Drosophila homolog of the myotonic dystrophy-associated gene, *SIX5,* is required for muscle and gonad development

Ruth J. Kirby*§, Graham M. Hamilton‡§, David J. Finnegan*, Keith J. Johnson‡ and Andrew P. Jarman*†

SIX5 belongs to a family of highly conserved homeodomain transcription factors implicated in development and disease [1–3]. The mammalian *SIX5/SIX4* gene pair is likely to be involved in the development of mesodermal structures [4–6]. Moreover, a variety of data have implicated human *SIX5* dysfunction as a contributor to myotonic dystrophy type 1 (DM1), a condition characterized by a number of pathologies including muscle defects and testicular atrophy [7–9]. However, this link remains controversial. Here, we investigate the *Drosophila* gene, *D-Six4*, which is the closest homolog to *SIX5* of the three *Drosophila* Six family members [10]. We show by mutant analysis that *D-Six4* is required for the normal development of muscle and the mesodermal component of the gonad. Moreover, adult males with defective *D-Six4* genes exhibit testicular reduction. We propose that D-Six4 directly or indirectly regulates genes involved in the cell recognition events required for myoblast fusion and the germline:soma interaction. While the exact phenotypic relationship between *D-Six4* and *SIX4/5* remains to be elucidated, the defects in *D-Six4* mutant flies suggest that human *SIX5* should be more strongly considered as being responsible for the muscle wasting and testicular atrophy phenotypes in DM1.

Addresses: *Institute of Cell and Molecular Biology, and †The Wellcome Trust Centre for Cell Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR, United Kingdom. ‡Institute of Biomedical and Life Sciences, Division of Molecular Genetics, University of Glasgow, Anderson College, 56 Dumbarton Road, Glasgow G11 6NU, United Kingdom.

Correspondence: Andrew Jarman
E-mail: andrew.jarman@ed.ac.uk

§These authors contributed equally to this work.

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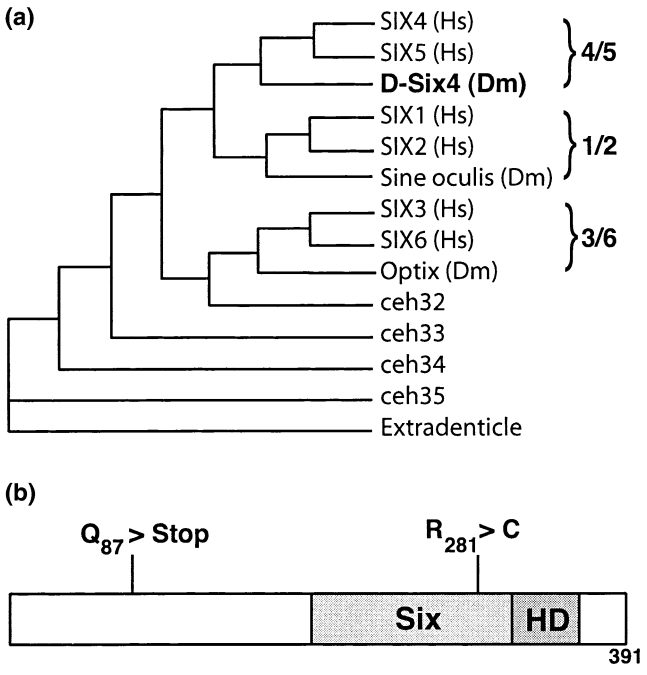
Results and discussion

A *Drosophila* homolog of *SIX5* was isolated by screening a genomic library with a *SIX5* RT-PCR probe. Sequence analysis showed that this homolog is identical to the recently reported gene *D-Six4* [10], except for four silent nucleotide differences. We found no evidence for additional *SIX5* homologs in our experiments or in the subsequent analysis of the *Drosophila* genome sequence [11]. As reported by Seo et al. [10], the comparison of the protein sequence of the combined Six- and homeodomains shows that D-Six4 is most similar to SIX4 and SIX5 (67% and 65% identity, respectively) (Figure 1a). Moreover, all three proteins have valine in homeodomain position 5, which is a potential contributor to DNA binding specificity, whereas all other Six proteins have serine or threonine [12]. It is likely that *D-Six4* is derived from the common ancestor of both *SIX4* and *SIX5*.

During embryogenesis, *D-Six4* mRNA is expressed in the developing head region, mesoderm, and CNS, (Figure 2a–c; see also [10]). Mesodermal expression becomes segmental and then becomes confined weakly to the somatic gonadal precursors (SGPs, also known as follicle cell precursors) in parasegments 10–12, which subsequently form the somatic sheath that surrounds the gonad [13] (Figure 2b). *D-Six4* expression then becomes strong in the SGPs after they have coalesced with the migrating germ cell precursors (pole cells) to form the immature gonad (Figure 2c). SGPs are crucial for gonad coalescence, and germ cells remain scattered if SGPs are dysfunctional [13]. Therefore, we examined the requirement for *D-Six4* in germ cell aggregation in double-stranded RNA interference (dsRNAi) experiments [14, 15]. When embryos were injected with *D-Six4* dsRNA, germ cells failed to coalesce in 100% of the embryos (n = 34), compared with 19% of the mock-injected embryos (n = 31) (Figure 3a,b). We concluded that *D-Six4* is required in SGPs for gonad formation.

This information was used to identify *D-Six4* mutations. From an EMS screen, two mutations were isolated that mapped to the *D-Six4* chromosomal location, failed to complement each other, and produced embryos that exhibited gonad coalescence defects. These mutants were found to harbor defective *D-Six4* genes (Figure 1b). Homozygotes of one mutation (*D-Six4*^{289}) fail to hatch, and it appears to be a null or a strong hypomorph of *D-Six4*. Consistent with this, the *D-Six4*^{289} gene has a nonsense point mutation ($C_{175\,3} > T$), resulting in a stop codon in

Figure 1



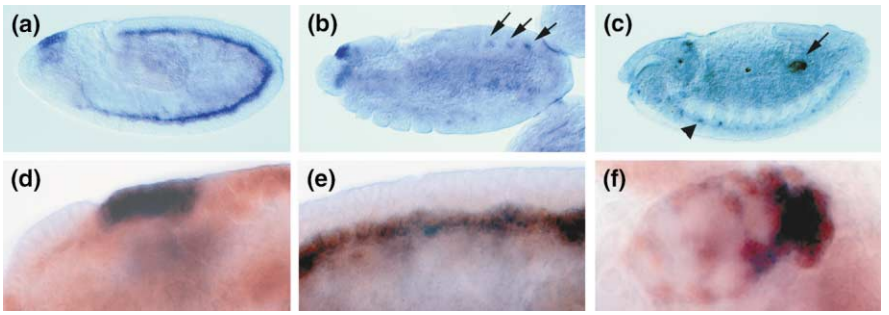
(a) A phylogenetic tree comparing the combined Six- and homeodomain amino acid sequences (174 amino acids) of all human (Hs) and *Drosophila* (Dm) Six proteins shows that they fall into three subfamilies. D-Six4 is a member of the SIX4/SIX5 subfamily. Extradenticle is the outgroup, while *ceh* represents predicted *Caenorhabditis elegans* Six protein homologs. The tree was constructed using PAUP [32]. **(b)** The predicted D-Six4 protein, including the Six- and homeodomains (HD), with the molecular lesions identified for the two mutants.

place of Gln_{87}. The second mutation (*D-Six4*^{131}) is less severe. *D-Six4*^{131} mutant embryos hatch normally, although many die during larval and pupal stages (data not shown). A small proportion survive to adulthood. The molecular defect of *D-Six4*^{131} is a point mutation ($C_{2404} >$ T), resulting in an amino acid substitution of Cys for Arg_{281}, which corresponds to position 102 within the Six domain. This Arg is conserved in all Six proteins, implying that it is important for structure or function of the Six domain. For some other Six proteins, including mouse Six4 and Six5, the Six domain has been shown to mediate the interaction with Eya proteins in vitro, resulting in a functional heterodimer [16, 17]. We found that *Drosophila* Eya is coexpressed with *D-Six4* in all the areas of the latter's expression (Figure 2d–f). This suggests that D-Six4 is a partner of Eya in these tissues. Consistent with this, *eya* mutant embryos also show lack of gonad coalescence [18]. The molecular defect in *D-Six4*^{131} is the first demonstration of an amino acid substitution in a Six domain being associated with a phenotypic effect and supports the importance of the Six domain for in vivo protein function.

In *D-Six4*^{289} homozygous embryos, initial germ cell internalization and migration are normal, but the cells then fail to coalesce to form a gonad (Figure 3c). The failure of gonad coalescence in *D-Six4* mutants is consistent with *D-Six4* expression and function in SGPs. To examine the SGPs themselves, we analyzed the expression of an SGP marker, the *412* retrotransposon [13]. This element is expressed in the head and the mesoderm (Figure 3d), the latter probably representing both the fat body precursors and the SGPs [19]; expression subsequently becomes prominent in the SGPs before and after they form the gonad (Figure 3e,f). In stage 10 embryos homozygous for *D-Six4*^{289}, the expression of this marker was entirely abolished (Figure 3g). Late in embryonic development, *412* expression could be observed in 1–5 scattered cells, which appear to be SGPs (Figure 3h). These experiments suggest that *D-Six4* is required for the correct pattern of gene expression within the mesoderm and SGPs. Genes involved in SGP-cell recognition are candidates for *D-Six4* target genes.

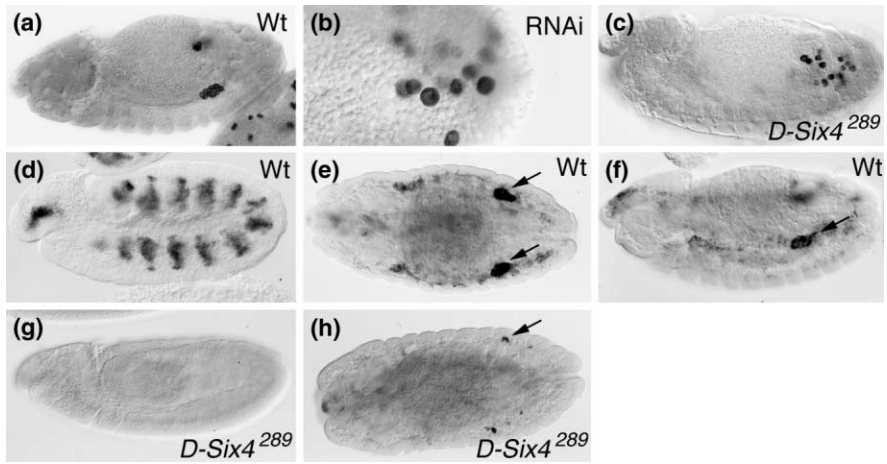
Germ cell coalescence was variably affected in the weaker

Figure 2



A summary of *D-Six4* expression during development. **(a–c)** In situ hybridization with an antisense *D-Six4* RNA probe. (a) Stage 9; transient general mesodermal expression. (b) Stage 13. Expression becomes refined briefly to developing SGPs (arrows). (c) Stage 15. *D-Six4* is expressed in SGPs after their coalescence with the germ cells in the gonad (arrow); some expression is also seen in the ventral nerve cord of the CNS (arrowhead). **(d–f)** *D-Six4* is expressed in a subset of the regions that express Eya. The detection of *D-Six4* mRNA (purple) and Eya protein (brown), with coexpression observed as a black coloration. Eya is expressed more widely than *D-Six4*, whereas all areas of *D-Six4* expression coincide with an area of Eya expression (hence, all areas of *D-Six4* expression are black). (d) Stage 10. *D-Six4* and Eya are coexpressed in the head. (e) Coexpression in the mesoderm. (f) Stage 13; coexpression in SGPs.

Figure 3



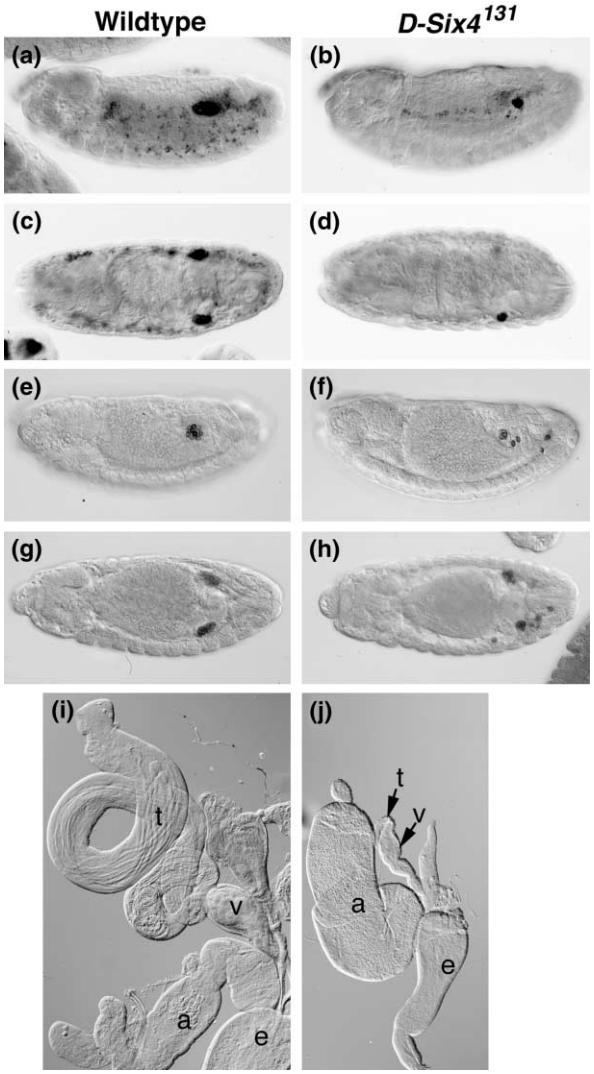
D-Six4 is necessary for SGP development. All are lateral views, except (e,h), which are dorsal. **(a–c)** Vasa protein marks the germ cells during their migration and coalescence with the SGPs to form the gonad. Stage 13 embryos are shown. (a) A wild-type embryo, showing coalesced germ cells. (b) *D-Six4* dsRNA interference. An embryo injected with *D-Six4* dsRNA, showing failure of coalescence. (c) An embryo homozygous for *D-Six4*^{289}, showing failure of coalescence. **(d–h)** In situ hybridization of embryos with the antisense *412* RNA probe as a marker of mesoderm and SGPs. (d) A wild-type stage 11 embryo, *412* labels the segmental groups of mesodermal cells. (e) A wild-type stage 15 embryo, showing the SGPs in the coalesced gonad (arrows). (f) A wild-type stage 13 embryo, showing the paired gonads. (g) A stage 11 homozygous *D-Six4*^{289} embryo; *412* expression is abolished. (h) A stage 15 homozygous *D-Six4*^{289} embryo; a few scattered *412*-expressing SGPs are seen.

D-Six4^{131} mutant. In most embryos, gonads were observed, as represented by distinct clusters of *412*-expressing SGPs, but these appeared consistently smaller than wild-type (Figure 4a–d). These gonads appeared to be populated by germ cells, although a proportion of the germ cells remained scattered (Figure 4e–h). Given the apparent formation of gonads, we examined the gonads of surviving *D-Six4*^{131} adults. Males exhibited severe testicular reduction, although other structures of the reproductive apparatus were present (being derived from the genital imaginal disc rather than the gonad) (Figure 4i,j). This suggests a degree of testicular atrophy after their formation in the embryo. Females exhibited strong ovarian reduction, although highly defective ovarioles were often present (data not shown).

We next looked at somatic muscle formation. In late stage wild-type embryos, an antibody to myosin reveals the regular pattern of myotubes (Figure 5a), but in homozygous *D-Six4*^{289} embryos, the somatic muscles were strongly disrupted. Muscles appeared disorganized in their arrangement or attachment (Figure 5b). Some muscles appeared to be entirely missing, although the number and location of such muscles varied between segments and between embryos. In *Drosophila*, somatic muscles are laid down by a distinct subset of myoblasts known as founder cells. Each founder cell seeds a muscle by fusing with "generic" fusion-competent myoblasts to form a syncytial myotube. Most prominently, in *D-Six4*^{289} embryos, many isolated rounded myosin-expressing cells were scattered among the muscles. These appear to be myoblasts that have not fused with developing myotubes. Thus, there appears to be a major defect in the fusion process. In some cases, elongated unfused founder cells could still be observed attempting to form a myotube (Figure 5c), suggesting that the mutant defect is primarily in fusion rather than in initial founder cell specification, although the latter has not been completely ruled out. Embryos homozygous for the weaker mutant, *D-Six4*^{131}, showed these muscle defects to a lesser extent (Figure 5d). Homozygous *D-Six4*^{131} adult escapers usually died within a few days, but preliminary examination did not reveal any gross muscle defect. However, they have bloated abdomens, owing to a hugely distended crop, which could be a consequence of visceral muscle defects. A number of known genes are required for myoblast fusion [20], either for the recognition event between founder cells and fusion-competent myoblasts (such as *dumbfounded* [21]) or for the events of fusion themselves (such as *myoblast city* [22]). While there are many explanations for the lack of fusion, it is intriguing that D-Six4 may regulate cell recognition processes in both muscle and gonad formation, suggesting that there are common features to these developmental events.

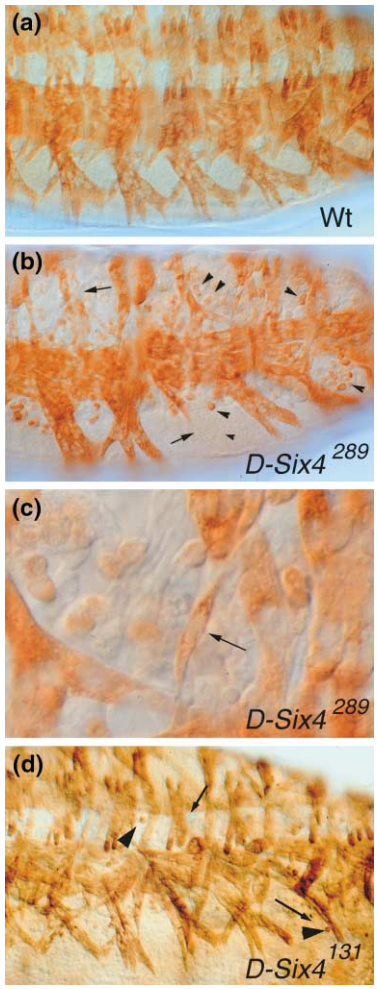
Given the functions we have uncovered for *D-Six4*, it seems likely that vertebrate *SIX4*, *SIX5*, or more likely a combination of both genes, will have important functions in the development of mesodermally derived tissues. Both are expressed widely, including somites [6], but mouse knockouts of *Six4* or *Six5* are viable, suggesting that there might be extensive redundancy between the two genes [23–27]. Human *SIX5* was originally identified (as *DMAHP*) as one of the genes adjacent to the CTG repeat expansion that causes DM1, and there is a variety of indirect evidence that haploinsufficiency of *SIX5* is a cause of some DM1 pathologies [8, 9, 24]. DM1 is a complex disease with a variety of pathologies, including

Figure 4



The gonad phenotype of *D-Six*^{131}. **(a–d)** *412* expression in stage 15–16 embryonic gonads. (a) Wild-type embryo, lateral view. (b) *D-Six*^{131} embryo, lateral view, showing a small but distinct gonad. (c) Wild-type embryo, dorsal view. (d) *D-Six*^{131} embryo, dorsal view, showing two small gonads, one out of focus. **(e–h)** Vasa protein expression in stage 14–15 embryos. (e) Wild-type embryo, lateral view. (f) *D-Six*^{131} embryo, lateral view, some germ cells have coalesced into a gonad, while others remain scattered. (g) Wild-type embryo, dorsal view. (h) *D-Six*^{131} embryo, dorsal view, showing some germ cell coalescence in the paired gonads as well as scattered germ cells. **(i,j)** Testicular loss in adults. (i) A wild-type male gonad and reproductive apparatus, showing one of the paired testes (t) with the seminal vesicle (v), one of the paired accessory glands (a), and the ejaculatory duct (e). (j) Male gonads and reproductive apparatus dissected from a homozygous *D-Six4*^{131} adult escaper. The ejaculatory duct, seminal vesicle, and accessory glands are present (one accessory gland was lost during dissection), while the testes are strongly reduced.

Figure 5



D-Six4 is necessary for somatic muscle formation. Muscle arrangement is detected using anti-myosin antibodies. Ventro-lateral views of abdomens of stage 16 embryos. **(a)** Wild-type embryo, showing the regular arrangement of syncytial myotubes. **(b)** A similar view of a homozygous *D-Six4*^{289} embryo, where muscles are highly disorganized; some are missing (arrows), and there are many unfused myocytes (arrowheads). **(c)** A higher power view of the region of (b), showing an apparently unfused (mononucleate) muscle founder cell (arrow). **(d)** A homozygous *D-Six4*^{131} embryo. Some muscle disruption and unfused myoblasts can be observed, even though such embryos are able to hatch.

myotonia, muscle wasting, testicular atrophy, and cataracts. Recent data strongly support a role in myotonia for RNA-mediated effects of the CUG repeat within the *DMPK* gene [25]. Other facets of the DM1 phenotype in muscle and heart tissues may be attributed to effects mediated by *DMPK* mRNA, but thus far, no compelling evidence has emerged. Recent initial reports of a *Six5* mouse knockout support a role for *SIX5* mutation in cataract formation [26, 27], but evidence for a role in mesodermally derived tissues is contradictory. The *Six5* mutant mice produced by one group are reported to have no muscle or reproductive defects [26], while a second group reports that their *Six5* mutant mice are sterile and show muscle wasting [28]. One possibility is that *SIX5* becomes

the more important of the *SIX4/5* gene pair later in life, while the more extreme effects of *D-Six4* mutants reflect important early functions of a semiredundant *SIX4/5* gene pair. How far the similarities between *SIX5* and *D-Six4* extend, therefore, remains to be determined, but it is a strong possibility that *D-Six4* may exhibit similar functional relationships to its vertebrate homologs. At the least, the immediate regulatory networks may be conserved. It is an exciting possibility that specific developmental functions may also be conserved, such as regulating target genes involved in cell recognition or association. This may allow the genetic dissection of such regulatory networks and target genes using *Drosophila* and may illuminate the role of human *SIX4/5* in development and disease.

Materials and methods

D-Six4 *cDNA cloning*

A *Drosophila* wild-type genomic library was probed with a human *SIX5* RT-PCR probe. RT-PCR primers were designed to positive clones (5′-GATATCCCACCCCCGACGAGAAG-3′ and 5′-CAGCCTCCTCA CATCGCAGTTTA-3′). A 200-bp product was amplified from 0–8 hr *Drosophila* wild-type embryonic cDNA extracted using a Dynal mRNA Direct kit. The RT-PCR product was used to screen a 4–8 hr *Drosophila* wild-type embryonic cDNA library [29]. The positive clones were sequenced on a Perkin Elmer 377 Automated Sequencer. Four nucleotide differences were found when compared with the previously reported *D-Six4* sequence (AF247709): $T_{238} > C$, $T_{313} > G$, $G_{424} > C$, and $C_{1024} > A$ (numbers refer to AF247709). The sequence has been submitted as GenBank accession number AF099185.

Fly stocks

The *D-Six4* gene is uncovered by the chromosomal deficiency, *Df(3L)ri*^{XT106} [30], as judged by the loss of *D-Six4* mRNA and DNA in homozygous embryos (data not shown). The *Df(3L)ri*^{XT106}*, ru*^{1} *st*^{1} *e*^{1} *ca*^{1}*/TM3, Ser* fly stock was a gift from C. Nüsslein-Volhard (breakpoints 77E1–2; 77E2–8). Wild-type flies were of the Oregon R strain.

Histology

For RNA in situ hybridization to imaginal discs and whole-mount embryos, we followed the protocol of Tautz [31], with modifications. Retrotransposon *412* DNA was obtained from R. White. Antibody staining of imaginal discs and whole-mount embryos was performed using standard methods. Primary antibodies that were used included: anti-Eya (mouse 1:500) [18], anti-myosin heavy chain (rabbit 1:500, provided by B. Paterson), and anti-Vasa (1:4,000, provided by R. Lehmann). Secondary antibodies were purchased from Jackson Research Laboratories.

RNA interference assay

For RNA interference experiments, we followed the protocol of R. Carthew (http://info.pitt.edu/∼carthew/manual/Manual.html). Full-length *D-Six4* cDNA was used as a template for making dsRNA. Embryos injected with *D-Six4* dsRNA were allowed to develop for 17 hr at 21°C for the detection of germ cells.

Mutagenesis screen

Mutations of *D-Six4* were isolated based on the assumption that such mutations would be homozygous lethal with gonad defects (indicated as a likely *D-Six4* function from RNAi experiments). Thus, a standard chemical mutagenesis screen using EMS was carried out. Oregon R males were fed 30 mM EMS overnight. These were mated to *Ly/TM3, Sb* females. Individual male offspring carrying the TM3 balancer were mated to pairs of females harboring the *D-Six4* deficiency (*Df(3L)ri*^{XT106}*, ru*^{1} *st*^{1} *e*^{1} *ca*^{1}*/TM3*). The offspring of these matings were scored for lack of non-TM3 flies, which would indicate the presence of a new lethal mutation that mapped to 77E1–2; 77E2–8. We isolated 23 such lethals from 3500 crosses. Stocks were established and then tested for complementation inter se and with *knirps* (*kni*). *kni* lies within *Df(3L)ri*^{XT106} and is known to have gonadal defects. Embryos from non-*kni* complementation groups were analyzed by anti-Vasa staining for gonad defects. One complementation group of two alleles showed a clear lack of gonad coalesence. The *D-Six4* exons were amplified from these mutant alleles and sequenced.

Sequencing of mutant alleles

DNA was isolated for PCR from homozygous mutant flies or embryos. The four exons were amplified using the following primers. Exon 1: 5′-TGGGATTAACCGAGTGATTT-3′ and 5′-CGGCTGTGAGATTGG ATA-3′; exons 2 and 3: 5′-GGGAATTTTAGGGGGATC-3′ and 5′-CTG TTCAAGATAGGATGTG-3′; exon 4: 5′-TCCCAGCCTGAACAGCAT AATA-3′ and 5′-CGAGCTCTTATTGCCCATTGAAAATCGTT-3′. The PCR products were sequenced directly using the same primers.

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