-2500x the drug IC50 or a dose of 5000nM of SU5402, which was previously used to inhibit FGF signalling (Bowles et al., 2010), we still did not observe any impact on germ cell mitotic arrest and the impact on Sertoli cell proliferation was not augmented. Given these data, the simplest explanation is that FGF9 is dispensable for germ cell mitotic arrest and for male germline differentiation and the modest impact on germ cell mitotic arrest achieved using FGFRi at E11.5 is due to disruption of testis development rather than a direct effect on germ cells. This is consistent with Jameson and colleagues' observations in *Wnt4/Fgf9* double knockout mice in which male germ cells developed normally (Jameson et al., 2012a).

FGF9 has also been proposed to inhibit the precocious entry of XY germ cells into meiosis (Bowles et al., 2010). Experiments using heterozygous *Fgf9* mice on a *Cyp26b1* null background, urogenital ridge cultures with the VEGF / FGFR inhibitor SU5402 and germ cell cultures with FGF9 indicated that FGF9 inhibits *Stra8* expression by making germ cells less responsive to retinoic acid (Bowles et al., 2010). Given this, we expected inhibition of FGFR to result in increased *Stra8* transcription. However, *Stra8* expression was not affected by FGFR inhibition, perhaps because FGFR inhibition did not reduce *Cyp26b1* transcription (our

observations) and therefore CYP26B1 mediated degradation of RA. This is consistent with previous experiments in urogenital ridge cultures in which FGFR inhibition alone did not affect *Cyp26b1* or *Stra8* expression, but inhibition of both FGFR and CYP26B1, or CYP26B1 alone led to robust *Stra8* transcription (Bowles et al., 2010). It therefore seems likely that the primary role of FGF9 in ensuring *Stra8* repression is mediated by ensuring an appropriate testicular environment and consequent *Cyp26b1* expression, rather than a direct effect of FGF9 on *Stra8*.

Together our study reveals a novel, essential role of MEK1/2 signalling in fetal male germline development, particularly in the promotion of germ cell mitotic arrest, the expression of an appropriate male germline transcriptional program and the activation of *de novo* DNA methylation. While FGF9 may be involved in priming male germline development, it appears to be dispensable for commitment of germ cells to male differentiation. Moreover, our data suggest that unknown ligands activate MEK1/2 signalling and promote germline differentiation through an indirect mechanism, but further work is required to determine how this may occur.

Materials and Methods

Mouse strains, animal housing, breeding and ethics

Mice were housed at Monash Medical Centre Animal Facility with controlled temperature and humidity, a 12 hours (h) light-dark cycle and food and water available *ad libitum*. Mouse embryos were obtained from inbred 129T2svJ *Oct4*-eGFP males crossed with Swiss females. Females were checked daily for vaginal plugs, with detection of a plug noted as E0.5. Animal work was undertaken in accordance with Monash Medical Centre Animal Facility Animal Ethics Committee approval.

Organ culture

E11.5 gonads were genetically sexed using PCR as previously described (McFarlane et al., 2013). E12.5 and E13.5 embryos were sexed visually by the presence (male) or absence (female) of testis cords in the gonad. Gonad-mesonephros samples were cultured on 30mm

Millicell Biopore membranes with 0.4µM pores (Merck Millipore; PICM03050) in 6-well plates, with each well containing 1400µL media (15mM Hepes, 0.1mM non-essential amino acids, 1mg/mL N-acetylcysteine, 1X penicillin/streptomycin, 55nM beta-mercaptoethanol and 10% fetal calf serum in DMEM/F12 with Glutamax). PBS was placed in between the wells to maintain humidity. Gonads were cultured in media containing DMSO (vehicle control, concentration 5000nM or less as appropriate for the concentration of each drug dilution), 125, 250, 500 1000nM of BGJ398 (FGFRi; SelleckChem, HY-13241), PD0325901 (MEKi; SelleckChem, S1036), GSK1059615 (PI3Ki; MedChem Express, HY-12036), PH797804 (p38i; MedChem Express, HY-10403). Additional inhibitors for FGFR (AZD4547; SelleckChem, S2801 and SU5402; MedChem Express, HY-10407), p38 (Ralimetinib dimesylate; MedChem Express, HY-13241) and PI3K (PF-04691502; MedChem Express, HY-15177) signalling were also used to ensure consistency of outcomes with each of the primary inhibitors used. All inhibitors were selected based on their high-specificity, potency and advancement in clinical trials (Table 1). Gonad-mesonephric complexes were randomly allocated to each culture treatment condition and cultured for 24, 48, 72 or 96h in 5% CO_2 at 37°C, with media refreshed daily. To facilitate analysis of cell proliferation, 5-ethynyl-2'-deoxyuridine (EdU) was added to each sample for the final two hours of culture at a final concentration of 20µM. After culture, gonads were photographed under brightfield and fluorescence optics, then processed for flow cytometry, immunofluorescence (IF) or florescence activated cell sorting (FACS). For flow cytometric experiments in which gonad only samples were examined and all experiments involving FACS purification of germ and somatic cells, gonads were dissected away from mesonephros at the end of the culture period.

Table 1: Summary of treatments and doses used in gonad cultures

Treatment	Dose	IC50 and target	Phase in clinical trials, clinicaltrials.gov identifier	Supplier, catalogue number	References
DMSO (vehicle control)	Comparable to DMSO used in drug treatments (~1/5000)	NA	NA	Thermo-Fisher, D12345	
BGJ398/ Infigratinib (FGFRi)	125, 250, 500, 1000 and 2500 nM	IC50 – FGFR1-3: 0.9-1.4 nM, FGFR4: 60 nM	Phase II, NCT02150967	SelleckChem, S2183	(Javle et al., 2018)
PD0325901/ Mirdametinib (MEKi)	125, 250, 500 and 1000 nM	IC50 – MEK: 0.33 nM	Phase II, NCT03962543	SelleckChem or MedChem Express, S1036 or HY-10254	(Weiss et al., 2021)
PH-797804 (p38i)	500 nM	IC50 – p38α: 26 nM; p38β 102 nM	Phase II, NCT00559910	MedChem Express, HY-10403	(MacNee et al., 2013)
GSK1059615 (PI3Ki)	500 nM	IC50 – PI3Kα/β/δ/γ: 0.4-5 nM, mTOR: 12 nM	Phase I, NCT00695448	MedChem Express, HY-12036	
AZD4547 (second FGFR inhibitor)	125, 250 and 500 nM	IC50 – FGFR1-3: 0.2-2.5 nM; FGFR4: 165 nM; VEGFR2: 24 nM	Phase II, NCT02465060	SelleckChem, S2801	(Chae et al., 2020)
SU5402 (third FGFR inhibitor)	5000nM	IC50 VEGFR: 20nM; FGFR1: 30nM	N/A	MedChem Express, HY-10407	(Sun et al., 1999)
Ralimetinib dimesylate/ LY2228820 (second p38 inhibitor)	500 nM	IC50 – p38α: 5.3 nM; p38β: 5.3 nM	Phase I, NCT01663857	MedChem Express, HY-13241	(Patnaik et al., 2016)
PF-04691502 (second PI3K inhibitor)	500 nM	IC50 – PI3Kα/β/δ/γ: 1.6-2.1 nM; mTOR: 16 nM	Phase II, NCT01420081	MedChem Express, HY-15177	(del Campo et al., 2016)

Flow cytometry

Gonad collection, dissociation, fixation, staining and flow cytometry were performed essentially as described previously (Wakeling et al., 2013), using eGFP or antibodies specific for MVH, DPPA4, SOX9, DNMT3L, STRA8 or H2AX. Mesonephros or limb samples were used as germ cell negative controls to set gates for eGFP, MVH or DPPA4 and E12.5 female gonads were used as a negative control to set gates for SOX9 and DNMT3L. Cultured male gonads were used as a negative control to set gates for STRA8 and H2AX. Representative gating and negative control gates can be found in S1. A rabbit IgG antibody was used as a negative control for determining staining intensities using specific rabbit antibodies. Primary antibodies used are listed in Table 2. Secondary antibodies used include Alexa Fluor Donkey anti Goat 488 (Thermo-Fisher, A11055), Alexa Fluor Donkey anti Goat 647 (Thermo-Fisher, A31573), Biotin Donkey anti Rabbit (Thermo-Fisher, A16027) and Biotin Donkey anti Goat (Thermo-Fisher, A16009). Cell cycle analysis was performed as previously described (Wakeling et al., 2013), with germ cells or Sertoli cells identified by their expression of MVH or SOX9, respectively. Cells were stained with 20µg/mL of propidium iodide, enabling quantitation of cellular DNA content. Proliferation was measured by gating EdU positive cells to identify cells in S-phase, while cells in G0/G1 or G2/M were respectively identified by DNA contents estimated as 2n or 4n in the EdU negative population compared to DMSO controls. All flow cytometry was performed on a BD FACS Canto II analyser (BD, Biosciences) and data analysed with FlowJo (v10.7.2) and Graphpad Prism (v9.2.0). Relative antibody staining intensities were calculated by removing background staining in negative control samples and normalising levels to the XY DMSO control set at 1.0. Data represents 3-16 biological replicates and statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons or unpaired two-tailed t-test, where appropriate. If variance was unequal, a non-parametric Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons was used. *P* values <0.05 were considered significant.

Tissue fixation, embedding, immunofluorescence and image analysis

Gonads were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Samples were washed three times in PBS before 70% ethanol processing, and embedded in paraffin. 4µm sections were cut in a compound series (typically four slides prepared per gonad sample with around 6 sections collected/slide), mounted on Superfrost Plus slides and dried at least

overnight before antibody incubation. Antigen retrieval was conducted using Dako Citrate buffer (pH 6.0) for 30 minutes at 98°C in a PT Link rinse station (Dako). Tissue sections were blocked in PBTx containing 5% BSA (Merck, A9647) and 10% donkey serum (Sigma; D9663) for 1 hour at RT. Primary antibody (Table 2) diluted in PBTx containing 1% BSA was left to incubate overnight at 4°C or for 2h room temperature (RT). Slides were incubated for 1h at RT in the dark in secondary antibody (Alexa Fluor, Thermo-Fisher, Donkey anti Goat 488 A11055 or Donkey anti Rabbit 488 A21206; Donkey anti Rabbit 647 A31573 or Donkey anti Mouse 647 A31571; Donkey anti Mouse 594 A21203; Donkey anti Goat 594 A11058 or Donkey anti Mouse 555 A31570) diluted at 1/300 in PBTx containing 1% BSA. Slides were mounted in ProLong Gold containing DAPI (Thermo-Fisher, P36931). Confocal images were taken using a Nikon C1 Confocal microscope, with images taken using either 10x lens or 40x oil immersion lens, or slides were scanned using a VS120 Virtual Slide microscope (Olympus), collecting single optical sections of the whole area for each section on the slide. Image analysis was conducted with QuPath (v0.3.0) (Bankhead et al., 2017). Data from at least three representative tissue sections from four biological replicates were averaged. Relative antibody staining intensity was calculated by normalising levels to the XY DMSO control set at 1.0. Data represents four biological replicates and statistical significance was determined with Graphpad Prism (v9.2.0) using one-way ANOVA with Tukey's multiple comparisons. If variance was unequal, a non-parametric Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons was used. *P* values <0.05 were considered significant.

Table 2: Antibodies for flow cytometry (F) and immunofluorescence (IF)

Protein	**Source and catalogue #**	**Species**	**Dilution**
Mouse Vasa Homologue (MVH)	R&D Systems, AF2030	Goat	F: 1/100 IF: 1/400
DPPA4	R&D Systems, AF3730	Goat	F: 1/100 IF: 1/400
OCT4	Santa Cruz, sc8628	Goat	IF: 1/400
MVH	Cell Signalling Technology, 8761S	Rabbit	IF: 1/400
SOX9	Sigma-Aldrich, AB5535	Rabbit	F: 1/200 IF: 1/1000
DNMT3L	Cell Signalling Technology, 13451S	Rabbit	F: 1/100

			IF: 1/100
STRA8	Abcam, ab49405	Rabbit	F: 1/500 IF: 1/400
Phospho-γH2AX	Cell Signalling Technology, 9718S	Rabbit	F: 1/100 IF: 1/800
IgG	Cell Signalling Technology, 3900S	Rabbit	
Laminin	R&D Systems, L9393	Rabbit	IF: 1/200
PIWIL2	Cell Signalling Technology, 5940S	Rabbit	IF: 1/200
SYCP3	Abcam, ab97672	Mouse	IF:1/200
Smooth Muscle Actin (SMA)	Sigma-Aldrich, A2547	Mouse	IF: 1/1000
NR2F2	R&D Systems, PP-H7147-00	Mouse	IF: 1/400
SOX2	Cell Signalling Technologies, 4900S	Mouse	IF: 1/100

Fluorescent activated cell sorting (FACS) of germ and somatic cells

After organ culture, the mesonephros was dissected from the gonads. Six to 15 gonads were pooled for each sample and were dissociated in trypsin. Germ and somatic cell populations were isolated as previously described (Western et al., 2008, Wakeling et al., 2013) using the BD FACSAria™ Fusion cell sorter. GFP positive germ and GFP negative somatic cell populations were isolated from E12.5 XX and XY control gonads cultured for 24 or 72h in DMSO or XY E12.5 gonads cultured for 24 or 72h in DMSO, FGFRi or MEKi ($n \geq 4$ for each treatment/group). Germ cells were defined as GFP positive, with dead propidium iodide positive germ cells excluded.

RNA-seq library construction and sequencing

RNA was isolated from 3-45 x 10^4 FACS-sorted germ cells using Macherey-Nagel NucleoSpin® RNA XS extraction kit (Scientifix, 740902.50) following manufacturer instructions. RNA quantity and RNA integrity (RIN) were assessed using Qubit and Bioanalyzer (Agilent Technologies). Libraries were prepared with 30ng of RNA from germ cells with RIN values greater than 7. The library was constructed as previously described by the MHTP Medical Genomics Facility (Grubman et al., 2021). Briefly, during initial poly(A) tail priming, an 8bp sample index along with a 10bp unique molecular identifier (UMI) was added. Samples were pooled and amplified with a template switching oligonucleotide. The Illumina P5 and P7 were added by PCR and Nextera transposase, respectively. The forward read (R1) utilises a custom

primer to sequence into the index while the reverse read (R2) uses a standard R2 primer to sequence the cDNA in the sense direction. I7 indexes were added to enable parsing of sample sets. Sequencing was performed on NextSeq2000 (Illumina) following Illumina protocol 1000000109376 v3.

Data pre-processing

FASTQ files were demultiplexed and mapped using scPipe (Tian et al., 2018) and Rsubread (Liao et al., 2019) (Bioconductor) packages in R studio. Briefly, FASTQ files were reformatted with *sc_trim_barcode* to incorporate barcode information from read 1 into read 2 into the header. Reads were aligned to a reference mouse genome (GENECODE GRCm39) using Rsubread. Reads were assigned to annotated exons with sc*_exon_mapping*, data were demultiplexed using *sc_demultiplex* and a gene count matrix was generated with UMI deduplication using *sc_gene_counting*. Gene count matrices from each set were combined into a DGEList object for analysis.

Downstream analysis of RNA-sequencing data

Differential gene expression was assessed using the Limma (Ritchie et al., 2015), Glimma (Su et al., 2017) and edgeR (Robinson et al., 2009) Bioconductor packages following a previously established workflow (Law et al., 2016). Briefly, gene count data was loaded into R studio and genes were annotated with any duplicates removed. Raw counts were transformed into counts per million (CPM). Lowly expressed genes were removed using the *filterByExpr* function in edgeR, and gene expression distributions were normalised using trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010). Multidimensional scaling (MDS) plots were generated to visualise sample clustering. Since there were many samples present, groups were subdivided into smaller groups to visualise clustering between different culture treatments and culture periods more clearly. Heteroscedasticity was removed from the count data with *voomWithQualityWeights* (Liu et al., 2015). Linear modelling and empirical Bayes moderations was used to test for differential expression. As there were many differentially expressed genes (DEGs) identified following empirical Bayes moderations, to identify DEGs of biological relevance, a $\log_2$fold-change cut-off was set at greater/less than 0.585 (equivalent to a FC of 1.5) using *treat* (McCarthy and Smyth, 2009). Genes were considered differentially expressed if they met the logFC cut-off and had a false discovery rate (FDR) adjusted p-value

less than 0.05. DEGs identified in XY E12.5+24h FGFRi vs XY E12.5+24h DMSO (24h FGFRi DEGs) and XY E12.5+24h MEKi vs XY E12.5+24h DMSO (24h MEKi DEGs) were compared, with genes commonly affected by FGFRi and MEKi assessed using Roast (Wu et al., 2010). Heatmaps were generated using ClustVis (Metsalu and Vilo, 2015).

Declarations

Competing interests:

The authors declare that they have no competing interests that affect this work

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Author contributions:

Conceived and designed experiments: ROB, PSW

Performed experiments and/or analysed data: ROB, EGJ, PSW

Bioinformatic analyses: ROB, RSL

Writing - original draft: ROB, PSW

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All authors critically read and approved the manuscript.

Ethics approval and consent to participate:

All animal work was undertaken in accordance with Monash University Animal Ethics Committee (AEC) approvals.

Data and materials availability: With exception of the RNA sequencing data generated in this study, all data are available in the main text and the Supplementary Materials. The RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) and are publicly available via accession number GSE221453.

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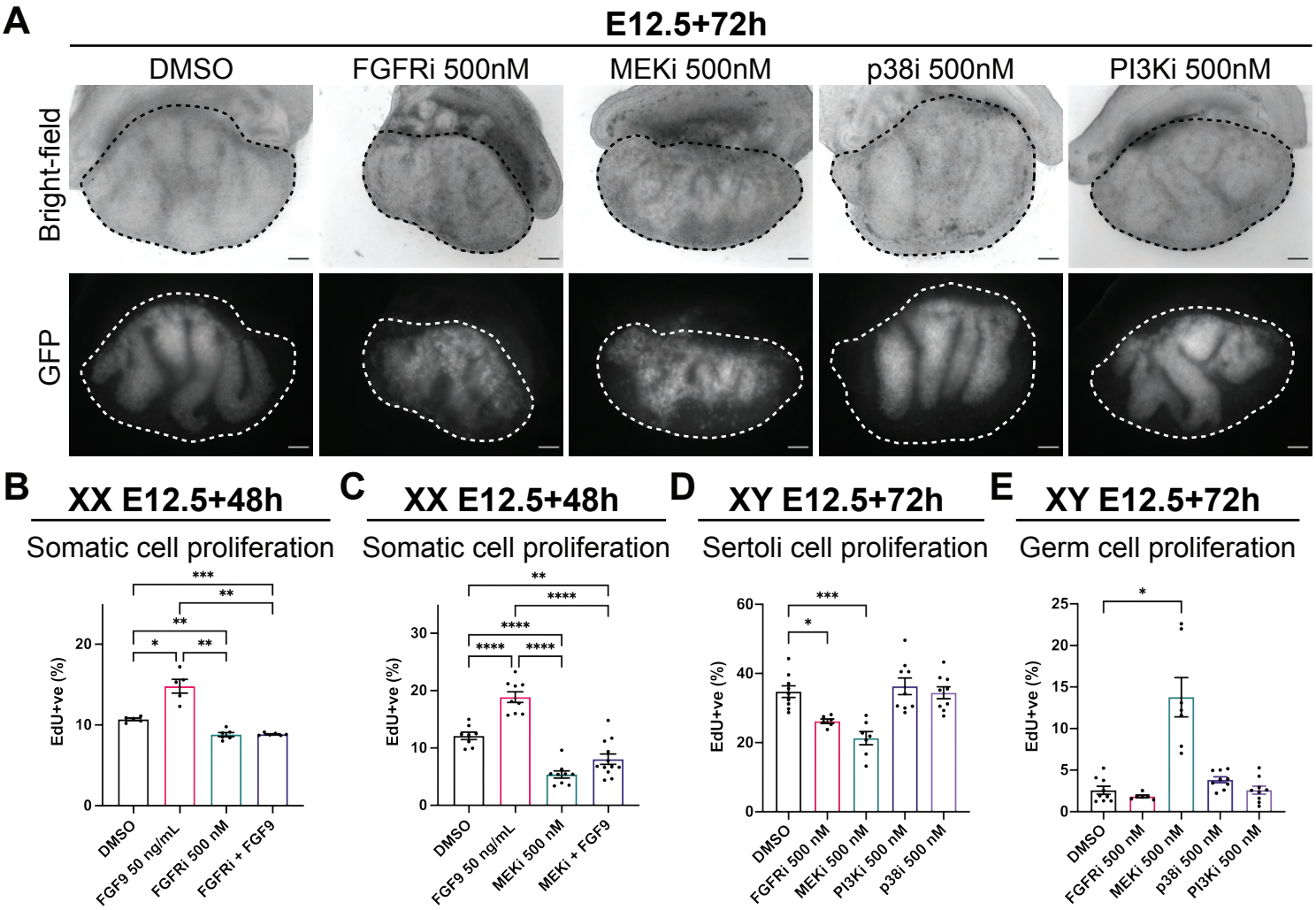

A
E12.5+72h
DMSO
FGFRi 500nM
MEKi 500nM
p38i 500nM
PI3Ki 500nM
Bright-field
GFP
B
XX E12.5+48h
Somatic cell proliferation
EdU+ve (%)
DMSO
FGF9 50 ng/mL
FGFRi 500 nM
FGFRi + FGF9
C
XX E12.5+48h
Somatic cell proliferation
EdU+ve (%)
DMSO
FGF9 50 ng/mL
MEKi 500 nM
MEKi + FGF9
D
XY E12.5+72h
Sertoli cell proliferation
EdU+ve (%)
DMSO
FGFRi 500 nM
MEKi 500 nM
PI3Ki 500 nM
p38i 500 nM
E
XY E12.5+72h
Germ cell proliferation
EdU+ve (%)
DMSO
FGFRi 500 nM
MEKi 500 nM
p38i 500 nM
PI3Ki 500 nM

Figure 1

Figure 1: FGF and MEK1/2 signalling inhibition disrupts fetal testis development. **A)** Bright-field and GFP images of E12.5 XY gonad-mesonephros tissue cultured for 72h with DMSO, or 500nM of FGFRi, MEKi, p38i or PI3Ki. Scale bar: 100µm. Dotted lines highlight the gonad. **B-C)** Flow cytometric analysis of gonadal somatic cell proliferation based on EdU incorporation in XX E12.5 gonads/mesonephros tissue cultured for 48h with DMSO, FGF9 (50ng/mL), 500nM FGFRi (B), FGF9+FGFRi (B), 500nM MEKi (C) or FGF9+MEKi (C). **D-E)** Flow cytometric analysis of Sertoli (D) or germ (E) cell proliferation based on EdU incorporation in XY E12.5 gonads/mesonephros tissue cultured for 72h with DMSO or 500nM of FGFRi, MEKi, p38i or PI3Ki. Replicates: n≥5; Statistics: B&E; Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons. C&D; Ordinary One-way ANOVA with Tukey's multiple comparisons; Error bars: Mean±SEM; Significance between treatments and controls; *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

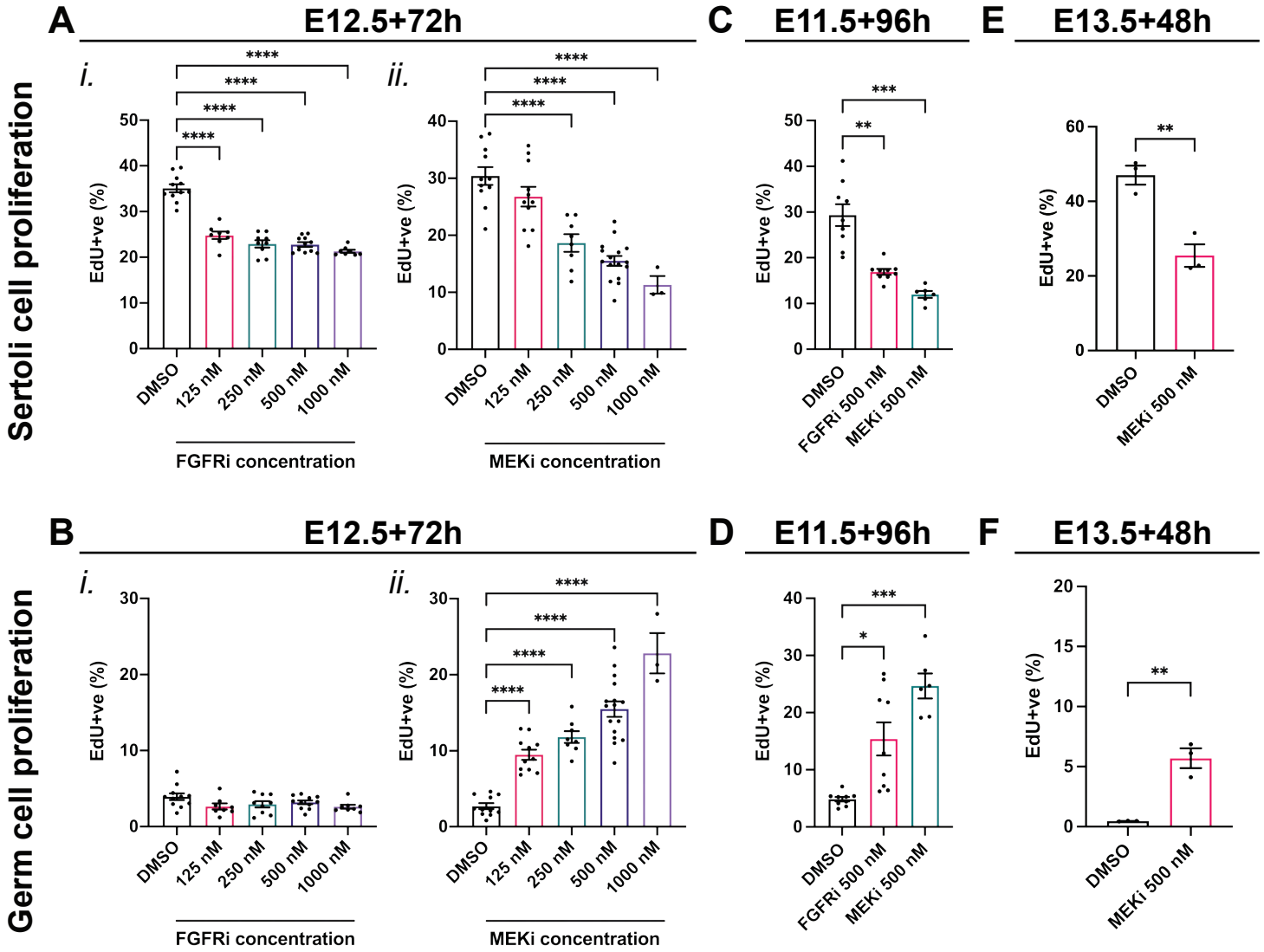

A
E12.5+72h
C
E11.5+96h
E
E13.5+48h
Sertoli cell proliferation
i.
ii.
EdU+ve (%)
DMSO
125 nM
250 nM
500 nM
1000 nM
FGFRi concentration
MEKi concentration
FGFRi 500 nM
MEKi 500 nM
B
D
F
Germ cell proliferation

Figure 2

Figure 2: FGF and MEK1/2 signalling inhibition reduce Sertoli cell proliferation, but only MEK1/2 inhibition disrupts germ cell mitotic arrest. A-B) Flow cytometric analysis of XY E12.5 gonad-mesonephros tissue cultured in DMSO, 125, 250, 500 or 1000nM of FGFRi or MEKi for 72h showing the percentage EdU incorporation in Sertoli (A) or germ (B) cells. **C-D)** Percentage EdU incorporation in Sertoli (C) or germ (D) cells from E11.5 XY gonad-mesonephros tissue cultured in DMSO or 500nM of FGFRi or MEKi for 96h. **E-F)** Percentage EdU incorporation in Sertoli (E) or germ (F) cells from E13.5 XY gonad-mesonephros tissue cultured in DMSO or 500nM MEKi for 48h. Replicates: n≥3. Statistics: A&B; Ordinary One-way ANOVA with Tukey's multiple comparisons. C&D; Brown-Forsythe and Welch ANOVA with Dunnett's T3 multiple comparisons. E&F; Unpaired two-tailed t-test. Error bars: mean±SEM. Significance between treatments and controls: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

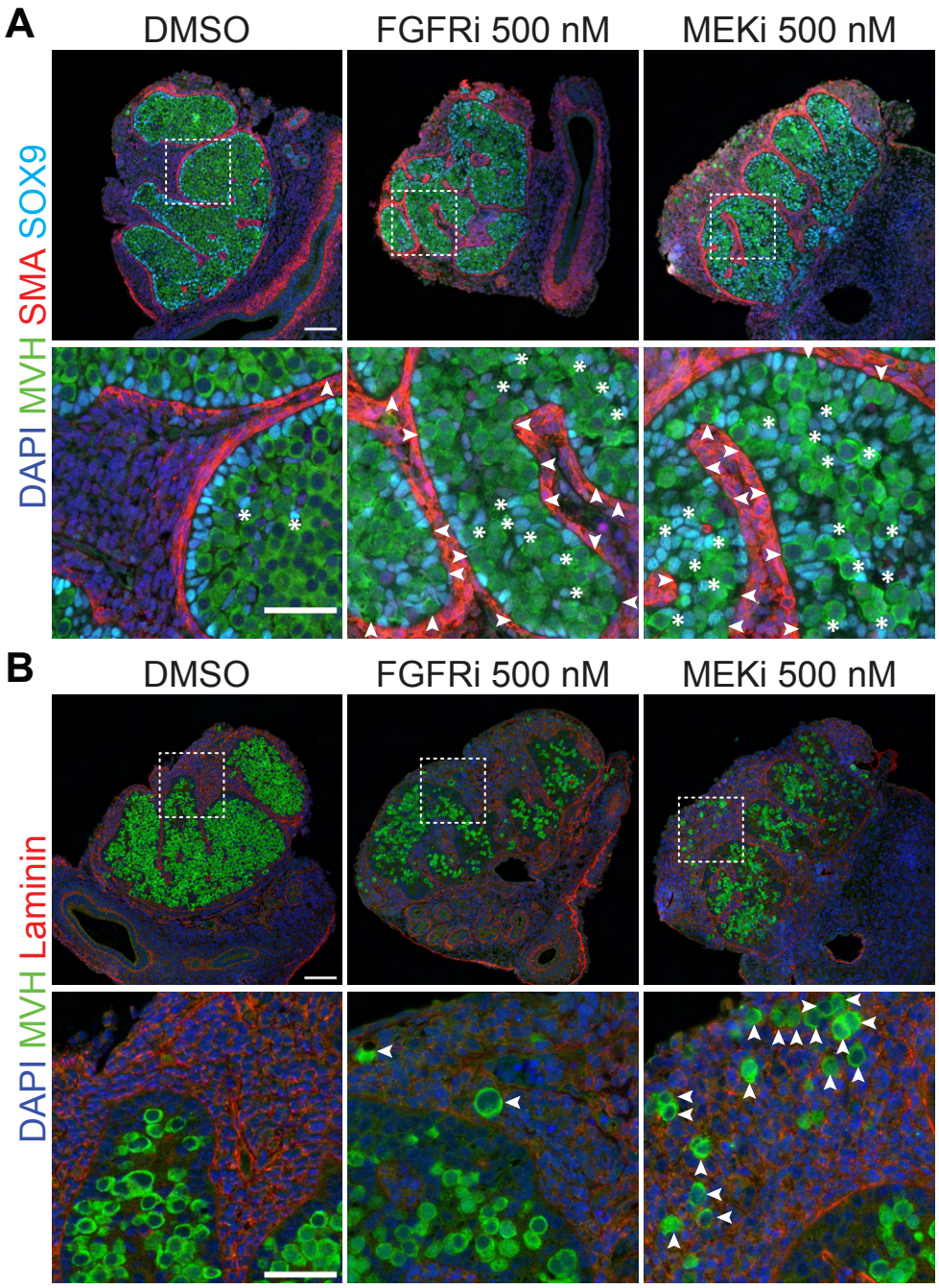

A
DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH SMA SOX9
B
DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH Laminin

Figure 3

Figure 3: FGF and MEK1/2 signalling is required for organisation of Sertoli cells within testis cords. Immunofluorescent images of XY E12.5 gonad-mesonephros tissue cultured with DMSO, 500nM FGFRi or MEKi for 72h demonstrating Sertoli (A) and germ (A and B) cell localisation. DAPI (blue; A&B), MVH (green; A&B), SMA (red; A), SOX9 (cyan; A) and Laminin (red; B). Scale bars: top panel 100µm, bottom panel 50µm. Replicates: n≥3. In A: White arrows identify gaps in the Sertoli cell layer at the testis cord basement; white asterisks identify Sertoli cells dispersed within the inner area of the testis cords. In B: White arrows identify germ cells localised outside of the testis cords.

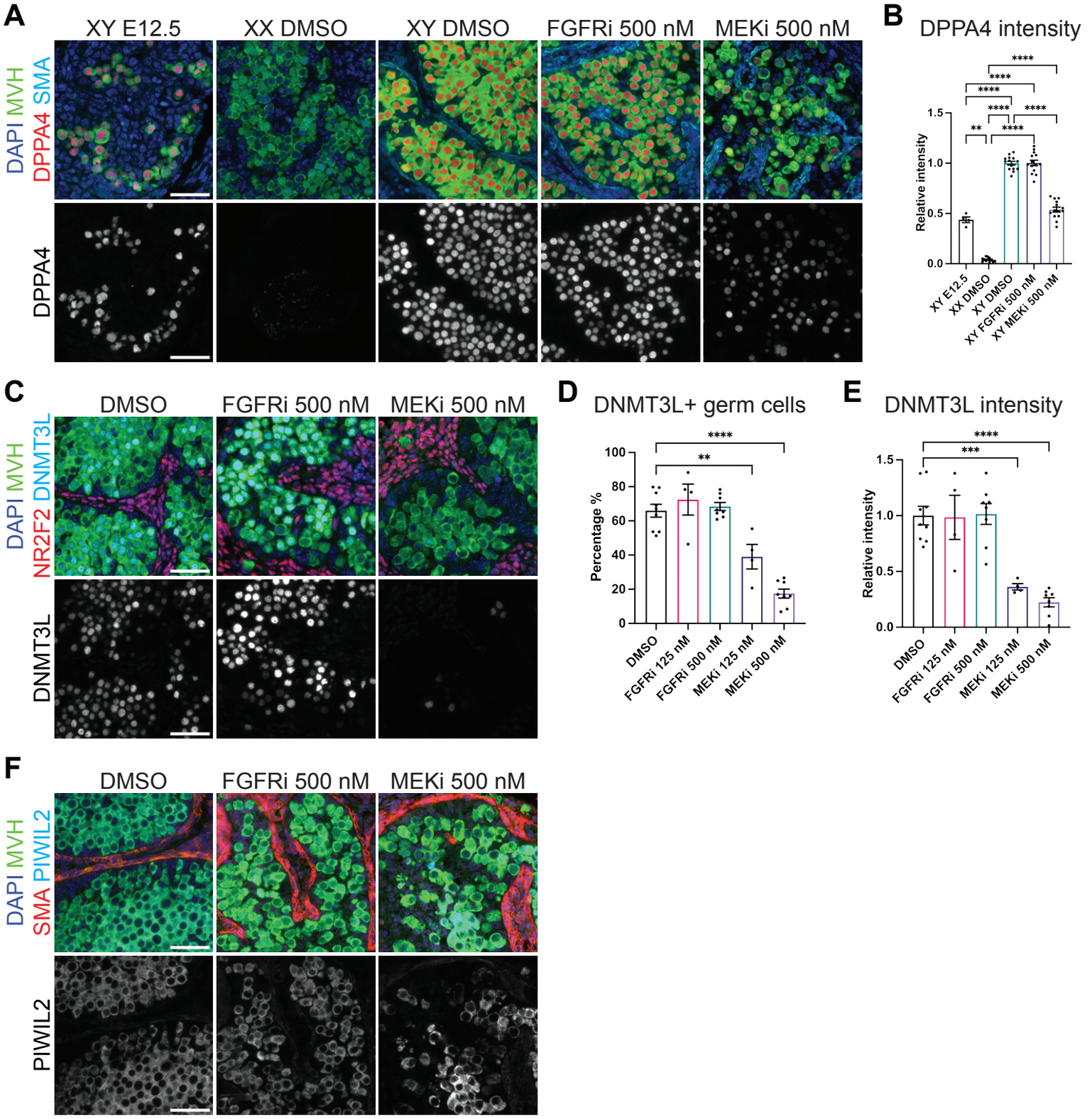

A
XY E12.5
XX DMSO
XY DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH
DPPA4 SMA
DPPA4
B
DPPA4 intensity
Relative intensity
XY E12.5
XX DMSO
XY DMSO
XY FGFRi 500 nM
XY MEKi 500 nM
C
DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH
NR2F2 DNMT3L
DNMT3L
D
DNMT3L+ germ cells
Percentage %
DMSO
FGFRi 125 nM
FGFRi 500 nM
MEKi 125 nM
MEKi 500 nM
E
DNMT3L intensity
Relative intensity
F
DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH
SMA PIWIL2
PIWIL2

Figure 4

Figure 4: MEK1/2 signalling is required for male germline differentiation. Analysis of XY E12.5 gonad-mesonephros tissue or XY or XX E12.5 gonad-mesonephros tissue cultured for 72h with DMSO or 125 or 500nM of FGFRi or MEKi. **A)** Immunofluorescent images demonstrating DPPA4 localisation. Top panel: DAPI (blue), MVH (green), DPPA4 (red) and SMA (cyan). Bottom panel: DPPA4 (white). **B)** DPPA4 staining intensity in germ cells determined by flow cytometry. **C)** Immunofluorescent images demonstrating DNMT3L localisation. Top panel: DAPI (blue), MVH (green), NR2F2 (red) and DNMT3L (cyan). Bottom panel: DNMT3L (white). **D-E)** Percentage of DNMT3L positive germ cells (D) and DNMT3L staining intensity (E) determined by flow cytometry. **F)** Immunofluorescent images demonstrating PIWIL2 localisation. Top panel: DAPI (blue), MVH (green), SMA (red) and PIWIL2 (cyan). Bottom panel: PIWIL2 (white). For A, C&F; Replicates: n≥3. Scale bar: 50µm. For B, D&E; Replicates: n≥3. Statistics: In B; Brown-Forsythe and Welch ANOVA with Dunnett's multiple comparisons. In D&E; Ordinary One-way ANOVA with Tukey's multiple comparisons. In B&E; Intensity is relative to E12.5+72h XY DMSO control sample set at 1.0. Error bars: mean±SEM. Significance between treatments and controls: **P<0.01, ***P<0.001, ****P<0.0001.

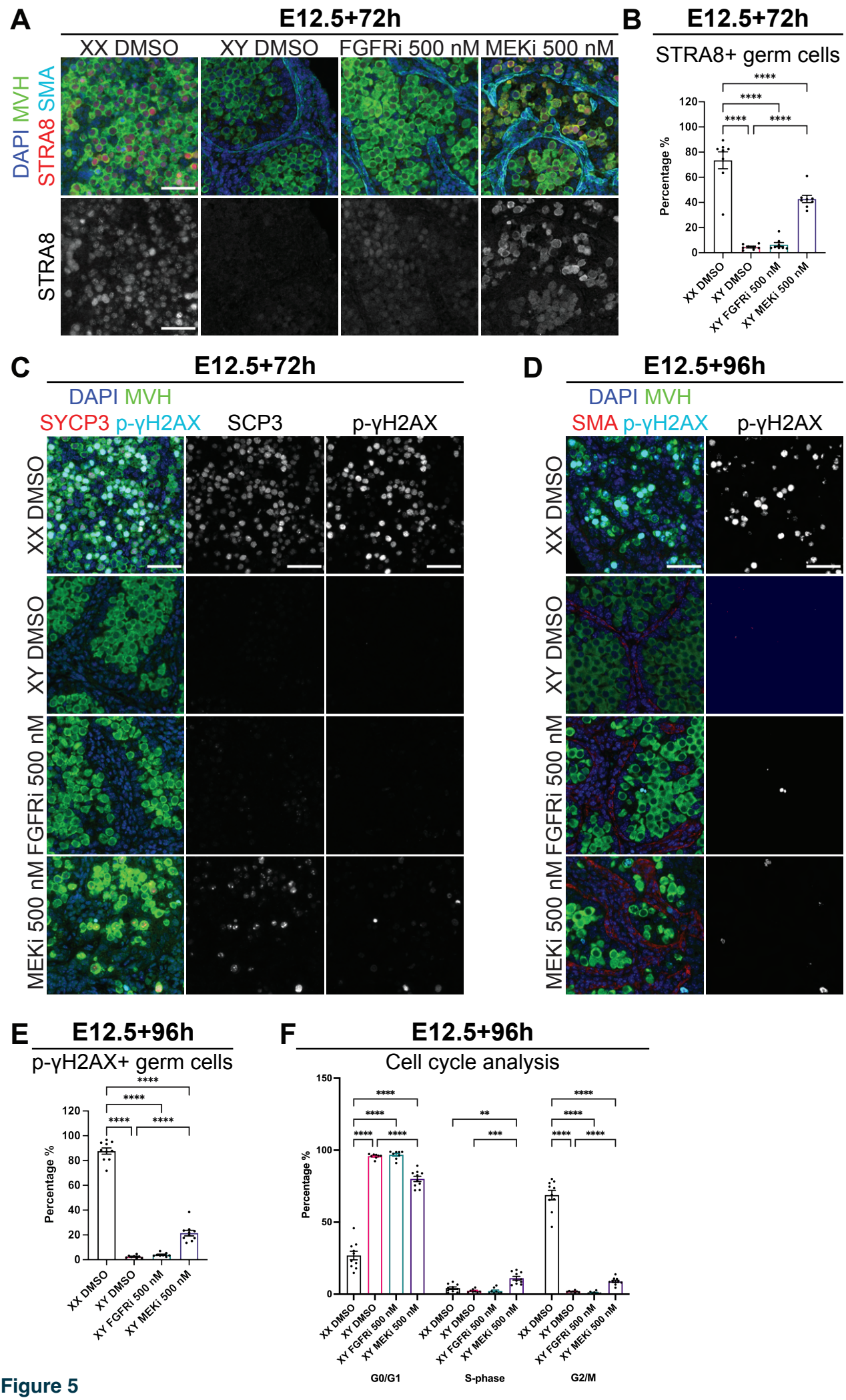

A
E12.5+72h
XX DMSO
XY DMSO
FGFRi 500 nM
MEKi 500 nM
DAPI MVH
STRA8 SMA
STRA8
B
E12.5+72h
STRA8+ germ cells
Percentage %
XX DMSO
XY DMSO
XY FGFRi 500 nM
XY MEKi 500 nM
C
E12.5+72h
DAPI MVH
SYCP3 p-γH2AX
SCP3
p-γH2AX
D
E12.5+96h
DAPI MVH
SMA p-γH2AX
p-γH2AX
E
E12.5+96h
p-γH2AX+ germ cells
F
E12.5+96h
Cell cycle analysis
G0/G1
S-phase
G2/M

Figure 5

Figure 5: MEK1/2 signalling inhibition permitted *Stra8* expression but failed to effectively induce meiosis in XY germ cells. Analysis of XY or XX E12.5 gonad-mesonephros tissue cultured with DMSO or 500nM of FGFRi or MEKi for 72h (A-C) or 96h (D-F). **A)** Immunofluorescent images demonstrating STRA8 localisation. Top panel: DAPI (blue), MVH (green), STRA8 (red) and SMA (cyan). Bottom panel: STRA8 (white). **B)** Percentage of STRA8 positive germ determined by flow cytometry. **C-D)** Immunofluorescent images demonstrating SYCP3 (C) and phospho-γH2AX (p-γH2AX) localisation. Left panel: DAPI (blue), MVH (green), SYCP3 (red; C), SMA (red; D) and p-γH2AX (cyan). Middle panel: SCP3 (white; C). Right panel: p-γH2AX (white). **E)** Percentage of p-γH2AX positive germ cells determined by flow cytometry. **F)** Cell cycle analysis of G0/G1, S-phase and G2/M based on the incorporation of EdU (S-phase) and propidium iodide (G0/G1 and G2/M) determined by flow cytometry. In A, C&D; scale bar: 50µm. Replicates: n≥3. In B, E&F; Replicates: n≥8. Statistics: In B; Ordinary One-way ANOVA with Tukey's multiple comparisons. In E; Brown-Forsythe and Welch ANOVA with Dunnett's multiple comparisons. In F; Repeated measure two-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons. Error bars: mean±SEM. Significance between treatments and controls: **P<0.05, ***P<0.005, ****P<0.0001.

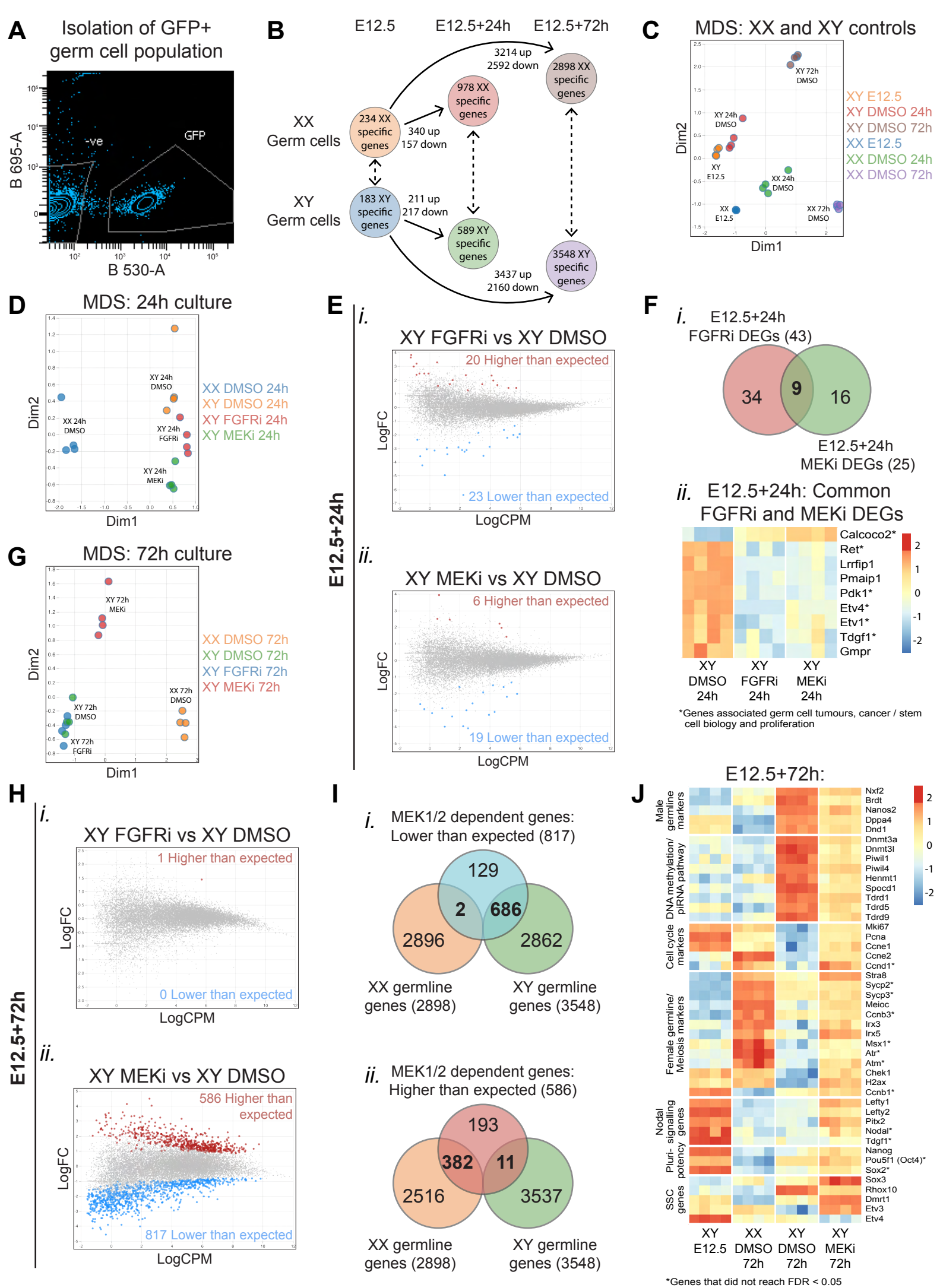

A
Isolation of GFP+ germ cell population
B 695-A
B 530-A
-ve
GFP
B
E12.5
E12.5+24h
E12.5+72h
XX Germ cells
XY Germ cells
234 XX specific genes
978 XX specific genes
2898 XX specific genes
183 XY specific genes
589 XY specific genes
3548 XY specific genes
3214 up
2592 down
340 up
157 down
211 up
217 down
3437 up
2160 down
C
MDS: XX and XY controls
Dim1
Dim2
XY E12.5
XY DMSO 24h
XY DMSO 72h
XX E12.5
XX DMSO 24h
XX DMSO 72h
D
MDS: 24h culture
XX DMSO 24h
XY DMSO 24h
XY FGFRi 24h
XY MEKi 24h
E
E12.5+24h
i.
XY FGFRi vs XY DMSO
20 Higher than expected
23 Lower than expected
LogFC
LogCPM
ii.
XY MEKi vs XY DMSO
6 Higher than expected
19 Lower than expected
F
i.
E12.5+24h FGFRi DEGs (43)
34
9
16
E12.5+24h MEKi DEGs (25)
ii.
E12.5+24h: Common FGFRi and MEKi DEGs
Calcoco2*
Ret*
Lrrfip1
Pmaip1
Pdk1*
Etv4*
Etv1*
Tdgf1*
Gmpr
XY DMSO 24h
XY FGFRi 24h
XY MEKi 24h
*Genes associated germ cell tumours, cancer / stem cell biology and proliferation
G
MDS: 72h culture
XX DMSO 72h
XY DMSO 72h
XY FGFRi 72h
XY MEKi 72h
H
E12.5+72h
i.
XY FGFRi vs XY DMSO
1 Higher than expected
0 Lower than expected
ii.
XY MEKi vs XY DMSO
586 Higher than expected
817 Lower than expected
I
i.
MEK1/2 dependent genes: Lower than expected (817)
129
2
686
2896
2862
XX germline genes (2898)
XY germline genes (3548)
ii.
MEK1/2 dependent genes: Higher than expected (586)
193
382
11
2516
3537
XX germline genes (2898)
XY germline genes (3548)
J
E12.5+72h:
Male germline markers
DNA methylation/ piRNA pathway
Cell cycle markers
Female germline/ Meiosis markers
Nodal signalling genes
Pluri-potency
SSC genes
Nxf2
Brdt
Nanos2
Dppa4
Dnd1
Dnmt3a
Dnmt3l
Piwil1
Piwil4
Henmt1
Spocd1
Tdrd1
Tdrd5
Tdrd9
Mki67
Pcna
Ccne1
Ccne2
Ccnd1*
Stra8
Sycp2*
Sycp3*
Meioc
Ccnb3*
Irx3
Irx5
Msx1*
Atr*
Atm*
Chek1
H2ax
Ccnb1*
Lefty1
Lefty2
Pitx2
Nodal*
Tdgf1*
Nanog
Pou5f1 (Oct4)*
Sox2*
Sox3
Rhox10
Dmrt1
Etv3
Etv4
XY E12.5
XX DMSO 72h
XY DMSO 72h
XY MEKi 72h
*Genes that did not reach FDR < 0.05 in XY E12.5+72h MEKi v XY E12.5+72h DMSO but are of interest

Figure 6

Figure 6: FGF-MEK signalling supports expression of genes associated with stem cells in early germ cells, but only MEK1/2 signalling is required for male germline differentiation. RNA sequencing analysis of germ cells from XX or XY E12.5 gonads, or XX or XY E12.5 gonads cultured for 24 or 72h with DMSO or 500nM of FGFRi or MEKi. **A)** Example of flow cytometric scatterplot depicting the isolation of the GFP positive germ cells via FACS. **B)** The number of differentially expressed genes (DEGs) identified in comparisons made between XX or XY E12.5 (time 0) and XX or XY DMSO controls from 24h and 72h cultures. **C)** Multidimensional scaling (MDS) of all control conditions. **D)** MDS of XX and XY gonads cultured for 24h. **E)** Differential gene expression analysis of XY E12.5+24h FGFRi XY (i) or XY E12.5+24h MEKi (ii) vs XY E12.5+24h DMSO. **F)** Venn-diagram of 24h FGFRi and MEKi DEGs (i) and heatmap of common DEGs (ii). Asterisks represents genes which are associated with germ cell tumours, cancer/stem cell biology and/or proliferation. **G)** MDS of XX and XY gonads cultured for 72h. **H)** Differential gene expression analysis of XY E12.5+72h FGFRi (i) or XY E12.5+72h MEKi (ii) vs XY E12.5+72h DMSO. **I)** Venn-diagram comparing MEKi 72h culture DEGs that are expressed lower (i) or higher (ii) than expected with XX or XY specific genes identified in B. **J)** Heatmap of DEGs identified in XY E12.5+72h MEKi v XY E12.5+72h DMSO associated with male germline differentiation, DNA methylation/piRNA pathway, the cell cycle, female germline differentiation/meiosis, Nodal signalling, pluripotency and spermatogonial stem cells (SSCs). Asterisks represents genes which are not differentially expressed but are of interest. For all comparisons, genes with an FDR <0.05 and |logFC| >0.585 (equivalent to |FC| >1.5) were considered differentially expressed.

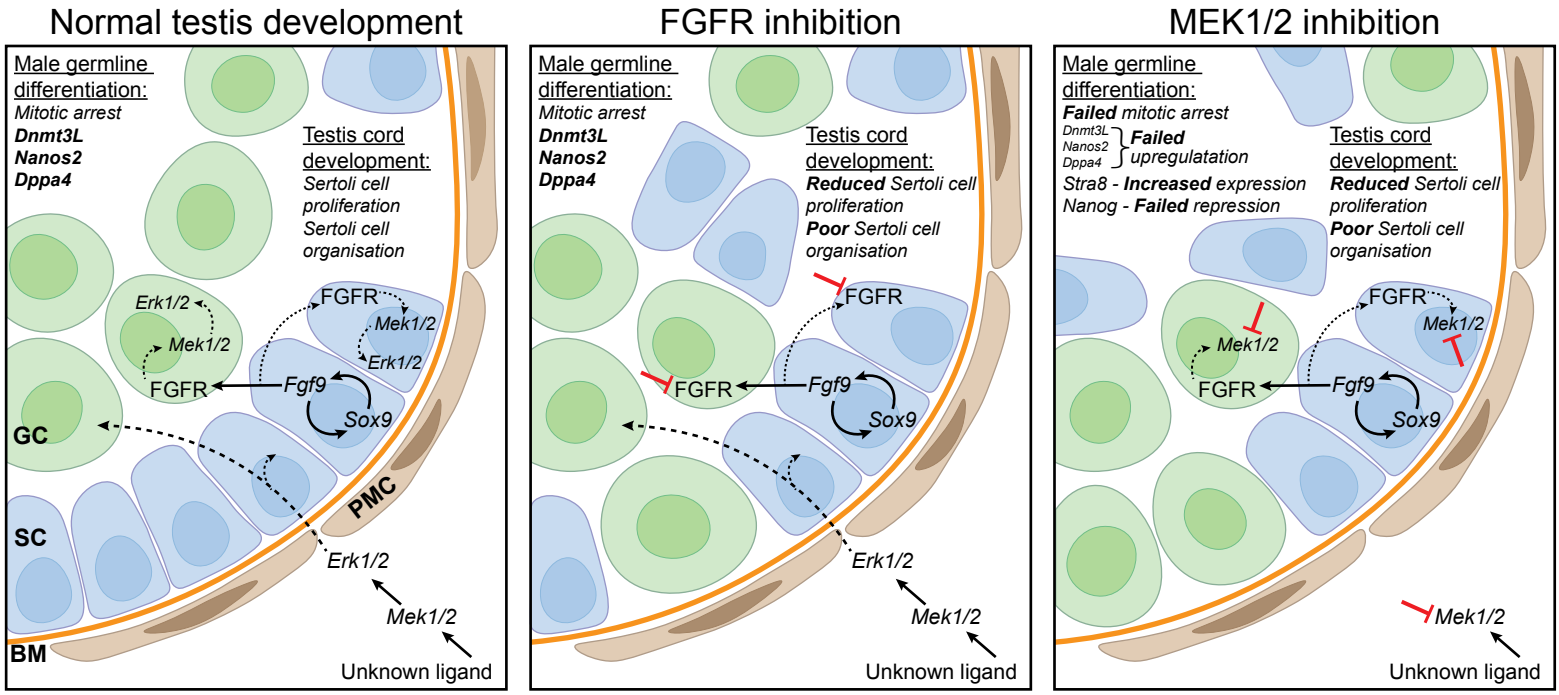

Normal testis development
Male germline differentiation:
Mitotic arrest
Dnmt3L
Nanos2
Dppa4
Testis cord development:
Sertoli cell proliferation
Sertoli cell organisation
Erk1/2
Mek1/2
FGFR
Mek1/2
Erk1/2
FGFR
Fgf9
Sox9
GC
SC
PMC
Erk1/2
Mek1/2
BM
Unknown ligand
FGFR inhibition
Male germline differentiation:
Mitotic arrest
Dnmt3L
Nanos2
Dppa4
Testis cord development:
Reduced Sertoli cell proliferation
Poor Sertoli cell organisation
FGFR
FGFR
Fgf9
Sox9
Erk1/2
Mek1/2
Unknown ligand
MEK1/2 inhibition
Male germline differentiation:
Failed mitotic arrest
Dnmt3L
Nanos2
Dppa4
Failed upregulatation
Stra8 - Increased expression
Nanog - Failed repression
Testis cord development:
Reduced Sertoli cell proliferation
Poor Sertoli cell organisation
Mek1/2
FGFR
Mek1/2
FGFR
Fgf9
Sox9
Mek1/2
Unknown ligand

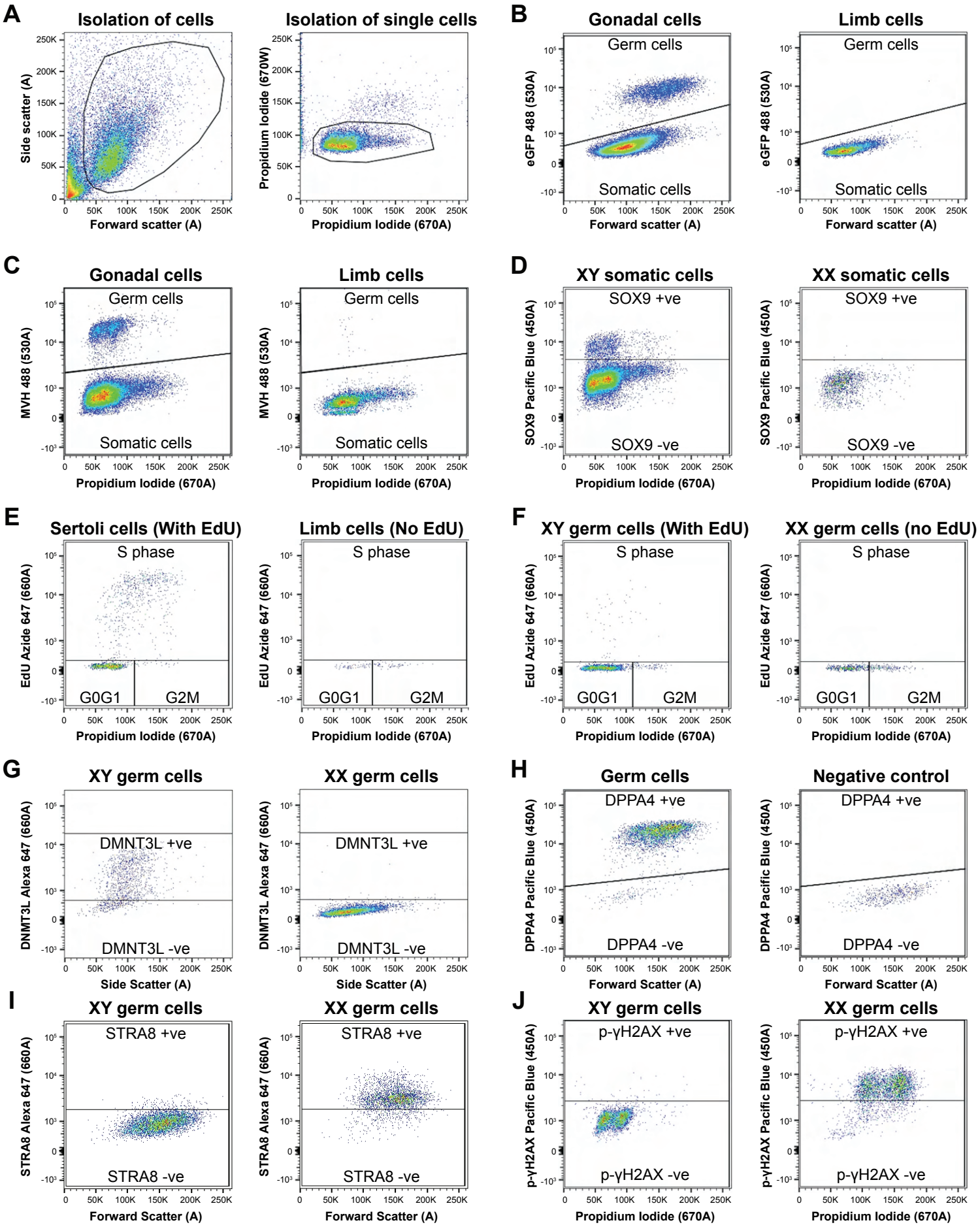
Figure 7

Figure 7: MEK1/2 and FGF signalling in male germline development. MEK1/2 signalling inhibition results in failed germ cell mitotic arrest, failed upregulation of male germline markers including *Nanos2,* DPPA4 and DNMT3L and aberrant expression of some female germline markers including STRA8 and aberrant expression of the pluripotency marker, *Nanog.* We propose that an unknown ligand activates the MEK1/2 signalling cascade which promotes key aspects of male germline differentiation including germ cell mitotic arrest and the expression of male germline and DNA methylation markers such as *Nanos2, Dppa4* and *Dnmt3L.* MEK1/2 signalling is also essential for aspects of testis cord development, including Sertoli cell proliferation and organisation. FGFR inhibition does not disrupt male germline differentiation, but results in poor Sertoli cell organisation and reduced Sertoli cell proliferation.

Supplementary Tables and Figures

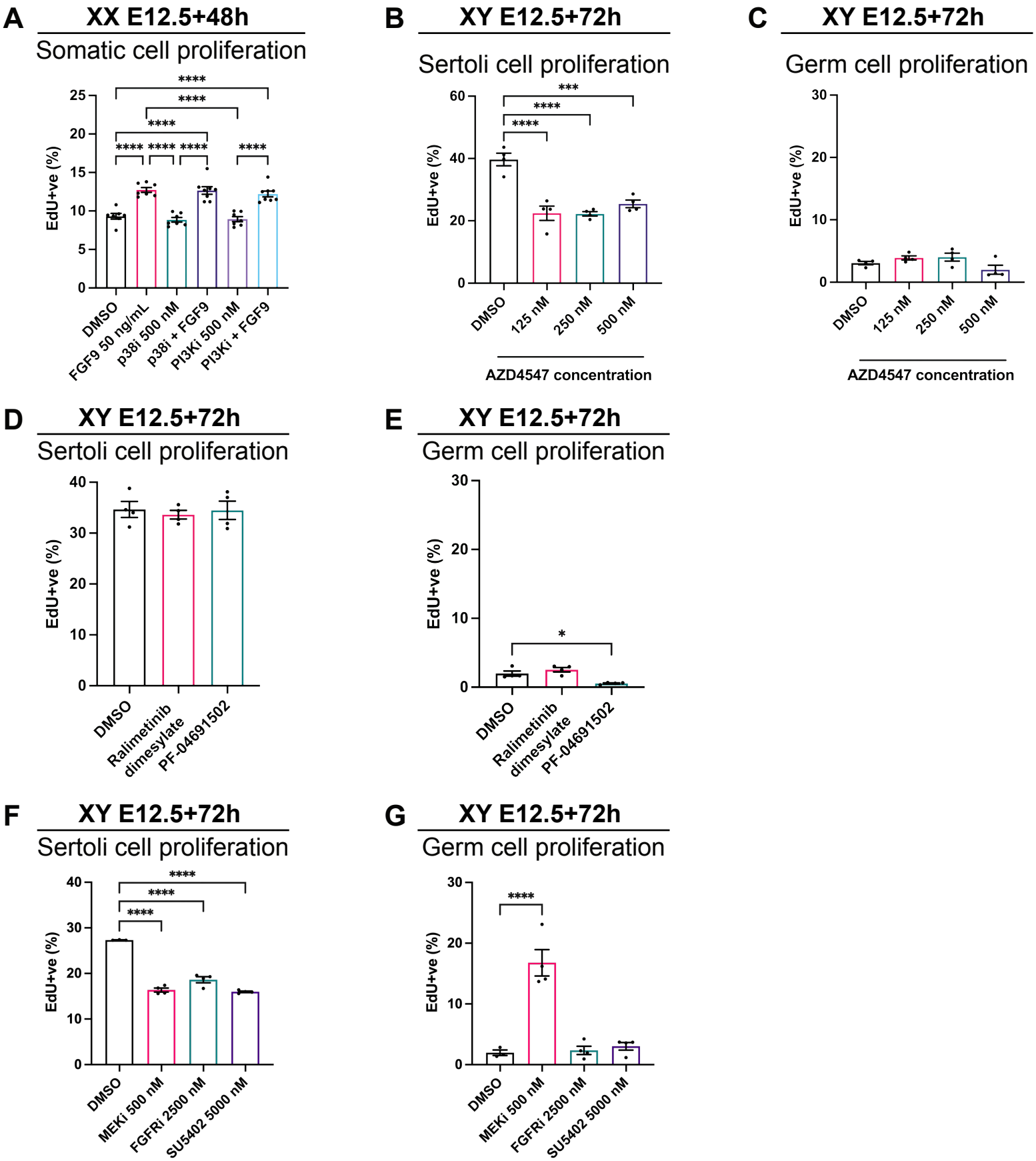
Supplementary Tables 1 and 2

Supplementary Tables 1 and 2 contain gene lists generated from RNA sequencing analyses performed in this study.



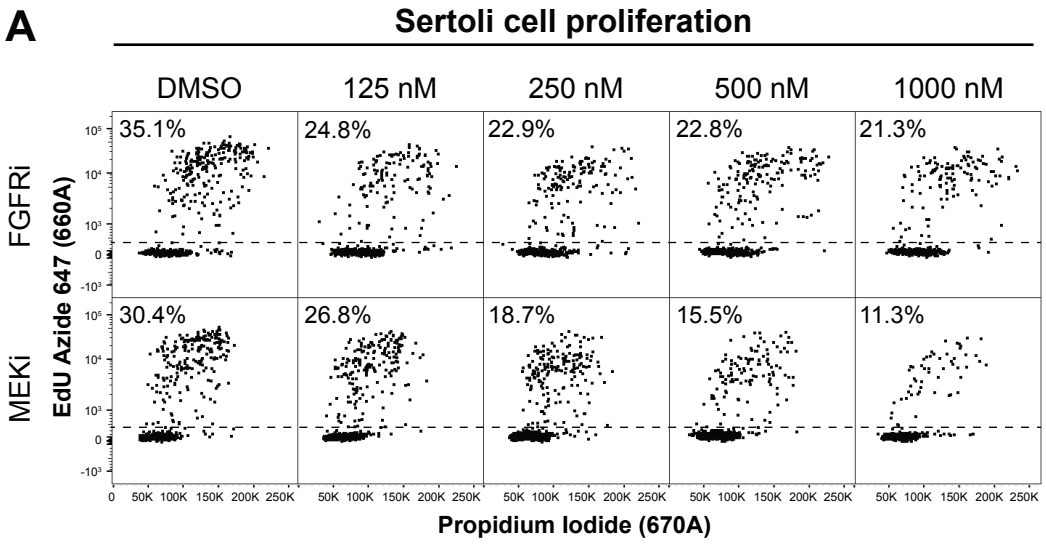
Supplementary Figure 1

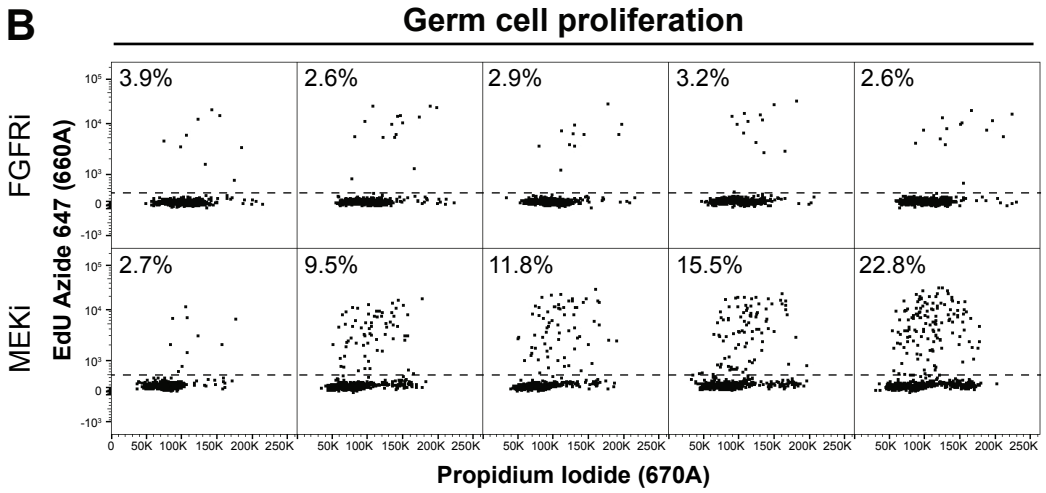
Supplementary figure 1: Representative plots depicting gating for antibodies used in flow cytometric analysis. A = Area, W = Width. **A)** Gates used to separate cells from debris (left) and to isolate single cells based on propidium iodide (PI) staining (right). **B)** Germ and somatic cells populations were identified by detecting Oct4GFP transgene (left). E12.5 mouse limb or mesonephros cells were used as a negative control (right). **C)** Germ and somatic cells populations were identified by detecting MVH staining (left). E12.5 mouse limb or mesonephros were used as a negative control (right). **D)** Sertoli cells were identified based on SOX9 staining (left). XX somatic cells were used as a negative control (right). **E-F)** Incorporation of EdU was used to identify proliferating Sertoli cells (E) or germ cells (F), with PI incorporation used to determine individual cell DNA content (left). E12.5 limb or mesonephros (E) or E12.5 XX germ cells not exposed to EdU (F) was used as a negative control for EdU (right). **G)** DNMT3L+ germ cells identified with DNMT3L staining (left). XX germ cells were used as a negative control (right). **H)** DPPA4+ germ cells were identified with DPPA4 staining (left). Cells not stained for DPPA4 were used as a negative control (right). **I-J)** E12.5+72h XY DMSO germ cells was used as a negative control for STRA8 (I) or p-γH2AX (J) staining (left). E12.5+72h XX DMSO germ cells was used as a positive control (right).



Supplementary Figure 2

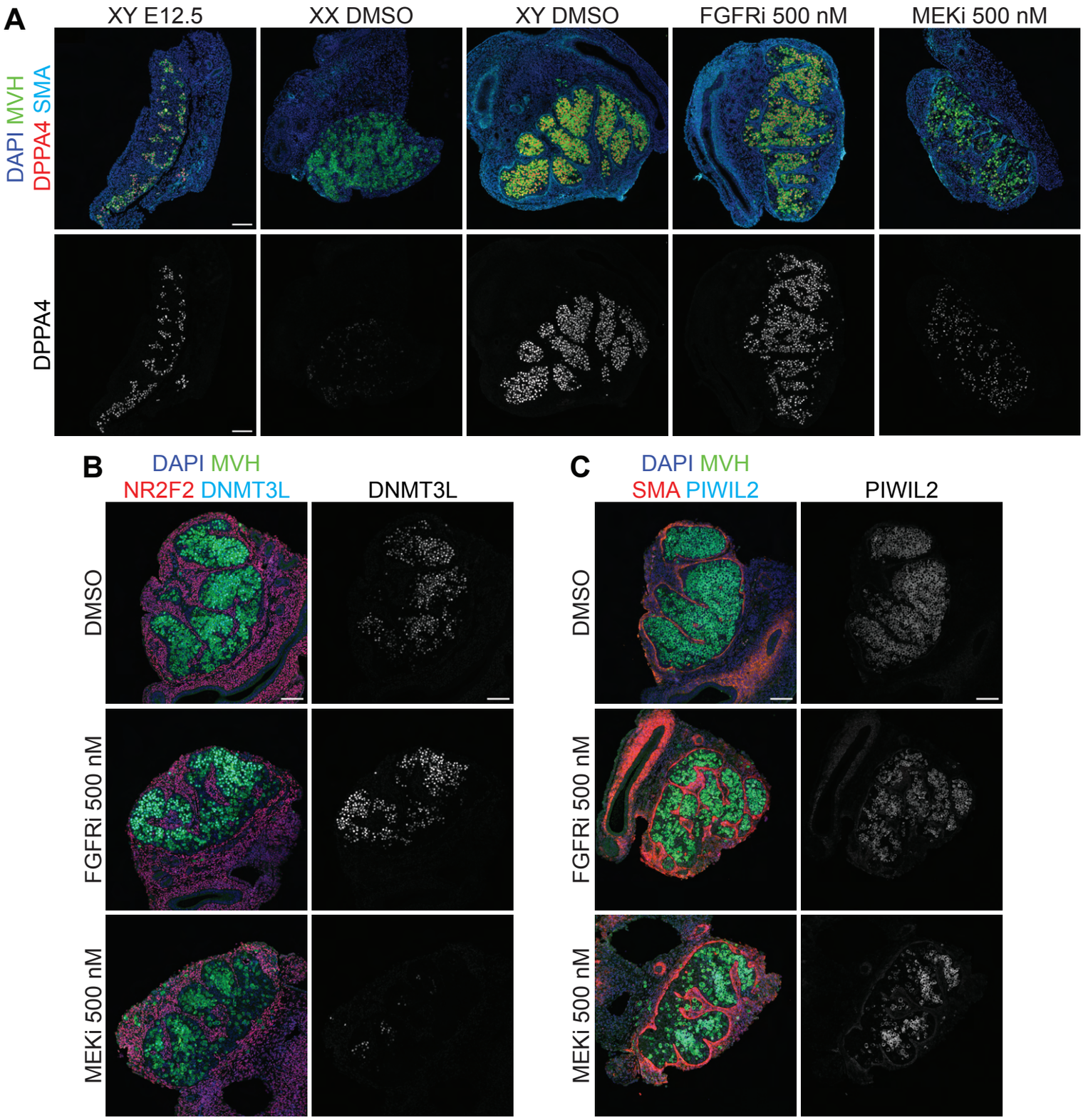
Supplementary figure 2: Supplementary material relating to figure 1 and 2. A) Flow cytometric analysis of gonadal somatic cell proliferation identified by EdU incorporation in E12.5 XX gonads/mesonephros tissue cultured for 48h with DMSO, FGF9 (50ng/mL), p38i (500nM), FGF9 + p38i, PI3Ki (500nM) or FGF9 + PI3Ki. **B-E)** A secondary inhibitor to target FGFR signalling (AZD4547; B-C), p38 signalling (ralimetinib dimesylate; D-E) or PI3K signalling (PF-04691502; D-E) was used to assess Sertoli (B&D) and germ cell (C&E) proliferation in E12.5 XY gonad-mesonephros cultured with DMSO or inhibitors for 72h. **F-G)** Sertoli (F) and germ cell (G) proliferation in E12.5 XY gonad-mesonephros cultured with DMSO, 500nM of MEKi, 2500nM of FGFRi or 5000nM of a third FGFR inhibitor (SU5402) for 72h. Replicates: n≥4. Statistics: Ordinary One-way ANOVA with Tukey's multiple comparisons. Error bars: mean±SEM. Significance between treatments and controls: *P<0.05, ***P<0.001, ****P<0.0001.





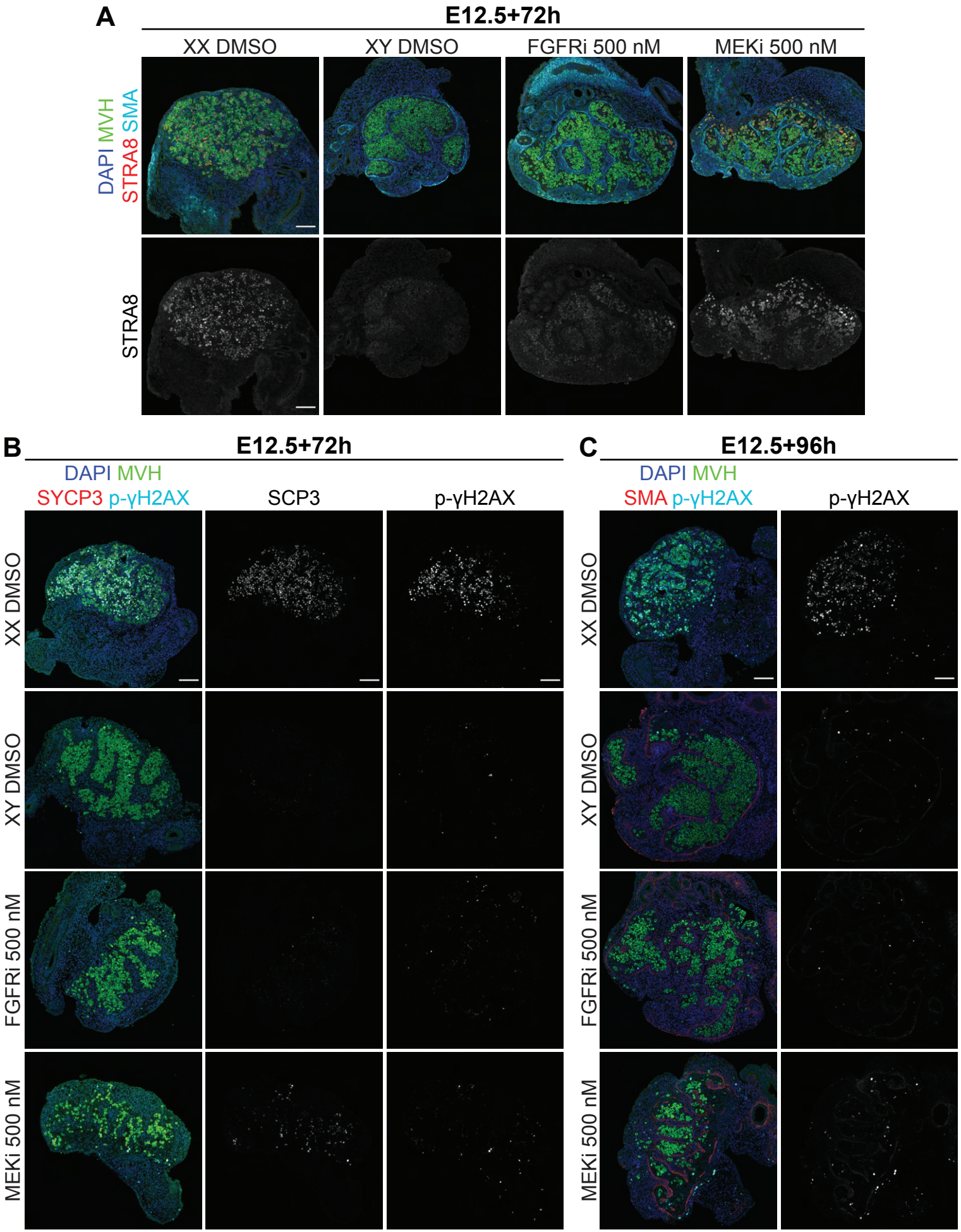
Supplementary Figure 3

Supplementary Figure 3: Flow cytometric scatterplot depicting Sertoli and germ cell proliferation. Flow cytometric scatterplots of XY E12.5 gonad-mesonephros tissue cultured in DMSO, 125, 250, 500 or 1000nM of FGFRi or MEKi for 72h showing the percentage EdU incorporation in Sertoli (A) or germ (B) cells. Percentage in top left corner of each graph represents the average proportion of Sertoli (A) or germ (B) cells in each treatment.



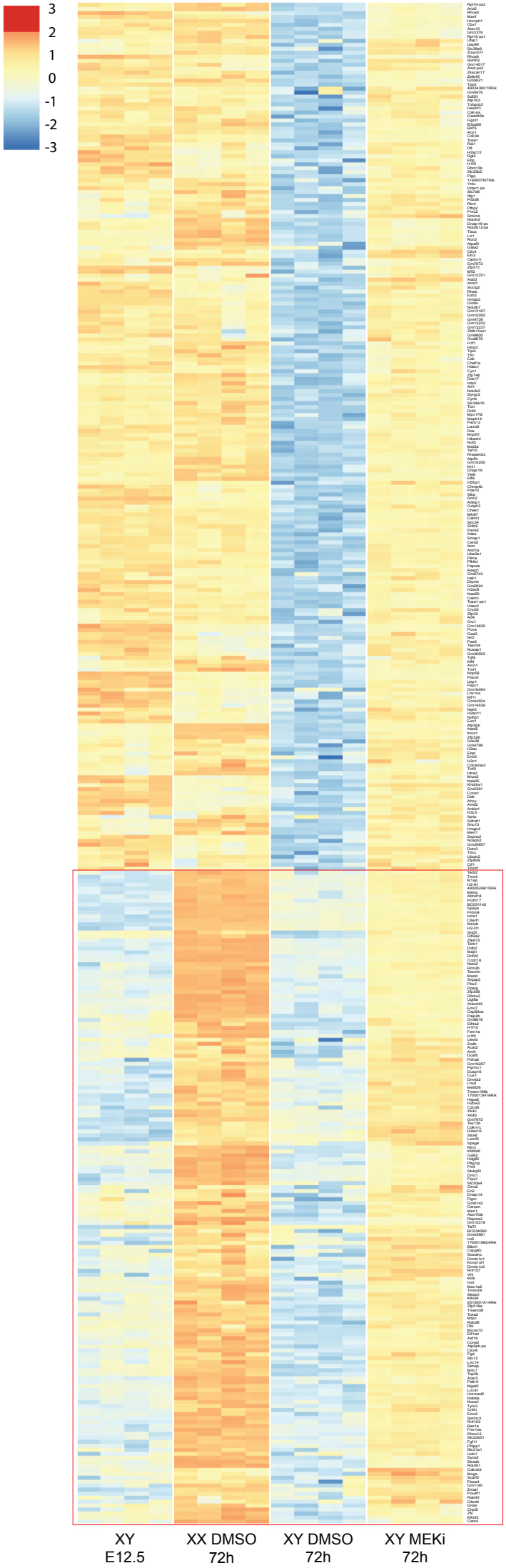
Supplementary Figure 4

Supplementary Figure 4: Whole view of images displayed in figure 4. Immunofluorescent images of XY E12.5 gonad-mesonephros or XY or XX E12.5 gonad-mesonephros tissue cultured with DMSO or 500nM of FGFRi or MEKi for 72h. **A)** Immunofluorescent images demonstrating DPPA4 localisation. Top panel: DAPI (blue), MVH (green), DPPA4 (red) and SMA (cyan). Bottom panel: DPPA4 (white). **B)** Immunofluorescent images demonstrating DNMT3L localisation. Left panel: DAPI (blue), MVH (green), NR2F2 (red) and DNMT3L (cyan). Right panel: DNMT3L (white). **C)** Immunofluorescent images demonstrating PIWIL2 localisation Left panel: DAPI (blue), MVH (green), SMA (red) and PIWIL2 (cyan). Right panel: PIWIL2 (white). Replicates: n≥3. Scale bar: 100µm.



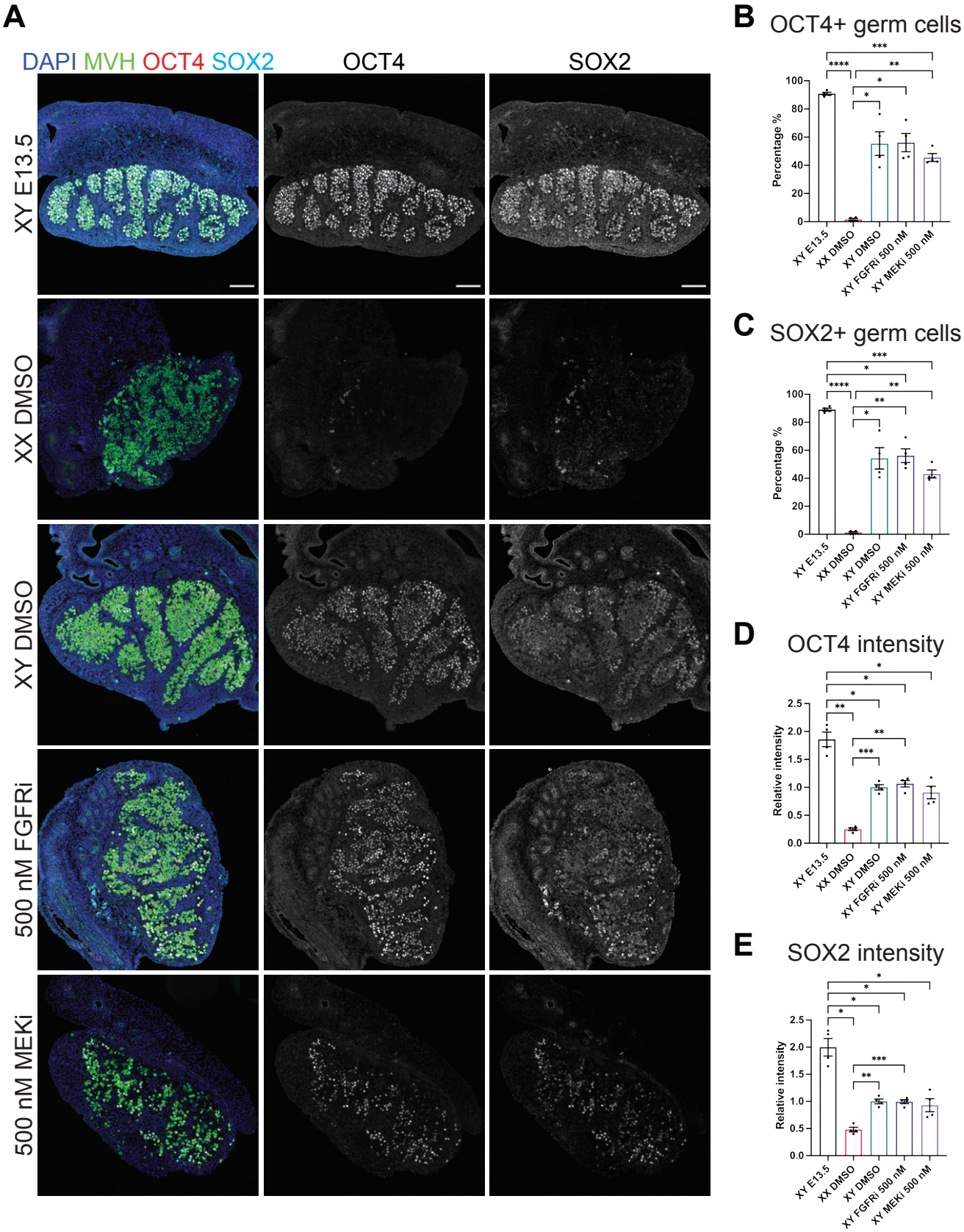
Supplementary Figure 5

Supplementary Figure 5: Whole view of images displayed in figure 5. Immunofluorescent images of XY or XX E12.5 gonad-mesonephros tissue cultured with DMSO, FGFRi or MEKi for 72h (A-B) or 96h (C). **A)** Immunofluorescent images demonstrating STRA8 localisation. Top panel: DAPI (blue), MVH (green), STRA8 (red) and SMA (cyan). Bottom panel: Stra8 (white). **B-C)** Immunofluorescent images demonstrating SCP3 and phospho-γH2AX (p-γH2AX) localisation. Left panel: DAPI (blue), MVH (green), SCP3 (red; B) or SMA (red; C) and phospho-γH2AX (cyan). Middle panel: SCP3 (white; B). Right panel: p-γH2AX (white). Replicates: n≥3. Scale bar: 100µm.



Supplementary Figure 6

Supplementary Figure 6: Heatmap of 382 72h MEK1/2 dependent genes expressed higher than expected common in 72h XX germline specific genes. Genes which were expressed higher than expected in XY E12.5+72h MEKi v XY E12.5+72h DMSO and were present in the 72h XX germline specific genes dataset (identified as genes which were upregulated in XX E12.5+72h DMSO v XY E12.5+72h DMSO) were assessed. Of these 382 genes, 218 genes were highly expressed in XY E12.5 germ cells and XX E12.5+72h DMSO germ cells and were therefore not considered informative. 164 genes were not or were lowly expressed in XY E12.5 germ cells compared to XX E12.5+72h DMSO germ cells and were therefore considered more reliable female germline differentiation genes (identified by red box). Genes with an FDR <0.05 and |logFC| >0.585 (equivalent to |FC| >1.5) were considered differentially expressed.



Supplementary Figure 7

Supplementary Figure 7: FGF and MEK1/2 inhibition does not result in abnormal maintenance of pluripotency markers. Immunofluorescent analysis of XY E13.5 gonad-mesonephros tissue or XY or XX E12.5 gonad-mesonephros tissue cultured with DMSO or 500nM of FGFRi or MEKi for 72h. **A)** Whole view immunofluorescent images demonstrating OCT4 and SOX2 localisation. Left panel: DAPI (blue), MVH (green), OCT4 (red) and SOX2 (cyan). Middle panel: OCT4 (white). Right panel: SOX2 (white). Scale bar: 100µm. **B-C)** Percentage of OCT4+ (B) or SOX2+ (C) germ cells calculated from immunofluorescent images. **D-E)** OCT4 (C) or SOX2 (E) intensity in germ cells relative to XY DMSO control set at 1.0, calculated from immunofluorescent images. Replicates: n=4. Statistics: Brown-Forsythe and Welch ANOVA with Dunnett's multiple comparisons. Error bars: mean±SEM. Significance between treatments and controls: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.