Loss of Mitogen-Activated Protein Kinase Kinase Kinase 4 (MAP3K4) Reveals a Requirement for MAPK Signalling in Mouse Sex Determination

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Abstract

Sex determination in mammals is controlled by the presence or absence of the Y-linked gene SRY. In the developing male (XY) gonad, sex-determining region of the Y (SRY) protein acts to up-regulate expression of the related gene, SOX9, a transcriptional regulator that in turn initiates a downstream pathway of testis development, whilst also suppressing ovary development. Despite the requirement for a number of transcription factors and secreted signalling molecules in sex determination, intracellular signalling components functioning in this process have not been defined. Here we report a role for the phylogenetically ancient mitogen-activated protein kinase (MAPK) signalling pathway in mouse sex determination. Using a forward genetic screen, we identified the recessive boygirl (byg) mutation. On the C57BL/6J background, embryos homozygous for byg exhibit consistent XY gonadal sex reversal. The byg mutation is an A to T transversion causing a premature stop codon in the gene encoding MAP3K4 (also known as MEKK4), a mitogen-activated protein kinase kinase kinase. Analysis of XY byg/byg gonads at 11.5 d post coitum reveals a growth deficit and a failure to support mesonephric cell migration, both early cellular processes normally associated with testis development. Expression analysis of mutant XY gonads at the same stage also reveals a dramatic reduction in Sox9 and, crucially, Sry at the transcript and protein levels. Moreover, we describe experiments showing the presence of activated MKK4, a direct target of MAP3K4, and activated p38 in the coelomic region of the XY gonad at 11.5 d post coitum, establishing a link between MAPK signalling in proliferating gonadal somatic cells and regulation of Sry expression. Finally, we provide evidence that haploinsufficiency for Map3k4 accounts for T-associated sex reversal (Tas). These data demonstrate that MAP3K4-dependent signalling events are required for normal expression of Sry during testis development, and create a novel entry point into the molecular and cellular mechanisms underlying sex determination in mice and disorders of sexual development in humans.


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Abbreviations: BSA, bovine serum albumin; dpc, days post coitum; ENU, N-ethyl-N-nitosourea; ERK, extracellular signal-related kinase; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase; MAP3K4, mitogen-activated protein kinase kinase kinase 4; PECAM, platelet/endothelial cell adhesion molecule; qRT-PCR, quantitative real time-PCR; SOX, SRY-like HMG box; SRY, sex-determining region of the Y; Tas, T-associated sex reversal; ts, tail somite; WMISH, wholemount in situ hybridisation.

Introduction

Sex determination is the process by which an embryo develops into a male or female, namely, the formation of testes in an XY embryo and ovaries in an XX embryo. In the mouse, this process begins with commitment of cells of the bipotential genital ridge to either the testicular or ovarian fate at 11.5 d post coitum (dpc) [1]. In mammals such as mice and humans, this commitment depends on the presence or absence of the Y-linked testis-determining gene, SRY [2–4].

During the search for the elusive mammalian testis-determining factor, it was a criterion of correct identification that any candidate gene be associated with mutations that cause pure (gonadal) XY sex reversal: the development of an ovary in an XY individual. Such mutations in SRY were readily discovered in mice [5] and humans [6] exhibiting sex reversal, and this link with sex reversal has been a constant theme in the subsequent identification of novel, mostly autosomal, genes functioning in sex determination.

Instances of XY sex reversal in the mouse associated with single gene mutations remain relatively uncommon. Excluding Sry, they include targeted mutations of Sox9 [7,8], Dax1 [9], Fgf9 [10], Fgf2 [11,12], Gata4/Fog2 [13,14], Cbx2 (M33) [15], and Wt1(KTS) [16]. Mice harbouring targeted mutations in three members of the insulin-receptor signalling pathway also exhibit XY sex reversal.
MAP3K4 Is Required for MouseSex Determination

**Author Summary**

In mammals, whether an individual develops as a male or female depends on its sex chromosome constitution: those with a Y chromosome become males because of the development of the embryonic gonad into a testes. The Y-linked sex determining gene *SRY* regulates this process by initiating a pathway of gene and protein expression, including the expression of critical autosomal genes such as *SOX9*. We identified a mouse mutant that causes embryonic gonadal sex reversal: the development of ovaries in an XY embryo. This mutant, which we called boygirl (*byg*), was shown to contain an early stop codon that disrupts the autosomal gene encoding MAP3K4, a component of the mitogen-activated protein kinase (MAPK) signaling pathway. Analysis of embryonic XYGonads suggests that sex reversal is caused by delayed and reduced expression of the sex-determining gene *SRY*. Our data indicate, for the first time, a requirement for MAPK signaling in the developing XY gonad in order to facilitate normal expression of *SRY* and the downstream testis-determining genes and also suggest that reduced dosage of MAP3K4 may be the cause of a previously described autosomal sex-reversing mutation in the mouse. We predict that loss of MAP3K4 or other MAPK components may underlie disorders of sexual development (DSD) in humans as well.

[17]. In several of these cases, variability exists in the degree of sex reversal observed, depending on genomic context. The C57BL/6J background often biases gonadal development in favour of ovarian tissue in mutant XY embryos and this “B6 sensitivity” increases background often biases gonadal development in favour of ovarian development, respectively, were established partly on the basis of systematic expression screens. As an alternative to expression-based screens, we have employed a forward genetic approach to identifying loci controlling sexual development in the mouse. Using N-ethyl-N-nitrosourea (ENU) mutagenesis and a three-generation (G3) breeding scheme, we screened for abnormalities of the developing gonads in embryos homozygous for induced mutations. In one mutant pedigree, RECB/31, we identified XY embryos exhibiting abnormal testis cord development and, in some cases, gonadal sex reversal. We have named this mutant line boygirl (*byg*). Genetic mapping placed *byg* on the proximal region of mouse Chromosome 17 and molecular studies revealed that the *byg* phenotype is caused by a point mutation in the *Map3k4* (*Mnk4*) gene. Embryos doubly heterozygous for both the *Map3k4* allele and a targeted null allele of *Map3k4* (*Map3k4*^*flv*^) exhibited neural tube defects and XY gonadal sex reversal, confirming that *Map3k4* is the causal gene. *Map3k4* encodes a mitogen-activated protein kinase (MAPK) kinase kinase, demonstrating for the first time a role for MAPK signalling in mammalian sex determination. We describe molecular and cellular studies on the *byg* mutant that demonstrate a requirement for mitogen-activated protein kinase kinase kinase 4 (MAP3K4) in regulating XY gonadal growth, mesonephric cell migration, and the expression of *Sry*, and hence *Sox9*, during XY gonad development. We also describe genetic experiments that suggest that loss of *Map3k4* is responsible for a previously reported autosomal sex reversal phenomenon, T-associated sex reversal (*Tai*) [40,41].

**Results**

**Identification and Molecular Characterisation of *byg* Mutant**

Line 31 (RECB/31) was identified in a forward genetic (phenotype-driven) screen for embryonic gonad abnormalities after ENU mutagenesis (see Materials and Methods for details). Embryos homozygous for ENU-derived mutations were isolated and examined for a variety of morphological abnormalities. One RECB/31 embryo, dissected at 13.5 dpc, exhibited spina bifida, mild oedema, and also contained gonads shaped like normal testes but with no visible testis cords (Figure 1A and 1B). A second, independent RECB/31 litter contained an embryo with spina bifida and testes that had fewer cords than normal with an irregular morphology (Figure 1C). Having identified these individuals, subsequent RECB/31 embryos were examined and gonads were collected for sexing and wholemount in situ hybridisation (WMISH). In this manner, another XY individual was identified in which the gonads were morphologically ovarian at the same stage (Figure 1D). WMISH analysis of gonads from these three abnormal embryos using the Sertoli cell marker *Sox9* confirmed the disruption to testis development and its variable severity as described above (Figure 1B–1D). In each case, *Sox9* expression was still prominent. However, in the case of the XY gonad with an ovarian appearance, expression was restricted to the central portions of the gonad and absent from the poles. This observed phenotypic variability, and that of subsequent mutants identified in the RECB/31 pedigree, is likely due to the mixed genetic background of the embryos examined. All embryos with abnormal XY gonads exhibited failure of neural tube closure, either spina bifida or exencephaly (unpublished data). Embryonic death of homozygous mutants was commonly observed after 15.5 dpc. Because of the observed gonadal abnormalities and apparent XY gonadal sex reversal, this mutant line was named boygirl (*byg*).

During subsequent generations of backcrossing onto C3H/HeH the gonadal phenotype was still robust, although the majority of RECB/31 XY gonads had the appearance of ovoestes, in which the central portion of the gonad shows evidence of cord formation, but the poles are ovarian in both appearance and marker expression (Figure 1E–1N). No overt abnormalities were observed in XX *byg/*byg gonads in these marker studies.
Identification of additional affected XY gonads permitted mapping of the byg mutation. Abnormal embryos (n = 9) were typed with a genome-wide panel of 55 SNP markers in order to identify chromosomal regions that were consistently homozygous for the C57BL/6-derived allele. Only one region, on proximal mouse Chromosome 17, showed this feature of genetic association with byg. This initial linkage was refined by subsequent backcrossing of byg carrier males with C3H/HeH females and intercrossing of carrier progeny, identified by SNP haplotype analysis. Additional SNPs were then used to identify a critical region, in which the byg mutation must reside, between 9.66 Mb (rs3665053) and 15.32 Mb (rs13482889) on Chromosome 17. We used an informatics-based approach to identify candidate genes in the critical region. One such candidate was the gene Map3k4 (also known as Mekk4, GenBank [http://www.ncbi.nlm.nih.gov/ Genbank] number NM_011948), which encodes a MAPK kinase kinase [42,43]. Mice lacking this gene, which were generated previously by gene targeting, are associated with perinatal lethality on the C57BL/6 background [44]. Because homozygous Map3k4 mutant embryos also exhibit neural tube defects and because Map3k4 is expressed in most embryonic tissues between 9.5 and 15.5 dpc [42,44,45], including the gonads (Figure 2A and 2B), we examined the sequence of Map3k4 in affected byg/byg embryos. A single nucleotide substitution at nucleotide position 1,144 of the Map3k4 open reading frame was identified in the homozygous form in two independent byg/byg mutants (Figure 2C and 2D). This substitution replaces an arginine with a premature stop codon at amino acid position 382 of the 1,597 amino acid MAP3K4 protein. The predicted truncated protein lacks the critical kinase domain of MAP3K4 and, therefore, lacks any MAPKKK function. Absence of full-length (180 kDa) MAP3K4 protein in byg homozygous mutants was confirmed by Western blotting with an anti-MAP3K4 antibody (Figure 2F). A kinase-inactive allele of Map3k4 has previously been shown to have very similar phenotypic consequences to the null allele [45]. Thus, because of the effect of the premature stop codon causing loss of the kinase domain, we conclude that the Map3k4<sup>byg</sup> allele is a null allele. The entire colony of byg mice was typed for the presence of the mutation in Map3k4 and all known byg carriers were heterozygous for the mutation. The mutation was not found in any wild-type C57BL/6j or C3H/HeH mice. We concluded, therefore, that the gonadal phenotype in mutant byg embryos is caused by loss of MAP3K4 function.

To confirm this, and discount the possibility that a second, closely linked mutation in an unrelated gene was responsible for the gonadal phenotype, we studied a second Map3k4 mutant allele.
Figure 2. The byg phenotype is caused by a point mutation in Map3k4. (A) WMISH of 12.5 dpc XY gonads with a Map3k4 probe revealing widespread expression, including in newly formed testis cords. (B) Longitudinal section through male gonad at 13.5 dpc, after WMISH, showing Map3k4 expression in testis cords. (C, D) Sequence traces from heterozygous (byg/+), C) and homozygous (byg/byg, D) individuals reveal an A to T transversion at nucleotide position 1,144 of the Map3k4 open reading frame of the byg allele. (E) This mutation replaces an arginine with a premature stop codon (asterisk) at amino acid position 382 of the 1,597 amino acid MAP3K4 protein. The predicted truncated protein lacks the critical kinase domain (S_TKc) and, therefore, any MAPKKK function. (F) Western blotting of protein extracted from byg/byg and +/+ embryos shows absence of full-length (180 kDa) MAP3K4 in mutant homozygotes. The position of size markers is shown on the left. The upper band found in both lanes is due to cross-reaction of the antibody with an unrelated protein. (G) A genetic complementation test was performed to confirm that homozygosity for the Map3k4 point mutation caused the byg gonadal phenotype. XY mutant embryos heterozygous for both the Map3k4byg and targeted Map3k4tm1Flv alleles were dissected at 14.5 dpc and contained gonads with an overt ovarian morphology (central gonad). XX and XY littermate controls are also shown. (H) Hybridisation of a Sox9 probe to a doubly heterozygous XY gonad (left) reveals little expression of the Sertoli cell marker, in contrast to an XY control (right). (I) XY gonad from 14.5 dpc embryo homozygous for the Map3k4tm1Flv allele (−/−) exhibits overt ovarian morphology and an absence of Sox9 (right), in contrast to XY littermate control (left). (J) XY gonad from homozygous knockout embryo (−/−) also expresses high levels of Wnt4 (central gonad). XY and XX control gonads are shown on left and right, respectively.

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confirm that Map3k4 of ovarian differentiation (Figure 2I and 2J). Thus, these data Embryos homozygous for the targeted allele, which was significant levels (Figure 2H). The absence of any overt testicular at 14.5 dpc and exhibited neural tube defects (unpublished data). Thus, we have performed a detailed examination of embryos homozygous for Map3k4 byg per剧场 targeted allele, which was maintained on the C57BL6/J background, also exhibited gonadal sex reversal, with affected XY embryos containing gonads with ovarian morphology, lacking Sox9 and expressing Wnt4, a marker of ovarian differentiation (Figure 2I and 2J). Thus, these data confirm that Map3k4 is the gene disrupted in the byg mutant and that MAP3K4 is required for testis determination in mice.

Phenotypic Characterisation of the byg Mutant

Evidence exists that the C57BL6/J background is sensitised to disruptions to XY gonad development, and this conclusion appeared to be supported by the increased severity of the XY gonadal phenotype observed in embryos homozygous for both Map3k4 flox and Map3k4 flox/flox, and homozygous for Map3k4 flox/flox, in which the contribution from C57BL/6J was greater. Thus, we performed a detailed examination of embryos homozygous for Map3k4 byg after backcrossing to C57BL6/J for at least two generations. We examined cell proliferation, mesonephric cell migration, and cellular differentiation in mutant and wild-type gonads because all these processes are required for normal testis development [1,46,47].

Cellular proliferation is an important component of the organogenetic programme of testis development [48,49]. Gonadal cell proliferation was examined at 11.5 dpc (17-18 tail somites [ts]), 12.0 dpc (20-22 ts), and 12.25 (24 ts) in the coelomic region of gonads from byg/byg and control littermates using immunostaining with an antibody for the mitotic marker phosphorylated histone H3 (pHH3). Somatic cell proliferation in XY byg/byg gonads appeared reduced in the coelomic region in comparison to wild-type XY embryonic gonads at all stages examined (Figure 3). Moreover, at the 22- and 24 ts stages (12.0-12.25 dpc), the coelomic region of control XY gonads was thickened and contained a larger number of somatic cells, in contrast to byg/byg XY gonads, which had fewer cells in this region and resembled wild-type XX gonads of the same stage (Figure 3). We conclude that cellular proliferation, and thus gonadal growth, in the coelomic region is severely compromised in byg/byg XY gonads at an early stage.

Increased levels of apoptosis have previously been described in the neural tube of mice lacking Map3k4 [44]. For this reason we examined levels of apoptosis in the byg/byg XY gonad and controls at 17 ts using an antibody to cleaved caspase 3. We observed very few positive cells in mutant and control gonads, although large numbers of apoptotic cells were observed in a positive control (interdigital mesenchyme of the developing limb) using this assay (unpublished data). Thus, we cannot attribute impaired gonadal growth in XY byg/byg embryos to increased levels of apoptosis.

Testis cord formation in the mouse requires cell migration from the associated mesonephros into the XY gonad in a male-specific fashion [50-53]. To examine mesonephric cell migration into the XY byg/byg gonad we first examined development of mutant gonads when explanted from the embryo at 11.5 dpc and cultured in vitro. Control gonads (wild-type and byg/+ littermates) formed clear testis cords after 2 d of culture and expressed the Sertoli cell marker, Sox9 (n = 3) (Figure S1A). In contrast, we did not observe any testis cords in cultured XY gonads from byg/byg embryos (n = 3) (Figure S1B and S1D). WMISH analysis revealed that these cord-free XY gonads failed to express Sox9 (Figure S1B), indicating a failure to execute the program of testis differentiation. In contrast, high levels of Wnt4 expression in the mutant XY gonads indicated an activation of the ovarian pathway (Figure S1D).

To examine whether the severe disruption to cord formation in the byg/byg gonad was associated with any loss of mesonephric cell migration, we performed recombination experiments in which mesonephroi ubiquitously expressing green fluorescent protein (GFP) were cultured adjacent to a byg/byg XY gonad from 11.5 dpc. Cell migration into control XY gonads was prominent after 48 h of culture (Figure S1E). In contrast, little or no cell migration was detected in cultured byg/byg gonads (Figure S1F). These data suggest that two early cellular processes associated specifically with XY gonad development, cell proliferation in the coelomic growth zone and mesonephric cell migration, are disrupted in the absence of MAP3K4.

In order to address the molecular basis of these defects, we next investigated the expression of key male- and female-determining genes and gene-products between 11.5 and 14.5 dpc, stages of gonad development between which the male and female fates are established and the programme of sexually dimorphic morphogenesis is executed. Several molecules have been shown to be required for normal testis determination, including SRY [5], fibroblast growth factor 9 (FGF9) [10], FGFR2 [11,12], and SRY-like HMG box 9 (SOX9) [7,8]. Current understanding suggests that SRY, in concert with SF1, acts to up-regulate Sox9 expression in the XY gonad at 11.5 dpc [54,55]. Sox9 expression is then maintained at a high level by a positive feedback loop with FGF9/FGFR2, and acts to antagonise function of the ovary-determining gene Wnt4 [56]. A role for prostaglandin D2 in the regulation of Sox9 expression has also been proposed [12,29,57,58]. Downstream of SOX9, genes such as Ambh [59] and Vasa-1 [31,32,60], with male-determining effects, are transcriptionally activated, and germ cell fate is established by modulation of retinoic acid signalling [36,37]. These molecular events are associated with precise spatial (cellular and subcellular) and temporal expression profiles of genes and their protein products, often in a sexually dimorphic manner.

Given its central role in testis development we began our study with an analysis of Sox9 expression. From 11.5 dpc onwards Sox9 transcription in control XY gonads is prominent, initially in pre-Sertoli cells and subsequently in Sertoli cells of the seminiferous cords/tubules. However, analysis of byg/byg homozygotes revealed dramatically reduced levels of Sox9 transcript (Figure 4). At 14.5 dpc the byg/byg XY gonad resembles an ovary morphologically and no significant Sox9 transcription was detectable (Figure 4A). This loss of a Sertoli cell marker in mutant XY gonads was accompanied by elevated expression of two known female-specific markers at the same stage, Stra8 and Wnt4 (Figure 4B and 4C). Expression of these genes indicates that the ovarian pathway of development, including entry of germ cells into meiosis, is activated in vivo in the absence of MAP3K4.

At 11.5 dpc, the sex-determining stage of gonadogenesis, little or no Sox9 transcript was observed (Figure 4E), and this loss of expression was confirmed by immunostaining of mutant and control gonads at the same stage with an anti-SOX9 antibody (Figure 4G-4H). However, Wnt4 expression was prominent in the XY byg/byg gonad at 11.5 dpc, in contrast to wild-type controls (Figure 4F). Interestingly, Sox9 transcription at 11.5 dpc in mutant gonads on the C3H/HeJ background was reduced in comparison.
Figure 3. Reduced gonadal growth in XY byg/byg embryos between 11.5 and 12.25 dpc. (A) Somatic cell proliferation in the coelomic region of control and XY byg/byg gonads was analysed by confocal imaging of wholemount organs after immunostaining with anti-PECAM (green) and anti-phospho-histone H3 (red) antibodies. Cell nuclei were visualised using DAPI staining (blue). All gonads were staged accurately by counting ts (ts number shown on left). The coelomic growth zone characteristic of XY gonads is shown with white brackets in the 22 ts and 24 ts samples. This thickened zone of proliferating somatic cells is not found in XY byg/byg or XX +/+ gonads at any of the stages analysed. (B) Counts in the coelomic region of total number of somatic cells and mitotic (pHH3-positive) cells in XX/XY wild-type (wt) and XY byg/byg (mut) gonads at the stages shown in (A). Cell counts were performed on limited numbers of XX byg/byg gonads at 18 ts and 20–22 ts. Numbers were comparable with wild-type XX and XY byg/byg samples (unpublished data). For details of cell counting methodology and statistical tests see Materials and Methods and Table 1.

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to controls (Figure 4D), but not to the same degree as the C57BL/6J-derived mutant gonads at the same stage. Loss of Sox9 expression is associated with XY sex reversal in a number of genetic contexts, and mice homozygous for a loss-of-function allele of Sox9 targeted to the developing XY gonads by Cre-mediated excision exhibit immediate, complete gonadal sex reversal, as evidenced by the expression of female-specific markers and the absence of testis cord formation [8]. Thus, loss of Sox9 expression is sufficient to explain the failure of male-specific events in XY gonadal enhancer (TESCO) in synergy with SRY [55]. We transcription in the early XY gonad by acting on a specific (NR5A-1) is thought to mediate up-regulation of ts) using immunohistochemical staining of transverse sections. SF-1 of male and female gonad development around 11.5 dpc (16 to 19 mesonephric cell migration, and testis cord formation.

**Table 1.** Cell proliferation in the XY byg/byg gonad.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>18 ts</th>
<th>20–22 ts</th>
<th>24 ts</th>
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<tr>
<td></td>
<td>N Mitotic Somatic</td>
<td>N Mitotic Somatic</td>
<td>N Mitotic Somatic</td>
</tr>
<tr>
<td>XY +/+</td>
<td>6 4.17 268.0</td>
<td>7 7.71 444.0</td>
<td>2 8.50 546.0</td>
</tr>
<tr>
<td>XY byg/byg</td>
<td>5 2.00* 281.4</td>
<td>8 5.50* 349.3*</td>
<td>2 3.00 277.0*</td>
</tr>
<tr>
<td>XX +/+</td>
<td>4 4.20 306.6</td>
<td>6 6.50 340.0*</td>
<td>4 4.25 317.8*</td>
</tr>
</tbody>
</table>

Cell counts of somatic cells in mitosis (mitotic) and total somatic cells (somatic) in coelomic region of wild-type (+/+) and byg(byg) gonads between 18 and 24 ts stages. N, independent gonad samples examined.

*, Values are significantly different from respective wild-type values using a two-tailed t-test where p≤0.05. Individual p-values: *0.01; *0.043; *0.001; *0.038; *0.002; *0.003.

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Expression of MAPK Signalling Components in the Developing XY Gonad

MAP3K4 activity results in activation of the p38 and JNK MAPK pathways as part of a three-kinase phosphorelay module [71]. This signalling module is thought to regulate, amongst other things, the cell’s response to stress including ultraviolet radiation, heat shock, and osmotic stress [72]. MAP3K4 regulates the MAPKs p38 and JNK via the phosphorylation of the MAP2Ks...
MKK3/MKK6 and MKK4/MKK7, respectively [42,43]. A reduction in the number of cells positive for activated MKK4 activity has been reported in the neuroepithelium of embryos lacking MAP3K4 [44]. Therefore, we assayed for the presence of activated MKK4 in wild-type XY gonads at 11.5 dpc using antibodies specific for the phosphorylated form of this protein (pMKK4). pMKK4-positive cells were observed in the gonad, but these were primarily found in the coelomic region of the gonadal periphery (Figure 6A and 6B), a profile reminiscent of pHH3-positive mitotic cells (Figure 3A). A similar distribution was observed when pMKK7-positive cells were imaged (Figure 6H). Given these observations, we assayed directly for co-expression of pMKK4 and pHH3 in the gonad at 11.5 dpc using immunostaining of sections. pMKK4-positive cells were found to be positive for pHH3 too, both in the gonad and adjacent mesonephros (Figure 6B–6D). We then assayed for the presence of activated p38 (pp38) and pMKK7-positive cells at 11.5 dpc express the ovarian somatic cell marker Wnt4 (top), in contrast to an XY control gonad (bottom). (G–I) Immunostaining of a transverse section from a control C57BL/6J XY gonad at 11.5 dpc with anti-PECAM (germ cell, red) and anti-SOX9 (pre-Sertoli cell, green) antibodies reveals large numbers of somatic cell nuclei (G), in contrast to XY byg byg (H), and XX wild-type control (I), in which SOX9 protein is not detected at significant levels.

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Figure 5. Loss of Sry transcript and protein expression in XY byg/byg gonads at 11.5 dpc on C57BL/6J. (A) At the 17 ts stage, Sry transcript is detected throughout an XY control gonad (left) but is absent from the C57BL/6J XY byg/byg gonad (right). (B) By 19 ts, the signal is diminishing in the XY control gonad (left) and is still not detectable in the XY byg/byg gonad (right). (C) qRT-PCR analysis of Sry, SF-1, and Fgf9 transcription in XY +/+ and byg/byg gonads at 11.5 dpc (17–18 ts). Error bars for the relative quantitation (RQ) values represent variation across four biological replicates for each genotype and three technical replicates for each sample. The 2.8-fold reduction in Sry levels in XY byg/byg gonads is significant (p = 0.02) based on a t-test calculated using average dCt values for the above genes and those of Hprt1. Differences in the levels of SF-1 (p = 0.28) and Fgf9 (p = 0.23) were not significant, although there is a trend for reduced expression of Fgf9 in byg/byg. (D) Immunostaining of a longitudinal section of control XY gonad at 17 ts with anti-SRY (green) and anti-PECAM (red) antibodies reveals abundant expression of SRY in somatic cell nuclei of the gonad. Tissue beneath the dotted white line in this and subsequent images is mesonephric. (E) In contrast, very few SRY-positive cells are detectable in a longitudinal section from a XY byg/byg gonad at the same stage. (F) Confocal imaging of a control XY gonad after whomomount immunostaining with anti-SRY and anti-PECAM antibodies reveals large numbers of SRY-positive somatic cells, in contrast to XY byg/byg (G) and control XX (H) gonads. (I) High magnification confocal image of wild-type gonad at 11.5 dpc showing large numbers of SRY-positive somatic cells (green) and germ cells (red). (J) Confocal image of SRY expression in XY byg/byg gonad at 11.5 dpc generated using the same settings as in (I). Note the greatly reduced number of SRY-positive cells and the reduction in signal intensity in the mutant gonad. (K) A wild-type XY gonad at 11.0 dpc (13 ts) showing SRY-positive cells (green) amongst germ cells (red). (L) In contrast, no SRY-positive cells are detected at 11.0 dpc in an XY byg/byg gonad.

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Figure 6. MAPK signalling and XY gonad development. (A–G) Gonadal expression of three activated MAPK signalling components was examined: phospho-MKK4 (pMKK4), a product of MAP3K4-mediated phosphorylation of MKK4, phospho-p38 (pp38), and phospho-MKK7 (pMKK7). (A) Anti-pMKK4 antibody detects activated MKK4 (red) in a number of somatic cells in the developing XY gonad at 11.5 dpc (21 ts) after whomomount immunostaining. Note the concentration of pMKK4-positive somatic cells at the gonadal periphery in the coelomic region. Germ cells are marked by anti-PECAM antibody (green) and cell nuclei by DAPI. (B–D) Transverse sections of 21 ts XY gonad showing co-expression of pMKK4 and the mitotic marker pHH3 in cells at the gonadal periphery. The gonad (left) is separated from the mesonephros (right) by a dotted white line. (E–G) Transverse sections of 21 ts XY gonad revealing co-expression of activated p38 (pp38) and pHH3 in cells at the gonadal periphery. (H–J) Transverse sections showing similar co-expression of pMKK7 and pHH3. (K–N) The effect of two specific inhibitors of MAPK signalling on XY gonad development in vitro was studied. (K) Culture of wild-type XY gonads from 11.5 dpc for 48 h in the presence anti-ERK inhibitor U0126 (+, upper three gonads in panel) has no obvious effect on Sox9 expression in comparison to gonads cultured with vehicle control (v, lower three gonads). Testis cord formation in treated samples is, however, not as pronounced as in controls. (L, M) Culture of wild-type XY gonads in the presence of p38 inhibitor SB202190 (+, upper rows of gonads results in striking, but variable, alterations to Sox9 expression patterns in contrast to vehicle control cultures (v, lower rows), ranging from loss of transcription in the gonadal poles (asterisks) to complete absence of transcription (gonads beneath brackets). (N) Analysis of XY gonads cultured in SB202190 also reveals up-regulation of Wnt4 transcription (open arrowheads, upper row) in contrast to vehicle controls, which lack Wnt4 (black arrowheads, lower row). These data suggest that, at least partial, XY gonadal sex reversal is caused by inhibition of p38 from 11.5 dpc. There were no signs of tissue necrosis or excessive cell death during these experiments. All gonads were from embryos on the C57BL/6J background.

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in the neural tube of embryos lacking MAP3K4 [44]. These data suggest that MAPK signalling is active in the developing XY gonad at early stages, and is associated with proliferating cells of the coelomic growth zone, but that alternative pathways exist for MAPK activation in the gonad in the absence of MAP3K4. Moreover, given our observation that mitotic somatic cells in the coelomic region are those cells with activated MKK4/7 and activated p38, the reduction in the number of proliferative cells in the XY byg/byg gonad (Figure 3) corresponds to a reduction in the number of pMKK4/7- and pp38-positive cells. Whilst it is possible that a gonadal somatic cell activates the MAPK pathway only once it enters mitosis, it is more consistent with the known role of MAPK signalling in cell proliferation to conclude that male-enhanced proliferation in the coelomic region is a MAPK-dependent process. The reduction of coelomic region growth in the XY byg/byg gonad at 11.5 dpc is thus explicable by a reduction in the number of cells exhibiting MAP3K4-mediated phosphorylation of MKK4/7, p38, and possibly other MAPK signalling components.

Inhibition of p38 MAPK Signalling Disrupts Testis Development In Vitro

In order to address whether disruption to components of MAPK signalling can disrupt testis development in vitro, we cultured wild-type embryonic XY gonads from 11.5 dpc for 48 h in the presence of highly selective inhibitors of the MAPKs extracellular signal-related kinase (ERK) (U0126) and p38 (SB202190) [73]. We then assayed for the expression of Sox9 using WISH (Figure 6K–6M). Similar experiments to address the role of JNK were not performed because of the unavailability of highly specific small molecule inhibitors. We observed little affect on Sox9 expression in gonads treated with ERK inhibitor when assayed by WISH, although testis cord formation did not occur in treated samples with the same efficiency as samples cultured in vehicle control (Figure 6K). These data are consistent with other reports that MEK1/ERK inhibitors fail to significantly disrupt testis development in vitro [74]. In contrast, culturing in the presence of p38 inhibitor resulted in dramatic reduction of Sox9 expression, including an almost complete loss of signal in 50% of treated samples (n = 8) (Figure 6L and 6M). Examination of Wnt4 expression in SB202190-treated cultured explants (n = 3) also revealed robust expression of this ovarian marker in contrast to vehicle controls (Figure 6N), suggesting that at least partial gonadal sex reversal was occurring during culture of XY explants because of abrogation of p38 activity. In this context, it is interesting to note that human SRY has been recently identified as a possible target of p38 MAPK signalling in cultured keratinocytes [75].

FGF9-Mediated Activation of Sox9 Transcription Is Not Abrogated In byg/byg Gonads

Given the importance of two components of the FGF signalling pathway, FGF9 and FGFR2, in XY gonad development, we next studied whether byg/byg gonads exhibited defects in this pathway by determining whether FGF9 was able to activate Sox9 transcription in XY byg/byg gonads. It has previously been reported that FGF9 is capable of activating Sox9 transcription in developing XX gonads if they are cultured in the presence of beads coated in this growth factor [56]. In an attempt to address the question of which upstream, extracellular signals employ MAP3K4-dependent phosphorylation during XY gonad development, we determined whether FGF9-mediated activation of Sox9 transcription was abrogated in MAP3K4-deficient gonads. XY gonads from byg/byg and control embryos were cultured at 11.5 dpc for 48 h in the presence of FGF9-coated beads (or beads coated in bovine serum albumin [BSA]) and then analysed for the presence of Sox9 transcripts in cells contacting the bead using in situ hybridisation. BSA-coated beads did not induce Sox9 transcription in any samples. In contrast, Sox9 transcripts were clearly detected in the vicinity of beads in both cultured wild-type XX gonads and in XY byg/byg gonads (Figure 7A and 7B). These data suggest that MAP3K4 is not an obligatory component of signal transduction pathways employed by FGF9 to activate transcription of Sox9 in the developing gonad. However, failure of normal SRY, and thus Sox9, expression in byg/byg XY gonads may result in failure to establish the positive feedback loop between Sox9 and FGF9/FGFR2 [56].

**Map3k4 Haploinsufficiency Contributes to Tas**

A locus on mouse Chromosome 17 associated with XY sex reversal and ovotestis formation has previously been described [40]. This mutation, known as *Tas*, was identified in a mouse stock carrying the hairpin-tail (Th6) deletion whilst being crossed to the C57BL/6J background. The presence of an AKR/J-derived Y chromosome is also required for the development of ovarian tissue in XY C57BL/6J Th6/+ individuals. It has been hypothesized that the *Tas* mutation resides within the region of the complex deleted in Th6 and hemizygosity for the relevant locus causes varying

**Figure 7. Exogenous FGF9 can activate Sox9 transcription in gonads lacking functional MAP3K4.** (A) Culture of a wild-type XX gonad (left) from 11.5 dpc for 48 h in the presence of a bead coated in BSA does not activate Sox9 transcription based on in situ hybridisation analysis. In contrast, a bead coated in FGF9 does result in up-regulation of Sox9 in both XX control gonads (centre) and XY byg/byg gonads (right). The small circle adjacent to each gonad shows the approximate position of the bead during culture. (B) In a separate experiment, FGF9-coated beads again activate Sox9 transcription in wild-type XX and XY byg/byg gonads, but a bead coated in BSA does not activate Sox9 transcription in XY byg/byg gonads. Note the increased size of gonads, both XX and XY, after exposure to exogenous FGF9. All gonads were from embryos on the C57BL/6J background.

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degrees of sex reversal when on the C57BL/6J YAKR background [41]. This genetic background is known to be very sensitive to disturbances in the early events of testis development induced by gene mutation [14], and thus one potential explanation for the Tas phenotype is haploinsufficiency for a t complex locus that is ordinarily testis determining. Map3k4 is located on proximal mouse Chromosome 17 in the region of the t complex and, in the form of the previously anonymous DNA marker D17Rp17 (still a synonym of Map3k4, see http://www.informatics.jax.org/searches/accession_report.cgi?id=MGI%3A1346875 and GenBank entry NM_011948), has been shown to map within the Tδ deletion [76]. Given this map position and the gonadal phenotype of XY embryos lacking functional Map3k4 on C57BL/6J, we hypothesized that haploinsufficiency for this gene might be, at least partially, responsible for the Tas gonadal sex reversal phenotype.

We tested this model in two ways. First, we generated embryos doubly heterozygous for the byg mutation and the Tδ deletion. If Map3k4 resides within the Tδ deletion these embryos will lack Map3k4 function because of failure of complementation and will recapitulate the phenotype of byg/byg homozygous embryos. Figure 8 shows that XY byg/+ Tδ/+ embryos exhibited abnormalities of testis development. XY gonads dissected from doubly heterozygous embryos at 13.5/14.5 dpc showed disruption to cord morphology or gonadal sex reversal, in which Sox9 transcription is lost (Figure 8A) and Wnt4 transcription is activated (Figure 8B). Doubly heterozygous mutants also exhibited neural tube defects (unpublished data). We performed this cross on the C3H/HeH background because this strain has not previously been associated with sensitisation to events disrupting testis development, even given the presence of the YAKR chromosome [41]. We confirmed, therefore, that Map3k4 resides in the Tδ deletion and that this deletion, combined with a loss-of-function allele of Map3k4, causes varying degrees of disruption to XY gonad development even in the absence of any other predisposing genetic factors.

Secondly, we performed a cross to test directly whether Map3k4 haploinsufficiency might account for the development of ovarian tissue in XYAKR Tδ/+ C57BL/6J individuals. We generated XYAKR mice after backcrossing of YAKR to C57BL/6J for six generations. These males were then crossed with females heterozygous for the targeted null allele of Map3k4 (Map3k4tm1Flv), also on C57BL/6J, to generate XYAKR Map3k4tm1Flv/+ heterozygotes on a C57BL/6J background. Nine of these individuals were generated in five litters and seven were scored as normal males based on examination of the external genitalia. However, two were scored as phenotypic females on the basis of external genitalia morphology. Examination of these sex-reversed individuals revealed the presence of ovaries and uterine horns. Histological examination of the ovaries from one of these

![Figure 8. Hemizygosity for Map3k4 contributes to Tas.](http://www.plosbiology.org/doi/figure/10.1371/journal.pbio.1000196.g008)

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Figure 8. Hemizygosity for Map3k4 contributes to Tas. (A, B) A genetic complementation test demonstrates that Map3k4 resides in the Thp deletion of proximal mouse Chromosome 17. XY embryos doubly heterozygous for the Map3k4byg allele and the Thp deletion (XY Thp+, byg+), on the C3H/HeH background, exhibit a range of defects of gonad development similar to byg/byg homozygous embryos, including testes with regions lacking clear cord morphology (bracket, A) and XY gonads with an overt ovarian appearance that lack Sox9 transcription (right-hand side gonad, A). Sex-reversed XY gonads express Wnt4, a marker of ovary development (central gonad, B), in contrast to XY controls. (C) Ovotestis development in embryos heterozygous for the Map3k4tm1Flv knockout allele, on the C57BL/6J XYAKR background (B6YAKR), characterised by gonadal poles (brackets) lacking markers of testis development (Sox9), and expressing markers of ovary development (Wnt4).

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individuals showed them to be smaller than controls and lacking clearly discernible follicles or ova (unpublished data). Examination of four other heterozygous males at approximately 11 wk of age revealed that these had testes of reduced size (ranging from 0.03 g to 0.00 g, mean = 0.06 ± 0.015); in contrast to wild-type controls (n = 6, ranging from 0.00 g to 0.11 g, mean = 0.093 g ± 0.009). Small testes are sometimes an indication of earlier ovotestis development.

To test this possibility, we performed timed matings in order to examine gonadal morphology in XY AKR Map3k4<sup>pA100/+</sup> embryos at 14.5 dpc. Of four XY<sup>AKR</sup> Map3k<sup>pA100</sup>/+ embryos examined, one contained gonads with an overt ovarian morphology, whilst three contained ovotestes identified by morphology and the familiar variegated expression of Sox9 and Wnt4 (Figure 8C). On the basis of the XY gonadal sex reversal, complete and partial, observed in adult embryos, we conclude that haploinsufficiency for Map3k4 is a major contributory factor to male-to-female sex reversal observed in XY<sup>AKR</sup> C57BL/6j<sup>T<sup>y</sup>/y</sup> individuals.

**Discussion**

Here we describe evidence demonstrating, for the first time to our knowledge, an in vivo role for the phylogenetically ancient MAPK signalling cascade in mammalian sex determination. XY embryos lacking functional MAP3K4 on a predominantly C57BL/6j background exhibit embryonic gonadal sex reversal associated with failure of a number of cellular and molecular events, paramount amongst these being failure to transcriptionally up-regulate Sry and, presumably as a consequence, Sox9 in the developing gonad at 11.5 dpc. Previous studies, often involving analyses of M<sub>us</sub> domesticus-derived Sry alleles on a C57BL/6 background, have suggested that the tests determining pathway is exquisitely sensitive to levels and timing of Sry; if a threshold level is not met in a critical time window, ovary development is likely to ensue [69,70,77]. Thus, attention is naturally focussed on the possibility of reduced Sry expression, at the transcript and protein levels, in XY<sup>byg</sup>/byg gonads. Three potential explanations exist: (i) that a transcriptional regulator (or regulators) required for transcription of Sry in pre-Sertoli cells does not function appropriately because of, either directly or indirectly, the absence of MAP3K4-mediated signalling; (ii) that insufficient numbers of pre-Sertoli cells are established in the XY<sup>byg</sup>/byg gonad; (iii) a combination of both of the above effects. With respect to the second hypothesis, the coelomic epithelium is thought to be a source of pre-Sertoli cells in the early XY gonad (prior to 11.5 dpc) [78]. Thus, the reduction in cell proliferation and gonadal growth in the coelomic region of XY<sup>byg</sup>/byg mutant embryos might be considered evidence of a wider range of defects in the developmental potential of the mutant coelomic epithelium and associated mesenchyme, perhaps extending to a reduction in the provision of pre-Sertoli cells, or the provision of pre-Sertoli cells competent to activate transcription of Sry. This hypothesis is consistent with the active MAPK signalling that we report in the coelomic region at 11.5 dpc in XY gonad. However, it should be noted that in other genetic contexts in which cell proliferation in the coelomic region of the developing XY gonad is disrupted, such as in gonads lacking Fgf9 [10,61], Sry transcription is reported to be unaffected [56]. Thus, there is no established mechanistic link between prior proliferative defects in the early XY gonad and subsequent loss of Sry expression. However, given the reported role of Fgf9 in promoting gonadal cell proliferation [61], it is possible that loss of MAP3K4 results in an inability of coelomic region cells to efficiently transduce Fgf9 signal produced by initial SRY-positive pre-Sertoli cells. This, in turn, would result in failure to establish a positive feedback mechanism by which cell proliferation and SRY expression mutually promote each other, causing insufficient provision of pre-Sertoli cells. This model would explain the reduced numbers of SRY-positive cells detected in XY<sup>byg</sup>/byg gonads between 11.0 and 11.5 dpc (Figure 5). In order to establish whether there is a paucity of cells migrating into the XY<sup>byg</sup>/byg gonad at around 11.2–11.4 dpc to populate the pre-Sertoli cell niche, it will be necessary to perform single-cell labelling experiments similar to those used to establish the role of the coelomic epithelium in this process [78]. However, establishing whether a marked cell was undergoing, or had undergone, active MAPK signalling of the appropriate sort would be technically daunting.

With respect to the first hypothesis, little is known about the transcriptional control of Sry, although several potential activators have been described including M33, WT1(+KTS), GATA4/FOG2, and SF1 [79]. This hypothesis is supported by the presence of a few SRY-positive cells in the XY<sup>byg</sup>/byg gonad at 11.5 dpc that exhibit a significant reduction in the intensity of the SRY signal, and also the existence of FOXL2-positive cells in the XY<sup>byg</sup>/byg gonad at 11.5 dpc, since this lineage is arguably the ovarian equivalent of the pre-Sertoli cell lineage of the testis. Evidence already exists for MAPK-dependent phosphorylation of SF1 [80,81] and GATA4 [82] in other contexts, as a means of increasing their transcriptional activation potency. It is also noteworthy that SRY, which is phosphorylated in humans [83], has recently itself been proposed to be a target of p38-mediated signalling pathways on the basis of cell line studies in vitro [75]. We are currently attempting to identify reduced phosphorylation of candidate testis-determining proteins in MAP3K4-deficient embryonic gonads. However, we cannot rule out the possibility that previously uncharacterised molecules are the key effectors of MAPK-mediated events during gonadogenesis. Moreover, MAP3K4-mediated events required for normal Sry transcription may occur in the progenitors of pre-Sertoli cells, in the form of programming, rather than pre-Sertoli cells themselves. In conclusion, the data suggest that the third hypothesis may best explain the observations concerning SRY expression.

The similarity in the phenotypes of mice lacking the Map3k4 gene [44] and those merely lacking a functional kinase domain of the same gene [45], strongly argues that MAP3K4 functions primarily to regulate MAPK signalling through its kinase domain. Thus, although we cannot formally exclude additional functions, we conclude that loss of functional MAP3K4 in the<sup>byg</sup> mutant results in disrupted MAPK signalling during gonad development. Although ours is the first report of a requirement for MAPK signalling in sex determination in vivo, our previous report has implicated a MAPK scaffolding protein, Vinexin-γ, in regulation of Sox9 transcription during gonad development [84]. However, the fetal gonads of both XX and XY embryos lacking Vinexin-γ are morphologically normal and adult mice of the same genotypes are viable and fertile. Moreover, Sox9 transcript levels in Vinexin-γ<sup>−/−</sup> XY gonads at 12.5 dpc are 75% that of Vinexin-γ<sup>−/+</sup> gonads, suggesting that any modulation of Sox9 transcription by Vinexin-γ is relatively modest. These data appear to be consistent with reported organ culture studies in which the MAPK inhibitor PD98059 did not significantly inhibit testis cord formation in XY gonad explants [74]. In contrast to the Vinexin-γ studies, we observe an almost complete absence of Sox9 at the sex determining stage of gonad development (11.5 dpc) in C57BL/6j XY embryos lacking MAP3K4 and a complete failure of testis cord formation at later stages.

One possible explanation of the apparent discrepancy in these observations with respect to the role of MAPK signalling in testis...
development is the focus in other studies on the MEK-ERK pathway of MAPK signalling, sometimes called the classical MAPK cascade [73]. It has been proposed that Vinexin-γ mediates its effects on Sox9 transcription in vitro via male-specific activation of the MAPK, ERK [84], and PD98059 is a specific MEK-ERK inhibitor [73,85]. The focus on MEK-ERK in other studies is likely a consequence of the inviting similarities between requirements for Sox9 up-regulation during gonad development and chondrogenesis. FGF-mediated activation of Sox9 transcription during chondrogenesis has been shown to be blocked by the MAPK inhibitor U0126 [86]. U0126 is also a specific MEK-ERK inhibitor [73,85]. Given that MAP3K4 is thought to act ultimately by activation of the MAPKs p38 and JNK [42,43], the focus on ERK activation and the consequences of its disruption as a means of determining the role of MAPK signalling during gonad development may have been overly restrictive and resulted in misleading conclusions. Our studies utilising specific small molecule inhibitors of MAPK signalling in organ culture assays corroborate previous observations that MEK-ERK inhibition does not significantly disrupt Sox expression in vitro. However, in contrast, they do suggest a possible role for p38 in gonadal Sox9 transcriptional regulation and testis cord formation. The significance of these in vitro observations for the possible role of p38 in the aberrant phenotype of the MAP3K4-deficient gonad is unclear, given that Sry transcription is already at its peak at 11.5 dpc, the approximate stage at which gonadal explants were employed for in vitro culture experiments. Inhibition of p38 at these stages may disrupt testis-determining events downstream of regulation of Sox9 expression, in a manner analogous to that reported for the IL-1β-dependent induction of Sox9 expression in human articular chondrocytes [87], or disruption of Sox9 function itself. Mice constitutively lacking the alpha isoform of p38 die at around 10.5 dpc, before gonadogenesis can be fully examined [88]. For this reason, it is important to remain open-minded about how many distinct steps in testis development require MAPK-depent events. Teasing these out genetically will require a conditional null allele of Map3k4 (and genes encoding other MAPK signalling elements) and inducible, cell-type-specific Cre lines. It will also be important to determine whether disruption to individual MAP2Ks and MAPKs also results in abnormal gonad development in vivo, or whether loss of MAP3K function is disruptive to a broader range of MAPK signalling events, including potential compensatory ones, and thus more likely to result in phenotypic abnormalities.

In addition to downstream events mediated by MAP3K4, it is not yet clear which upstream signals employ MAP3K4 for their transduction. Analogies with chondrogenesis, as described above, have tended to focus attention on the role of FGF signalling and its use of MAPK for its transduction. Moreover, FGFR9 is known to be required for the male-specific elevated proliferation rate in the gonadal coelomic region at around 11.5 dpc [61]. However, we have demonstrated that the ability of exogenous FGF9 to activate Sox9 transcription during gonad culture remains unaltered in the absence of MAP3K4. These data do not definitively demonstrate that FGFR9 does not employ MAP3K4-mediated signal transduction during regulation of Sox9 expression during male gonad development in vivo, but they do suggest that such a pathway is not obligatory. Moreover, initial up-regulation of Sox9 transcription, along with Sry transcription, proceeds as normal in embryonic gonads lacking FGFR9 [56]. It is, rather, the maintenance phase of Sox9 transcription in developing male gonads that is disrupted in the absence of FGFR9. Taken together, these observations suggest that we should look at other pathways, in addition to FGF, for the activating signals that require MAP3K4 for their transduction. Although activation of MAPK is a widespread phenomenon, ligation binding to receptor tyrosine kinases (RTK) is commonly associated with activation of this intracellular signalling cascade [89]. Interestingly, the insulin receptor tyrosine kinase gene family (I, Igf1r, and Inr) has previously been shown to be required for testis determination through its regulation of Sry expression [17], and a number of reports describe a requirement for MAPK in signal transduction through this family of receptors in different biological contexts [90,91]. Similarly, loss of another RTK, PDGFRα, also disrupts testis development [21] and PDGF signalling is reported to employ MAPK [92]. Finally, in addition to RTK activity, proteaglandin D2 (PGD2) has been shown to influence Sertoli cell differentiation and SOX9 activity [39,57,58], presumably through its G-protein coupled receptors, DP and CRTH2 [93], although this is not established. PGD2 signalling in other contexts has been shown to require MAPK [94,95]. Although the details of MAPK activation in these disparate systems vary, they are all potentially relevant to the phenotype of MAP3K4-deficient gonads because evidence suggests cross-talk between distinct MAPK pathways [75].

Despite the above observations, we cannot rule out the possibility of a role for a hitherto unrecognised growth factor or other extracellular signal in the employment of MAP3K4 during testis development. One virtue of invoking a requirement for MAP3K4 in FGF9-mediated signalling during gonadogenesis in vivo is that this model does not predict a requirement for sexually dimorphic expression of MAP3K4, consistent with Map3k4 expression data. We observed near ubiquitous expression of Map3k4, including male and female gonads, although higher levels were detected in particular cell types. Because of a lack of the relevant antibodies, we were unable to assay for the presence of activated MAP3K4 specifically in XY gonads, although such activation is predicted by the existence of MAP2Ks [96]. It should also be noted that the same explanatory virtue applies to invoking a requirement for MAP3K4 in activation of Sry transcription.

We also report here data indicating that haploinsufficiency for Map3k4 is sufficient to account for Tas [40], a phenomenon that has remained unexplained at the molecular level since its discovery more than 20 y ago. XY embryos heterozygous for the Map3k4<sup>pre<sub>β</sub></sup> mutation on the C57BL/6j-Y<sup>kk</sup> background exhibit testicular abnormalities, including XY ovary and ovotestis development, reminiscent of XY<sup>AKR</sup> C57BL/6j<sup>T<sup>pre<sub>β</sub></sup></sup> embryos [40]. Moreover, two adult XY Map3k4<sup>pre<sub>β</sub></sup>/+ individuals developed as phenotypic females, and both contained ovaries. Four others exhibited testicular hypoplasia, which is associated with prior ovotestis development. It is unclear, however, despite the role for Map3k4 haploinsufficiency established here, whether additional testis-determining genes exist in the region deleted in T<sup>pre<sub>β</sub></sup>, or whether chromosome deletions themselves predispose XY embryos to sex reversal by inhibitory effects on fetal growth [97].

Significantly, it has been demonstrated that, on the appropriate genetic background, the loss of a single copy of a male-determining gene can result in XY gonadal sex reversal [14]. It has been proposed that such phenotypic effects in mice caused by a single disrupted allele mimic the more common situation in humans, where loss of a single functional copy of genes such as Sfi1, SOX9, or WT1 can result in the development of XY females [98,99]. Our findings suggest that the loss of a single copy of Map3k4, caused by the T<sup>pre<sub>β</sub></sup> deletion or targeted gene deletion, is another example of such a case. Thus, we propose that haploinsufficiency of MAP3K4 could be the cause of previously unassigned cases of XY gonadal dysgenesis in humans [100]. A second, independent case of Tas on
the C57BL/6J XY^AKR background (B6-TAS) is caused by the T-Orleans deletion (T^Or^), which overlaps with the hairpin tail deletion and also includes Map3k4/D17Rsp17 [41]. Interestingly, it has been proposed that B6-TAS in T^Or^/+ XY^AKR mice is due to biologically insufficient levels of Sry expression [101]. An analogous explanation of the mechanism underlying the T^Or^/+ XY^AKR phenotype is consistent with the report here of delayed 11.5 dpc. Levels of Sry transcription in byg XY gonads at 11.5 dpc. Levels of Sry transcription in XY embryonic gonads of Map3k4^byg^ or Map3k4^byg^ heterozygotes on the C57BL6/j-Y^AKR background have yet to be determined, but this experiment will form part of a more extensive analysis of gonadogenesis in these individuals.

Our data have opened a novel entry point into the molecular genetic control of mammalian sex determination and, in particular, the regulation of Sry expression. We know of no other higher organisms in which MAPK signalling is thought to regulate sexual development, although pheromone response during mating in yeast and other fungi is known to require a highly related pathway of kinase activity [102–104]. We are currently investigating the role of other proteins required for MAPK signalling in mouse gonad development, utilising in vivo and in vitro methods. The ultimate aim of these studies is to clarify the pathway of MAPK signalling that operates during gonadogenesis and determine precisely how it interacts with the molecular events constituting sex determination. Finally, our study suggests that forward genetic screens in the mouse should be considered as another important tool for identifying vertebrate sex determining genes.

Materials and Methods
Forward Genetic Screen, Mouse Breeding, and Embryo Generation
We have previously described the mutagenesis and screening methodology employed here [105]. Briefly, a three-generation (G3) recessive mutagenesis screen was used in which C57BL/6j males were injected with ENU and outcrossed to C3H/HeH females; F1 (founder) males were used to establish pedigrees by mating to C3H/HeH and F2 female offspring were backcrossed to their father. Using this breeding scheme it is expected that approximately one in eight embryos in a pedigree will be homozygous for any given ENU-induced mutation. G3 embryos were examined at 13.5 and 14.5 dpc for developmental abnormalities. Examination of pedigree RECB/31 (byg) revealed several embryos with abnormal male gonad development. Affected embryos were used for genetic mapping with a 55-marker genome wide SNP panel (sequences available on request). byg was maintained by backcrossing to C3H/HeH and, following identification of the Map3k4 mutation, genotyped for the mutant SNP by pyrosequencing. Timed matings were used to generate embryos at specific stages. Breeding pairs were set up at approximately 3 pm and vaginal plugs were checked the following morning. Noon on the day of the plug was counted as 0.5 dpc. Embryos were typed for chromosomal sex as previously described [106].

Genotyping for the byg mutation was performed using a PCR-based pyrosequencing assay using the following primers: Forward PCR primer: 5'-AGGACTATGAACCGTGACTG-3'; Reverse PCR primer: 5'-Bio-CGCAAGTCGTCTAGTATGAGC-3'; Sequencing primer 5'-GCCAAGGACCTTTGAGG-3'. byg was backcrossed to C3H/HeH and C57BL/6j. Analysis of byg/byg embryos on C57BL/6j was performed between generations n = 2 to n = 5.

The generation and maintenance of mice lacking Map3k4 has been previously described [44,107]. Map3k4-deficient mice utilised here were maintained on a C57BL/6j background. Hairpin tail (T^h^) mice, originally archived on a mixed genetic background, were rederived using independent in vitro fertilisation (IVF) with both C57BL6/j and C3H/HeJ oocytes. T^h^ was maintained on both C57BL/6j and C3H/HeJ. XY sex reversal was observed on the former, but not the latter, genetic background. T^h^ carriers were identified by the shortened tail [108]. Confirmation of the presence of the AKR-derived Y chromosome was performed by using a PCR assay based on that described in [109], which exploits a Zf-2 polymorphism between M. domesticus and M. musculus.

Expression Studies
WMISH to explanted gonads was performed as previously described [31,106]. The following probes were used for WMISH: Sox9 [110]; Oct4 [111]; 3β-HSD [112]; Wnt4 (IMAGE clone 40044945), Sry [113], Stra8 (IMAGE clone 40045823), Map3k4 (IMAGE clone 5705378).

qRT-PCR
Total RNA and protein were extracted from individual 11.5 dpc (17–18 ts) mouse urogenital ridges (comprising gonad and mesonephros) using the Nucleospin RNA/protein isolation kit (MACHEREY-NAGEL) following manufacturer’s instructions. The quantity and quality of the RNA was assessed using the NanoDrop ND1000 (Bogen Life Science) and by gel electrophoresis. A two-step real-time analysis approach was taken. First, cDNA was synthesised using the AB High Capacity cDNA Reverse Transcription Kit using 1 μg of total RNA. The following TaqMan assays (Applied Biosystems [AB]) were used: Sfi (Mm00496060_m1); Fgf9 (Mm00442795_m1); Syp (Mm00441712_s1); Hprt1 (Mm01545399_m1). For each assay, reactions were performed in triplicate using AB Fast Mastermix in a final volume of 20 μl (5 μg of cDNA added); Real-time amplification was performed on an AB 7500 Fast machine, using the manufacturer’s recommended program for Fast Mastermix. Analysis of the results was performed using AB software, employing a ddCt method with the gene Hprt1 as the endogenous control. For each assay four biological replicates and three technical replicates were performed. Statistical analysis was performed using a non-paired t-test on the average Ct values calculated for the three technical replicates of each independent sample (biological replicate).

Immunohistochemistry and Confocal Imaging
The following antibodies were used in this study: SRY (Santa Cruz number sc-122); SF1, a kind gift from K. Morohashi; FOXL2: antibodies were raised in rabbits against the peptides MNASYPEPDEAAAGAALL and WDHDSKTGALHSRLDL, previously utilised in [114]. Antibodies were affinity purified and tested prior to use: platelet/endothelial cell adhesion molecule (PECAM) (BD Bioscience number 553708); phospho-histone H3 (pHH3, Sigma number HH1908 or Upstate number 06-570); phospho-MKK4 (Cell Signalling number 9151); phospho-MKK7 (Cell Signalling number 4171); phospho-p38 (Cell Signalling number 4631); phospho-JNK/SAPK (Cell Signalling number 9251); cleaved caspase-3 (Cell Signalling number 96618); MAP3K4 (Sigma m7194). Wholemount immunohistochemistry was performed as previously described [106]. Section immunohistochemistry was performed on the basis of protocols described in [39]. Whole-mount samples were imaged using a Leica TCS SP5 confocal microscope. Sections were visualised using a Zeiss Axioshot 2.
Gonadal Cell Proliferation

After immunostaining with anti-PECAM and anti-pH3 (Upstate, number 06-570) and nuclear counterstaining with aqueous DAPI, the central third of each gonad was imaged using a Leica TCS SP5 confocal microscope (40×). A Z-stack series (10 μm steps) was generated for each sample and then three central sections were chosen for cell counts in the coelomic region (pH3-positive cells and DAPI-stained nuclei). Sections were separated by 20 μm to ensure that no cell was counted twice. Differences between samples were assessed using a two-tailed t-test.

Organ Culture

Culturing of embryonic gonads and recombination experiments between subdissected gonads and marked mesonephroi were performed based on methodologies described in [50] and [106]. Briefly, XY urogenital ridges (UGRs), consisting of gonad and attached mesonephros, were collected at 11.5 dpc (16–19 ts stage) and cultured to establish conditions under which testis cords formed reliably after 48 h culture. Samples were incubated on 1.5% agar blocks at 37 °C/5% CO2 in Dulbecco’s Minimal Eagle’s Medium (DMEM)/10% fetal calf serum (FCS)/50 μg/ml ampicillin/200 mM L-glutamine in the presence of MAPK inhibitors or vehicle control. For recombination cultures, 11.5 dpc XY male UGRs from byg/byg mutant embryos were subdissected into component gonad and mesonephros in PBS. The gonads were recombined with mesonephros from XY Tg(GFPU)5Nagy/J embryos (ubiquitously expressing GFP) and cultured for 48 h, as above. Migration from the marked mesonephros into the attached gonad was imaged using a Leica TCS SP5 confocal microscope. No migration was observed into control XX gonads during these experiments.

The following MAPK signalling inhibitors were used: SB202190 (p38 inhibitor, Sigma) and U0126 (ERK [Mek1] inhibitor, Sigma). SB202190 was used at a final concentration of 25 μM in culture medium, in line with previously reported in vitro studies employing this inhibitor [115–117]. U0126 was also used at a final concentration of 25 μM [86,110,119].

To examine the effects of exogenous FGF9 expression in XX gonad development we employed the methodology described in [56]. Briefly, agarose beads (Sigma-Aldrich) were incubated in culture medium containing 50 ng/ml FGF9 protein (R&D Systems), or 0.1% BSA, in a humidified chamber at room temperature for at least 5 h. Beads were then placed adjacent to gonads (n = 3 for each genotypic class) and cultured for approximately 42 h.

Ethics Statement

Animal procedures employed in this study were authorized by UK Home Office Project License PPL 30/2381.

Supporting Information

Figure S1 Gonadal sex reversal and failure of mesonephric cell migration into XY byg/byg embryonic gonads during organ culture. (A) In vitro culture of wild-type XY embryonic gonads at 11.5 dpc for 48 h results in testis cord formation visualised by in situ hybridisation with the Sertoli cell marker, Sox9. The asterisk indicates Sox9 expression in the metanephric kidney, which was still attached to this explant when dissected prior to culture. (B) Culture of XY byg/byg gonads results in no testis cord formation and very low levels of Sox9 transcription, which is limited to the gonadal region adjacent to the mesonephros, as in wild-type female gonads. (C) Wild-type explants do not express Wnt4 in the developing gonad after culture, but do express this marker in the mesonephros. (D) XY byg/byg explants exhibit high levels of Wnt4 expression in the gonad, similar to XX gonads at 13.5 dpc, indicating gonadal sex reversal in cultured XY mutant gonads. (E) Culture of a wild-type XY gonad adjacent to a stage-matched mesonephros derived from a line expressing GFP (recombination) reveals migration of endothelial cells into the gonad to form cord-like structures and an aggregation of cells in the coelomic region. (F) Culture of an XY byg/byg gonad with a marked mesonephros reveals negligible cell migration into the control gonad indicated by the region within the white dotted line.

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Figure S2 Analysis of SF1, FOXL2, and FGFR2 protein expression in XY control and byg/byg gonads at 11.5 dpc. (A, B) Transverse section showing SF1 expression (green) in a large number of somatic cells of control XY gonads (A) and XY byg/byg gonads (B). SF1 signal is nuclear in contrast to the cytoplasmatic staining of germ cells with PECAM (red). (C–E) FOXL2 is not detected in control XY gonads at this stage (C) but nuclear signal (green) is detected in somatic cells of XY byg/byg (D) and control XX gonads (E, F–J). FGFR2 (green) is expressed in somatic cell nuclei of control XY gonads (F, G), White arrowhead indicates individual nucleus on section counterstained with DAPI (blue). FGFR2 is still detected in XY byg/byg gonads (H, I), but signal is restricted to the cytoplasm of somatic cells (arrowhead, I). This cytoplasmatic localisation is reminiscent of FGFR2 expression in control XX gonads of the same stage (J). All gonads were from embryos on the C57BL/6j background.

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Figure S3 Immunohistochemical analysis of pMMK4, pMKK7, pp38, and pJNK on transverse sections of wild-type and byg/byg XY gonads at 11.5 dpc. In each case, the activated MAPK signalling molecule is detected in somatic cells (red), whilst germ cells and endothelial cells are detected by PECAM staining (green). Counterstaining is with DAPI (blue). The gonad is to the left of the dotted line in each image and the mesonephros is to the right.

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: DB PS RB NW DW MC. Analyzed the data: DB PS RB NW DW MC. Contributed reagents/materials/analysis tools: DW PK R AF HC HO. Wrote the paper: AG.
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