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The polyubiquitin gene *Ubi-p63E* is essential for male meiotic cell cycle progression and germ cell differentiation in *Drosophila*

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SUMMARY

The ubiquitin proteasome system (UPS) regulates many biological pathways by post-translationally ubiquitylating proteins for degradation. Although maintaining a dynamic balance between free ubiquitin and ubiquitylated proteins is key to UPS function, the mechanisms that regulate ubiquitin homeostasis in different tissues through development are not clear. Here we show, via analysis of the *magellan* (*magn*) complementation group, that loss of function of the *Drosophila* polyubiquitin *Ubi-p63E* results specifically in meiotic arrest sterility in males. Ubi-p63E contributes predominantly to maintaining the free ubiquitin pool in testes. The function of *Ubi-p63E* is required cell-autonomously for proper meiotic chromatin condensation, cell cycle progression and spermatid differentiation. *magn* mutant germ cells develop normally to the spermatocyte stage but arrest at the G2/M transition of meiosis I, with lack of protein expression of the key meiotic cell cycle regulators Boule and Cyclin B. Loss of *Ubi-p63E* function did not strongly affect the spermatocyte transcription program regulated by the testis TBP-associated factor (tTAF) or meiosis arrest complex (tMAC) genes. Knocking down proteasome function specifically in spermatocytes caused a different meiotic arrest phenotype, suggesting that the *magn* phenotype might not result from general defects in protein degradation. Our results suggest a conserved role of polyubiquitin genes in male meiosis and a potential mechanism leading to meiosis I maturation arrest.

KEY WORDS: Ubiquitin, *Drosophila*, Spermatogenesis, Meiosis, Spermatocyte

INTRODUCTION

In eukaryotic cells, proteins destined for degradation by the 26S proteasome are marked with covalently linked chains of ubiquitin (Ub) (Hochstrasser, 1996). The crucial role of Ub in protein degradation necessitates that cellular Ub is maintained at a homeostatic balance between free Ub monomer and Ub-conjugated proteins (Kimura and Tanaka, 2010). Although Ub can be recycled from Ub chains attached to protein substrates by deubiquitylating enzymes (DUBs) before tagged proteins enter the 26S proteasome for degradation (Reyes-Turcu et al., 2009), levels of free Ub are maintained primarily by *de novo* synthesis from ubiquitin genes (Ryu et al., 2008).

Conserved from yeast to mammals, ubiquitin genes are of two structural types: polyubiquitin genes and monomeric ubiquitin fusion genes. Polyubiquitin genes encode a precursor protein with many head-to-tail tandem Ub repeats, which is cleaved by the ubiquitin C-terminal isopeptidase activities of DUBs to produce monomeric Ub molecules (D'Andrea and Pellman, 1998; Lee et al., 1988; Özkaynak et al., 1984). Mono-ubiquitin genes encode a single Ub moiety fused directly at its C-terminus to ribosomal protein subunits, either RpS27 or RpL40 (Cabrera y Poch et al., 1990; Finley et al., 1989; Lee et al., 1988; Redman and Rechsteiner, 1989), with the single Ub moiety released post-translationally by ubiquitin C-terminal isopeptidase activities. Studies from *S. cerevisiae* suggest that the mono-ubiquitin genes are the primary contributors to cellular Ub synthesis under normal conditions, whereas expression of the polyubiquitin gene is highly stress inducible (Finley et al., 1987). In mouse, however, the polyubiquitin gene *Ubc* is required for normal embryonic development (Ryu et al., 2007).

Here we show that one of the three polyubiquitin genes of *Drosophila*, *Ubi-p63E*, is required specifically for meiotic cell cycle progression and spermatid differentiation in the male germ line. Males null for *Ubi-p63E* are viable, but show striking phenotypic similarities to the clinical pathology of meiosis I maturation arrest azoospermia, a common form of idiopathic male infertility in humans (Meyer et al., 1992). In both mammals and *Drosophila*, male germ cells descended from germline stem cells (GSCs) go through spermatogonial mitotic divisions and then switch to differentiation as spermatocytes. Primary spermatocytes grow in volume, turn on cell type-specific transcription and then undergo meiotic divisions before initiating spermatid differentiation (Fuller, 1993; Kierszenbaum and Tres, 1978).

Much of our knowledge of the molecular mechanisms coordinating the gene expression program for spermatid differentiation and meiosis in *Drosophila* stems from analysis of meiotic arrest mutants. Most of the previously identified meiotic arrest genes fall into two functional classes: genes that encode testis-specific paralogs of TBP-associated factors (tTAFs) or genes that encode protein components of the testis meiosis arrest complex (tMAC) (Beall et al., 2007; Hiller et al., 2004). The functions of both classes of genes are required to establish the spermatocyte transcription program that drives the expression of hundreds of spermatid differentiation genes. Meiotic arrest genes involved in other cellular processes, such as nucleolar integrity (Moon et al., 2011), are also beginning to emerge.

We found that a complementation group of *Drosophila* meiotic arrest mutants, *magellan* (*magn*), required for meiotic cell cycle

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DEVELOPMENT

progression and spermatid differentiation *in vivo*, map to the polyubiquitin gene *Ubi-p63E*, which is a major source of ubiquitin expression in spermatocytes. Consistent with the viable, female fertile but male sterile *magn* phenotype, *Ubi-p63E* appeared necessary for Ub homeostasis in testes but not in the adult body or ovary. The function of *Ubi-p63E* is required cell-autonomously in germ cells for normal chromatin condensation during meiotic prophase and for progression of the meiotic cell cycle through the G2/M transition of the first meiotic division. However, unlike the previously studied tTAF and tMAC meiotic arrest mutants, the spermatocyte transcription program was largely unaffected in *magn* mutants. The defects observed in *magn* mutants were unlikely to be due to general defects in protein degradation by the proteasome, as knockdown of proteasome function in male germ cells by RNAi had much more severe effects on the spermatocyte transcription program.

MATERIALS AND METHODS

Fly husbandry

Drosophila stocks were raised on cornmeal/dextrose or cornmeal/molasses media at 25°C. Fly strains were obtained from the Bloomington Stock Center and the Vienna *Drosophila* RNAi Center. Wild-type control flies were *y,w* unless otherwise stated.

RNA interference (RNAi)

Virgin UAS-Dicer2;;BAM-GAL4 females were crossed to *y,w* males (control) or males carrying RNAi hairpin against each of the proteasome subunits: *Rpt2*, *Prosα3*, *Prosα7* or *Prosβ7*. Crosses were grown at 29°C. Effectiveness of the RNAi knockdown of individual proteasome subunits was assessed by directly comparing the mRNA level with that of control testes samples by *in situ* hybridization (supplementary material Fig. S4).

Deficiency mapping, cloning of *magn* and generation of *magn* null alleles

The original *magn* alleles, $magn^{Z3\text{-}4216}$, $magn^{Z3\text{-}3969}$, $magn^{Z3\text{-}2534}$ and $magn^{Z3\text{-}5802}$, were identified in the M.T.F. laboratory from the Zuker collection of viable but male sterile lines (Wakimoto et al., 2004). $magn^{Z3\text{-}5802}$ was mapped by deficiency complementation to the 11.94 kb gap region (3L: 3905091-3893148) between two adjacent non-overlapping end point-defined deficiencies Df(3L)ED208 and Df(3L)ED4341. Df(3L)Exel6098, which uncovered this gap region, failed to complement all Zuker alleles. The Zuker alleles were sequenced from 1 kb upstream of the start codon to the end of the first 5′ ubiquitin unit and from the beginning of the last 3′ ubiquitin to 580 bp after the stop codon. No mutations were identified in these regions in any of the *magn* Zuker alleles.

Excision of the p[EPGY2]Ubi63E^{EY07341} insertion was carried out by crossing p[EPGY2]Ubi63E^{EY07341}/TM3Δ2-3 males to *w*;;TM3/TM6B females, collecting TM6B balanced white-eyed male progenies and individually crossing to *w*;;TM3/TM6B females to establish stock before backcrossing to p[EPGY2]Ubi63E^{EY07341}/TM6B females to examine the meiotic arrest phenotype in progeny. Two imprecise excision null mutations, $magn^{12c}$ and $magn^{23b}$, genetically behaved the same as Df(3L)Exel6098 when placed in *trans* to p[EPGY2]Ubi63E^{EY07341}. The lesions in the *Ubi-p63E* region were sequence verified for both alleles (Fig. 1C). $magn^{12c}$ and $magn^{23b}$ were both homozygous lethal due to additional lesions other than loss of function of *Ubi-p63E*. The exact cause of $magn^{23b}$ lethality was not identified. The lethality of $magn^{12c}$ was fully rescued by an *Sc2* genomic rescue construct.

To construct the *Ubi-p63E* rescuing plasmid, a *Pst*I-*Bam*HI fragment containing the *Ubi-p63E* coding region including 1.3 kb of promoter and 1 kb of downstream sequences, was released from pUB#3 (Lee et al., 1988) and inserted into pCaSpeR4 (Thummel and Pirrotta, 1992). The *Sc2* rescuing plasmid was constructed by inserting PCR-amplified *Sc2* genomic sequences into the *Xba*I-*Xho*I sites of pCaSpeR4. Primers used were: forward, 5′-CAAGGCTTGTTAGGATTGGTT; reverse, 5′-TGGCGCGT-TGTTTCAAAATG.

RT-PCR

Total RNA was extracted from dissected testes with Trizol reagent (Invitrogen) and DNase I treated. First-strand cDNA was generated with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) using oligo(dT) primers. cDNA derived from 30 pairs of testes was used for each PCR reaction, with 25 amplification cycles. A *magn*-specific cDNA fragment was amplified with the following primers: 5′-ATCAGCAGCGTCTGATCTTC and 5′-ATTTAGAGTGAAGTGCGTGC. *boule* cDNA was amplified with 5′-CAAAAAGCAGCCCAATCCTC and 5′-TGCGAACTGATCCATGGG. RT-PCR of *RpL32* provided a loading control across all samples. *magn* RT-PCR and *RpL32* RT-PCR were performed in the same tube.

In situ hybridization of adult testis

RNA *in situ* hybridization was performed following a standard protocol (Morris et al., 2009). Primer pairs used to generate gene and isoform-specific probes are listed in supplementary material Table S1. Primer pairs amplifying full-length cDNA were used for *gdl* and *CG9975*. PCR-amplified fragments were subcloned into TOPO-pCR4 (Invitrogen) or pBluescript II SK+ for *in vitro* transcription and DIG labeling (Roche). Antisense probes for *Cyclin A*, *Cyclin B*, *twine*, *fzo* and *aly* were generated as previously described (White-Cooper et al., 1998).

Western blotting

Testis or ovaries were dissected in cold PBS containing Protease Inhibitor Cocktail (Roche). Gonadoectomized male flies were generated by surgical removal of the entire male reproductive tissue. Samples were homogenized in 50 μl cold PBS containing 1×SDS sample buffer, boiled and loaded onto a 10% SDS-PAGE gel (Bio-Rad). Primary antibodies anti-Ub (clone 6C1, Sigma), anti-uH2A (clone D27C4, Cell Signaling), anti-uH2B (clone D11, Cell Signaling), anti-actin (clone C4, Millipore) and anti-Boule (Cheng et al., 1998) were used at 1:2000 dilution.

Microscopy and immunofluorescence

For live squash, dissected testes were cut open in PBS containing 2 μg/ml Hoechst 33342, squashed with a coverslip and examined on a Zeiss Axioplan microscope. Immunofluorescence was performed as previously described (Chen et al., 2005), using the following antibodies: mouse anti-GFP (1:200, Roche), rabbit anti-HA (1:200, Bethyl Laboratories), rabbit anti-Myc (1:1000, Santa Cruz Biotechnology), anti-Topi (1:1000) (Perezgasga et al., 2004), anti-Cyclin B (1:50, Developmental Studies Hybridoma bank) and anti-Fibrillarin (1:200, Cytoskeleton). Anti-uH2A (Millipore) staining was performed as described (Rathke et al., 2007). Alexa Fluor-conjugated goat or donkey secondary antibodies were used (Molecular Probes). Images were captured with a Leica SP2 confocal microscope and processed with Adobe Photoshop software. Stem cell numbers were scored as described (Srinivasan et al., 2012).

Clonal analysis

Homozygous mutant clones in a heterozygous background were induced using Flippase (FLP)-mediated mitotic recombination. *hs-flp*^{122};;FRT2A, Ubi-GFP virgin females were crossed to p[sc-2];FRT2A or p[Sc-2];FRT2A, $magn^{12c}$ males. The progeny were grown at 25°C and heat shocked at 37°C for 2 hours each on day 8 and 9. Testes were dissected on the indicated days after the second day of heat shock and examined for the presence of GFP-negative clones in germ cells. Clonal analyses were repeated independently three times and the data are shown as the average of each of the experiments.

Microarray

magn, *red,e*, *aly* and *sa* testes without seminal vesicle and accessory glands were dissected in ice-cold PBS containing 0.1% DEPC. Total RNA was extracted using TRIzol (Invitrogen). Affymetrix 2.0 *Drosophila* chips were processed by the Stanford Protein and Nucleic Acid (PAN) Facility. Data on three replicates of *red,e*, $aly^{2/5p}$ and $sa^{1/2}$ samples were from previous studies (X. Chen and M.T.F., unpublished). Two replicates of $magn^{12c/23b}$ and two replicates of $magn^{12c}$;[pSC2] were prepared for this study. Data were normalized using the R program (v2.13.2) with the BioConductor package GCRMA (Bolstad et al., 2003). 4306 probe sets without gene annotation were discarded. To obtain differentially expressed genes, two-sample *t*-tests were

DEVELOPMENT

performed and false discovery rate (FDR)-adjusted *P*-values were calculated with the Benjamini-Hochberg (BH) procedure. Genes showing more than 2^3-fold (i.e. 8-fold) differences and BH FDR-adjusted $P<0.05$ were chosen as differentially expressed genes in each comparison. Statistical tests and plots were performed with MATLAB software (MathWorks, R2011b). Microarray data are available at GEO with accession number GSE48837.

RESULTS

The *magn* meiotic arrest locus encodes the *Drosophila* polyubiquitin Ubi-p63E

Four mutants from the C. Zuker collection of EMS-induced viable but male sterile *Drosophila* strains (Wakimoto et al., 2004) formed a single complementation group, in which males transheterozygous for any pair of the Zuker alleles displayed a meiotic arrest phenotype. In testis of mutant males, germ cells developed into mature spermatocytes but cell cycle progression was blocked prior to entering the first meiotic division (Fig. 1A). The locus identified by the complementation group was termed *magellan* (*magn*), after the effect of loss of function on meiotic chromosome condensation in spermatocytes (as described below).

Genetic mapping and complementation tests identified *magn* as the *Drosophila* polyubiquitin gene *Ubi-p63E*. The mutant allele $magn^{z3\text{-}5802}$ was mapped by deficiency complementation to an 11.94 kb genomic region (see Materials and methods). An EP transgene insertion in this genomic region (p[EPGY2]Ubi63E), located in the first intron of the longest transcript of *Ubi-p63E* (Fig. 1C), showed the male meiotic arrest phenotype when in *trans* to any of the Zuker *magn* alleles or Df(3L)Exel6098, which removes the *Ubi-p63E* locus. Although flies homozygous for the EP insertion were male sterile, they did not show meiotic arrest, suggesting that the EP line was a weak allele. Consistent with this, RT-PCR showed that mRNA was still produced from the *Ubi-p63E* locus in testes from EP/EP homozygotes (Fig. 1D). Mobilization of the P-element in the EP line (see Materials and methods) produced two null *magn* alleles, $magn^{12c}$ and $magn^{23b}$. PCR from genomic DNA revealed that the $magn^{12c}$ allele had a 2.2 kb deletion that removed the transcription start site of all four *Ubi-p63E* mRNAs plus most of the 5′UTR, stopping at 34 bp before the ATG. The 2.2 kb deletion also removed the promoter and first 583 bp of protein coding sequence of a neighboring essential gene, *Sc2* (Fig. 1C) (Wohlwill and Bonner, 1991). However, when a genomic fragment containing the entire *Sc2* gene was introduced, $magn^{12c}/magn^{12c}$;[pSc2] flies were completely viable but male sterile and displayed the *magn* meiotic arrest phenotype, indicating that the lethality of $magn^{12c}$ homozygotes was due to loss of function of *Sc2* rather than *Ubi-p63E*. The $magn^{23b}$ allele retained a 43 bp insertion in the *Ubi-p63E* 5′UTR consisting of a fragment of the starting P-element remaining after incomplete excision (Fig. 1C). No *Ubi-p63E* mRNA was detected by RT-PCR from $magn^{12c}$;[pSc2] or $magn^{12c}/magn^{23b}$ testes (Fig. 1D) or whole mutant flies (data not shown), indicating that both of the new alleles were null. The highly repetitive nature of the polyubiquitin gene, which encodes ten tandem copies of the Ub monomer, prevented PCR amplification and sequencing of the polyubiquitin coding region of *Ubi-p63E* from the Zuker alleles. All four Zuker alleles behaved genetically as nulls in terms of their meiotic arrest phenotype, both in combination with each other and with the new null mutations induced by imprecise excision, although low levels of *magn* transcript were detected in testis of the Zuker alleles (supplementary material Fig. S1). Finally, confirming the identity of *magn* as *Ubi-p63E*, a transgene containing the *Ubi-p63E* genomic region (Fig. 1C) completely rescued the meiotic arrest and the male sterility when introduced into any *magn* allele (Fig. 1B).

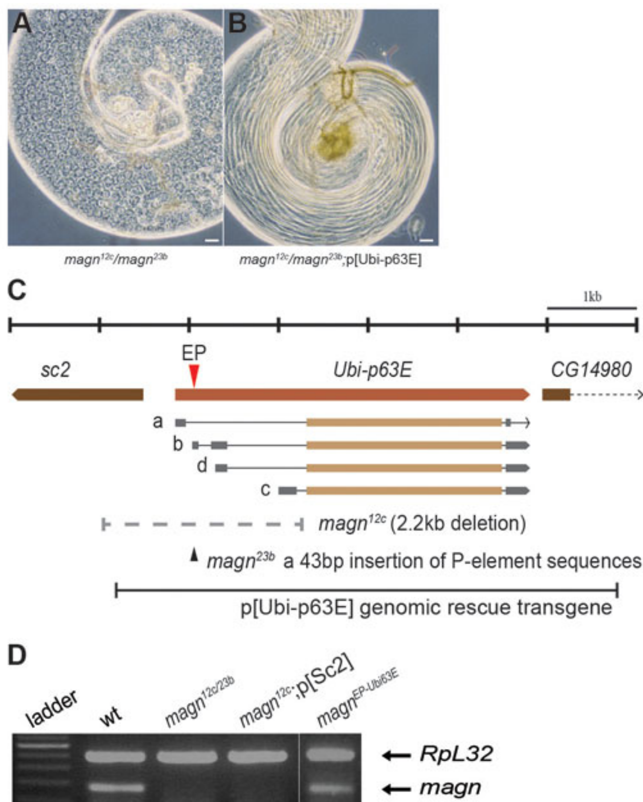

Fig. 1. The *magn* meiotic arrest locus encodes the *Drosophila* polyubiquitin Ubi-p63E. (**A**,**B**) Phase contrast images of testis from (A) $magn^{12c/23b}$ and (B) $magn^{12c/23b}$;p[Ubi-p63E] flies. Scale bars: 25 μm. (**C**) Genomic region showing the four *magn* transcripts (a, b, c and d). Red triangle, P-element insertion site in $magn^{EP}$; black triangle and dotted line, nature of the *magn* null mutations. The *Ubi-p63E* genomic rescue construct is illustrated beneath. The *Sc2* gene is upstream of *Ubi-p63E* and is transcribed in the opposite orientation. *CG14890* starts ~200 bp downstream of *Ubi-p63E*. (**D**) RT-PCR to detect *magn* mRNA from wild-type and *magn* null mutant testes. *RpL32* RT-PCR provided a loading control.

Ubi-p63E is the main ubiquitin gene expressed in spermatocytes

Surprisingly, $magn^{12c}/magn^{23b}$ and $magn^{12c}$;p[Sc2] null flies were fully viable, developed at a normal pace into adulthood, and females were robustly fertile (data not shown), indicating that under normal non-stress growth conditions *Ubi-p63E* gene function is predominantly required for male fertility. Consistent with this requirement, analysis of the expression patterns of the genes encoding mono-ubiquitin and polyubiquitin in *Drosophila* suggested that *Ubi-p63E* is the major ubiquitin gene expressed in spermatocytes. *In situ* hybridization using isoform-specific RNA probes revealed that the four annotated transcripts of *Ubi-p63E* were all expressed in adult testes (Fig. 2A,C,E), with highest expression in primary spermatocytes. Of the four predicted mRNA isoforms, *Ubi-p63E-a* (Fig. 2A) and *Ubi-p63E-b*/*Ubi-p63E-d* (Fig. 2C), as detected by a common probe, were expressed at high levels in spermatocytes, whereas the level of the *Ubi-p63E-c* isoform was much lower (Fig. 2E).

Three loci in *D. melanogaster* encode polyubiquitin: *Ubi-p63E*, with ten consecutive Ub repeats; and two closely linked

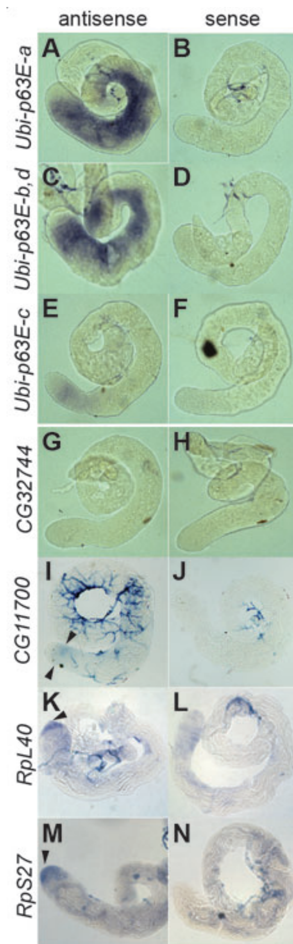

Fig. 2. *Ubi-p63E* is the major ubiquitin gene expressed in spermatocytes. *In situ* hybridization on wild-type testes using transcript-specific antisense and sense RNA probes for (**A**-**F**) the four isoforms of *Ubi-p63E*, (**G**,**H**) *CG32744*, (**I**,**J**) *CG11700*, (**K**,**L**) *RpL40* and (**M**,**N**) *RpS27* mRNA. Arrowheads indicate early spermatocytes (I) or the tip of the testis where GSCs and spermatogonia reside (K,M). Tip of the testis is to the left in all panels.

polyubiquitin genes on the X chromosome, namely *CG32744* (*Ubi-p5E*, previously named *Ubi-p5E4*) and *CG11700* (previously named *Ubi-p5E5*), with seven and four consecutive Ub repeats, respectively. *In situ* hybridization with probes designed to best distinguish the two X-linked transcripts (see Materials and methods) detected no transcripts from *CG32744* (Fig. 2G,H) and possibly only very low levels of transcripts from *CG11700* in early spermatocytes (Fig. 2I,J, arrowheads). Consistently, although RNA-Seq data from ModENCODE (source: FlyBase) showed that *CG11700* was expressed primarily in testis, imaginal discs and larval gut, expression of *Ubi-p63E* was 32-fold higher than *CG11700*. *D. melanogaster*, similar to other higher eukaryotes, also has two mono-ubiquitin genes, *RpL40* and *RpS27*, which encode fusion proteins containing a single Ub moiety fused directly upstream of the ribosomal proteins L40 and S27, respectively. Interestingly, both the mono-ubiquitin *RpL40* and the mono-ubiquitin *RpS27* transcripts were detected by *in situ* hybridization in the tip spermatogonial region of adult testes, but were at much lower levels or not detected in the spermatocyte region of wild-type testes (Fig. 2K-N). Thus, Ubi-p63E might serve a crucial role in spermatocytes as the main source of newly synthesized Ub.

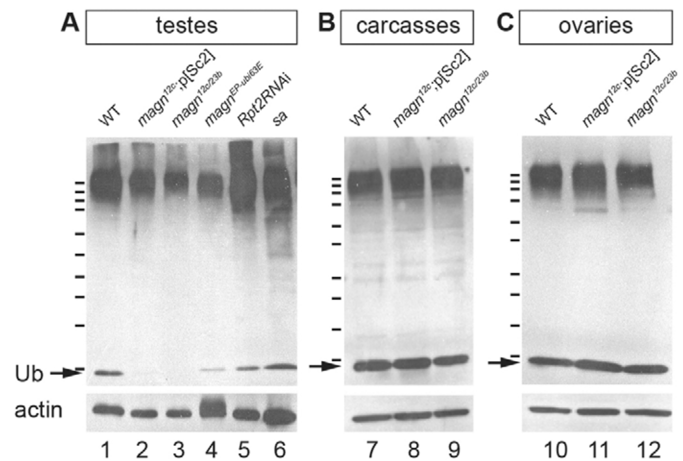

Fig. 3. Ubiquitin homeostasis in the testis is affected in *magn* mutants. Anti-ubiquitin western blot showing levels of free ubiquitin (arrows) and ubiquitylated proteins in (**A**) testes, (**B**) gonadoectomized males (carcasses) and (**C**) ovaries. Anti-actin provided a loading control. Thirty pairs of testes (A), five male carcasses (B) and ten pairs of ovaries (C) were used as starting material across the different genotypes. Molecular weight markers, from top to bottom: 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa. Ub, ubiquitin.

Ubi-p63E is required for ubiquitin homeostasis in the testis

Consistent with the expression patterns of the *D. melanogaster* ubiquitin genes and the specificity of the *magn* null mutant phenotype, loss of function of *Ubi-p63E* drastically reduced the pool of free Ub protein in testes, but not in ovaries or the bodies of gonadoectomized adult males (Fig. 3). In western blots of protein extracts from testes dissected from wild-type versus *magn* mutant flies probed with an antibody against Ub, the amount of free Ub (running as an ~8 kDa band) dropped to almost undetectable levels in the *magn* null mutant samples (Fig. 3A, lanes 2 and 3) under conditions in which free Ub was clearly seen in parallel samples of wild-type testes (Fig. 3A, lane 1). High molecular weight ubiquitylated proteins were still abundant in the extracts from *magn* null mutant adult testes, suggesting that lack of *Ubi-p63E* function had its greatest effect on the levels of the free Ub pool. This effect was not due to the lack of post-meiotic stages in *magn* mutant testes, as free Ub levels were not reduced in *sa* mutant testis, which also exhibit meiotic arrest and lack of spermatid stages (Fig. 3, lane 6). Likewise, lack of spermatids caused by RNAi against the proteasome subunit *Rpt2* did not result in dramatic reduction of free Ub levels (Fig. 3, lane 5). Testes from flies homozygous for the $magn^{EP}$ allele had moderately decreased levels of free Ub (Fig. 3A, lane 4), consistent with the behavior of $magn^{EP}$ as a weak allele. Male germ cells in $magn^{EP}$ homozygotes progressed through meiosis and spermatid elongation but did not produce functional sperm, suggesting that lowered free Ub levels either directly affected a late differentiation event or affected the accumulation, in spermatocytes, of particular transcripts needed for spermatid differentiation. By contrast, the levels of free Ub and ubiquitylated proteins in gonadoectomized males or ovaries were similar in extracts from *magn* null animals and wild type (Fig. 3B,C), suggesting that under non-stress conditions Ubi-p63E function is not required to maintain Ub homeostasis in tissues other than the testis, consistent with the viable, but male sterile, phenotype of *magn* null mutant flies.

Ubi-p63E is required in male germ cells for meiotic chromatin condensation and cell cycle progression

The early stages of male germ cell differentiation appeared to proceed normally in *magn* null mutant testes, consistent with the expression of mono-ubiquitin-RpL40 and mono-ubiquitin-RpS27 during the spermatogonial stages. Testes from *magn* null mutant males had similar numbers of GSCs to wild-type controls (supplementary material Fig. S2). Likewise, mature spermatocyte cysts had the normal number of 16 germ cells per cyst in *magn* null mutant testes, indicating that spermatogonia proceeded through the correct number of transit-amplifying divisions prior to entering the spermatocyte differentiation program. However, mature spermatocytes from *magn* mutant males were all arrested at the G2/M transition of meiosis I and failed to initiate spermatid differentiation (Fig. 4A,A′).

The first defects observed in *magn* mutant male germ cells were abnormalities in chromatin condensation during the spermatocyte growth stages. In wild-type growing spermatocytes, the major autosomal bivalents begin to condense, forming distinct domains near the nuclear periphery as nuclear volume increases (Fig. 4B,B′). By contrast, in *magn* mutant spermatocytes, the autosomal bivalents had subtle defects in organization even at such immature stages (Fig. 4F,F′). These defects became more apparent as spermatocyte growth and maturation progressed. In wild-type mature spermatocytes, the autosomal bivalents formed a pair of crescent-shaped structures near the nuclear periphery, and the sex bivalent associated with the nucleolus (Fig. 4C,C′). In *magn* mature spermatocytes, however, although the major autosomal and sex bivalents occupied separate domains of the nucleus, overall the chromatin appeared less uniformly condensed, forming variable structures lacking clear boundaries (Fig. 4G,G′).

In wild-type primary spermatocytes entering the first meiotic division, the spermatocyte nucleolus breaks down as the compacted bivalents move towards the middle of the nucleus (Fig. 4D,D′), prior to clustering tightly at the metaphase plate. *magn* null mutant spermatocytes never reached this state, but arrested with partially fragmented nucleoli and incompletely condensed chromatin, forming abnormal structures resembling the Magellanic clouds (Fig. 4G-H′) – hence the mutant name *magellan*. Whereas spermatocytes undergoing meiotic division were observed in wild-type testis squashes (Fig. 4E,E′), spermatocytes undergoing meiotic division were never observed in *magn* mutant testes. Likewise, *magn* mutant testes never displayed any signs of spermatid differentiation visible by phase contrast imaging of live squashed preparations. Instead, arrested spermatocytes accumulated throughout the basal end of *magn* null testes and eventually began to degenerate (Fig. 1A).

Clonal analysis revealed that *Ubi-p63E* function is required cell-autonomously in germ cells for spermatocytes to progress to spermatid differentiation. Negatively marked clones of germ cells homozygous for the null allele *magn*^{12c} were generated in heterozygous males using the FLP-FRT-based mitotic recombination system, and the percentage of testes showing GFP-negative spermatocyte or post-meiotic spermatid clones was scored at various time points post-clone induction (PCI) (Fig. 5A,B). By day 4 PCI, 100% of both FRT control and *magn* testes had GFP-negative homozygous spermatocyte cysts. However, whereas wild-type control clonally marked germ cysts progressed to spermatid stages, such that 56.5% of control testes at day 8 PCI and 43.9% at day 12 PCI had at least one spermatid clone (Fig. 5A), absolutely no *magn* null mutant germ cell clones were observed at the spermatid stage, even at 12 days PCI (Fig. 5B). Instead, *magn* null mutant spermatocyte clonal cysts accumulated, with the characteristic chromatin condensation defects, failure to reach metaphase and failure of complete nucleolar breakdown, and eventually degenerated (data not shown).

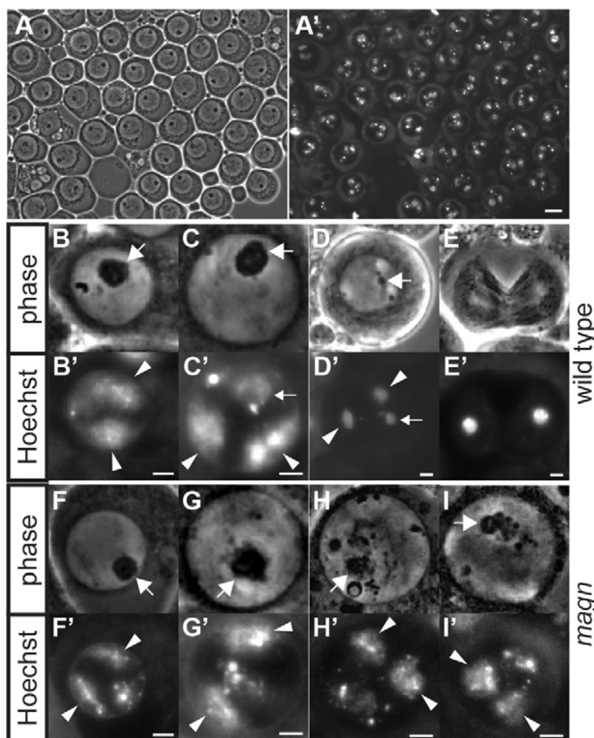

Fig. 4. *magn* mutant spermatocytes arrest at the G2/M transition with chromatin condensation defects. (**A**,**A′**) Arrested *magn* mature spermatocytes: (A) phase contrast, (A′) Hoechst. (**B-I′**) Stage-matched spermatocytes from (B-E′) wild-type and (F-I′) *magn* mutant testes squashes. (B-I) Phase contrast, (B′-I′) Hoechst. Large arrows, nucleolus; small arrows, sex bivalents; arrowheads, condensing autosomal bivalents. (B,B′,F,F′) Wild-type and *magn* young spermatocytes with autosomal bivalents starting to condense. (C,C′,G,G′) Wild-type and *magn* mature spermatocytes with autosomal bivalents forming crescents at the nuclear periphery. (D,D′) Wild-type spermatocytes entering meiosis, with condensed bivalents moving towards the middle of the nucleus and nucleolus breaking down. (H-I′) Arrested *magn* spermatocytes with partially fragmented nucleolus and defectively condensed sphere-shaped chromatin, resembling Magellanic clouds. (E,E′) Wild-type spermatocyte undergoing meiosis I; the corresponding stage was never observed in *magn* mutant testes. Scale bars: 10 μm in A,A′; 2 μm in B-I′.

The spermatocyte transcription program is largely unaffected in *magn* mutant testis

Although the meiotic arrest observed in *magn* mutant testes resembled the G2/M meiotic arrest previously described for loss of function of either the tTAF or tMAC genes, the arrest in *magn* mutants was unlikely to be due to loss of function of tTAF or testis-specific tMAC components. The tTAF proteins Sa and Can, as marked by Sa-GFP or Myc-Can (Chen et al., 2005), respectively, were still expressed and localized normally in *magn* mutant spermatocytes, appearing weakly on the condensing chromatin and strongly within a subcompartment of the nucleolus interdigitating with the nucleolar marker Fibrillarin, as in wild type (Fig. 5C-C‴, Fig. 8E,F). Likewise, staining of *magn* testis with antibodies against Topi or Aly, the protein products of two tMAC genes (Perezgasga

DEVELOPMENT

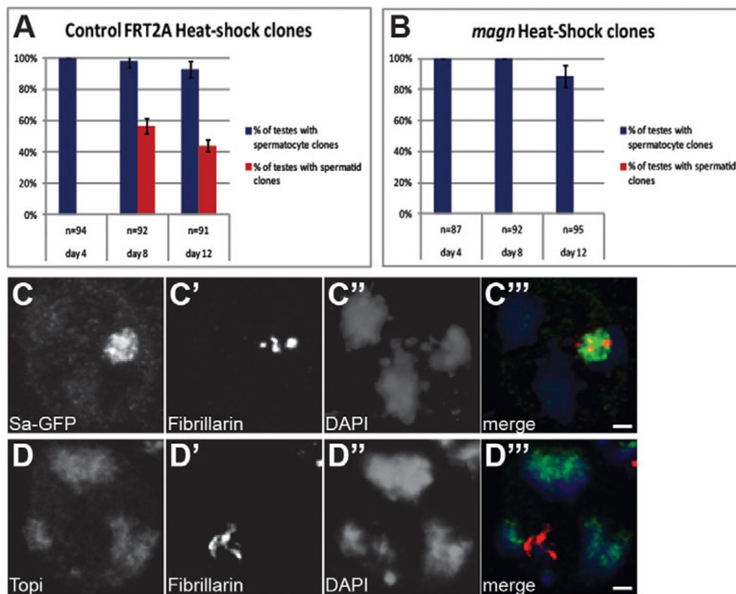

Fig. 5. *Ubi-p63E* is required cell-autonomously for spermatocyte cell cycle progression and spermatid differentiation without affecting tTAF or tMAC localization. (**A**,**B**) *Ubi-p63E* function is required cell-autonomously in germ cells for cell cycle progression. Germline clonal analysis of (A) FRT2A controls compared with (B) $magn^{12c}$. Blue columns indicate the percentage of testis with GFP-negative germ cells reaching the spermatocyte stage; red columns indicate the percentage of testis with GFP-negative germ cells reaching post-meiotic spermatid stages. Error bars indicate s.d. from three independent clone inductions. (**C-D‴**) Indirect immunofluorescence of arrested $magn^{12c/23b}$ spermatocytes stained for (C) the testis TAF fusion protein Sa-GFP, (D) the tMAC protein Topi or (C′,D′) Fibrillarin and stained with (C″,D″) DAPI. (C‴,D‴) Merge, showing Sa-GFP (green in C‴) or Topi (green in D‴), Fibrillarin (red) and DAPI (blue). Scale bars: 2 μm.

et al., 2004; White-Cooper et al., 2000), showed that they were expressed in spermatocytes and localized to the condensing chromatin, as in wild type (Fig. 5D-D‴; data not shown). In addition, mono-ubiquitylated histone H2A (uH2A) was present and localized to a subnuclear structure in *magn* mutant testis, as in wild type (supplementary material Fig. S3A-C). Likewise, mono-ubiquitylated histone H2B (uH2B) was also present at similar levels in *magn* mutant testis and wild type (supplementary material Fig. S3D).

Consistent with the normal expression and localization of tTAFs, Aly and Topi in the mutant spermatocytes, *magn* mutant testes did not display the widespread defects in the transcription of spermatid differentiation genes characteristic of tTAF or tMAC mutants. *In situ* hybridization analysis showed that the representative differentiation genes *fzo* and *gdl*, which are expressed robustly in wild-type spermatocytes but only at low levels in tTAF or tMAC mutant spermatocytes (Hiller et al., 2004; White-Cooper et al., 1998), were expressed normally in *magn* mutant spermatocytes (Fig. 6A-D). Transcripts of *twine* and *Cyclin B* (*CycB*), which are expressed in wild-type spermatocytes dependent on the function of tMAC genes, were also expressed at levels comparable to wild type in *magn* mutant spermatocytes (Fig. 6E,F, Fig. 8A,B). In addition, genes such as *aly*, *Cyclin A* (*CycA*), *Rbp4* and *CG9975*, which are upregulated in wild-type spermatocytes and independent of either the tMAC or the tTAFs, were expressed normally in *magn* spermatocytes (Fig. 6G-N).

Global gene expression analysis also indicated that the majority of transcripts that are dependent on either *aly* or *sa* for normal expression in testis did not require *Ubi-p63E* function. Using a 2^3-fold cut-off, microarray data showed 1476 genes downregulated in *aly* compared with wild type (Fig. 7A, red). Of those, 82.5% (1217/1476) were not dependent on *Ubi-p63E* (Fig. 7B). Similarly, of the 766 genes downregulated in *sa* compared with wild type (Fig. 7C, magenta), 67.5% (517/766) were not dependent on *Ubi-p63E* (Fig. 7D). These numbers suggest that *Ubi-p63E* function is, for the most part, not required for regulation of transcription by tMAC or the tTAFs.

The majority of the genes downregulated in *magn* mutant testes were also downregulated in *aly* or *sa*. Only 309 genes were more than 2^3-fold lower in *magn* testis than in wild type (Fig. 7E, green). Of those, 84% (260/309) and 79% (244/309) were also *aly* and *sa* dependent, respectively (Fig. 7F,G). No significant gene ontology (GO) terms were associated with *Ubi-p63E*-dependent genes that did, versus those that did not, depend on the function of *aly* and *sa*. The 225 transcripts that were downregulated in all three genotypes (*magn*, *aly* and *sa*) are likely to include mRNAs that are either

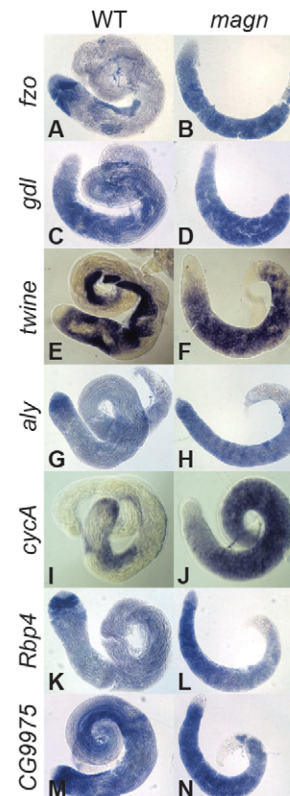

Fig. 6. *Ubi-p63E* is not required for the expression of tTAF- and tMAC-dependent transcripts. *In situ* hybridization on wild-type and *magn* null mutant testes processed in the same tube using antisense RNA probes for (**A**,**B**) *fzo*, (**C**,**D**) *gdl*, (**E**,**F**) *twine*, (**G**,**H**) *aly*, (**I**,**J**) *CycA*, (**K**,**L**) *Rbp4* and (**M**,**N**) *CG9975* transcripts. The testis apical tip is to the left in all panels.

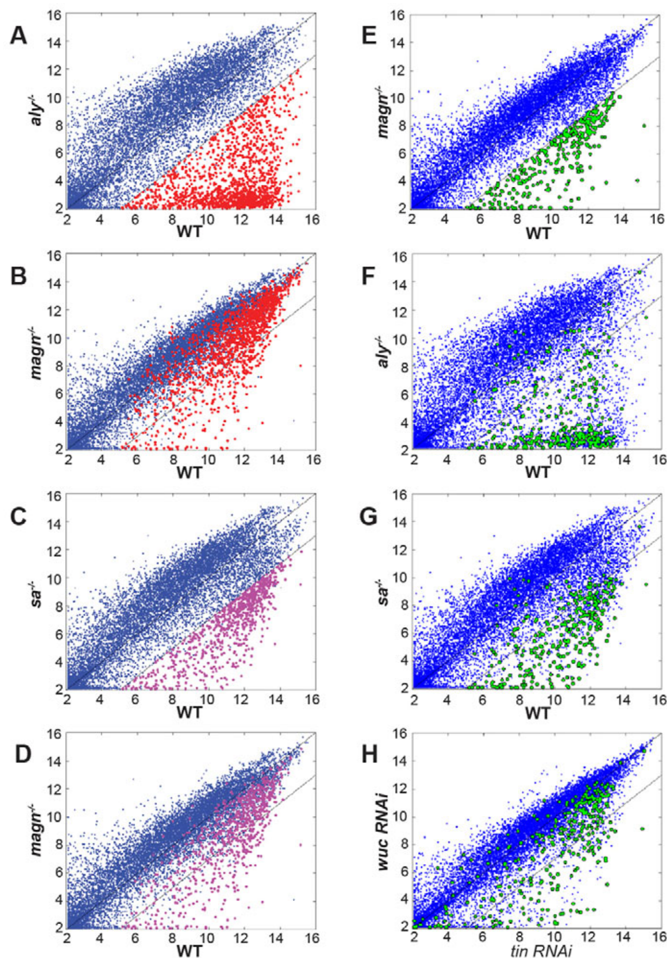

Fig. 7. *Ubi-p63E* is dispensable for the tMAC- and tTAF-dependent spermatocyte transcription program. (**A-G**) Scatter plots comparing transcript levels in wild-type testes for (A,F) *aly*, (B,D,E) *magn* and (C,G) *sa*. (**H**) Scatter plot comparing transcript levels between *wuc* RNAi and *tin* RNAi [data from Doggett et al. (Doggett et al., 2011)]. *x* and *y* axes show $\log_2$ transformed gene expression values. Red, 1476 *aly*-dependent transcripts (≥2^3-fold higher in wild type than in $aly^{-/-}$); magenta, 766 *sa*-dependent transcripts (≥2^3-fold higher in wild type than in $sa^{-/-}$); green, 309 *magn*-dependent transcripts (≥2^3-fold higher in wild type than in $magn^{-/-}$). FDR adjusted to $P<0.05$ for all samples.

expressed or stabilized at later stages of spermatogenesis that are not present in *magn* or the other meiotic arrest mutant testes.

Loss of function of a testis-enriched *lin-52* homolog, *wake-up-call* (*wuc*), by RNAi also showed meiotic arrest but had very mild effects on the spermatocyte transcription program when knocked down specifically in spermatocytes (Doggett et al., 2011). Only 28% (88/309) of the transcripts that were $\geq 2^3$-fold downregulated in *magn* compared with wild type were also $\geq 2^3$-fold downregulated in *wuc* (Fig. 7H), suggesting that *Ubi-p63E* and *wuc* are unlikely to act together, or in the same pathway, to control gene expression in spermatocytes.

Ubi-p63E is required for expression of key G2/M cell cycle regulatory proteins in spermatocytes

Key cell cycle regulatory proteins that are normally expressed in advance of the G2/M transition in mature spermatocytes were not expressed in *magn* null mutant spermatocytes, suggesting a mechanism underlying the arrest in meiotic cell cycle progression

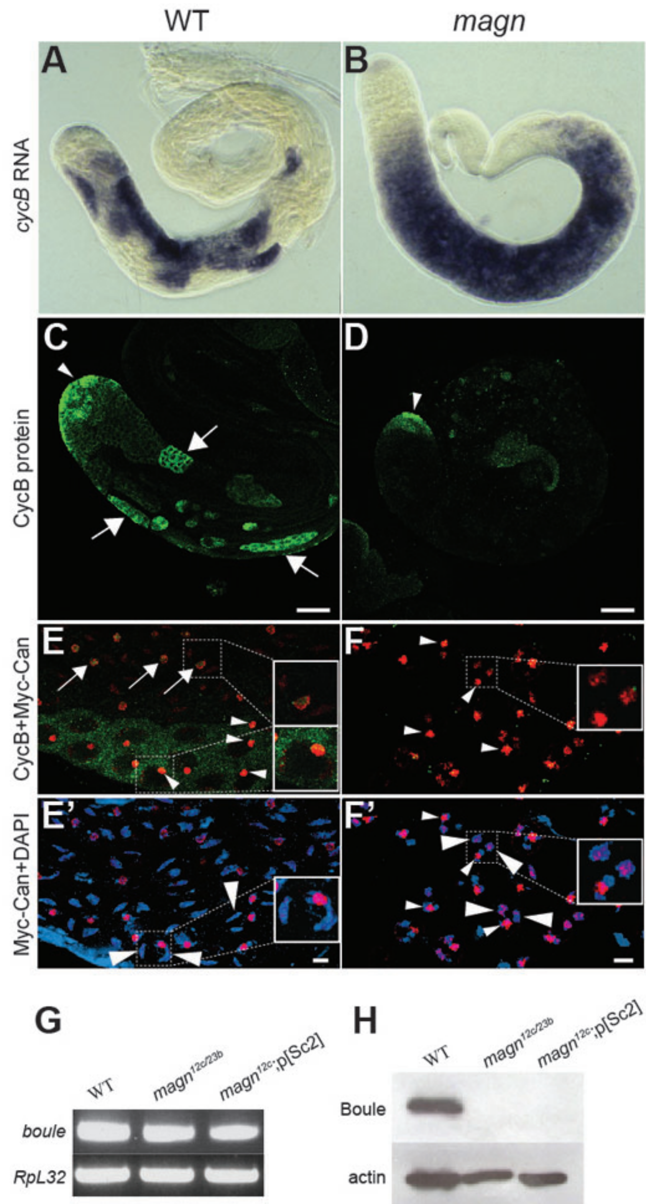

Fig. 8. Protein, but not transcripts, of key cell cycle regulators fails to accumulate in *magn* spermatocytes. (**A**,**B**) *In situ* hybridization to (A) *magn* mutant and (B) wild-type testes with antisense RNA probes for *CycB* transcripts. (**C**,**D**) CycB protein stain in wild-type (C) and *magn* (D) testis. Arrowheads, CycB protein expression in spermatogonia; arrows, CycB protein expression in wild-type mature spermatocytes. (**E**,**E′**) Timing of CycB protein accumulation in wild-type mature spermatocytes. (**F**,**F′**) *magn* spermatocytes arrest at a stage past that of normal spermatocyte CycB accumulation. (E,F) Merge of the Myc-Can and CycB channels. Green, CycB; red, Myc-Can. (E′,F′) Merge of Myc-Can and DAPI channels. Red, Myc-Can; blue, DAPI. Large arrowheads indicate crescent-shaped autosomal bivalents in wild-type mature spermatocytes (E′) or globular arrested *magn* chromatin (F′). Arrows (E) indicate the less compacted circular Myc-Can nucleolar stain associated with low levels of CycB. Small arrowheads indicate the solid nucleolar Myc-Can stain associated with high levels of CycB (E) or compacted nucleolar Myc-Can stain as solid dots (F,F′). (**G**) RT-PCR showing mRNA expression of *boule* in wild-type and *magn* null mutant testes. *RpL32* RT-PCR provided a loading control. (**H**) Western blot of Boule protein in wild-type and *magn* testes. Anti-actin provided a loading control. Thirty pairs of testes were used as starting material across the different genotypes in G and H. Scale bars: 10 μm in C,D; 4 μm in E′,F′.

DEVELOPMENT

in the mutant. Although transcripts of the cell cycle regulators *CycB* and *boule* were expressed in *magn* mutant spermatocytes at levels comparable to wild type (Fig. 8A,B,G), Cyclin B and Boule proteins did not accumulate in *magn* spermatocytes. Immunofluorescence staining of wild-type testes revealed that Cyclin B protein was expressed at the tip (Fig. 8C, arrowhead), where the stem cells and spermatogonia undergo mitotic divisions, declined to very low levels in newly formed and growing spermatocytes, and accumulated again in mature spermatocytes preceding the onset of the G2/M transition (Fig. 8C, arrows), as described previously (White-Cooper et al., 1998). Although expression of Cyclin B protein at the testis tip was not affected in *magn* null testes (Fig. 8D, arrowhead), Cyclin B protein was not detected by immunofluorescence in the mature spermatocytes that accumulate to fill *magn* mutant testes (Fig. 8D). Two lines of evidence indicate that the lack of expression of Cyclin B protein in *magn* mutant spermatocytes was not simply a consequence of the *magn* cell cycle arrest but that the *magn* mutant spermatocytes arrest at a stage after the point when Cyclin B would have accumulated in wild type. First, when Cyclin B levels became high in wild-type spermatocytes, the condensing autosomal bivalents were still crescent shaped and localized to the nuclear periphery (Fig. 8E′, large arrowheads), a stage much earlier than the *magn* arrest point, when autosomal bivalents were more condensed, adopting smaller spherical shapes inside the nucleus (Fig. 4H′,I′; Fig. 8F′, large arrowheads). Second, wild-type testes co-stained for Cyclin B and Myc-Can (Chen et al., 2005) showed the accumulation of Cyclin B protein in mature spermatocytes coinciding with compaction of the Myc-Can nucleolar stain from small circles into solid dots (Fig. 8E, compare arrows and small arrowheads). By contrast, Cyclin B expression was still not observed in the arrested *magn* mutant spermatocytes even though the Myc-Can nucleolar stain had already compacted (Fig. 8F,F′, small arrowheads).

Likewise, *magn* loss of function blocked the accumulation of Boule, an RNA-binding protein required for translation of the G2/M cell cycle regulator *twine* in spermatocytes. RT-PCR showed the presence of *boule* transcript in testes from flies null mutant for *magn* (Fig. 8G). However, no Boule protein was detected on western blots of *magn* mutant testes (Fig. 8H). In wild type, Boule protein expression normally begins in early spermatocytes, well before the arrest point of *magn* spermatocytes. Unlike *boule* or *twine* mutant germ cells, which eventually skip meiosis and initiate spermatid differentiation (Maines and Wasserman, 1999), *magn* mutant spermatocytes never displayed any sign of spermatid differentiation, suggesting that Ubi-p63E function is required for more than just meiotic cell cycle progression.

The meiotic arrest in *magn* mutants does not resemble defects caused by knocking down proteasome subunits

Interfering with the function of the proteasome in spermatocytes did not cause the same phenotype as *magn* loss of function, suggesting that the effects observed in *magn* mutants might not be due to general defects in protein degradation of unwanted proteins. Proteasome function was impaired by expressing UAS-RNAi hairpins against each of several key proteasome subunits, *Rpt2*, *Prosα3*, *Prosα7* or *Prosβ7*, specifically in late spermatogonia and spermatocytes under control of the Bam-GAL4 expression driver. *In situ* hybridization with gene-specific probes showed that this RNAi method effectively abolished messenger expression of the targeted proteasome subunits in spermatocytes (supplementary material Fig. S4G-L). Although germ cell-specific knockdown of each generally expressed proteasome subunit tested resulted in a meiotic arrest phenotype with lack of spermatid stages (supplementary material Fig. S4A-D), the arrested spermatocytes displayed phenotypes that were strikingly different from the *magn* null phenotype. For example, knocking down proteasome function resulted in spermatocytes with a largely intact nucleolus (supplementary material Fig. S4E,F, arrows) and poorly condensed chromatin adjacent to the nuclear periphery (supplementary material Fig. S4E′,F′, arrowheads), suggesting an earlier arrest than in *magn* mutants, in which the spermatocyte nucleolus had begun to break down and chromatin condensation had proceeded to a spherical shape (Fig. 4H-I′).

Consistent with this difference, analysis of transcript expression by *in situ* hybridization revealed that knockdown of the key proteasome component *Rpt2* had very different effects on the spermatocyte transcription program than *magn* loss of function. For example, transcripts for the differentiation genes *fzo* and *gdl* were not detected and transcripts of the cell cycle genes *CycB* and *twine* remained at background levels in *Rpt2* knockdown spermatocytes (Fig. 9A-H), whereas they were expressed in *magn* null spermatocytes (Fig. 6A-F). The tTAF- and tMAC-independent transcripts *aly*, *CycA*, *Rbp4* and *CG9975* were expressed at either lower (*CycA*) (Fig. 9I,J) or at wild type-comparable levels (*aly*, *Rbp4* and *CG9975*) in *Rpt2* RNAi knockdown spermatocytes (Fig. 9K-P), indicating that only the later events of the spermatocyte transcription

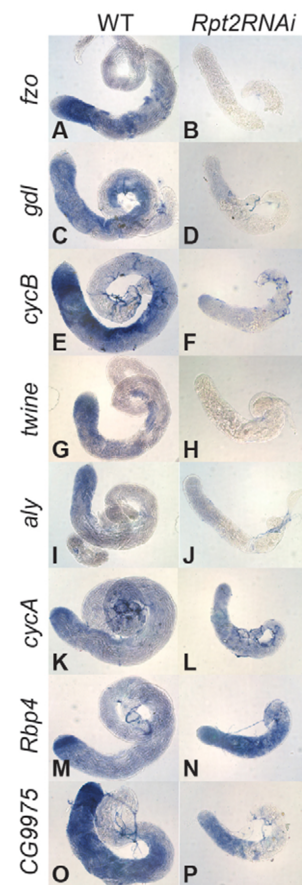

Fig. 9. Function of the 26S proteasome is required for the spermatocyte transcription program. *In situ* hybridization on wild-type and *Rpt2* RNAi knockdown testes processed in the same tube with antisense RNA probes for (**A**,**B**) *fzo*, (**C**,**D**) *gdl*, (**E**,**F**) *CycB*, (**G**,**H**) *twine*, (**I**,**J**) *CycA*, (**K**,**L**) *aly*, (**M**,**N**) *Rbp4* and (**O**,**P**) *CG9975*. The tip of the testis is to the left in all panels.

program were affected in *Rpt2*-depleted spermatocytes. Notably, knockdown of *Rpt2* in spermatocytes did not deplete the pool of free Ub (Fig. 3A, lane 5). Together, these findings suggest that *magn* might affect germ cell differentiation due to defects in Ub homeostasis rather than through disrupted protein degradation.

DISCUSSION

The *Drosophila* polyubiquitin gene *Ubi-p63E*, identified here as the *magellan* (*magn*) locus needed specifically for male fertility, is required in spermatocytes for normal chromatin condensation, cell cycle progression through the G2/M transition of meiosis I and for the onset of spermatid differentiation. *magn* loss of function causes a male meiotic arrest phenotype similar to meiosis I maturation arrest, the most common form of idiopathic male infertility in humans (Meyer et al., 1992): testes fill with mature spermatocytes that do not progress into meiotic division and lack all spermatid stages.

The cell type-specific expression patterns of *Drosophila* ubiquitin genes may underlie the male germ cell-specific phenotype of *magn* loss of function, as *Ubi-p63E* was by far the most abundantly expressed ubiquitin gene in spermatocytes. Although *Ubi-p63E* is generally expressed in all fly tissues (Lee et al., 1988), its function is not required for viability or female fertility. It is likely that the two mono-ubiquitin genes and the other polyubiquitin genes provide a sufficient cellular Ub supply throughout development and in other adult tissues, including the female germ line. However, we expect that because *Ubi-p63E* is highly stress inducible (Lee et al., 1988), it might be required for the stress response in most fly tissues.

The requirement for polyubiquitin gene function for meiosis is evolutionarily conserved. The fission yeast polyubiquitin *ubi4*$^{+}$ and budding yeast polyubiquitin *Ubi4* are both highly expressed in meiotic cells (Okazaki et al., 2000; Treger et al., 1988), although the level of free Ub was not raised in meiotic cells. Loss of function of polyubiquitin caused defects specifically in meiosis (fission yeast) or sporulation (budding yeast) (Okazaki et al., 2000; Treger et al., 1988). As with *magn* mutant testes in *Drosophila*, the level of free Ub was greatly reduced in cells entering meiosis in the yeast *ubi4* mutant. The mouse genome has two polyubiquitin genes: *Ubb* and *Ubc* (Wiborg et al., 1985). Whereas *Ubc* is needed for embryonic liver development, *Ubb* null mice grow normally into adulthood but are male and female infertile (Ryu et al., 2007; Ryu et al., 2008). *Ubb* mutant male germ cells arrest in meiotic prophase, resembling the *magn* arrest point. The level of free Ub was also significantly lower in testes from *Ubb* null mice. The similarity of the phenotypes in yeast, flies and mammals suggests that a conserved, but unknown, mechanism consumes free Ub during meiosis, generating a requirement for compensation from induced polyubiquitin gene expression to maintain Ub homeostasis in meiotic prophase. The specific events that demand high levels of free ubiquitin expression remain to be determined. However, our results indicate that the *magn* phenotype does not simply reflect defects in proteasome-mediated protein degradation, but a depleted reservoir of free Ub.

It is possible that the conserved requirement for polyubiquitin might arise from a stress response mechanism that is unique to male meiosis. A large number of genes required for spermatid differentiation are transcribed in spermatocytes but translation of their mRNAs is delayed until after meiosis. The delayed translation of so many terminal differentiation genes in spermatocytes might trigger stress conditions demanding high levels of free ubiquitin. In yeast, it was shown that ubiquitin overexpression confers resistance to overall inhibition on translation (Hanna et al., 2003).

It has been suggested that high activity of the ubiquitin proteasome system (UPS) during the dramatic events of cellular differentiation, such as the replacement of histones by protamines, towards the end of spermatogenesis might stimulate the expression of polyubiquitin genes in the testis (Baarends et al., 2000). However, *magn* mutant spermatocytes arrested well before spermatid differentiation or translation of protamines, suggesting that the meiotic arrest phenotype is not due to defects in these later events. The meiotic chromatin condensation defects observed in growing spermatocytes raised the possibility that some features of chromatin compaction and rearrangement during meiosis are sensitive to free Ub levels.

Most of the previously identified meiotic arrest mutants characterized in *Drosophila* affected the spermatocyte transcription program that controls the expression of a large number of spermatid differentiation gene transcripts. Most of the meiotic arrest genes characterized to date encode either tTAFs or components of the tMAC complex. *Ubi-p63E* does not seem to belong to either of these classes of transcriptional regulators, as many tTAF-dependent and tMAC-dependent genes were expressed at relatively normal levels in *magn* mutant testes (Figs 6, 7).

The arrest in meiotic cell cycle progression in *magn* mutant spermatocytes might be due to failure of the key G2/M regulators Boule and Cyclin B to accumulate. One possibility is that defects in chromatin condensation or other events in *magn* mutant spermatocytes trigger a checkpoint mechanism that blocks the translation of Boule and Cyclin B. In *Drosophila*, spermatid differentiation does not require prior completion of the meiotic divisions, as germ cells mutant for the cell cycle-activating phosphatase *cdc25/twine* or for the CDK-1 kinase fail to undergo the meiotic divisions but proceed with extensive spermatid differentiation with 4N nuclei (Alphey et al., 1992; Sigrist et al., 1995). It is possible that high levels of free Ub are required to pass through a checkpoint that blocks both meiotic cell cycle progression and the onset of spermatid differentiation. In mammals, a checkpoint elicited by transcriptionally active unpaired chromatin in mature spermatocytes blocks both cell cycle progression at the onset of the first meiotic division and the initiation of spermatid differentiation (Burgoyne et al., 2009).

Strikingly, analysis of testis biopsies of 32/32 patients with meiosis I maturation arrest male infertility showed lack of Boule protein expression in testes, although no defects in the *boule* gene itself were identified (Luetjens et al., 2004), suggesting a shared arrest mechanism involving regulation of Boule protein translation or stability. The similarity in the meiosis I arrest phenotype of *magn* mutant *Drosophila* males, *Ubb* mutant mice and human meiosis I maturation arrest patients (Luetjens et al., 2004; Ryu et al., 2008) raises the possibility that Ub homeostasis and the regulatory pathways that depend on a robust pool of free Ub might be compromised in some patients with meiosis I maturation arrest idiopathic human azoospermia.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

C.L. performed the experiments. J.K. analyzed and discussed the microarray data. C.L. and M.T.F. designed the study, interpreted data and wrote the manuscript.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.098947/-/DC1

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