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Dmp53 is sequestered to nuclear bodies in spermatogonia of *Drosophila melanogaster*

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Abstract

p53 family members have been implicated in regulation of genomic integrity and apoptosis in a variety of tissues. The *Drosophila* family member, Dmp53, primarily functions to regulate apoptosis in developing and regenerating tissues but loss of function mutants are viable and fertile. Dmp53 exhibits a striking expression pattern in the male germline with high levels found in nuclear bodies in pre-meiotic germ cells. The localisation of Dmp53 to nuclear bodies is dependent upon Dmp53 complexes being able to bind DNA and although *dmp53* mutants do not affect germline stem cell (GSC) maintenance or differentiation GSCs are sensitive to overexpression of Dmp53 but maturing spermatogonia are not. Dmp53 thus has differential effects depending upon the stage of male germline maturation.

Keywords:

Dmp53, p53, Drosophila, spermatogonia, testis

Introduction

Dmp53 is the sole *Drosophila melanogaster* member of the larger p53 superfamily and was originally regarded as a structural and functional orthologue of vertebrate p53 (Brodsky et al. 2000; Ollmann et al. 2000). The vertebrate p53 family consists of three paralogues: p53, p63 and p73, reviewed in (Marcel et al. 2011). p53 is well known for its role in responding to cellular stresses by activating expression of genes that trigger cell cycle arrest, e.g. p21, (Li et al. 1994) or apoptosis, e.g. Puma, (Nakano and Vousden 2001; Yu et al. 2003) and hence as a "genomic gatekeeper" that is frequently mutated during tumourigenesis. $p73^{-/-}$ and $p63^{-/-}$ mice display several developmental defects that have led to the view that the primary roles of p63/p73 are in the regulation of cell differentiation (Stiewe 2007; Oswald and Stiewe 2008). New data from a variety of sources have indicated that p53/p63/p73 proteins physically interact and that p73 also has an important role in tumourigenesis (Stiewe 2007; Oswald and Stiewe 2008; Rosenbluth and Pietenpol 2008; Tomasini et al. 2008a; Tomasini et al. 2008b; Tomasini et al. 2009). Analysis of p63/p73 gene structure indicates that they share similar domains with p53: transactivation (TA), DNA-binding (DBD), and oligomerisation (OD) domains. They also encode multiple splice forms, some of which contain C-terminal Sterile

Alpha Motif (SAM) and transcriptional inhibitory (TID) domains (Deyoung and Ellisen 2007).

Vertebrate p53, p63 and p73 have all been associated with nuclear bodies known as PML bodies (Gostissa et al. 2003; Bernassola et al. 2005; Lunardi et al. 2009), named after Promyelocytic Leukaemia Protein, a key organiser of the structures (reviewed in (Lallemand-Breitenbach and de The 2010). They are components of the nuclear matrix that anchor a wide variety of proteins and have been associated with DNA replication, transcription and epigenetic silencing (Lallemand-Breitenbach and de The 2010).

Recent phylogenetic studies have concluded that radiation of p53, p63 and p73 proteins occurred specifically within vertebrates and that the invertebrate p53 family members represent a more ancient p63-like structure (Rutkowski et al. 2010). This is also supported by the genetic analysis of Dmp53 function in *Drosophila*. Dmp53 has been shown to play a role in DNA-damage induced cell cycle arrest only in a specific circumstance, tissue regeneration following damage to imaginal discs (Wells et al. 2006), but is required in most instances of DNA-damage induced apoptosis (Brodsky et al. 2000; Ollmann et al. 2000; Lee et al. 2003; Sogame et al. 2003; Sutcliffe and Brehm 2004; LaRocque et al. 2007; Mehrotra et al. 2008; Titen and Golic 2008). Overexpression of Dmp53 in transgenic strains results in induction of apoptosis and loss-of-function mutant animals are largely defective in apoptosis (Ollmann et al. 2000; Lee et al. 2003; Sogame et al. 2003). Hence, expression of endogenous Dmp53 is tightly regulated and generally cannot be easily detected using Dmp53-specific antibodies in wild-type tissues. Genetic analysis has also shown that Dmp53 must be endogenously expressed in a number of tissues as loss of Dmp53 function has a variety of effects in different tissues. It has been demonstrated to be protective against UV-light induced apoptosis in the retina (Jassim et al. 2003) as well as a regulator of lifespan in muscle/fat body and neurons (although the effects are opposite in the respective tissues) (Bauer et al. 2005; Bauer et al. 2007). Dmp53 also appears to have a function in the female germline as a reporter gene generated by fusing the Dmp53-binding sites upstream of the pro-apoptotic gene *reaper* to GFP, is activated upon meiotic recombination (Lu et al. 2010). *dmp53* mutants were also observed to have lower levels of meiotic recombination frequencies. This would appear to be a function specific to females as recombination does not occur during male gametogenesis (Morgan 1914).

The *Drosophila* male germline is established by a small number of stem cells that produce spermatozoa throughout adult life. In contrast to many tissues, the identity of the germline stem cells in *Drosophila*, their morphology and physical relationship to surrounding somatic cells are known. Each testis contains 8-10 germline stem cells (GSCs) which lie clustered around a central, somatically-derived hub located at the apical tip of the testis (Lindsley and Tokuyasu 1980). When the stem cells divide, one daughter retains stem cell identity, while the other daughter (gonialblast) becomes committed to differentiation (Spradling et al. 2001). Each GSC is also adjacent to a pair of somatic cyst stem cells (CySCs) which divide asymmetrically to regenerate themselves and produce two cyst cells which encapsulate the gonialblast. The gonialblast then undergoes four rounds of mitosis accompanied by incomplete cytokinesis to produce a cyst of 16 spermatogonia that enter a pre-meiotic S-phase prior to differentiating into spermatocytes (Fuller 1993). The spermatogonial mitoses are regulated by a number of proteins that ultimately lead to accumulation of the Bag-of-marbles (Bam) protein to a threshold level that triggers mitotic exit and differentiation (Insco et al. 2009). *bam* mutants have a tumourous phenotype due to failure of mitotic exit and accumulation of late spermatogonia (McKearin and Spradling 1990). Bam protein function depends upon association with the DExH family protein, Bgcn (Lavoie et al. 1999), and *bgcn* mutants have a similar phenotype to *bam* mutants (Gonczy et al. 1997). GSC maintenance and differentiation is governed by a relay of signals such that secretion of Upd from the hub acts on CySCs which in turn secrete BMP ligands (Gbb/Dpp) that prevent GSC differentiation (Leatherman and DiNardo 2010). Overexpression of Upd results in increased numbers of CySCs and hence also produces a tumourous phenotype due to failure of GSC differentiation (Leatherman and DiNardo 2010).

Genome wide transcriptional analysis of adult *Drosophila* testes has indicated that although *dmp53* mRNA levels are relatively low in wild type, they appear upregulated in *bgcn* mutant testes, and even more so in *bgcn* mutant testes, in which *upd* expression is induced in the germline (Fig. 1). In both of these cases subsets of pre-meiotic spermatogonia are over-represented and we therefore resolved to determine the specific localisation of *dmp53* expression within the regenerating germ cell population of the adult testis.

Materials and methods

Drosophila strains

Fly strains used for this study include w^{1118} (Bloomington Drosophila Stock Center), *bam*-GFP (Chen and McKearin 2003), *nos*-GAL4::VP16 (Van Doren et al. 1998), *bam*-GAL4::VP16 (Chen and McKearin 2003), GUS-*dmp53 (Brodsky et al. 2000)*, UAS-$dmp53^{R155H.Ex}$ (BDSC) and GUS-$dmp53^{H159N}$ (Brodsky et al. 2000), $dmp53^{-ns}$ (Sogame et al. 2003) and P{GD11134}v45138 (*dmp53* shRNA, Vienna Drosophila RNAi Center). Flies were raised at 29ºC to maximise GAL4 activity, unless otherwise stated.

Immunostudies

Testes were fixed and immunostained as per (Bunt and Hime 2004). Serial confocal sections were taken on a Zeiss LSM510 Confocal Microscope. Antibodies were used at the following concentrations: 1:50 mouse anti-Dmp53 (25F4, Developmental Studies Hybridoma Bank, DSHB), 1:500 rat anti-DE-Cadherin (DSHB), 1:100 goat anti-Vasa (Santa Cruz), 1:5000 rabbit anti-Zfh-1 (R. Lehmann), 1:10 mouse anti-Bam-C (DSHB), 1:500 rat anti-Topi (H. White-Cooper). Statistical analyses were reported as mean $\pm$ standard error of the mean. P-values were obtained by Student's t test.

NCBI GEO Database analysis

Microarray data were accessed at

http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2228.

Mean values from 5-6 replicate samples of wild-type, *bam* mutant or *nosGAL4, UAS-Upd* testes were determined.

Results

Expression of *dmp53* mRNA is upregulated in mutant testes with excess spermatogonia

Microarray data from the study of (Terry et al. 2006) in which mRNA has been profiled in testes of wild type, *bgcn* mutants, and *bgcn* mutants with induced germline-specific expression of *upd* are publically available on the NCBI GEO database (http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS2228). We examined these

datasets and found that expression of *dmp53* was upregulated 6.6-fold in *bgcn* mutant testes and 10.6-fold in *bgcn*, Upd-overexpressing testes (Fig. 1). These data indicate that *dmp53* is most highly represented within the spermatogonial population of adult testes but may also be present in the various somatic cell populations. The very high levels observed in *bgcn*, Upd-overexpressing testes indicate specific enrichment in GSCs and/or somatic CySCs.

Dmp53 is specifically localised to nuclear bodies of germ cells

Four mRNA isoforms are produced by the *dmp53* locus from a combination of different transcriptional start sites and alternative splice sites (www.flybase.org). We used a monoclonal antibody (25F4) raised against amino acids (282-386) of Dmp53-PA, which is common to all isoforms (Brodsky et al. 2004), to examine expression of Dmp53 within adult testes. Dmp53 was visibly expressed in GSCs and spermatogonia but was notably absent (or at very low levels) from somatic lineages (hub, CySCs and cyst cells) (Fig. 2a-e). The antibody also demonstrated that Dmp53 was only present in the nuclei where it was confined to several nuclear bodies in each cell that did not overlap with DAPI staining, or the markers of repressed chromatin H3K9me3 and H3K27me3 (van Leeuwen and van Steensel 2005) (Fig. 2b-c). This indicated that the nuclear staining was not generally localised to heterochromatin but was specifically localised to distinct nuclear bodies. Expression was not limited to GSCs and their immediate daughters but was also found in 4-16 cell stage spermatogonia in which it co-localized with Bam (Fig. 2d). Expression ceased prior to spermatogonial differentiation as no co-localisation was observed with spermatocyte-specific anti-Topi (Fig. 2g). Hub cells (Fig. 2e) expressed low levels of Dmp53 but staining in cyst cells was not above background as seen in loss-of-function mutants (Fig. 2e, 2h-i).

The antibody could not detect Dmp53 in testes from animals where a short hairpin RNA molecule directed against *dmp53* was expressed from the *nos*-GAL4 driver to knockdown Dmp53 levels (Fig. 2i). It also showed no staining in testes from a *dmp53* loss of function mutant (*dmp53*$^{-ns}$, (Sogame et al. 2003)) generated by ends-in homologous recombination (Fig. 2h). These data indicate that the nuclear expression observed using the antibody in GSCs and spermatogonia is specific to Dmp53 protein.

Dmp53 expression in nuclear bodies was observed in all spermatogonial stages and this could be most easily observed in testes from 3rd instar larvae. All germ cells from GSCs to 16-cell stage spermatogonia showed Dmp53 expression (Fig. 2j).

Dmp53 germline expression was not confined to males but was also observed in ovaries. As in spermatogonia the protein was localised to nuclear bodies, however, in contrast to the male germline expression did not cease prior to meiosis. Meiotic exchange occurs in region 2 of the germarium (Carpenter 1975) and cells in this region were observed to express Dmp53 as were germ cells in later egg chambers (Fig. 2k).

Dmp53 is not required for GSC/spermatogonial maintenance or differentiation

Prior studies have indicated that *dmp53* mutant animals are viable and fertile (Lee et al. 2003). Dmp53 acts as a tetrameric protein complex and function can be disrupted by expression of dominant-negative mutant proteins. $Dmp53^{R155H}$ and $Dmp53^{H159N}$ mutant proteins retain the ability to form tetrameric complexes but contain disruptions to the DNA-binding domain such that tetramers incorporating these proteins cannot bind DNA (Brodsky et al. 2000). Germline (*nos*-GAL4) driven expression of $Dmp53^{H159N}$ did not result in any disruption to normal testis cellular architecture. GSCs surrounded the hub and produced spermatogonia which differentiated into spermatocytes. Bam protein was expressed in its normal domain approximately 3 cell diameters from the hub (Fig. 3a). We also drove the p53 shRNA construct (that resulted in severe knockdown of Dmp53 protein levels, Fig. 2i) using *nos*-GAL4 and *bam*-GAL4, neither of which showed any effect on spermatogonial differentiation (Fig. 3b-c).

We also examined testes from homozygous $dmp53^{-ns}$ mutants. This strain was generated by ends-in homologous recombination and is missing most of the Dmp53 TA domain, the oligomerisation domain and part of the DNA-binding domain. It is predicted to be a loss-of-function mutant and does not induce apoptosis upon DNA damage (Sogame et al. 2003). Testes appeared phenotypically normal when lacking Dmp53 function and the stem cell niche was identical to testes from wildtype animals (Fig. 3d-e). GSCs are normally found in a ring abutting the hub and can be unambiguously counted by immunostaining testes with hub (DE-Cadherin) and germ cell (Vasa) markers. $dmp53^{-ns}$ mutant testes exhibited no difference in numbers of GSCs to those from wildtype animals (Fig. 3f).

The expression of the dominant-negative proteins did have an effect on the endogenous localisation of Dmp53. Germline specific expression of $Dmp53^{R155H}$ altered the nuclear distribution of Dmp53 protein detected by the monoclonal antibody. The protein was no longer observed in nuclear bodies as per control testes (Fig. 4a-d) but diffusely distributed

throughout nuclei, indicating that localisation to the nuclear bodies is dependent upon DNA-binding (Fig. 4e-h).

Overexpression of Dmp53 can induce loss of GSCs and spermatogonia

The failure to observe a significant developmental phenotype upon loss of Dmp53 activity not only indicated that Dmp53 may not function in regulation of spermatogonial differentiation but prompted the question as to whether Dmp53 can function in the male germline to induce apoptosis. Overexpression of Dmp53 in somatic tissues has previously been demonstrated to result in apoptosis (Jin et al. 2000; Ollmann et al. 2000). High levels of Dmp53 activity can be induced in the male germline by expression of UAS-*dmp53* from the *nos*-GAL4 driver (*nos*>Dmp53). In testes derived from animals that contain both of these transgenes, germ cell loss was observed to varying extents. Testes were either rudimentary in size with no GSCs surrounding an expanded hub (Fig. 5a), or of approximately normal size but containing a reduced number of pre-meiotic germ cells (Fig. 5b). The expansion of the hub observed in the rudimentary testes is a known phenotype associated with loss of GSCs. In mutants that lose GSCs the somatic CySCs proliferate and begin to express markers characteristic of hub cells (Gonczy and DiNardo 1996). The rudimentary testes do contain some germ cells but these are most likely all spermatocytes as they do not express the spermatogonial specific Bam protein (Fig. 5a''). The germ cells are of varying sizes but spermatocytes increase in volume approximately 25-fold as they mature (Fuller 1993). In the normal sized testes large spermatocytes can be observed in an abnormal position near to the hub due to the loss of spermatogonia (Fig. 5b). These testes have not lost cells to the same extent as the rudimentary testes and cells that express Dmp53 (from either the transgene or endogenously) can be observed (Fig. 5b''). These cells appear to have an abnormal morphology and are not as clearly defined by Vasa staining (Fig. 5b) as the spermatocytes present in Fig. 5a. GSCs and spermatogonia appear sensitive to high levels of Dmp53 with the GSCs being more sensitive as they are lost even though spermatogonia may still be present (Fig. 5b''').To determine whether germ cells overexpressing Dmp53 were being lost from the germline via cell death, we performed an acridine orange (AO) stain on unfixed adult testes. Many *nos*>Dmp53 adult testes were agametic when grown at 29ºC and this impaired our ability to measure cell death in these testes as germ cells were frequently not present. Therefore we raised flies at 25ºC for this experiment to lessen the impact of Dmp53 overexpression. Control testes (*nos*>+) did not show any specific AO-positive staining (Fig, 5c), whereas 63% of *nos*>Dmp53 testes (n=8) showed AO-positive staining at the anterior tip

of the testis (fig. 5d), indicating that overexpression of Dmp53 in the early male germline results in cell death.

To further illustrate the point that GSCs and early spermatogonia are more sensitive to overexpression of Dmp53 than later germ cells, we drove UAS-*dmp53* expression in late-stage spermatogonia from the *bam*-GAL4 promoter. In this case no effect was observed upon spermatogonial differentiation (Fig. 5e) even though very high levels of Dmp53 protein persisted into the spermatocyte stage (Fig. 5f") suggesting that later spermatogonia and spermatocytes are refractory to the effects of Dmp53 overexpression.

Discussion

Dmp53 was found to be much more highly expressed in pre-meiotic spermatogonia than in somatic tissues, with highest levels observed in GSCs (Fig. 1, 2). The subcellular localisation of Dmp53 was also distinct with a concentration in nuclear bodies (Fig. 2). This pattern of expression raises several questions regarding localisation and function of Dmp53 in germ cells.

What are the nuclear bodies?

Proteins found in PML bodies are conjugated to SUMO (sumoylated), a ubiquitin-like protein that is known to regulate protein interactions (reviewed in (Hay 2005). *In vitro* cell culture studies have indicated that Dmp53 can be sumoylated and co-localises with markers of PML bodies (Mauri et al. 2008). Mutation of Dmp53 sumoylation sites inhibits the ability of Dmp53 to act as a transcriptional activator or to induce apoptosis suggesting that localisation to PML-like bodies is critical to its function. A direct orthologue of PML does not exist in *Drosophila* but it is a member of the TRIM/RBCC family which includes the *Drosophila* proteins Brat and Mei-P26, both of which play roles regulating cell division and differentiation in different tissues (Liu et al. 2009; Harris et al. 2011).

Is the Dmp53 protein observed in germ cells a specific isoform?

A recent study of alternative RNA-splicing in *Drosophila* gonads has indicated that the *dmp53-RA* mRNA isoform is 2-fold enriched in ovaries compared to testes while the longer *dmp53-RB* form is 14.6-fold more enriched in testes compared to ovaries (Gan et al. 2010). *dmp53-RB* is therefore much more highly prevalent in testes and it is tempting to suggest that

the unique N-terminal extension of Dmp53-RB is associated with localisation to nuclear bodies but this specific localisation was also observed in female germ cells (Fig. 2k). Dmp53-RA is sometimes referred to as a "DeltaN isoform" structurally related to the vertebrate DeltaNp63/p73 isoforms transcribed from internal promoters that act as endogenous dominant-negative molecules (Marcel et al. 2011). This nomenclature is confusing as Dmp53-RA has an intact TA domain and is capable of inducing transcriptional activation of target genes and apoptosis. Phylogenetic analysis of Dmp53-RB suggests that this isoform may be specific to the *melanogaster* and *obscura* groups of the *Sophophora*. The RB isoform has been annotated in the *D. melanogaster, D. simulans, D. sechellia, D.yakuba and D.erecta* genomes and can be identified as a (mistakenly) assigned separate gene in *D.pseudoobscura* (GA27049) and *D.persimilis* (GL27355). We were unable to identify this isoform in any more distantly related species.

What is the function of Dmp53 in nuclear bodies?

We were unable to discern a function for Dmp53 in pre-meiotic germ cells but recruitment to PML bodies has been shown to be associated with vertebrate p53 function (reviewed in (Gostissa et al. 2003). *Drosophila* spermatogonia are affected by overexpression of Dmp53 but the most sensitive cells were the GSCs where we also observed highest expression of endogenous Dmp53. Dmp53 has been previously shown to play an important role in primordial germ cells where it is required to induce apoptosis of germ cells that fail to correctly migrate into the embryonic gonad (Yamada et al. 2008). A role for Dmp53 in adult female germ cells has also been established. The Dmp53 transcriptional response is activated upon generation of DNA double strand breaks that occur during meiosis and loss of Dmp53 reduces the level of meiotic crossover (Lu et al. 2010). A similar role cannot be required in testes as meiotic recombination does not occur in male germ cells.

Mouse p53 is highly expressed in testes where it also regulates meiosis (Rotter et al. 1993; Lu et al. 2010) but it also has a role in mediating DNA-damage induced spermatogonial apoptosis (Hasegawa et al. 1998). p63 appears to be a more ancestral member of the p53 family and it has been shown to have a role in maintaining genomic integrity in the female germline by causing apoptosis of damaged oocytes (Suh et al. 2006). p63 has also been associated with regulation of mitotic and apoptotic properties of immature male germ cells (gonocytes) in the developing mouse testis (Petre-Lazar et al. 2007). The expression of p53 family members show some evolutionary conservation of expression within germ cells and ultimately may have similar properties regulating germline integrity. It is of course essential

that the germline maintains strict controls over genomic integrity as any alterations in DNA sequence will be inherited by the next generation. We hypothesize that Dmp53 plays a role in maintaining integrity in the male germline by perhaps eliminating cysts of germ cells that have accumulated DNA damage. This hypothesis awaits large scale mutagenesis studies to be conducted in *dmp53* mutant animals in order to determine if the loss of *dmp53* results in increased mutagenesis rates or decreases in male fertility.

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Figure Legends

Fig 1 *dmp53* expression is enriched in the early male germline. Analysis of testes from WT, *bgcn* mutants with or without overexpression of Upd. When compared to WT (green), *dmp53* expression is higher in *bgcn* testes (blue), which are enriched in spermatogonia ($p<0.0001$). When compared to WT testes, *dmp53* expression is higher in *bgcn* mutants which overexpress Upd (red), which are enriched in germline and somatic stem cells ($p<0.0001$). Data are compiled from microarray studies reported in (Terry et al. 2006).

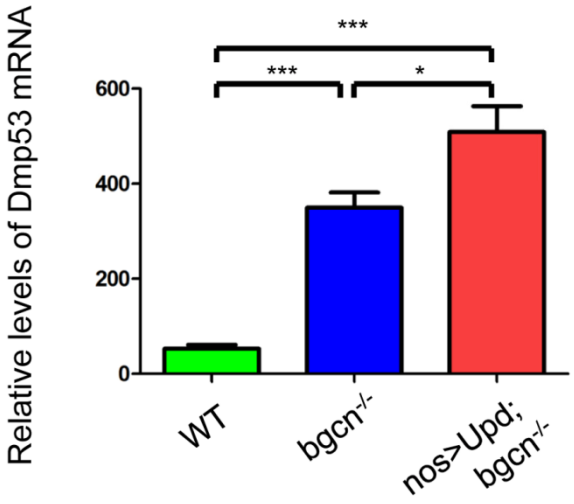
Fig 2 Dmp53 is expressed in the early male germline. (a) Anti-Dmp53 (white) appears in the Vasa-positive GSCs (magenta) surrounding the somatic hub cells (green, yellow star), and is also detected in all spermatogonial stages (magenta). The transmembrane protein DE-Cad marks germline and somatic cell membranes (green). (b) Anti-Dmp53 staining (white) is located adjacent to DAPI regions (red), which stains more intensely in spermatogonia than spermatocytes, and does not colocalise with markers of repressed chromatin, anti-H3K9me3 (green), or (c) anti-H3K27me3 (green). An anti-GFP stain (green) on a *bam*-GFP strain, which expresses GFP in the 4 to 16-cell spermatogonial stages, shows that the *bam* (green, d and d'') and Dmp53 (white, d'') expression patterns overlap. (e) Dmp53 (white) is expressed adjacent to DAPI regions (red) in GSCs (magenta) surrounding the DE-Cad-positive hub cells (green), and is also detected at low levels in the hub cells (e'). (f) Dmp53 (white) does not colocalise with cells expressing the early cyst cell marker Zfh-1 (green). (g) Dmp53 is detected in the germline until the end of the spermatogonial region, but is not detected in Topi-expressing germ cells (green), a marker of spermatocytes. (h-j) Testes stained with anti-Vasa (magenta) and DE-Cad (green). (h) Dmp53 levels (white) are not detectable in testes carrying the $dmp53^{-ns}$ mutation. (i) Dmp53 levels (white) are much reduced following germline-specific RNAi knockdown of *dmp53* using the *nos*-Gal4 driver. (j) Anti-Dmp53 staining (white) appears similar in 3rd instar larval gonads as in the adult testis. (k) Dmp53 is also detectable in the female germline in Vasa-positive (magenta) germ cells. DE-Cad (green) marks the somatic lineage in the ovary. Scale bar 5µm. Hub position (testes) or cap cell region (ovaries) marked with yellow star.

Fig 3 Reduction in Dmp53 levels results in no observable phenotype. (a) Germline-specific overexpression of a UAS dominant negative Dmp53 construct (*nos*>p53^{R259H}) results in testes appearing WT. DE-Cad staining (green) shows that hub and somatic cells of the testis appear normal, while Vasa-expressing germ cells (magenta) retain their normal organisation. The Bam expression domain (red), marking spermatogonial differentiation, is similar to WT Bam expression. (b) *dmp53* mRNA levels were reduced using a UAS-*dmp53*^{RNAi} construct driven from the *nos*:Gal4 driver (*nos*>*dmp53*^{RNAi}), which drives in GSCs and early spermatogonial cells. Germline and somatic cell architecture are similar to WT testes. (c) The same UAS-*dmp53*^{RNAi} construct was also driven off the *bam*-Gal4 driver (*bam*>*dmp53*^{RNAi}), which drives expression from the 4 to 16-cell spermatogonial stage. Germline and somatic cell expression were similar to WT. (d) WT (*nos*-Gal4) testes contain 11.0±0.5 (n=11) Vasa-positive GSCs (magenta) abutting the somatic hub cells (green). (e) Testes carrying the *dmp53*$^{-ns}$ mutation, which do not have observable Dmp53 levels, appear similar to WT and contain 10.9±0.4 (n=15) GSCs per testis. (f) Graphical representation comparing the number of GSCs per testis in WT (*nos*-Gal4) and p53KO testes. There is no significant difference in GSC number in these two genotypes (p=0.9). Hub marked with yellow star. Scale bar 5µm.

Fig 4 Expression of a dominant-negative form of Dmp53 prevents localization of endogenous Dmp53 in nuclear bodies. *nos*>+ testes show a normal intracellular distribution of Dmp53 concentrated in nuclear bodies (a-d). A UAS-Dmp53^{R155H} construct driven in the male germline (green, e (*nos*>p53^{R155H})) results in anti-Dmp53 localisation (red, f) that does not predominantly localise to nuclear bodies as per WT GSCs and early spermatogonia, and is instead distributed throughout the entire nucleus, and overlaps the DAPI staining (blue, g). Scale bar 5µm.

Fig 5 Overexpression of WT Dmp53 in the early germline results in GSC loss via apoptosis. (a) Germline expression of a UAS:Dmp53 construct using the *nos*-Gal4 driver (*nos*>Dmp53), which drives expression in the GSCs and early spermatogonia results in testes which have lost GSCs, and have reduced numbers of Vasa-positive germ cells (magenta). The loss of germline is largely noticeable in the early cells, as seen by the reduction in Bam staining (red), which is normally expressed in 4-16 cell spermatogonia. Somatic and hub cell architecture (green) is also altered in these testes. (b) A less severe phenotype observed in a *nos*>Dmp53 adult testis showing mild early germ cell loss. To confirm that Dmp53 is specifically overexpressed, an anti-p53 stain on these testes shows that p53 levels are increased (red). (c) Acridine orange (AO) does not stain *nos*>+ testes, whereas in

nos>Dmp53 testes (d), AO staining is visible near the hub. These testes were raised at 25ºC (e) Overexpression of the UAS-Dmp53 construct using the *bam*:Gal4 driver (*bam*>p53), which drives expression in 4-16 cell spermatogonia (yellow arrow), produces adult testes which appear normal in germ cell (magenta) and somatic cell (green) architecture. Bam expression (red) also appears normal. (f) To confirm that overexpression is occurring in Bam-expressing cells, anti-Dmp53 staining (red) shows that the Dmp53 expression domain is extended and expression levels are higher in the male germline in *bam*>Dmp53 testes, indicating that overexpression of Dmp53 in later spermatogonia has no obvious effect on the adult germline. Hub marked with yellow star. Scale bar 5µm for a-d, 10µm for e,f.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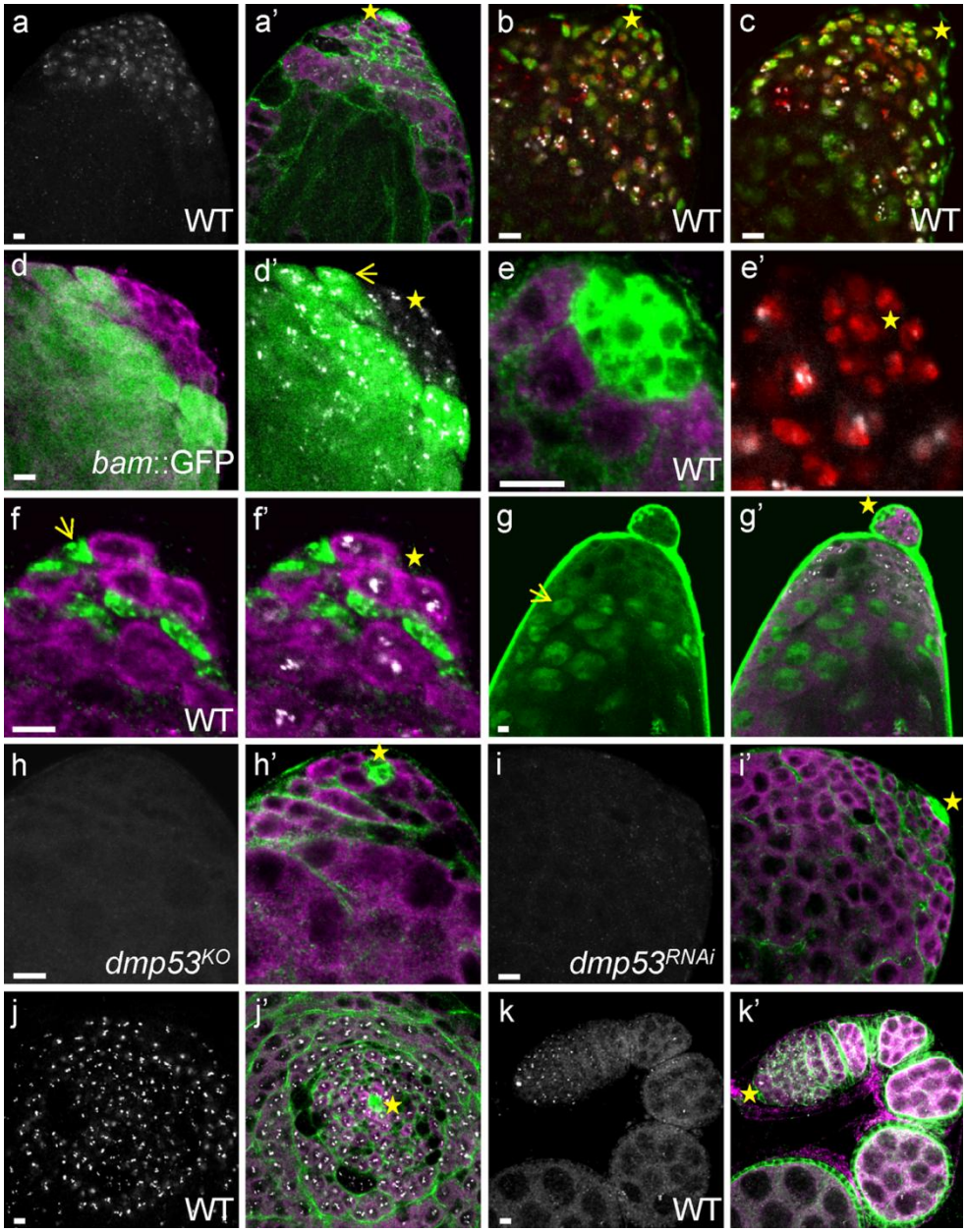
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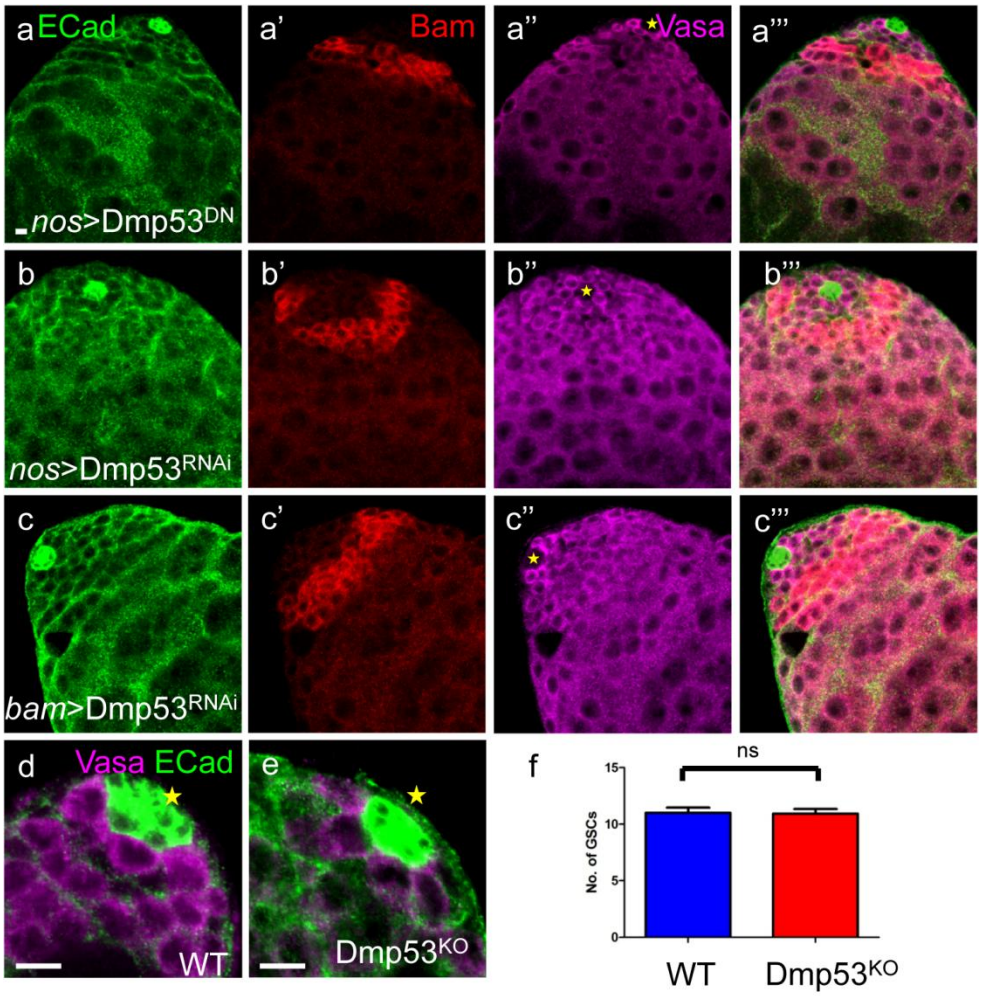
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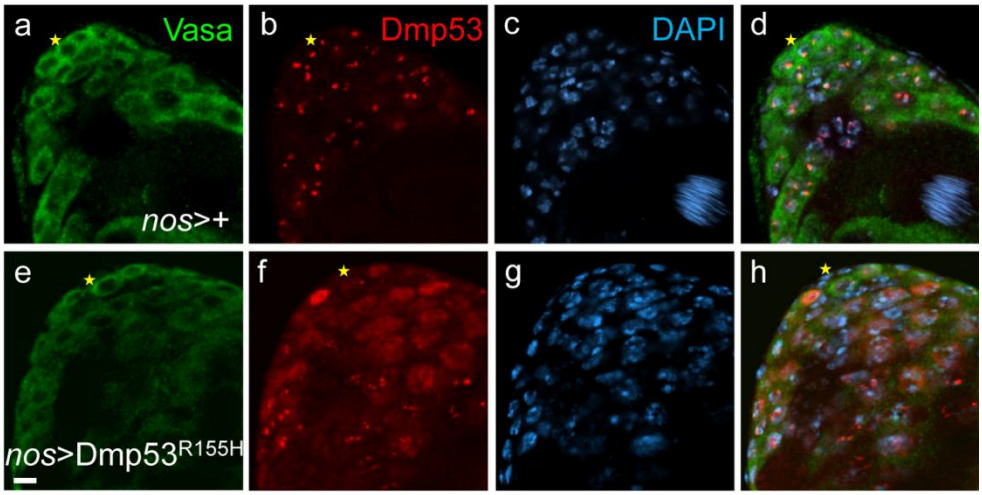
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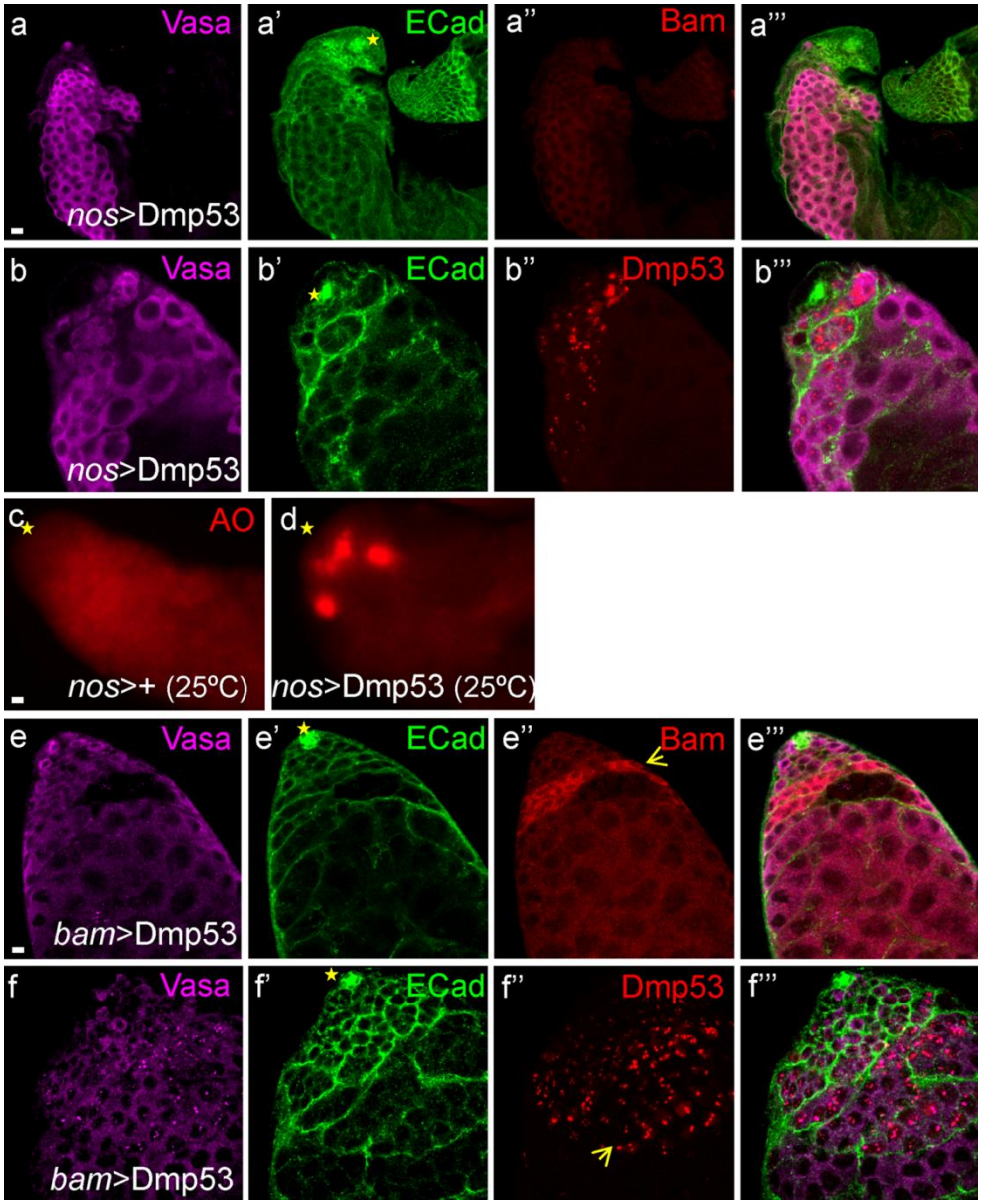
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