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ORIGINAL ARTICLE

RNA immunoprecipitation identifies novel targets of DAZL in human foetal ovary

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STUDY QUESTION: Can novel meiotic RNA targets of DAZL (deleted in azoospermia-like) be identified in the human foetal ovary?

SUMMARY ANSWER: *SYCP1* (synaptonemal complex protein-1), *TEX11* (testis expressed 11) and *SMC1B* (structural maintenance of chromosomes 1B) are novel DAZL targets in the human foetal ovary, thus DAZL may have previously unrecognised roles in the translational regulation of RNAs involved in chromosome cohesion and DNA recombination in the oocyte from the time of initiation of meiosis.

WHAT IS KNOWN ALREADY: The phenotype of Dazl deficiency in mouse is infertility in both sexes and DAZL has also been linked to infertility in humans. Few studies have explored targets of this RNA-binding protein. The majority of these investigations have been carried out in mouse, and have focussed on the male thus the basis for its central function in regulating female fertility is largely unknown.

STUDY DESIGN SIZE, DURATION: We carried out RNA sequencing after immunoprecipitation of endogenous DAZL from human foetal ovarian tissue (17 weeks of gestation, obtained after elective termination of pregnancy) to identify novel DAZL targets involved in meiosis ($n = 3$ biological replicates).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Using quantitative RT-PCR, we examined the expression of selected RNAs identified by our immunoprecipitation across gestation, and visualised the expression of potential target *SMC1B* in relation to DAZL, with a combination of *in situ* hybridisation and immunohistochemistry. 3′ untranslated region (3′UTR)-luciferase reporter assays and polysome pro-file analysis were used to investigate the regulation of three RNA targets by DAZL, representing key functionalities: *SYCP1*, *TEX11* and *SMC1B*.

MAIN RESULTS AND THE ROLE OF CHANCE: We identified 764 potential RNA targets of DAZL in the human foetal ovary (false discovery rate 0.05 and log-fold change ≥ 2), with functions in synaptonemal complex formation (*SYCP1*, *SYCP3*), cohesin formation (*SMC1B*, *RAD21*), spindle assembly checkpoint (*MAD2L1, TRIP13*) and recombination and DNA repair (*HORMAD1, TRIP13, TEX11, RAD18, RAD51*). We demonstrated that the translation of novel targets *SYCP1* ($P = 0.004$), *TEX11* ($P = 0.004$) and *SMC1B* ($P = 0.002$) is stimulated by the presence of DAZL but not by a mutant DAZL with impaired RNA-binding activity.

LARGE SCALE DATA: The raw data are available at GEO using the study ID: GSE81524.

LIMITATIONS, REASONS FOR CAUTION: This analysis is based on identification of DAZL targets at the time when meiosis starts in the ovary: it may have other targets at other stages of oocyte development, and in the testis. Representative targets were validated, but detailed analysis was not performed on the majority of putative targets.

WIDER IMPLICATIONS OF THE FINDINGS: These data indicate roles for DAZL in the regulation of several key functions in human oocytes. Through the translational regulation of novel RNA targets *SMC1B* and *TEX11*, DAZL may have a key role in regulating chromosome cohesion and DNA recombination; two processes fundamental in determining oocyte quality and whose establishment in foetal life may support lifelong fertility.

Introduction

DAZL (deleted in azoospermia-like) and its homologues DAZ (deleted in azoospermia) and BOLL (bol-like RNA-binding protein) comprise a family of RNA-binding proteins that have essential roles in gametogenesis. *DAZ* gene expression is only found in humans and Old World monkeys; transcription is limited to the male germline (Ruggiu and Cooke, 1999) and *DAZ* is deleted in 12–15% of azoospermic men (Reijo *et al.*, 1995). In all other vertebrates, DAZ is replaced by an autosomal single-copy gene: *DAZ-like* (*DAZL*). Targeted disruption of *Dazl* in mice results in infertility in both males and females (Ruggiu *et al.*, 1997; Saunders *et al.*, 2003) and in the human *DAZL* is expressed in germ cells in ovary and testis in both foetal and adult life (Habermann *et al.*, 1998; Dorfman *et al.*, 1999; Anderson *et al.*, 2007). Polymorphisms in the promoter and coding sequence of *DAZL* have been correlated with total sperm count and sperm motility in infertile men (Teng *et al.*, 2002; Tung *et al.*, 2006a), while altered methylation of the *DAZL* promoter has been associated with male infertility (Navarro-Costa *et al.*, 2010; Li *et al.*, 2013). In women, however, there has been only one report concerning the relevance of DAZL to fertility, with polymorphisms in *DAZL* shown to influence the age at premature ovarian insufficiency and menopause (Tung *et al.*, 2006a); however, this has not been reported in other studies (Bartoloni *et al.*, 2004; Zerbetto *et al.*, 2008). These observations suggest that hypomorphic polymorphisms and single nucleotide polymorphisms that quantitatively reduce DAZL expression or function in human germ cells could potentially have consequences for fertility and reproductive lifespan in women.

Deletion of *Dazl* in mice causes loss of germ cells in the gonads of both sexes, with increased apoptosis, reduced expression of germ-cell markers and aberrant chromatin structure (Ruggiu *et al.*, 1997; Schrans-Stassen *et al.*, 2001; Lin and Page, 2005). *Dazl* null mice also have a reduction in the number of post-migratory primordial germ cells, and germ cells fail to sexually differentiate and properly erase genomic methylation imprints (Haston *et al.*, 2009; Gill *et al.*, 2011). Of the few cells that are able enter meiosis, none are able to proceed beyond leptotene of Prophase I, as complete synaptonemal complexes fail to form in *Dazl*$^{-/-}$ mice, thus giving a precise point beyond which meiosis cannot progress without *Dazl* (Saunders *et al.*, 2003). Furthermore, germ cells fail to induce the expression of meiotic genes in response to retinoic acid (Lin and Page, 2005). *Dazl* deficiency specifically compromises germ-cell function, as the transplantation of ROSA/*LacZ*-labelled spermatogonia into the testes of *Dazl*$^{-/-}$ mice confirms that the somatic tissues are capable of supporting gamete development (Rilianawati *et al.*, 2003).

DAZL acts post-transcriptionally, as demonstrated by its association with actively translating polysomes (Tsui *et al.*, 2000b) and its ability to activate translation of bound mRNAs (Collier *et al.*, 2005). The involvement of DAZL in translational control is key for gametogenesis. During their growth phase, mammalian oocytes are transcriptionally active, however, mRNA synthesis drops to very low or undetectable levels at the onset of meiosis (Clarke, 2012). From this point onwards, gene expression in the oocyte and subsequent protein production is highly dependent upon a well-orchestrated programme of dynamic modulation of mRNA poly(A)-tail length, recruitment to polysomes and translational activation or repression (Seydoux and Braun, 2006; Radford *et al.*, 2008). However, in order to understand the growing spectrum of processes in gametogenesis that this gene may be involved in, a knowledge of DAZL mRNA targets is required. Efforts to uncover *in vivo* mRNA targets of mammalian DAZL have mainly been focussed on mouse, and the male. Analysis of gene expression in the *Dazl*$^{-/-}$ mouse foetal ovary has identified mRNAs whose abundance is downstream of Dazl function, but this analysis does not identify mRNAs which are directly regulated by Dazl (Soh *et al.*, 2015). Three direct mRNA targets of Dazl characterised *in vivo* are *Sycp3* (synaptonemal complex protein 3), *Mvh* (mouse vasa homologue) (formally known as *Ddx4* or *Vasa*) and *Tex19.1* (testis expressed 19.1) (Reynolds *et al.*, 2005; Reynolds *et al.*, 2007; Chen *et al.*, 2011), and *Tet1* (tet methylcytosine dioxygenase 1) has also been demonstrated to be dependent on Dazl for its translation in cultured mouse embryonic stem cells (Welling *et al.*, 2015). *Sycp3*-deficient spermatocytes arrest in meiosis due to defects in chromosome synapsis, as do spermatocytes from *Mvh* and *Tex19.1* null males (Tanaka *et al.*, 2000; Ollinger *et al.*, 2008). This is a similar point at which meiotic progression ceases in male mice lacking Dazl (Lin and Page, 2005). This would suggest that a loss or reduced expression of these genes, and possibly also additional transcripts that are dependent on Dazl for their translation, contributes to the infertility phenotype observed in *Dazl* null male mice. In contrast, whereas *Dazl* null female mice also arrest meiotic progression in early prophase during foetal development (Saunders *et al.*, 2003), neither *Sycp3*, *Mvh* nor *Tex19.1* null female mice have such severe defects that oogenesis arrests at this stage (Tanaka *et al.*, 2000; Yuan *et al.*, 2002; Ollinger *et al.*, 2008). Additionally, currently unknown Dazl targets in foetal oocytes may therefore be contributing to the *Dazl* null phenotype in female mice. In humans, even less is known about DAZL targets and their impact on female fertility (Rosario *et al.*, 2016).

Here we identify novel RNA targets of DAZL in the human ovary, using foetal tissue to allow analysis during the onset and early stages of Meiosis I. Our data confirm the meiotic role of DAZL in the human foetal ovary, and also reveal novel potential functions for DAZL through translational regulation of RNA targets involved in establishment of chromosome cohesion and DNA recombination during early meiosis in the foetal oocyte, key pathways implicated in the determination of lifelong oocyte quality.

Materials and Methods

Ethics statement

Ethical approval for this study was obtained from Lothian Research Ethics Committee (study code LREC 08/S1101/1), and women gave informed

written consent. Experiments involving mice were approved by the University of Edinburgh Animal Research Ethics Committee and performed according to the UK Animal (Scientific Procedures) Act 1986.

Collection of human foetal ovaries

Human fetuses (8–20 weeks gestational age (wga)) were obtained after elective termination of pregnancy, and all fetuses used in this study were morphologically normal. Gestational age was determined by ultrasound scan, and confirmed (for second trimester fetuses) by direct measurement of foot length. The sex of first trimester foetal gonads was determined by PCR for the SRY gene (Childs and Anderson, 2012). Extra-ovarian tissue was removed from dissected ovaries, which were then either snap frozen on dry ice and stored at −80°C for subsequent RNA extraction, or fixed in Bouins or 4% neutral buffered formalin for 2–3 h before processing into paraffin blocks for immunohistochemical analysis. Thirty-two foetal ovarian samples were obtained and used in this study.

RNA immunoprecipitation

RNA immunoprecipitation was carried out using the Magna RIP™ Kit (Millipore, Livingstone, UK) according to manufacturer's instructions. Immunoprecipitations were performed in lysates made from 2 homogenised 17-week human foetal ovaries, from different fetuses. Separate lysates were made for 3 biological replicates, thus 12 ovaries from a total of 6 fetuses were used for these experiments. RNA–protein complexes were immunoprecipitated using an antibody against human DAZL (dilution 1:100) (#8042, Cell Signalling Technology, Leiden, The Netherlands) (see Supplementary Fig. S1 for antibody validation) or a control matched-IgG. Following RNA purification, the Ovation® RNA-Seq system V2 (NuGEN, The Netherlands) was utilised to generate sufficient quantities of oligo-T primed cDNA (average size 350 bp), which was then subjected to paired-end RNA sequencing using the Illumina HiSeq platform, performed by Edinburgh Genomics, University of Edinburgh, UK. Paired-end sequencing data were processed using TrimGalore v0.4.1 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove the CTTTGTGTTTGA Ovation RNA-Seq adapter using—stringency 5 and—length 30 command line parameters, then mapped to the hg19 assembly of the human genome and Ensembl transcriptome using Tophat v2.1.0 (http://ccb.jhu.edu/software/tophat) with command line parameters-g 1—mate-inner-dist 260—mate-std-dev 110—no-coverage-search—b2-sensitive. Mapped reads that were primary alignments and overlapped Ensembl transcript co-ordinates were counted using Bedtools intersect v2.25.0 (https://github.com/arq5x/bedtools) in-split mode. Paired reads that mapped within the same transcript were counted as a single read. Read counts were imported into R v3.3.2 (https://www.r-project.org/) and biomaRt v2.30.0 (https://bioconductor.org/packages/release/bioc/html/biomaRt.html) used to map Ensembl transcripts to Ensembl genes using the Homo sapiens GRCh38.p7 data set. Genes with multiple transcripts were collapsed into the transcript containing the most read counts, the total number of reads mapped to Ensembl genes counted. Low abundance genes containing fewer than one count per million mapped reads in at least three samples were discarded. Differential analysis was performed using DESeq2 v1.14.0 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) using the biological replicate as a blocking factor in the design model to identify differences between control and DAZL immunoprecipitations. False discovery rate (FDR)-adjusted P-values of ≤0.05 were considered significant, and genes significantly enriched more than 4-fold in DAZL relative to control immunoprecipitations were considered DAZL targets. This was filtered further to include only Ensembl genes with an HGNC gene symbol in the bioMart database. Relationships between significantly differentially expressed RNAs were further explored using GeneSetDB (Araki et al., 2012) using all genes represented in the control and DAZL immunoprecipitations as the universe.

Site-directed mutagenesis

The R115G mutant DAZL overexpression construct was generated by PCR of the R115G coding sequence from pMS2-hDAZL (Collier et al., 2005) harbouring the R115G mutation (a gift from Prof. Nicola Gray), which was introduced with the QuikChange Lightening Site-Directed Mutagenesis kit (Agilent Technologies, Edinburgh, UK). The PCR product was cloned into pCMV6-entry (referred to as vector only control) (OriGene Technologies, Rockville, MD, USA) using SgfI and XhoI. The mutant SMC1B 3′ untranslated region (3′UTR)-luciferase construct and the mutant TEX11 3′UTR-luciferase construct were created by mutating G in the mouse Dazl-binding site to A using the QuikChange Lightening Site-Directed Mutagenesis kit (Agilent Technologies, Edinburgh, UK). A second mutant SMC1B 3′UTR-luciferase construct was also created using site-directed mutagenesis to delete the entire mouse Dazl-binding site. All constructs were confirmed to be correct using an ABI 3730XL capillary Sanger sequencing instrument.

Luciferase assays

HEK293T cells (human embryonic kidney cells) were cultured in DMEM + GlutaMAX™ supplemented with 10% foetal bovine serum and maintained at 37°C in 5% CO$_2$. For the luciferase assays, cells were seeded at a density of 20 000 cells per well of a 96-well plate. Cells were transfected with 1ng of the 3′UTR-luciferase construct (wildtype or mutant) or a 3′UTR-luciferase empty vector control (Genecopoeia, Rockville, MD, USA), plus 50 ng of either a wildtype DAZL overexpression construct, a R115G mutant DAZL overexpression construct or a vector only control (OriGene Technologies, Rockville, MD, USA), using TransIT-LT1 transfection reagent (Mirus Bio, Cambridge, UK). Luciferase expression was detected 48 h post transfection using the Luc-Pair Luciferase Assay Kit (Genecopoeia, Rockville, MD, USA) according to manufacturer's instructions.

Sucrose gradient analysis

HEK293T cells were transfected as described above. At 48 h post transfection, cells were treated with 150 µg/ml of cycloheximide before lysis in a polysome lysis buffer (20 mM Hepes pH 7.6, 2 mM MgCl$_2$, 150 mM KCl, 0.5% Nonidet P40, 2 mM dithiothreitol (DTT), 100 U/ml RNasin (Promega, South Hampton, UK)), 150 µg/ml cycloheximide, 1× protease inhibitor (Sigma Aldrich, Dorset, UK) and 1× Halt phosphatase inhibitor (Thermo Fisher Scientific, Paisley, UK). The KCl concentration of the lysates was adjusted before being loaded onto a 10-ml linear sucrose gradient (10–50%) containing 20 mM Hepes pH7.6, 2 mM MgCl$_2$, 250 mM KCl, 0.5% Nonidet p40, 2.5 mM DTT and 0.5 µg/ml heparin. Gradients were centrifuged for 120 min at 4°C in a Beckman SW41 Rotor at 181 781g. Following centrifugation, 10 fractions of 1 ml were collected from the gradient with a KD Scientific peristaltic pump (Holliston, MA, USA) and a Foxy Jr® fraction collector (Teledyne Isco, Lincoln, NE, USA). Absorption traces were recorded with a UA6 Absorbance Detector with an A$_{254}$ filter (Teledyne Isco, Lincoln, NE, USA). Fractions 1–2 and 6–10 were pooled, respectively, for luciferase RNA analysis.

RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA from human foetal ovaries was extracted using the RNeasy Micro Kit (Qiagen, Crawley, UK) according to manufacturer's instructions. Cytoplasmic RNA from luciferase-transfected HEK293T cells was extracted with the Cytoplasmic and Nuclear RNA Purification Kit (Norgen-Biotek, Ontario, Canada) according to manufacturer's instructions. RNA was precipitated

from sucrose gradients using phenol–chloroform (Life Technologies, Paisley, UK), 0.3 M sodium acetate and isopropanol. RNA was reverse transcribed to cDNA using concentrated random primers and Superscript III reverse transcriptase (Life Technologies) according to manufacturer's instructions, and the cDNA synthesis reaction was diluted appropriately before proceeding. Primers for quantitative RT-PCR (RT-qPCR) were designed to amplify all transcript variants and are exon-spanning. Primer pair efficiencies were calculated with the LinReg PCR applet (Ramakers *et al.*, 2003). Each reaction was performed in a final volume of 10 μl, with 1× Brilliant III SYBR Green qPCR Master Mix (Agilent, Santa Clara, CA, USA), 20 pmol of each primer and 2 μl of diluted cDNA. Each cDNA sample was analysed in triplicate. For expression analyses in human foetal ovary, target genes were normalised to the geometric mean expression of *B2M* (Beta-2 microglobulin) and *RPL32* (ribosomal protein L32). For *Luciferase* expression, the *Renilla* sequence which was located on the same construct was used for normalisation. Data analysis for relative quantification of gene expression and calculation of SDs was performed as outlined (Livak and Schmittgen, 2001; Vandesompele *et al.*, 2002).

RNA *in situ* hybridisation and immunohistochemistry

Multiplex detection of *SMC1B* and DAZL protein was performed by Aquila Histoplex, University of Edinburgh. RNA *in situ* hybridisation was carried out using RNAscope® designed probes and reagents (Advanced Cell Diagnostics, Hayward, CA, USA). Briefly, slides were dewaxed and tissue permeabilised with hydrogen peroxide, heat retrieval and proteinase K. RNA probes (*SMC1B* or *DapB* (RNAscope® universal negative control)) were hybridised at 40°C for 2 h, followed by six amplification steps to amplify the signal. For detection, tissue was stained with FITC tyramide at 1:100 for 10 min. Immunohistochemistry was carried out using a mouse DAZL antibody (Abd Serotec, Oxford, UK) at 1:200, a peroxidase secondary antibody at 1:200 and Cy3 tyramide signal amplification at 1:50; counterstained with DAPI.

Results

RNA immunoprecipitation and sequencing identifies novel RNA targets of DAZL

To discover novel and human DAZL targets in the human foetal ovary, we carried out RNA-Seq after immunoprecipitation with foetal ovaries at 17 weeks gestation, when many oocytes are in the early stages of meiosis. Differential gene expression analysis identified a total of 764 RNAs that were significantly enriched by DAZL immunoprecipitation relative to the IgG control (FDR ≤ 0.05 and log-fold change ≥ 2) (see Supplementary Table S1). Among these were validated RNA targets of murine Dazl, *SYCP3* and *TEX14*, indicating technical success.

We performed a gene set enrichment analysis using GeneSetDB (Araki *et al.*, 2012) to investigate whether any disease/phenotype or gene ontology was overrepresented in our list of DAZL-bound RNAs (FDR < 0.05)(Table I and Supplementary Table S2). The majority of the disease/phenotypes that were enriched were related to germ cells, meiosis, gamete production and infertility, but only in males, which likely reflects the lack of female/ovary-related high-throughput data available for enrichment analyses. The role of DAZL in gametogenesis was also evident in the gene ontologies, as the ontology meiosis was significantly enriched (GO:0007126). Within this ontology, the DAZL-enriched RNAs could be divided into different functionalities in meiosis such as synaptonemal complex formation (*SYCP1*, *SYCP3*), cohesin formation (*SMC1B*, *RAD21*), spindle assembly checkpoint (*MAD2L1*, *TRIP13*), and recombination and DNA repair

Table I Gene set enrichment analysis.

Class	Set name	FDR
Disease/ phenotype	Arrest of male meiosis	7.80E-06
	Abnormal male meiosis	1.70E-03
	Azoospermia	2.50E-03
	Decreased testis weight	4.80E-03
	Abnormal spermatocyte morphology	5.80E-03
	Male infertility	5.90E-03
	Small testis	6.80E-03
	Male germ-cell apoptosis	7.30E-03
	Testicular atrophy	8.10E-03
	Abnormal apoptosis	1.70E-02
	Abnormal synaptonemal complex	3.90E-02
Gene ontology	Mitotic cell cycle (GO:0000278)	1.40E-04
	G1/S transition of mitotic cell cycle (GO:0000082)	2.50E-03
	DNA repair (GO:0006281)	8.10E-03
	Protein K63-linked ubiquitination (GO:0070534)	1.70E-02
	GTP catabolic process (GO:0006184)	1.70E-02
	Protein K48-linked ubiquitination (GO:0070936)	2.60E-02
	Meiosis (GO:0007126)	2.60E-02
	G1 phase of mitotic cell cycle (GO:0000080)	4.70E-02
	Ubiquitin-dependent protein catabolic process (GO:0006511)	4.80E-02

False discovery rate (FDR) is <0.05. For a list of RNAs associated with different Subclasses, refer to Supplementary Table S2.
Enrichment of specific disease/phenotypes and gene ontologies was performed using GeneSetDB (Araki *et al.*, 2012).

(*HORMAD1*, *TRIP13*, *TEX11, RAD18*, *RAD51*). The enrichment analysis also highlighted the role DAZL targets may have in the mitotic cell cycle (GO:0000278) and the G1/S transition (GO:0000082).

Given the well-established role of DAZL in meiosis (Saunders *et al.*, 2003), we decided to focus our investigation by identifying other RNA targets of DAZL with meiotic roles in the human foetal ovary. *SYCP1*, *TEX11* and *SMC1B*, which represent RNAs from different meiotic functions, were selected for further investigation (Supplementary Fig. S2). We validated these novel RNA targets *in vitro* using RT-qPCR on a separate immunoprecipitation experiment to confirm that DAZL can bind these RNAs, and calculated the enrichment ratios for each target (Supplementary Fig. S3). Furthermore, we assessed the abundance of reference genes *RPL32* and *GAPDH*, and the granulosa cell marker *FOXL2*, showing these were not enriched after DAZL immunoprecipitation, thus highlighting the specificity of our RNA immunoprecipitation experiment.

DAZL and novel target RNA expression across gestation

Expression of a selected number of novel meiotic RNA targets in the human foetal ovary identified by DAZL immunoprecipitation was analysed across gestation, which was divided into three periods, including
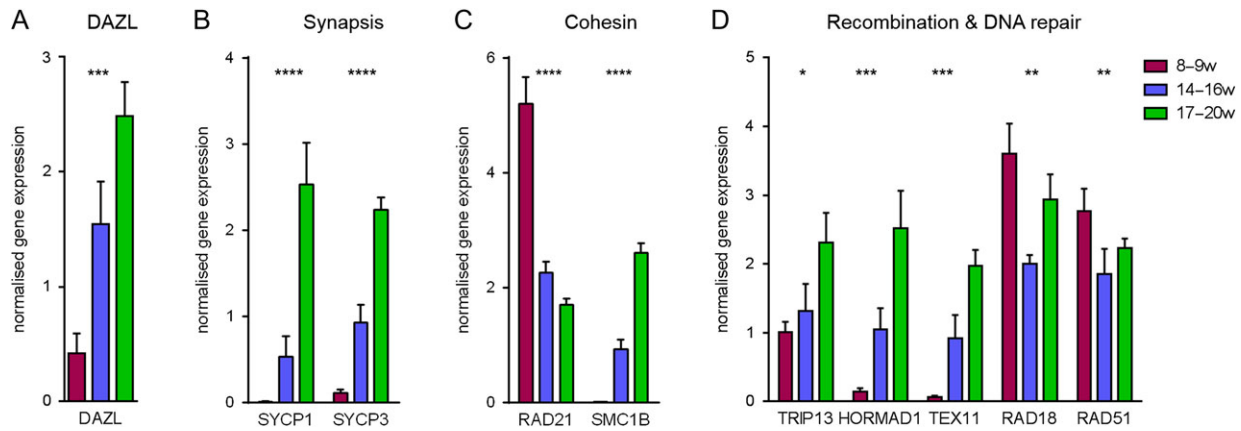
Figure 1 Selected RNA expression in the human foetal ovary across gestation. RNAs have been grouped into different functions based on published literature (**A–D**); however, some RNAs have overlapping functions. Target gene expression has been normalised to the geometric mean expression of *RPL32* (ribosomal protein L32) and *B2M* (beta-2 microglobulin). Mean ± SEM, $n = 5$. $*P < 0.02$, $**P < 0.002$, $***P < 0.0002$, $****P < 0.0001$ by ANOVA.

germ cells in mitosis before the onset of meiosis (8–9 wga), at the commencement of meiosis (14–16 wga) and later in meiosis including the onset of meiotic arrest and primordial follicle formation (17–20 wga). *DAZL* transcript levels dramatically increased at 14–16 wga, and continued to rise at 17–20 wga (Fig. 1A). This pattern of expression across gestation was also observed in RNAs that have a role in synaptonemal complex formation (Fig. 1B), as well as with the meiotic cohesin *SMC1B*. The expression of the mitotic cohesin *RAD21*, however, was highest at 8–9 wga and fell significantly during gestation (Fig. 1C). Those RNAs with functions in recombination and DNA repair showed two different patterns of expression (Fig. 1D). *TRIP13*, *HORMAD1* and *TEX11* mimicked the expression trend observed for the other meiotic RNAs, while *RAD18* and *RAD51* transcript levels dropped between 8–9 and 14–16 wga, but then increased again at 17–20 wga.

As chromosome cohesion is an example of a role not previously associated with DAZL, we visualised expression of the meiotic cohesin subunit *SMC1B* across gestation in relation to DAZL using RNA *in situ* hybridisation for *SMC1B* with immunohistochemistry for DAZL (Fig. 2). At all gestational ages examined, *SMC1B* RNA was exclusively present in germ cells, and predominantly co-localised with DAZL protein, although there are areas of germ cells expressing neither, and a few DAZL positive cells that lacked *SMC1B* were also identified. At 14 wga, *SMC1B* staining was punctate, with expression being mainly located in the germ-cell cytoplasm; only a few germ cells showed some nuclear staining. This changed at 16 wga, as *SMC1B* transcripts were detected in markedly more germ cells. There was no significant difference in *SMC1B* expression between 16 wga and 19 wga, however in the 19 wga tissue, *SMC1B* staining appeared more punctate again.

Stimulation of translation by DAZL via 3′UTR sequences

Previous investigations into the function of Dazl have shown this RNA-binding protein to either stimulate (Reynolds *et al.*, 2005; Reynolds

et al., 2007) or, more rarely, to repress (Chen *et al.*, 2014) the translation of specific transcripts. To ascertain what translational effect DAZL had on the selected targets *SYCP1*, *TEX11* and *SMC1B*, representing members of different functional groups, we used a 3′UTR-*luciferase* reporter assay in HEK293T cells. All three novel RNA targets showed significantly increased luciferase activity in the presence of DAZL, compared to the vector only control (Fig. 3). No stimulation of translation was observed with a *GAPDH* 3′UTR or in the absence of a 3′UTR, indicating this effect is specific to the novel target 3′UTRs. The DAZL R115G mutant, identified in a woman with spontaneous premature ovarian failure (Tung *et al.*, 2006b), has a significantly impaired ability to bind RNA, as the mutation is located within the DAZL RNA recognition motif (RRM) (Jenkins *et al.*, 2011). Expression of the R115G mutant in HEK293T cells resulted in no increase in luciferase activity compared with the vector only control for all the novel RNA targets (Fig. 3). This indicates that the translation of *SYCP1*, *TEX11* and *SMC1B* is dependent on the RNA-binding ability of DAZL.

As the human DAZL-binding site has not been identified, we mutated instances of the consensus mouse Dazl-binding site, i.e. $U_{(2–10)}G/CU_{(2–10)}$ (Venables *et al.*, 2001) in these novel target 3′UTR's to elucidate whether DAZL is acting via this site in humans. To do this, we mutated the G base to an A in *SMC1B* and *TEX11*, which has previously been demonstrated to significantly weaken DAZL binding (Venables *et al.*, 2001). However, upon using these mutant 3′UTR constructs in a luciferase reporter assay, we were unable to see a significant difference in the stimulation of translation in comparison to the wildtype 3′UTR (Supplementary Fig. S4). In addition, we repeated this assay using a mutant *SMC1B* 3′UTR-luciferase construct with deleted mouse Dazl-binding sites, and were also unable to observe any significant differences (Supplementary Fig. S4).

As mRNA translation and mRNA stability are often linked, and DAZL has been reported to mediate the stabilisation of *HuB* mRNA in germ cells of zebrafish embryos (Wiszniak *et al.*, 2011), we examined the steady-state levels of *luciferase* mRNA in the cytoplasm to determine whether the change in luciferase activity in the presence of
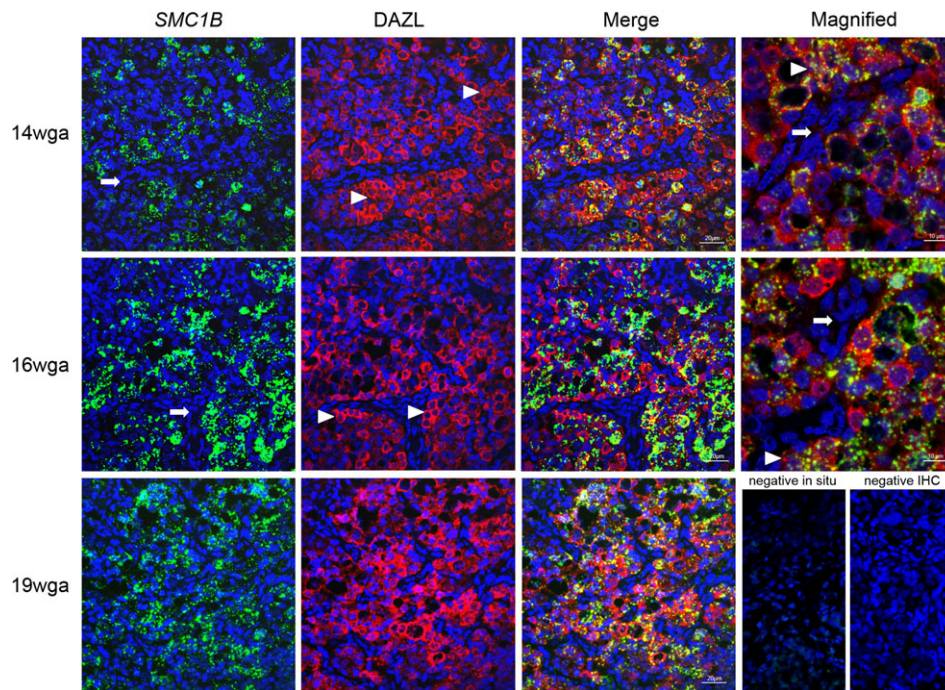
Figure 2 Localisation of *SMC1B* mRNA with DAZL in human foetal ovary, by RNA *in situ* hybridisation in combination with immunohistochemistry. Columns show *SMC1B* (structural maintenance of chromosomes 1B) or DAZL (deleted in azoospermia-like), and merged images as indicated, with the right hand column showing higher power images at the indicated gestations and negative controls. Arrows identify somatic cell streams. Arrowheads identify germ-cell nests. Merge image; scale bar is 20 μm (the scale is the same for columns 1 and 2). Magnified image; scale bar is 10 μm.
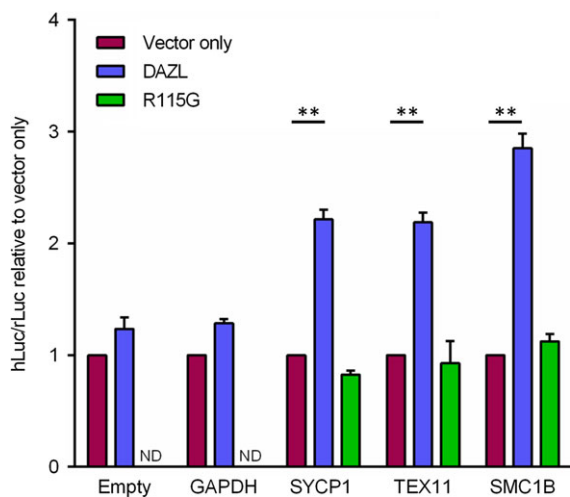

Figure 3 3′UTR-luciferase reporter assay. Increased luciferase activity is observed for target RNAs, but not an empty 3′ untranslated region (3′UTR) or *GAPDH* 3′UTR, in the presence of DAZL (blue bar) relative to an empty vector control (magenta bar). No stimulation is observed when the R115G DAZL mutant is overexpressed (green bar). Firefly luciferase signals were normalised to Renilla luciferase signals. Mean ± SEM, $n = 4$, **$P < 0.01$ (Student's t-test).

DAZL (Fig. 3) was a consequence of altered *luciferase* mRNA stability. For each of the target mRNAs that showed a dependence on DAZL for increased luciferase activity, there appeared to be no significant change in *luciferase* mRNA levels, as assessed by RT-qPCR, when compared to the control (Fig. 4A), indicating a specific effect on translation.

We complemented the mRNA stability analysis with polysome profiling to study the translational state (i.e. ribosome loading) of novel target RNAs in the presence and absence of DAZL. We used the 3′UTR-*luciferase* reporter system with added cycloheximide to 'freeze' actively translating ribosomes on mRNAs (Fig. 4B). After ultracentrifugation to separate the cellular extracts into efficiently translated (ribosome bound) and untranslated (ribosome free) RNAs, we used RT-qPCR to calculate the proportion of *luciferase* RNA being translated. The proportion of *SYCP1*, *TEX11* and *SMC1B luciferase* RNA that was ribosome bound was significantly higher in the presence of DAZL (Fig. 4C) while the control *GAPDH* 3′UTR mRNA was unaffected, suggesting this effect is specific to the novel target 3′UTRs and confirms the ability of DAZL to stimulate the translation of these RNAs.

Discussion

We adopted a global approach combining the immunoprecipitation of endogenous DAZL with RNA-sequencing technology to identify novel transcripts bound by DAZL in the human foetal ovary, and identified 764 potential RNA targets of DAZL. Among these targets were RNAs
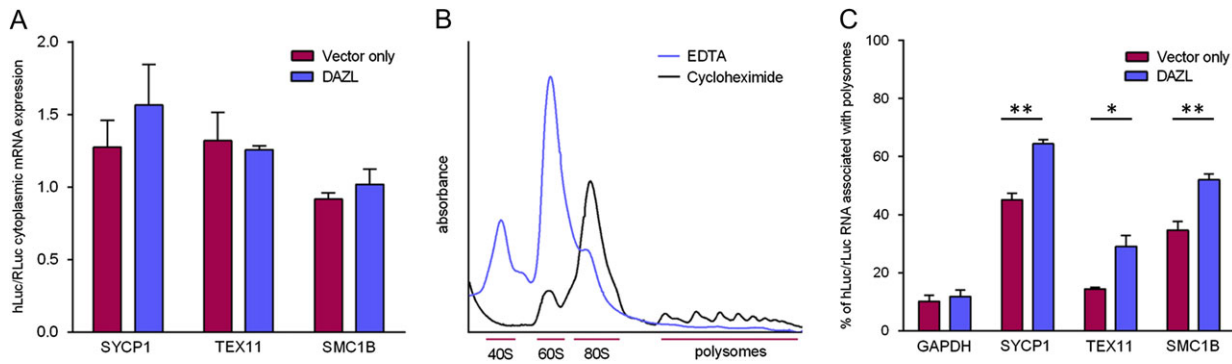
Figure 4 (**A**) DAZL stimulates the translation of *SYCP1*, *TEX11* and *SMC1B*. Quantitative RT-PCR of 3′UTR-*luciferase* expression shows that RNA stability is not affected by the presence of DAZL. Mean ± SEM, $n = 4$. (**B**) Polysome profiles. With the use of cycloheximide, polysomes are 'frozen' on RNA and can be seen as small bumps in the trace. Polysomes are no longer present with EDTA. (**C**) Percentage of 3′UTR-*luciferase* RNA associated with translating polysomes. In the presence of DAZL, the 3′UTRs of *SYCP1* (synaptonemal complex protein-1), *TEX11* (testis expressed 11) and *SMC1B* were significantly more ribosome bound than ribosome free, implicating DAZL in the translation of these targets. Mean ± SEM, $n = 4$–6. *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

involved in synaptonemal complex formation, including *SYCP1*. More importantly, these data identified novel potential roles for DAZL function during early meiosis in the human oocyte, specifically in chromosome cohesion establishment, through regulation of targets including *SMC1B*, and in DNA recombination, via targets including *TEX11*. These are fundamental processes in determining oocyte quality and whose establishment in foetal life may support lifelong fertility. We report changes in the levels of a range of novel DAZL mRNA targets across gestation in the human foetal ovary, over the period including germ-cell mitosis, entry into meiosis and subsequent meiotic arrest and follicle formation. Furthermore, using translation assays we showed that DAZL stimulates translation of its novel targets *SYCP1*, *TEX11* and *SMC1B* (representing different meiotic processes) via their 3′UTRs. This effect was not seen using a DAZL mutant with impaired RNA-binding activity, indicating that translation of these mRNAs is indeed regulated by DAZL.

In order to understand the role of DAZL in fertility, several groups have carried out investigations to identify *in vivo* mRNA targets of DAZL. These inquiries have mainly relied on microarray analysis with or without immunoprecipitation of recombinant/endogenous Dazl from wildtype and *Dazl*^{−/−} male mice (Jiao *et al.*, 2002; Reynolds *et al.*, 2005). The limited information on the function of Dazl in the female indicates that it is important in later stages of oocyte maturation through zygote formation, where Dazl-dependent translation is necessary for spindle assembly, the Metaphase I–II transition and early embryo development (Chen *et al.*, 2011). More recently, expression profiling of the *Dazl*^{−/−} mouse foetal ovary has identified 104 genes expressed specifically in pre-meiotic to pachytene germ cells and the use of single-cell, single-transcript measurements has demonstrated that *Dazl* was required for the initial induction of nearly all identified meiotic prophase genes (Soh *et al.*, 2015), but whether the mRNA transcripts of these genes were bound and/or translated by Dazl was not investigated. Both these studies were conducted in mouse, and no studies have addressed DAZL and its mRNA targets in human female meiosis.

In this investigation, the limited availability of fresh human foetal ovaries and the need to pool snap-frozen foetal ovaries to create a lysate for immunoprecipitation precluded the use of CLIP-Seq (UV crosslinking prior to immunoprecipitation and sequencing), currently the gold standard for large-scale identification of protein–RNA interactions (Murigneux *et al.*, 2013). While the potential for post-lysis association of RNAs is a concern, Supplementary Fig. S2 shows no enrichment of the reference RNAs *GAPDH* and *RPL32*, or the granulosa cell marker *FOXL2* with DAZL. Furthermore, our use of RNA *in situ* hybridisation with immunohistochemistry confirms that *SMC1B* is co-localised with DAZL in germ cells. However, we cannot exclude the possibility of post-lysis interactions between germ-cell expressed mRNAs containing a DAZL-binding site and DAZL protein. In addition, DAZL may bind different variations of its binding site with different affinities, and mRNAs containing low affinity sites may be missed in the absence of cross-linking.

Interestingly, only a few of the validated RNAs that have been previously identified as direct Dazl targets in mouse also appear in our human data set; namely *Sycp3* and *Tex14* but not *Tex19*, *Mvh* or *Tet1*. However, the majority of the previous work was done using testis, and the differences we observe may reflect species differences (mouse versus human), but also possibly reflect the different requirements of female and male germ cells. Indeed, there is some evidence of a sex-dependent function of DAZL: the *Dazl*^{−/−} phenotype can be partially rescued by a human DAZL transgene in mouse testis, but not ovary (Vogel *et al.*, 2002). Additionally, several DAZL allelic variants have been correlated with different phenotypic effects in men and women (Tung *et al.*, 2006b). In their study, Soh *et al.* (2015) identified 104 genes whose expression at the mRNA level during prophase is *Dazl*-dependent (Soh *et al.*, 2015). Intriguingly, a small number of these transcripts, including *SYCP1*, *TEX11* and *SMC1B*, were also identified in our study. This indicates that full expression of a subset of genes may be dependent on DAZL at two distinct stages: early on for initial induction and accumulation of mRNA via a poorly understood DAZL activity (Lin *et al.*, 2008; Soh *et al.*, 2015) and at a later stage for efficient activation

of translation (Fig. 3), as previously demonstrated for mouse Dazl targets (Reynolds *et al.*, 2005; Reynolds *et al.*, 2007; Chen *et al.*, 2011).

The human DAZL-binding element has yet to be elucidated, therefore we carried out analysis of the effect of mutating the mouse Dazl-binding site in *SMC1B* and *TEX11*, but found that the specific mutations we introduced, based on work by Venables *et al.* (2001), did not affect the ability of DAZL to stimulate translation. However, a few RNAs identified by our DAZL immunoprecipitation did not contain the murine Dazl-binding site, one of these being *SYCP3*, a major component of the lateral elements of the synaptonemal complex in meiotic germ cells (Schramm *et al.*, 2011). This is intriguing given the evidence implicating it as a direct Dazl target in mouse (Reynolds *et al.*, 2007). In primordial germ cells derived from human embryonic stem cells, overexpression of DAZL resulted in the upregulation of SYCP3 and formation of synaptonemal complexes, consistent with *SYCP3* being a DAZL target in human (Kee *et al.*, 2009). Potentially, translational regulation of *SYCP3* mRNA in humans occurs via a non-consensus DAZL-binding site located in the 3′UTR, or regulation may occur indirectly through the association of DAZL with a partner protein that is also an mRNA-specific binding protein. A known example is DAZL associated protein-1 (Tsui *et al.*, 2000a; Smith *et al.*, 2011). This may offer a possible explanation for the absence of a significant effect of our Dazl-binding site mutagenesis. While a detailed identification and analysis of the molecular interaction between DAZL and the novel targets identified here is outwith the scope of this study, both these lines of evidence indicate the need for such studies in the human.

These data confirm the involvement of DAZL in synaptonemal complex formation in humans through the translation of *SYCP1*, and evidence suggests this may have important implications for lifelong female fertility. SYCP1 is thought to function as a molecular framework, which enables other proteins to attach and complete synaptonemal complex assembly and progression of recombination (Schramm *et al.*, 2011). Indeed, *Sycp1* null mice of both sexes are infertile due to germ-cell loss through apoptosis despite normal axial element formation and chromosome alignment (de Vries *et al.*, 2005). Importantly, these data also suggest novel roles for DAZL (which may be indirect) during early meiosis in the foetal oocyte, namely in establishment of chromosome cohesion through the regulation of *SMC1B*, and in DNA recombination via *TEX11*. There are no previous data implicating DAZL in the translation of RNAs with these functions, which are increasingly recognised to be key pathways that underpin lifelong oocyte quality. Recent data have highlighted the cohesin complex as a major contributor to age-related aneuploidy. Mice that are deficient in *Smc1β* show loss of cohesion and aneuploidy in their oocytes (Gilliland and Hawley, 2005; Hodges *et al.*, 2005), while oocytes of older women have decreased SMC1β (Tsutsumi *et al.*, 2014) show an increased inter-kinetochore distance (Duncan *et al.*, 2012) and increasingly show premature predivision of sister chromatids (Handyside, 2012; Ottolini *et al.*, 2015). Intriguingly, the cohesion complex is also implicated in DNA repair (Watrin and Peters, 2006). Similarly, mutations in TEX11 have been reported in infertile men with non-obstructive azoospermia (Yang *et al.*, 2015). Meiotic arrest in these patients resembled the phenotype of $Tex11^{-/-}$ male mice (Yang *et al.*, 2008), which have spermatocytes with asynapsed chromosomes and reduced cross-over formation, leading to apoptosis at the pachytene stage of Prophase I. Thus, these data suggest the potential involvement of DAZL in the subsequent risk of age-dependent aneuploidy through translational regulation of *SMC1B* in early meiosis,

and as well as through its regulation of several other factors of established importance in synapsis and recombination.

Conclusion

The RNA-binding protein DAZL is well established as an important regulator of germ-cell development in both male and female although most data derive from studies of mouse testis. Here we have identified 764 potential RNA targets of DAZL in the human foetal ovary, and confirmed in translational assays that three of these, *SYCP1*, *TEX11* and *SMC1B* are DAZL-dependent targets. Through regulation of these RNAs, we have uncovered novel potential functions for DAZL in the foetal oocyte, in establishment of chromosome cohesion and in DNA recombination, in addition to roles in synaptonemal complex formation. Consequently, we suggest DAZL has a key role in regulating fundamental processes that underpin oocyte quality, which may in turn influence a woman's fertility and reproductive lifespan.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Conflict of interest

The authors' declare no conflict of interest.

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