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The Fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice

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Abstract

Background: Specification of primordial germ cells in mice depends on instructive signalling events, which act first to confer germ cell competence on epiblast cells, and second, to impose a germ cell fate upon competent precursors. *fragilis*, an interferon-inducible gene coding for a transmembrane protein, is the first gene to be implicated in the acquisition of germ cell competence.

Results: Here, we describe four additional *fragilis*-related genes, *fragilis2–5*, which are clustered within a 68 kb region in the vicinity of the *fragilis* locus on Chr 7. These genes exist in a number of mammalian species, which in the human are also clustered on the syntenic region on Chr 11. In the mouse, *fragilis2* and *fragilis3*, which are proximate to *fragilis*, exhibit expression that overlaps with the latter in the region of specification of primordial germ cells. Using single cell analysis, we confirm that all these three *fragilis*-related genes are predominant in nascent primordial germ cells, as well as in gonadal germ cells.

Conclusion: The Fragilis family of interferon-inducible genes is tightly associated with germ cell specification in mice. Furthermore, its evolutionary conservation suggests that it probably plays a critical role in all mammals. Detailed analysis of these genes may also elucidate the role of interferons as signalling molecules during development.

Background

Germ line determination in the mouse is thought to occur through instructive signalling in the gastrulating post-implantation embryo [1,2]. First, proximal epiblast cells acquire germ cell competence at E6.5, partly in response to extraembryonic ectoderm-derived signalling molecules. A subset of these competent cells then acquire a primordial germ cell (PGC) fate and a population of approximately 45 founder germ cells are detected in the posterior proximal region of the embryo at the base of the incipient allantoic bud on E7.5 [1,2]. The secreted signalling molecules, BMP4, BMP8b and BMP2 as well as components of the BMP signal transduction pathway, including Smad1 and Smad5, appear to be involved in the specification of PGCs [3–7]. However, *in vitro* culture studies and analysis of *BMP4*-deficient mice suggest that an additional signal may also be required for the acquisition of PGC fate, but its identity is yet unknown [2,3].

We recently identified *fragilis*, a putative interferon-inducible gene, which codes for a transmembrane protein that is apparently associated with the acquisition of germ cell

competence by epiblast cells [8]. Extraembryonic ectoderm is able to induce *fragilis* expression in epiblast tissue, and BMP4 is required for this induction [8]. *fragilis* is expressed in proximal epiblast at E6.5, the region in which PGC-competent cells reside according to clonal analysis [1]. As these proximal cells move to the posterior proximal region during gastrulation, *fragilis* expression increases within a community of cells at the base of the incipient allantoic bud. Cells with the highest expression of *fragilis* initiate the germ cell-characteristic expression of *TNAP* and *stella*/*PGC-7* [8–10]. These nascent founder PGCs with high expression of *fragilis* also show repression of *Hox* genes, including *Hoxb1* [8].

In view of the strong association of *fragilis* with PGC specification, we have started to investigate further how this gene may be regulated and what precise function it serves during germ cell development. Towards this objective, we now report that *fragilis* belongs to a novel murine gene family, comprising five members, which code for five highly similar transmembrane proteins. Importantly, the genes are clustered within a 68 kb genomic region. As we found several homologues of the Fragilis family in human, cow and rat, they seem to be evolutionarily conserved amongst mammalian species. Most if not all homologous genes have been reported to be responsive to interferon signalling, which is in agreement with the presence of conserved interferon stimulable response elements (ISREs) within at least the murine and human loci. Furthermore, our in situ hybridisation and single cell expression analysis reveal that the two members located close to *fragilis*, *fragilis2* and *fragilis3*, are also expressed in founder PGCs, although their overall expression pattern in post-implantation embryos in other respects is distinct. Studies on the Fragilis family of genes could therefore be crucial for our understanding of PGC specification, especially since their homologues have been implicated in mediating homotypic cell adhesion and lengthening of the cell cycle time [11,12]. These studies may also show how interferons act as signalling molecules, which has hitherto not been considered in the context of embryonic development.

Results

The Fragilis gene family

Using the cDNA sequence of *fragilis* as a template to search the Ensembl genome browser http://www.ensembl.org, we identified eight mouse genes with moderate to high DNA sequence similarity to *fragilis* (45–74%). ESTs from a variety of embryonic and adult tissues have been reported for five of these genes, of which four possess a two-exon structure similar to *fragilis*. Analysis of the genomic location of the latter revealed that the four genes cluster around the *fragilis* locus within a 68 kb region on the distal tip of mouse Chr 7 (F5). We therefore named the four novel genes *fragilis2-5*, reflecting their genomic location, their similarity to *fragilis* and their germ cell associated expression pattern (see below; Fig. 1). The four remaining putative genes that we detected have few or mostly no reported ESTs and are coded by a single exon unlike *fragilis*. We therefore consider them to be pseudogenes.

To determine whether the Fragilis genes are evolutionarily conserved, we searched the human genome using Ensembl and found four homologues on Chr 11 (p15.5), a region which is indeed syntenic to the Fragilis family locus on mouse Chr 7 (Fig. 1). Three of these genes, *Ifitm1* (*9–27*), *Ifitm2* (*1-8D*) and *Ifitm3* (*1-8U*), share 58–65% similarity to the fragilis gene cluster and are located within an 18 kb genomic stretch [13]. They are responsive to type1/2 interferons and code for interferon induced transmembrane (Ifitm) proteins, involved in antiproliferative signalling and homotypic cell adhesion [11,12,14,15]. The fourth gene, *ENSG142056*, a novel gene with two exons, is highly similar to mouse *fragilis4* (83% DNA sequence similarity) and neighbours *Ifitm2*. The human Fragilis family homologues hence form a similar genomic cluster as the five Fragilis genes in the mouse (Fig. 1). Phylogenetic tree analysis suggests however, that only two Fragilis genes, *fragilis4* and either *fragilis*, *fragilis2* or *fragilis3*, have been conserved from mouse to human (data not shown). Subsequent gene duplications may therefore have occurred independently in both species. We also identified two Fragilis family-like genes in cow (bovine *1-8U*, bovine *9–27*) and four genes in rat (*P26376*, *JC1241*, *NP110460*, *AAD48010*). While the rat genes have been annotated as putative interferon-inducible, the two bovine genes that are similar to the human Ifitm genes, have been reported to respond to interferon signalling [16,17]. Due to limited mapping information of the cow and rat genomes, we cannot, at this stage, deduce whether these homologous genes are also organised in a cluster. Interferon stimulable response elements (ISREs, GGAAAN(N)GAAAC) within the human Ifitm locus confer the responsiveness of the three human Ifitm genes to interferons [11,18]. Similar ISRE consensus sequences are also found within the Fragilis family cluster in the mouse, associated in particular with *fragilis*, *fragilis2* and *fragilis5* (Fig. 1).

The murine family of *fragilis* and related genes code for five highly similar transcripts of 104 to 144 amino acids, each containing two predicted transmembrane domains (Fig. 2). The sequence similarity to human, cow and rat *fragilis*-like genes is equally high (overall 68% amino acid similarity). It should be noted, that the first transmembrane domain as well as the following stretch to the beginning of the second transmembrane domain constitute the regions of highest intra- and inter-species conservation.

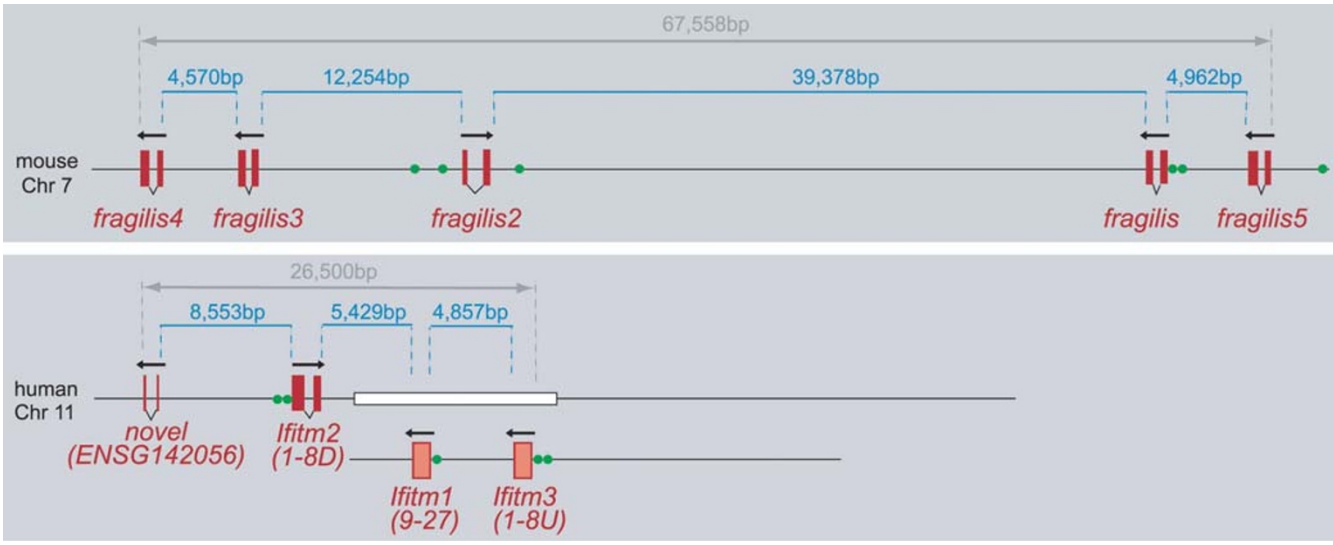

Figure 1
The Fragilis family cluster on mouse Chr7, and the human homologues in the syntenic region on Chr11. In the mouse, the five Fragilis genes are clustered within a 68 kb region. All genes are encoded by two exons, and apart from *fragilis2*, they are located on the minus strand. In human, the four homologous genes, *ENSG142056*, *Ifitm1* (9–27), *Ifitm2* (*1-8D*) and *Ifitm3* (*1-8U*), are clustered within a 26.5 kb stretch. No sequence information is currently available for the genomic region downstream the *Ifitm2* gene (blank bar), although a mapped genomic DNA clone containing the three Ifitm genes has been reported [13]. The four human homologues are each encoded by two exons, but the length of the intronic sequence for *Ifitm1* and *Ifitm3* is not known. Apart from *Ifitm2*, all human genes are encoded on the minus strand. The green circles represent ISRE consensus sequences.

fragilis, fragilis2 *and* fragilis3 *are expressed during early post-implantation development*

We analysed the expression pattern of the five Fragilis family genes by whole mount *in situ* hybridisation using probes that span the 3' region (150–200 bp) of the corresponding mRNAs. These probes show no significant cross-hybridization between members of the Fragilis family as judged by dotblot analysis (data not shown). As reported, we saw expression of *fragilis* restricted to the epiblast at E5.5 and E6.5. More importantly, around E7.5, expression of *fragilis* is intense within a population of cells at the base of the allantois in the region where PGC specification occurs (Fig. 3a,3b,3c) [8]. *fragilis2* and *fragilis3* are also expressed within the epiblast of E5.5 embryos (Fig. 3g, data not shown). While expression of *fragilis2* is thereafter significantly downregulated, *fragilis3* remains expressed at a similar level in the embryonic tissues. At E7.5, *fragilis2* is detected in the posterior mesoderm, while *fragilis3* expression is seen throughout the epiblast. More significantly, like *fragilis*, both *fragilis2* and *fragilis3* show high expression in the region where the cluster of founder PGCs originates (Fig. 3i/i',3n/n'). Thus, these three members of the Fragilis family show significant expression at the time and site of PGC specification.

At E8.5, *fragilis* expression is seen in cells at the base and within the proximal third of the allantois (Fig. 3d). Additionally, a signal is detected in the latero-anterior aspects of the developing brain (Fig. 3e). At this stage, *fragilis2* is expressed in the mesoderm in the caudal half of the embryo (Fig. 3j,3k), whereas *fragilis3* appears present throughout the entire embryo (Fig. 3p,3q,3r). It is noteworthy, that expression seems significantly stronger in cells at the base and within the proximal third of the allantois at this stage (Fig. 3q). At E9.5, *fragilis* signal is seen in the mesoderm and the pharyngeal arches (Fig. 3f). At this stage, *fragilis2* expression appears restricted to the tailbud, the mesoderm caudal to the 15th somite and the developing lung tissue (Fig. 3l).

In order to further analyse *fragilis2* expression, we carried out in situ hybridisation on transverse and sagittal sections of E9.5 and E10.5 embryos, respectively. This confirmed the pattern seen in whole mount preparations, namely presence of staining in the presomitic, somatic and bodywall mesoderm in the caudal regions of the embryo at E9.5 (Fig. 4a,4b,4c,4d). It appears that *fragilis2* expression is lost in differentiating mesodermal or somatic tissue, hence explaining the caudal-to-rostral gradient of staining seen in the caudal region of the whole mount

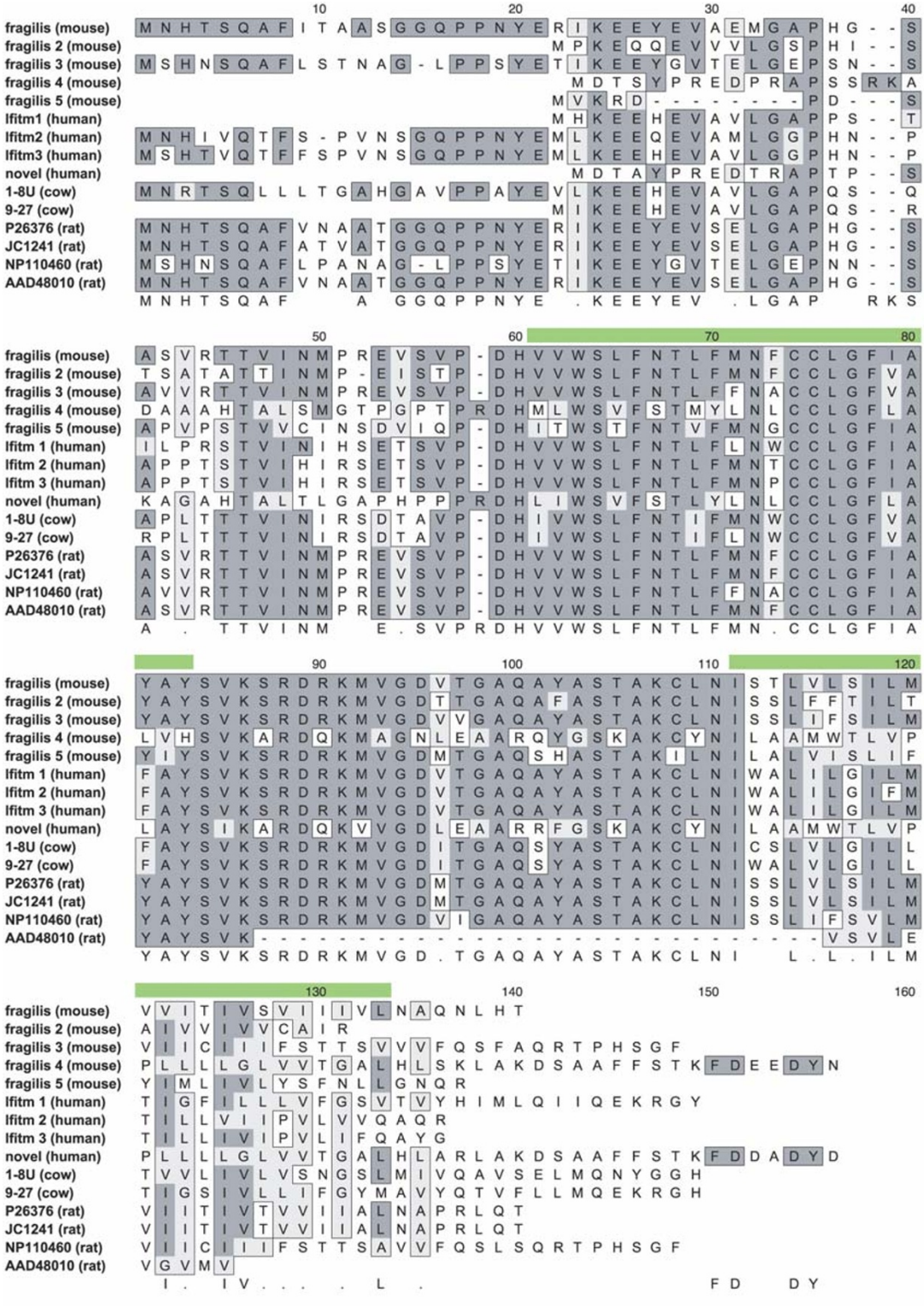

Figure 2
Protein alignment of the Fragilis family and their homologues in human, cow and rat. Green bars indicate the location of the two predicted transmembrane domains, of which the first as well as the inter-domain stretch appear to be highly conserved throughout the four mammalian species. Identical amino acids are highlighted in dark grey, similar amino acids in light grey. The alignment was done using ClustlW.

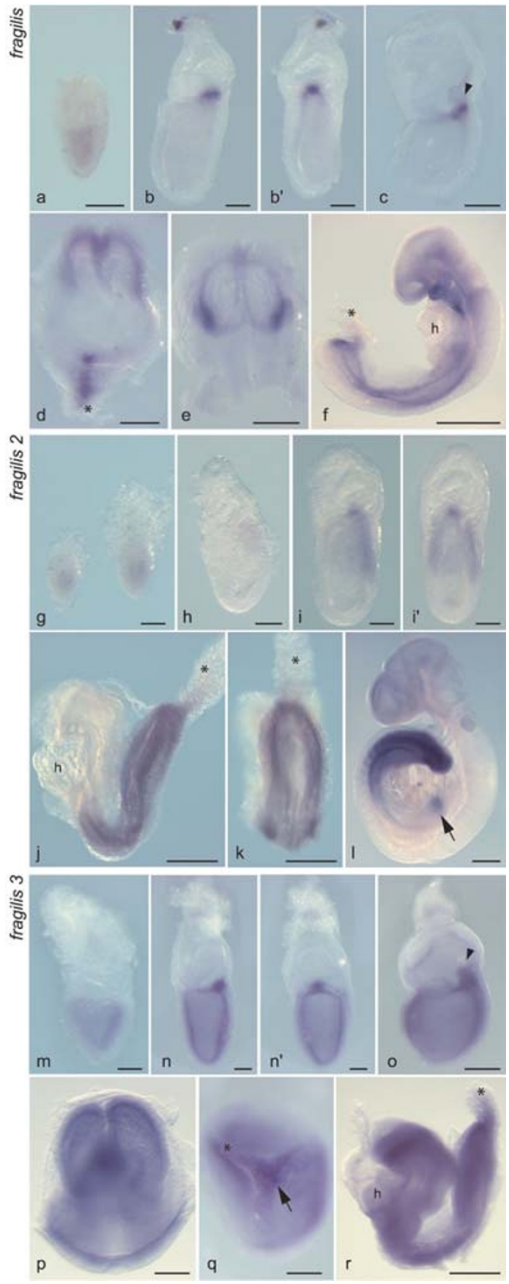

Figure 3
Expression analysis of *fragilis* (a-f), *fragilis2* (g-l) and *fragilis3* (m-r) by whole mount in situ hybridisation. Pictures are taken as lateral view unless otherwise stated, with anterior to the left and posterior to the right. *fragilis* is expressed throughout the epiblast in E5.5 embryos (a) and in the region of germ cell specification at the base of the incipient and early allantoic bud at E7.5 (b, b' posterior view, c). At E8.5, signal is detected at the base and in the proximal third of the allantois as well as in the latero-anterior aspects of the brain (d rostral view, e anterior view). At E9.5, *fragilis* appears expressed in the mesoderm as well as in the pharyngeal arches (f). *fragilis2* is detected throughout the epiblast at E5.5 (g). Expression seems thereafter downregulated but becomes again detectable in the posterior mesoderm and at the base of the incipient allantoic bud in E7.0 and E7.5 embryos (h, i, i' posterior view). At E8.5, expression is seen in caudal mesoderm (j, k posterior view), while at E9.5 expression is seen in the tailbud, the mesoderm caudal to the 15th somite and the developing lung tissue (arrow, l). *fragilis3* is expressed throughout the epiblast at E6.5 (m) and around E7.5 additionally in the region of PGC specification (n, n' posterior view, o). At E8.5, *fragilis3* expression is seen throughout the embryo and appears intense in cells (arrow in q posterior view) at the base and within the proximal region of the allantois (p posterior view, q, r). asterix: allantois; black arrowhead: allantoic bud; h: developing heart; scale bars: 100 μm (a, b, g-i, m, n); 200 μm (c-e, o-q); 400 μm (f, j-l, r).

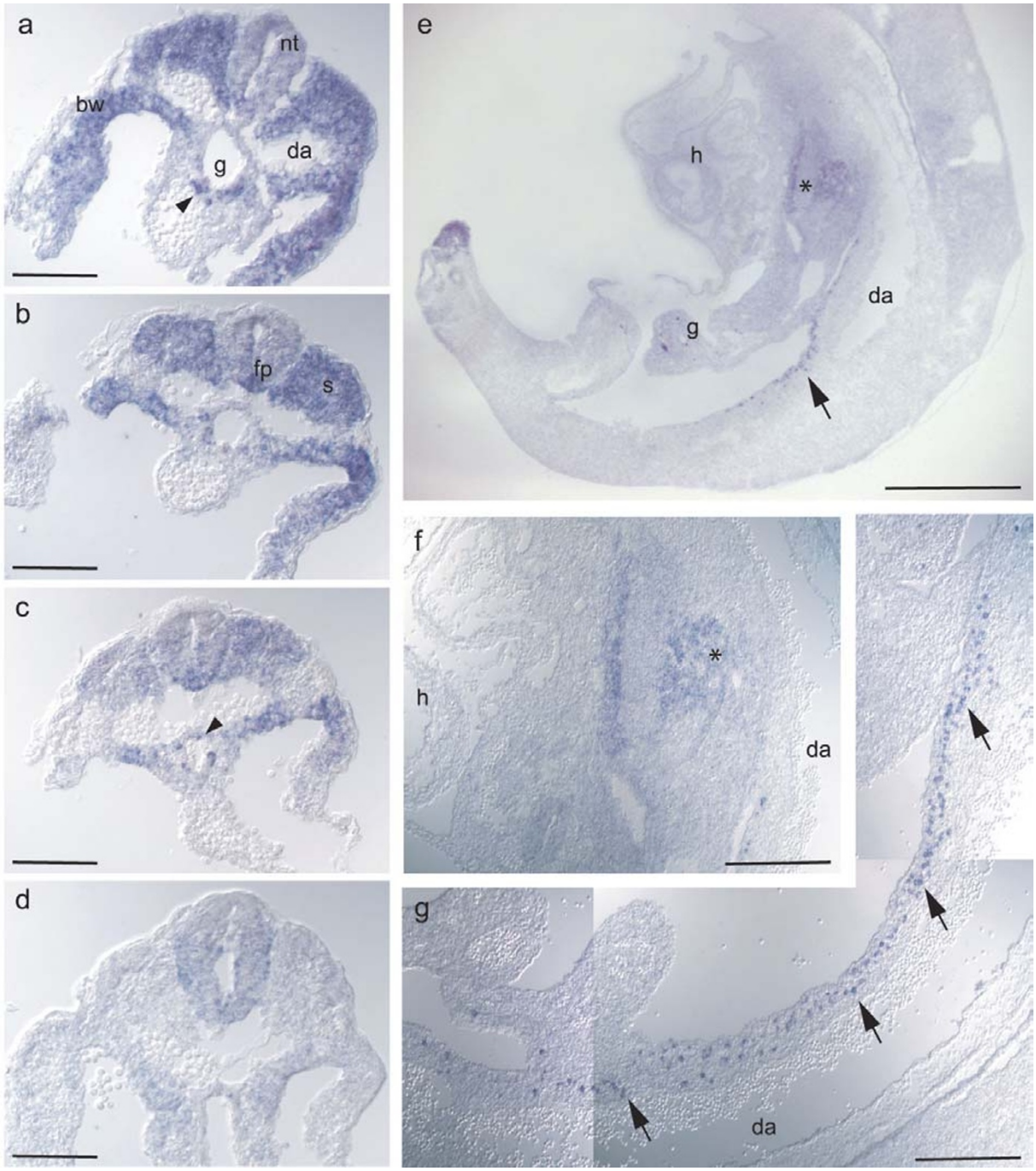

Figure 4
Expression analysis of *fragilis2* by in situ hybridisation on sections. (a-d) transverse sections through the caudal region of an embryo at E9.5 (approx. 25 somites) at progressively rostral levels. At most caudal levels, *fragilis2* expression is seen in cells of the neural tube, in the presomitic mesoderm, in single cells within the hindgut (arrowhead) and in the body wall. (b) staining at approx. 23rd somite level is present within the forming somite, the body wall mesoderm and cells within the hindgut as well as the floorplate. (c) at approx. 21st somite level, expression in the differentiating somites is reduced, while cells in the floor plate and within the hindgut remain *fragilis2* mRNA positive. (d) at approx. the 13th somite level, *fragilis2* expression is absent from the somatic mesoderm as well as the neural tube. (e) sagittal section through an E10.5 embryo shows *fragilis2* expression in developing lung tissue (asterix; higher magnification in f) and migrating cells along the hindgut anterior to the dorsal aorta (arrow). (g) shows a magnified view of *fragilis2* mRNA expressing, migrating cells. da: dorsal aorta; fp: floor plate; g: gut; h: developing heart; nt: neural tube; s: somite; bw: body wall; scale bars: 150 μm (a-d); 1 mm (e); 400 μ m (f, g).

embryos (see Fig. 3l). In addition, the sections show expression in the ventral neural tube, as well as in single cells within the wall of the hindgut. As the sagittal sections of E10.5 embryos demonstrate, these latter cells seem to belong to a population of *fragilis2* mRNA positive cells, which are migrating along the hindgut and ventral to the dorsal aorta (Fig. 4e,4g). Their location suggests strongly that these cells are at least in part PGCs, migrating from their site of origin into the developing genital ridges. Furthermore, *fragilis2* expression is seen in the developing lung tissue posterior to the embryonic heart and ventral to the dorsal aorta (Fig. 4e,4f).

In contrast to the first three members of the family, neither *fragilis4* nor *fragilis5* showed expression at early post-implantation stages (E7.0-E8.5, data not shown). Consequently, only the three genes at the centre of the family cluster, that is *fragilis*, *fragilis2* and *fragilis3* are expressed in the embryo between E5.5 and E9.5. While their expression pattern is distinct, there is a striking overlap within the region where founder germ cells are located. This could suggest that the three neighbouring genes, *fragilis*, *fragilis2* and *fragilis3*, may share regulatory elements that might be present within the cluster. Such regulatory elements may be responsible for the genes' overlapping expression pattern specifically around the region of PGCs specification.

Single cell analysis of fragilis, fragilis2 *and* fragilis3 *in PGCs and somatic neighbours*

To obtain more precise information on the expression of the new Fragilis family members in the context of germ cell specification, we tested single cell cDNAs from PGCs and surrounding somatic cells sited at the base of the incipient allantoic bud in E7.5 embryos. Both *fragilis2* and *fragilis3* were expressed in founder PGCs, which show transcription of the germ cell marker *stella*/*PGC7* (Fig. 4a) [8,10]. The two Fragilis family members were also detected in surrounding somatic cells that lack expression of *stella*/*PGC7* [8]. Notably, semi-quantitative analysis using Southernblotting showed that *fragilis2* and *fragilis3* are expressed predominantly and at higher levels in PGCs compared to the neighbouring somatic cells (Fig. 4b,4c). This mimics the pattern seen for *fragilis*, although expression of the latter is more specific to germ cells. Combined with the in situ hybridisation data, these observations further support the notion that certain common control elements may be involved in the upregulated expression of the three Fragilis genes in the founder PGCs.

During the developmental stages directly subsequent to PGC specification, all three Fragilis family genes are expressed in a population of cells associated with the allantois and in a location where premigrating PGCs are thought to reside (Fig. 3,3d,3k,3q). The precise gene expression during migration of PGCs is not clear at this stage from our analysis. However, using in situ hybridisation and PCR analysis of cDNAs from single cells within the genital ridge, we found clear expression of *fragilis*, *fragilis2* and *fragilis3* in the gonadal germ cells at E11.5/12.5 (Fig. 5). While *fragilis3* expression extends to the mesonephros, *fragilis* and *fragilis2* signal was restricted to the genital ridge. A punctate staining pattern was seen for *fragilis*, mimicking the germ cell restricted expression of *stella*/*PGC7* (Fig. 5b). This pattern in addition to the PCR analysis suggests that *fragilis* is expressed predominantly if not solely in germ cells at E11.5. As was the case in earlier embryos, neither *fragilis4* nor *fragilis5* were detected in gonadal germ cells (data not shown).

Discussion

In this study we describe the identification of the murine Fragilis gene family, which appears to be conserved amongst mammalian species, and whose members code for five highly similar transmembrane proteins. Three members of the Fragilis family, *fragilis*, *fragilis2* and *fragilis3*, exhibit expression, which is associated with germ cell specification and development. Located at the cell membrane, the Fragilis proteins may be important for mediating interactions amongst germ cells and their surrounding neighbours. While the three genes are expressed earlier at E5.5 and thereafter to a varying extent, they all show upregulation of expression within nascent PGCs. It is possible that a cis control element exists within the locus that is required for this expression, which continues within gonadal PGCs. Future studies will elucidate whether such control elements exist, where they are located and how they could regulate expression of the *fragilis*-related genes.

Although the five Fragilis family members are clustered within a small genomic region, it appears that neither *fragilis4* nor *fragilis5* show expression in early embryos or embryonic germ cells. It is striking that these two members are located at the periphery of the cluster in contrast to the centrally located *fragilis*, *fragilis2* and *fragilis3* genes. This lack of expression may be due to the presence of boundary elements, which might restrict the action of control elements to genes present within the centre of the cluster. The four human homologues of the Fragilis family are also arranged in a genomic cluster. However, sequence comparison suggests that gene duplications at the mouse Fragilis and the human Ifitm locus may have occurred independently in the two species. Hence, it appears that a certain evolutionary constrain may exist on duplication and maintenance of the duplicated genes within immediate neighbourhood. Since the human homologues are clustered in the syntenic region and are highly similar to the family genes, it is tempting to speculate that they may also serve similar functions as in mouse and man.

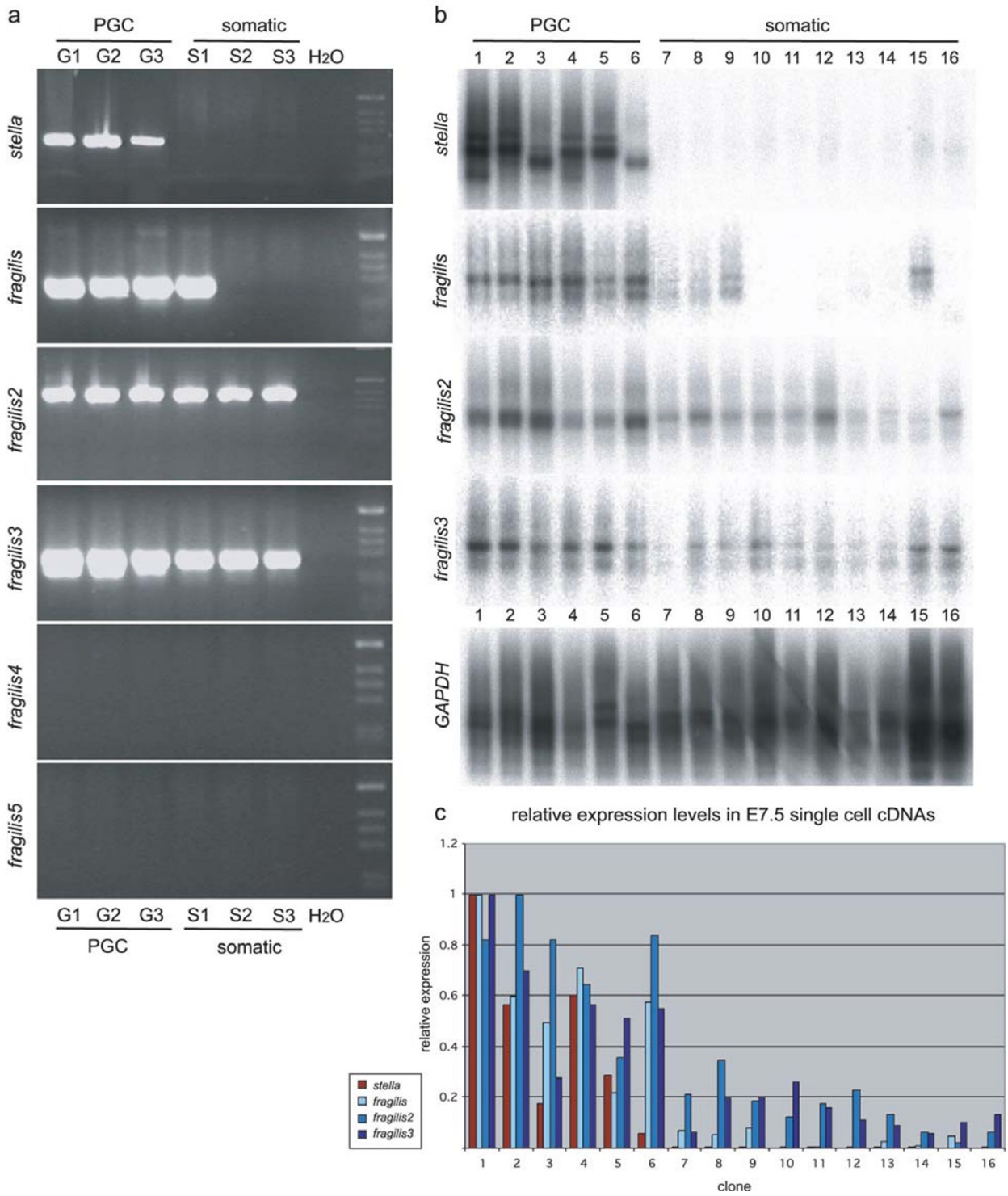

Figure 5

Expression analysis of the Fragilis family genes in single cells from the region of germ cell specification of E7.5 embryos. (a) shows PCR analysis of cDNAs from three, *stella*-positive founder PGCs and three surrounding, *stella*-negative somatic cells. Note that *fragilis, fragilis2 and fragilis3* are expressed in PGCs and somatic cells, while *fragilis4* and *fragilis5* are not detected in any of the cells. (b) shows expression of *fragilis*, *fragilis2* and *fragilis3* in single cell cDNAs using Southernblot analysis. GAPDH was used as blotting control. (c) Semi-quantitative expression analysis of the Southernblot data shows that all three Fragilis genes are predominantly expressed in nascent PGCs compared to the somatic cells within the region.

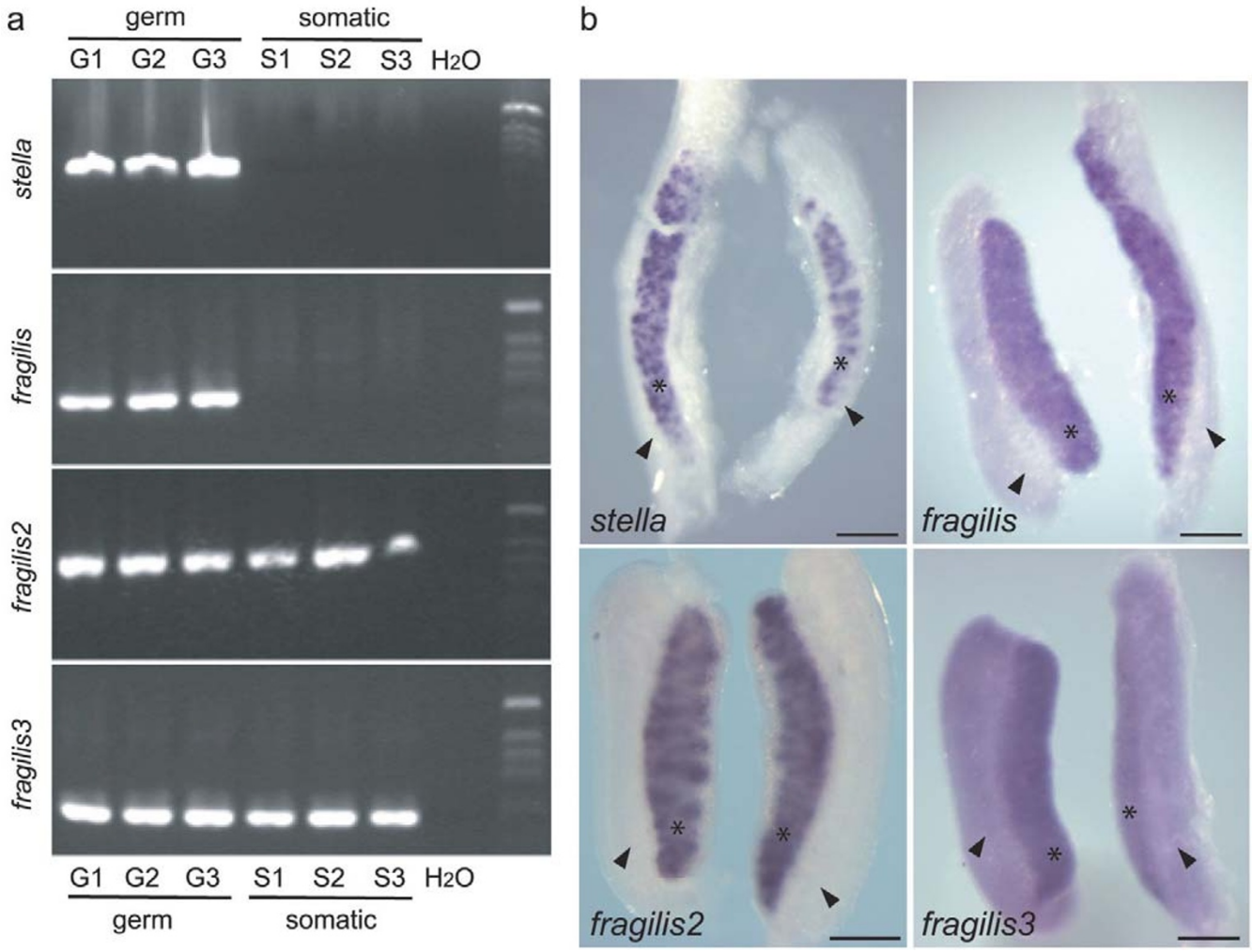

Figure 6
Expression analysis of *fragilis*, *fragilis2* and *fragilis3* at E11.5/E12.5 in single cells from the genital ridge and by in situ hybridisation. (a) shows PCR analysis of cDNAs from three gonadal *stella*-positive germ cells and three surrounding, *stella*-negative somatic cells. While *fragilis* is detected only in the three germ cell clones, *fragilis2* and *fragilis3* are expressed in the germ cells as well as the somatic cells. (b) shows in situ hybridisation of urogenital ridges of E11.5/E12.5 embryos. While *fragilis3* is expressed in the mesonephros as well as the genital ridge, *fragilis* and *fragilis2* are restricted to the genital ridge. The staining pattern for *fragilis* appears punctate and restricted to single cells mimicking the pattern seen for the germ cell-specific *stella* gene. asterix: genital ridge; black arrowhead: mesonephros; scale bars: 400 μm.

The presence of several interferon stimulable response element (ISRE) consensus sequences within the Fragilis locus, together with the similarity of the genes to their interferon-inducible human and bovine counterparts, suggest very strongly that *fragilis* and the *fragilis*-related genes are responsive to interferons or possibly other cytokines. Indeed, the ISRE tandem repeat present in the 5' flanking region of human *Ifitm1*, *Ifitm2* and *Ifitm3* genes is also present in the 5' flanking region of *fragilis* exon 1 [13]. Interferons, as secreted signalling molecules, have so far been implicated mainly in the process of immune response, the inhibition of cellular growth and the control of apoptosis [19]. Although interferons are expressed in the post-implantation embryo, their role during development has not been addressed in detail [20,21]. Our studies have pointed to a possible involvement of interferons in germ cell development. Future work will determine whether the Fragilis genes respond to interferon signals in all or some instances where the genes are expressed.

Conclusion

We have identified the Fragilis family of interferon-inducible genes, which code for transmembrane proteins. The five members are arranged in a cluster within a genomic region of 68 kb in the mouse that also contains ISRE elements. The centrally located *fragilis*, *fragilis2* and *fragilis3* genes are of particular interest, because they are expressed in the region and at the time where germ cell specification occurs. The family is evolutionarily conserved amongst mammalian species where it may serve similar functions. Detailed studies of the Fragilis family may also show what role interferons have in embryonic development.

Methods

Accession numbers

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases as primary sequences under the accession numbers: AY082484 (*fragilis*), AY082486 (*fragilis3*), AY247203 (*fragilis5*); and in the Third Party Annotation Section under the accession numbers: BK001123 (*fragilis2*), BK001124 (*fragilis4*).

Database searches and animals

Ensembl and partly NCBI genome browsers have been used for data retrieval. Embryos and genital ridges used for in situ hybridisation experiments came from 129/SvEv × 129/SvEv or B6CBAF1 × Oct4-GFP matings. Embryos and genital ridges used for single cell analysis came from 129/SvEv × 129/SvEv or MF1 × Oct4-GFP matings, respectively. The noon of the day of the vaginal plug was designated as E0.5. Embryos were staged according to Downs and Davies [22]. Work on animals was performed under Home Office project licence PPL80/1706.

In situ hybridisation

3'-fragments of *fragilis* and *fragilis2–5* cDNAs were PCR amplified using the primers described below, and cloned into pGEM-T vector (Promega). DIG-labelled antisense RNA probes were synthesized using DIG RNA labelling kit (Sp6/T7; Roche). In situ hybridisation on whole embryos and urogenital ridges was performed as described [23,24]. Hybridisation was carried out using 1 μg/ml DIG-labelled RNA probe in hybridisation buffer (50% formamide, 1.3 × SSC (pH 5), 5 mM EDTA (pH 8), 50 μg/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS, 100 μg/ml heparin in DEPC treated H_2O) at 70°C over night. In situ hybridisation on sections was performed as described [25]. Prior to cryosectioning at 12–15 μm thickness, embryos were fixed in 4% paraformaldehyde in PBS and cryoprotected in 20% sucrose in PBS. Hybridisation was carried out using 200–400 μg/ml DIG-labelled RNA probe in hybridisation buffer (50% formamide, 0.1 mg/ml yeast total RNA, 10% dextran sulphate, 1 × Denhardt's, 0.2 M NaCl, 5 mM EDTA, 10 mM Tris (pH 7.5), 5 mM $NaH_2PO_4 \times 2H_2O$, 5 mM Na_2HPO_4 in dH_2O). Hybridised probe was detected using alkaline phosphatase conjugated anti-DIG Fab fragments (Roche) and BM Purple alkaline phosphatase substrate (Roche) for whole mounts and 0.35 mg/ml NBT, 0.175 mg/ml BCIP, 0.24 mg/ml levamisole in 0.1 M Tris (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ for sections, respectively.

Preparation, PCR and Southernblot analysis of single cell cDNAs

Early bud stage embryos (E7.5) and genital ridges (E11.5) were isolated in DMEM/10% fetal calf serum/25 mM HEPES (pH 7.4). Fragments bearing primordial and gonadal germ cells, respectively, were dissected out and dissociated into single cells. The latter were picked using mouth pipettes and their cDNAs were amplified as described previously [26]. The following primers were used in order to PCR amplify *stella* cDNA, parts of *fragilis2* cDNA and 3'-fragments of *fragilis* and *fragilis3–5* cDNAs (25 cycles of amplification): *stella*: 5'CTCACAGCTTGAGGCTTCTAA3', 5'GCGATTCAGATGTCTCTGCAC3', *fragilis*: 5'GTTATCACCATTGTTAGTGTCATC3', 5'AATGAGTGTTACACCTGCGTG3'; *fragilis2*: 5'CCTTCCTTATTCTCACTCTG3', 5'GTTGCAAGACATCTCACATC3'; *fragilis3*: 5'GATCTTCAGCATCCTTATGGTC3', 5'GAAGGTAACATTTGCATACGCG3'; *fragilis4*: 5'AACTTGGAGGCTGCAAGGCAG3', 5'CTCGGAACTCTTAGTTATAGTC3'; *fragilis5*: 5'TGCTCTGGTCATCTCCCTCA3', 5'CAGGATAAGGGGCAACTCTG3'. PCR products were run on 1.5% agarose/TBE electrophoresis gels. For Southernblot analysis, single cell cDNAs were blotted onto Hybond-N+ membranes (Amersham) and probed with $^{32}\alpha$P dCTP-labelled DNA probes comprising the 3' regions of *fragilis*, *fragilis2* (primers for amplification: 5'AGCTCCCTGTTCTTCACCAT3', 5'GCAAATGGTCAGGACTAAGTC3') and *fragilis3* cDNAs and full length *stella* cDNA. The 3' fragment of *GAPDH* cDNA was used as loading control (primers for amplification: 5'TTGGGCTACACTGAGGACCAG3', 5'GATAGGGCCTCTCTTGCTCAG3'). Blotting signal was detected using a Fuji film FLA 5000 scanner. Signal strength was semi-quantified in relation to GAPDH signal, whereby relative gene expression was calculated as ratio of gene signal to GAPDH signal and this ratio was subsequently normalized by division through the highest hybridisation signal per blot (as described previously [8]). For dotblot analysis, full length fragilis cDNAs were blotted and probed with $^{32}\alpha$P dCTP-labelled 3' probes.

Authors' contributions

U.C.L. carried out the in situ hybridization studies, PCR and Southernblot analysis, sequence alignment and genomic cluster identification, participated in the database

searches and drafted the manuscript. M.S. constructed the single cell cDNAs of E7.5 embryos, participated in the in situ hybridisation studies and the database searches. P.S.W. constructed the single cell cDNAs of the genital ridges of E11.5 embryos. S.C.B. participated in the construction of single cell cDNAs. U.C.L., M.S. and M.A.S conceived of the study, participated in its design and coordination and were primarily involved in writing the manuscript.

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