Redd1 Is a Novel Marker of Testis Development but Is Not Required for Normal Male Reproduction

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Key Words

Gonadogenesis • Murine • *Redd1* • Sex determination • Testis

Abstract

In an effort to identify novel candidate genes involved in testis determination, we previously used suppression subtraction hybridisation PCR on male and female whole embryonic (12.0–12.5 days post coitum) mouse gonads. One gene to emerge from our screen was *Redd1*. In the current study, we demonstrate by whole-mount in situ hybridisation that *Redd1* is differentially expressed in the developing mouse gonad at the time of sex determination, with higher expression in testis than ovary. Furthermore, *Redd1* expression was first detected as *Sry* expression peaks, immediately prior to morphological sex determination, suggesting a potential role for *Redd1* during testis development. To determine the functional importance of this gene during testis development, we generated *Redd1*-deficient mice. Morphologically, *Redd1*-deficient mice were indistinguishable from control littermates and showed normal fertility. Our results show that *Redd1* alone is not required for testis development or fertility

in mice. The lack of a male reproductive phenotype in *Redd1* mice may be due to functional compensation by the related gene *Redd2*.

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The key sex-determining gene in most mammals is the Y-linked gene *Sry* (sex-determining region on the Y chromosome) [Sinclair et al., 1990]. In the mouse, *Sry* is expressed briefly in the pre-Sertoli cells of the genital ridge from 10.5 days post coitum (dpc) to 12.5 dpc and this initiates testis determination and differentiation [Koopman et al., 1990]. The genetic pathways downstream of *Sry* that further regulate testis formation and differentiation, however, remain largely unknown. In an effort to identify novel genes involved in these pathways, we previously performed a differential expression screen, which revealed 54 transcripts that were upregulated in embryonic mouse testis at 12.0–12.5 dpc. This included 8 genes that were novel or of unknown function [McClive et al., 2003].

One gene to emerge from our screen was *Redd1* (regulated in development and DNA damage responses 1). Male-specific up-regulation of *Redd1* in the gonad at 12.5

dpc was confirmed by whole-mount in situ hybridisation (WISH). Furthermore, within the gonad, *Redd1* expression localised to the testis cords suggesting that this gene might play a role in male sex determination or early differentiation of the testis.

Previous studies reported that *Redd1* (also known as *RTP801*, *Ddit4* and *Dig2*) showed strong up-regulation in response to a variety of cellular stressors, including hypoxia, DNA damage and glucocorticoid treatment [Ellisen et al., 2002; Shoshani et al., 2002; Wang et al., 2003]. The *Redd1* gene encodes a widely expressed, highly conserved, low-molecular-mass protein that contains no known structural domains or functional motifs [Ellisen et al., 2002; Shoshani et al., 2002]. *Redd1* has been described as a transcriptional target of HIF-1 α (hypoxia-inducible factor 1 alpha) that can act as a pro- or anti-apoptotic factor, depending on the cellular type [Shoshani et al., 2002]. It has also been described as a transcriptional target of the tumour suppressor genes, *p53* and *p63*, involved in *p53*-dependent DNA damage response and *p63*-dependent epithelial differentiation [Ellisen et al., 2002]. Furthermore, *Redd1* has been shown to act as a negative regulator of the mammalian target of rapamycin (mTOR) pathway which controls cell growth and proliferation by modulating the activity of the tuberous sclerosis tumour suppressor complex [Brugarolas et al., 2004; Corradetti et al., 2005]. A recent study has demonstrated that *Redd1* is an early responsive retinoic acid target gene associated with myeloid differentiation [Gery et al., 2007]. Taken together, these findings suggest that *Redd1* may potentially play a role in testis cell growth.

The *Redd1* paralogue *Redd2* shares approximately 50% sequence identity with *Redd1*, but little homology to other known proteins [Corradetti et al., 2005]. The *Drosophila* orthologues of *Redd1* and *Redd2* are termed *scylla* and *charybdis*, respectively. *Scylla* and *charybdis* act as negative regulators of growth and simultaneous loss of these genes in *Drosophila* results in increased susceptibility to hypoxia and mild overgrowth phenotypes [Reiling and Hafen, 2004].

Here, we present a detailed analysis of *Redd1* expression during fetal testis development. Our data demonstrates that within the developing testis cords, *Redd1* is expressed in the germ cells and the key testis-determining cell type, pre-Sertoli cells. Since *Redd1* shows a sexually dimorphic expression pattern at the time of testis determination and is expressed in the key testis-determining cell type, we generated an in vivo model to investigate the functional importance of this gene during testis development.

Materials and Methods

Whole-Mount in situ Hybridisation

Outbred CD1 mice were used for all expression studies. WISH was carried out with digoxigenin-labelled probes, as described previously [Andrews et al., 1997; McClive and Sinclair, 2003; McClive et al., 2003]. A 320-bp *RsaI* cDNA fragment corresponding to *Redd1* (NM_029083) was obtained from the subtraction procedure. The probes for *Redd1* and *Sry* (NM_011564) were generated using SP6 and T7 RNA polymerases as described previously [Andrews et al., 1997; Hurley et al., 2004]. For WISH experiments using gonads from *Redd1*-deficient mice, the template for the 797-bp *Redd1* riboprobe was generated by PCR from adult mouse spleen cDNA using the primers 5'-GGACAGCAGCAACAGTGGCT-3' and 5'-CCTGTGTGACTCCTAAGCCTT-3'. To visualise staining in sections, tissues were left in chromogen solution over 2 days at room temperature. Tissues were cryoprotected in 30% sucrose in PBS (overnight at 4°C), snap frozen in OCT embedding compound and 14- μ m sections were cut.

Redd1-Deficient Mice

Redd1-deficient mice were generated by Ozgene Pty Ltd (Canning Vale, W.A., Australia) using the Cre/loxP system. Briefly, 3 DNA fragments (a 3.1-kb 5' homology arm containing exon 1, a 2.2-kb floxed arm containing exons 2 and 3 and a 3.1-kb 3' homology arm) were generated by nested PCR from C57BL/6 mouse genomic DNA and cloned into the pOzIII vector (Ozgene). The 5' homology arm was cloned into an *AscI* site, upstream of the first loxP site. The loxP arm was cloned into a *SalI* site upstream of the PGK-neo cassette. The 3' homology arm was cloned into a *KpnI* site downstream of the PGK-neo cassette and the second loxP site. The targeting vector was linearised at the 3' end by *NotI* digestion and electroporated into Bruce-4 embryonic stem cells (derived from murine strain C57BL/6J) [Kontgen et al., 1993]. Genomic DNA was isolated from individual neomycin (G418)-resistant colonies, digested with *BglII* and screened for homologous recombination events by Southern blotting using a 450-bp 5' probe. Targeted embryonic stem cell clones were microinjected into BALB/c blastocysts and transferred to pseudopregnant foster mothers. This resulted in the generation of 26 chimeric males, which were backcrossed to *PGK-Cre* transgenic (on a C57BL/6 background) mice to confirm germline transmission and to generate heterozygous *Redd1*-deficient mice. Mice were maintained on a C57BL/6 background as this strain has been shown to be sensitive to sex reversal [Eicher et al., 1996]. Heterozygous *Redd1*-deficient mice were intercrossed in order to generate homozygous *Redd1*-deficient mice. Embryos were collected from timed matings, with noon of the day on which the mating plug was observed recorded as 0.5 dpc. Genomic DNA was isolated from tail biopsies of 3-week-old mice and embryos and used as a template for multiplex PCR genotyping using primers specific for the *Redd1* wild-type allele (wt β : 5'-CGACTTTGAGCTGCTCAGTGA-3' and wt γ : 5'-GCTCGGAGCTGTAGAGTTTCT-3') and the *Redd1* knockout allele (ko β : 5'-GCACCAGTTCGCTCACCTT-3' and ko γ : 5'-GCCTTCTCACCTGGAGAGCA-3').

Ethics Statement

All procedures involving mice were performed with the approval of the Murdoch Children's Research Institute Animal Ethics Committee (Project Numbers A527 and A619).

Tissue Collection, Histology and Immunofluorescence

Embryonic gonads were dissected and fixed in 4% paraformaldehyde in PBS for 20 min on ice. For histological examination, tissues were embedded in paraffin, 5- μ m serial sections were cut and stained with haematoxylin and eosin (H&E) using standard protocols. For immunofluorescence, tissues were embedded in OCT embedding compound and 10- μ m serial sections were cut, dried, postfixed for 1 min in 4% paraformaldehyde and permeabilised in 1% Triton X-100 for 10 min. Non-specific antibody binding was blocked in PBS containing 5% BSA and 10% normal goat serum overnight at 4°C. Primary antibodies used were WT1 (Wilms tumour 1) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) diluted 1:200, laminin (Sigma, St. Louis, Mo., USA) diluted 1:200, P450scc (P450 side-chain cleavage) (Chemicon, Temecula, Calif., USA) diluted 1:400, and MVH (mouse vasa homologue) (Abcam, UK) diluted 1:10,000. Primary antibody binding was detected using goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, Calif., USA) diluted 1:500. Adult mice were killed by cervical dislocation, tissues were immediately dissected and wet weight was determined.

Serum Hormone Measurements

Concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by heterologous radioimmunoassay using reagents kindly provided by Dr. Parlow at the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, Md., USA). Iodinated preparations and antisera used in the FSH and LH radioimmunoassays were: rFSH-I-9 and anti-rFSH-S-11; and rLH-I-9 and anti-rLH-S-10, respectively. Results are expressed in terms of NIDDK mFSH-RP (AFP-5308D) and NIDDK mLH-RP (AFP-5306A). The iodination preparations were iodinated using Iodogen reagent (Pierce, Rockford, Ill., USA). Goat anti-rabbit IgG (GAR#12 – Monash Institute of Medical Research, Monash University, Melbourne, Vic., Australia) was used as second antibody for the FSH and LH radioimmunoassays. The lower limits of detection were 1.83 ng/ml (FSH) and 0.13 ng/ml (LH). The within-assay % coefficients of variances were 8.0% (FSH) and 4.8% (LH). Testosterone was measured using a commercial radioimmunoassay kit (DSL-4100; Diagnostic Systems Laboratories, Webster, Tex., USA) according to manufacturer's instructions. The lowest limit of detection was 0.094 ng/ml and the within-assay % coefficient of variance was 4.2%. All samples were measured in a single assay for each hormone.

Reverse Transcription PCR (RT-PCR)

Total RNA was isolated from fetal and adult mouse gonads using RNeasy Mini kit (Qiagen, Valencia, Calif., USA) and treated with DNaseI using DNA-free (Ambion, Austin, Tex., USA). 1 μ g of DNaseI-treated RNA was reverse transcribed using random hexamers and 10 units of Transcriptor High Fidelity Reverse Transcriptase (Roche, Mannheim, Germany) according to manufacturers' instructions. To determine the expression level of *Redd1*, 50 ng of cDNA was subject to multiplex PCR using primers specific for *Redd1* (fwd: 5'-GCACCAGTTCGCTCACCCTT-3' and rev: 5'-GGCACACAGGTGCTCATCCT-3') and *Hprt* (hypoxanthine guanine phosphoribosyl transferase, NM_013556) (fwd: 5'-TGCTGACCTGCTGGATTACA-3' and rev: 5'-GCTTAACCGAGGAAAGCAAAG-3').

Quantitative Real-Time PCR (qPCR)

For experiments using cDNA isolated from pure cell populations, testes were dissected from *Oct4-GFP* transgenic mice and germ and somatic cell populations were separated by fluorescence activated cell sorting (FACS) as described previously [Western et al., 2008]. qPCR was performed in triplicate using up to 50 ng cDNA in each 10- μ l reaction, using the mouse Universal Probe Library (UPL) system (Roche), LightCycler 480 Probes Master (Roche) and a LightCycler 480 instrument (Roche). Relative expression was determined using the comparative C_T method ($\Delta\Delta C_T$) [Livak and Schmittgen, 2001], with samples normalised against *Sdha* (succinate dehydrogenase complex, subunit A, flavoprotein) and expressed relative to the sample showing the highest level of expression for each experiment. Primers for *Redd1*, *Redd2* (NM_030143) and *Sdha* (NM_023281) were designed using the UPL Assay Design Centre (<https://www.roche-applied-science.com/sis/rtpcr/upl/adsc.jsp>).

Fertility Study

The fertility of *Redd1*-deficient males and females aged 7–11 weeks, was assessed by matings with wild-type female and male mice, respectively. At least 6 different breeding pairs were set up for each group. The number of pups born per litter, the percentage of pups weaned per litter and the percentage of males weaned per litter were recorded. The effect of genotype on group means for pups born/litter and pups weaned/litter was analysed by one-way analysis of variance. The effect of genotype on percentage of male offspring per total number of mice born was analysed by χ^2 analysis.

Results

Redd1 Is Differentially Expressed during Embryonic and Adult Gonad Development

We previously identified *Redd1* as a novel gene that is differentially expressed in the developing mouse gonad at the time of sex determination, with higher expression in testis than ovary [McClive et al., 2003]. In the present study, differential expression of *Redd1* in male and female gonads from 11.5 to 16.5 dpc was confirmed by WISH (fig. 1A–H). *Redd1* expression in the testis was maintained throughout gonad development (fig. 1A–D) and was localised within the cords from 12.5 dpc (fig. 1B–D). Expression was also observed in the Wolffian and Müllerian ducts in both male and female embryos (fig. 1B and G). In the ovary, weak *Redd1* expression was observed from 11.5 dpc (fig. 1E–H). We next compared temporal expression of *Redd1* and *Sry* using tail somite (ts) staged testis pairs. *Redd1* mRNA expression was first detected just as *Sry* expression peaked at 19 ts (fig. 1I–P) and immediately prior to morphological sex determination, suggesting that *Redd1* could be involved downstream in the *Sry* pathway. We also examined *Redd1* expression levels in adult versus embry-

Fig. 1. Expression analysis of *Redd1* during mouse gonadogenesis. **A–H** Temporal expression analysis of *Redd1* by WISH. **A–D** Male and **E–H** female gonads at 11.5 dpc, 12.5 dpc, 13.5 dpc and 16.5 dpc. Dotted lines show the mesonephric-gonadal boundaries. md = Müllerian duct; wd = Wolffian duct. **I–P** Temporal expression analysis of *Redd1* versus *Sry* in left and right gonad pairs by WISH. **I–L** *Sry* and **M–P** *Redd1* expression at 11 tail somites (ts), 19 ts, 20 ts and 21 ts. Bars = 500 μ m. **Q** qPCR analysis of *Redd1* cDNA expression (normalised against *Sdha* and relative to 12.5 dpc testis) in whole gonads from 12.5 dpc embryos and 8-week-old mature mice. Fetal gonads were pooled according to sex (1 litter per pool). Values are mean $\pm$ SEM (n = 3).

onic gonads by qPCR. Differential expression of *Redd1* at 12.5 dpc was confirmed using this approach. Furthermore, we observed a reduction in the expression levels of *Redd1* in the adult gonad compared to the embryonic gonad (fig. 1Q). These results indicate that *Redd1* may be important at various stages during testis development.

Redd1 Is Expressed in Pre-Sertoli and Germ Cells

In order to identify which cell types within the developing testis cords express *Redd1*, we performed WISH on 12.5 dpc male gonads. Sections from WISH analysis demonstrated that within the developing testis, *Redd1* expression is confined to the testis cords indicating that *Redd1* is expressed by the Sertoli and/or germ cells (fig. 2A, B). The expression of *Redd1* in somatic and germ cell populations was ascertained by qPCR using sorted cell populations derived from the testes of *Oct4-GFP* transgenic mice. We have previously demonstrated the sorted cell populations to be 99% pure by FACS analysis and qPCR using germ- and somatic cell-specific markers [Western et al., 2008]. In this study, we found that *Redd1* expression was detected in both the somatic and germ cell populations between 12.5 and 15.5 dpc (fig. 2C). The level of *Redd1* expression in the germ cell populations at 12.5 and 13.5 dpc was as high or higher

than the levels in the somatic cell population (fig. 2C), suggesting that *Redd1* may also play a role in germ cell development.

Generation and Validation of Redd1-Deficient Mice

To investigate the function of *Redd1*, mice carrying a *Redd1* floxed allele (fig. 3A) were generated by standard gene targeting techniques. Offspring resulting from matings between floxed *Redd1* chimeric males and *PGK-Cre* transgenic mice were screened by Southern analysis to confirm germline transmission (fig. 3B). Heterozygous *Redd1*-deficient mice carrying *PGK-Cre* were backcrossed to C57BL/6 to remove the *Cre* transgene. Heterozygous *Redd1*-deficient mice (negative for *Cre*) were intercrossed and the resulting progeny were genotyped by PCR. Using this approach, we observed that heterozygous *Redd1*-deficient matings produced offspring with a normal Mendelian distribution of genotypes (data not shown). Furthermore, *Redd1*-deficient mice were grossly indistinguishable from littermate controls, suggesting that *Redd1* does not play a critical role in embryonic or neonatal development. Multiplex RT-PCR on total testis cDNA from wild-type and heterozygous *Redd1*-deficient mice resulted in a 402-bp band corresponding to *Redd1* cDNA and a 230-bp band corresponding to *Hprt* cDNA (fig. 3C). In

Fig. 2. *Redd1* is expressed in both Sertoli and germ cell populations. **A, B** Overstained and sectioned WISH of 12.5 dpc wild-type testis. *Redd1* expression is localised to the testis cords, indicative of a Sertoli and/or germ cell expressed transcript. Bars = 50 μ m. **C** qPCR analysis of *Redd1* cDNA expression (normalised against *Sdha* and relative to 13.5 dpc germ cell population) in pure germ cell populations and somatic cell populations, sorted from the testes of 12.5 to 15.5 dpc *Oct4-GFP* transgenic mice. Values are mean $\pm$ SD for pooled samples (n = 3) measured in triplicate.

contrast, only the 230-bp *Hprt* band could be amplified from *Redd1*-deficient mice (fig. 3C), verifying that Cre-mediated deletion of the floxed *Redd1* allele was complete.

Redd1-Deficient Mice Display Normal Fetal Testis Development

H&E-stained testis sections from 14.5 dpc wild-type and *Redd1*-deficient mice showed no obvious differences in cord formation or testis development (fig. 4A, B). Furthermore, no gross differences in general tissue organisation or cellular composition were observed following immunofluorescence on 14.5 dpc testes with cell-type-specific markers for WT1 (Sertoli cells), laminin (basement cell membrane), P450scc (Leydig cells) and MVH (germ cells) (fig. 4C–J).

Fig. 3. Generation of *Redd1*-deficient mice. **A** Schematic diagram of wild-type, floxed and *Redd1* knockout alleles. *Redd1* is comprised of 3 exons (numbered 1 to 3) and the ATG is located within exon 2. LoxP sites are represented as black triangles. The position of the 450-bp 5' probe used for Southern blotting and the primers (wt_f, wt_r, ko_f, ko_r) used for PCR genotyping are indicated. B = *Bgl*II restriction site; B* = newly created *Bgl*II restriction site. **B** Southern blot analysis of wild-type (+/+), heterozygous floxed (+/flox) and heterozygous knockout (+/-) mice using *Bgl*II-digested tail DNA and the 450-bp 5' probe to confirm germline transmission. **C** RT-PCR using total testis cDNA from wild-type, heterozygous knockout and homozygous knockout adult mice. *Redd1* primers generate a 402-bp band and *Hprt* primers generate a 230-bp band.

Redd1-Deficient Mice Are Fertile

Both *Redd1*-deficient males and females demonstrated normal fertility with respect to the number of pups born per litter, the proportion of pups weaned per litter and the percentage of males weaned per litter (table 1). No cases of ambiguous genitalia or sex reversal were observed among the offspring. Furthermore, serum LH, FSH and testosterone levels were normal in *Redd1*-deficient adult males (table 2).

Redd2 Is Expressed in the Somatic Cells during Early Testis Development

The possibility of functional redundancy by *Redd2* (a *Redd1* paralogue) was investigated as an explanation for the mild phenotype in our knockout model. The levels of *Redd2* expression in sorted somatic and germ cell popula-

Fig. 4. Immunofluorescence of wild-type and *Redd1*-deficient male gonads. Wild-type (**A**, **C**, **E**, **G**, **I**) and homozygous *Redd1*-deficient (**B**, **D**, **F**, **H**, **J**) fetal mouse testis sections at 14.5 dpc stained with H&E and immunofluorescence performed with WT1 (a Sertoli cell marker), laminin (a basement cell membrane marker), P450sc (a Leydig cell marker) and MVH (a germ cell marker). Bars = 50 μ m.

Fig. 5. qPCR analysis of *Redd2* in wild-type and *Redd1*-deficient male gonads. **A** qPCR analysis of *Redd2* cDNA levels (normalised against *Sdha* and relative to 12.5 dpc somatic cell population) in pure germ cell and somatic cell populations, sorted from the testes of 12.5 to 15.5 dpc *Oct4*-GFP transgenic mice. Values are mean $\pm$ SEM for pooled samples ($n = 3$) measured in triplicate. **B** qPCR analysis of *Redd2* cDNA levels (normalised against *Sdha* and relative to 12.5 dpc knockout sample) in wild-type and *Redd1*-deficient whole gonads at 12.5 and 14.5 dpc. Fetal gonads were pooled according to sex and stage (1 litter per pool) and each sample was measured in triplicate. Values represent the mean of technical triplicates.

tions were ascertained by qPCR. *Redd2* expression was detected exclusively in the somatic cell populations between 12.5 and 15.5 dpc (fig. 5A), suggesting that this gene may compensate for *Redd1* loss in the supporting cell lineage, but not in germ cells. We also performed qPCR to determine the levels of *Redd2* in *Redd1*-deficient gonads compared to wild-type controls at 12.5 dpc and 14.5 dpc. Fetal gonads were pooled according to sex and stage (1 litter per pool) and each sample was measured in triplicate. qPCR analysis revealed no change in the level of *Redd2* expression in *Redd1*-deficient gonads compared to controls at either stage (fig. 5B).

Discussion

Causative mutations in known sex-determining genes such as *SRY* account for approximately 20% of human XY male-to-female sex reversal cases. In the remaining 80%

Table 1. Reproductive phenotype of *Redd1*-deficient mice

Mating (male × female)	Number of days until first litter ^a (n)	Pups born/litter ^a (n)	Pups weaned/litter, % ^a (n)	Males weaned ^b (%)
Wild-type × wild-type	20.7 ± 0.9 (6)	7.6 ± 0.4 (17)	74.3 ± 7.2 (17)	45/96 (46.9)
<i>Redd1</i> ^{-/-} × wild-type	26 ± 2.9 (14)	7.3 ± 0.5 (29)	74.8 ± 6.6 (29)	83/171 (48.5)
Wild-type × <i>Redd1</i> ^{-/-}	28.4 ± 6.7 (5)	7.2 ± 0.5 (19)	69.5 ± 7.2 (19)	48/99 (48.5)

^aValues are mean ± SEM. n = Number of litters analysed per group. p > 0.05, calculated using one-way analysis of variance.

^bValues are number of males/number of pups weaned. p > 0.05, calculated using χ^2 analysis.

Table 2. Serum hormone measurements of *Redd1*-deficient mice

Genotype	LH, ng/ml	FSH, ng/ml	Testosterone, ng/ml
<i>Redd1</i> ^{+/+}	0.20 ± 0.01	9.6 ± 0.4	1.0 ± 1.6
<i>Redd1</i> ^{-/-}	0.18 ± 0.00	9.1 ± 0.2	1.9 ± 13.9

All values are mean ± SEM. Ten mice were analysed per group.

of cases, mutations in known sex-determining genes have not yet been found, suggesting that other, as yet unidentified genes are likely to play an important role in the sex determination/differentiation pathway. We previously used a suppression subtraction hybridisation screen to identify novel sexually dimorphic genes expressed in the mouse gonad at the time of sex determination [McClive et al., 2003]. One gene revealed by our screen was *Redd1*, a recently cloned stress response gene. We present here a detailed analysis of *Redd1* expression during mouse gonadogenesis and functional analysis of a mouse model with targeted disruption of this gene.

Expression analysis using WISH and qPCR revealed higher levels of *Redd1* expression in the testis than in the ovary at 12.5 dpc. Furthermore, expression localised to the pre-Sertoli and germ cells of the testis cords and was upregulated immediately following peak *Sry* expression. Based on the temporal and spatial expression pattern observed for *Redd1*, we investigated the functional importance of this gene during gonadogenesis. To our surprise, *Redd1*-deficient mice were grossly indistinguishable from littermate controls and showed normal fertility. Fetal testis development appeared normal with respect to cord formation, cell composition and expression of cell type-specific markers. No differences in hormone production were detected in *Redd1* null mice and the litter sizes and

sex ratios of offspring born to knockout mice were also normal. Interestingly, although we observed no phenotypic differences in the cellular composition or reproductive function of testes in *Redd1* null mice, there appeared to be a small (<20%) increase in testis weight, compared to wild-type controls. The significance of this difference remains unknown, but is consistent with a role for *Redd1* in controlling testis growth.

One explanation for the normal reproduction and fertility of *Redd1*-deficient mice may be the potential for *Redd2* to functionally compensate for the loss of *Redd1*. A study in *Drosophila* has revealed the functional redundancy of *scylla* and *charybdis* (*Redd1/Redd2* orthologues) for head involution during larval development [Scuderi et al., 2006]. In this study, we demonstrate that *Redd2* is expressed exclusively in the somatic cells of the developing gonad, although further investigation is required to determine the precise somatic cell type(s) that expresses *Redd2*. Since *Redd1* is expressed in both somatic and germ cells of the developing testis, this data implies that if *Redd2* can compensate for loss of *Redd1*, this compensation is restricted to the somatic cell compartment and any effect on germ cell development is indirect.

A double knockout of *Redd1* and *Redd2* may reveal a more pronounced phenotype and role for these genes during testis development.

Acknowledgements

We would like to thank members of our laboratories for valuable discussions; Anne O'Connor for serum hormone measurements; and Anna Piper and Christine Hall for animal husbandry. This research was supported by The National Health and Medical Research Council (Program Grant #334314) and the Victorian Government's Operational Infrastructure Support Program.

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