Rpl22e, but not Rpl22e-like-PA, is SUMOylated and localizes to the nucleoplasm of Drosophila meiotic spermatocytes

Michael G. Kearse, Jill A. Ireland,† Smrithi M. Prem,‡ Alex S. Chen§ and Vassie C. Ware*

Department of Biological Sciences; Lehigh University; Bethlehem, PA USA

†Current affiliation: University of Central Florida College of Medicine; Orlando, FL USA; †University of Medicine and Dentistry of New Jersey; Robert Wood Johnson Medical School; New Brunswick, NJ USA; ‡University of Medicine and Dentistry of New Jersey; Robert Wood Johnson Medical School; New Brunswick, NJ USA; §Section on Cellular Neurobiology; NICHD; National Institutes of Health; Bethesda, MD USA

Keywords: Rpl22e, Rpl22e-like-PA, Drosophila, ribosomal protein paralogues, duplicated ribosomal proteins, SUMOylation, phosphorylation, post-translational modification, male germline

Abbreviations: bam, bag-of-marbles; IHC, immunohistochemistry; IP, immunoprecipitation; m, molecular mass; Rp, ribosomal protein; SUMO, small ubiquitin-like modifier

Introduction

Duplicated ribosomal protein (Rp) gene families often encode highly similar or identical proteins with redundant or unique roles. Eukaryotic-specific paralogues Rpl22e and Rpl22e-like-PA are structurally divergent within the N terminus and differentially expressed, suggesting tissue-specific functions. We previously identified Rpl22e-like-PA as a testis Rp. Strikingly, Rpl22e is detected in immunoblots at its expected molecular mass (m) of 33 kD and at increasing m of ~43–55 kD, suggesting Rpl22e post-translational modification (PTM). Numerous PTMs, including N-terminal SUMOylation, are predicted computationally. Based on S2 cell co-immunoprecipitations, bacterial-based SUMOylation assays and in vivo germline-specific RNAi depletion of SUMO, we conclude that Rpl22e is a SUMO substrate. Testis-specific PTMs are evident, including a phosphorylated version of SUMOylated Rpl22e identified by in vitro phosphatase experiments. In ribosomal profiles from S2 cells, only unconjugated Rpl22e co-sediments with active ribosomes, supporting an extra-translational role for SUMOylated Rpl22e. Ectopic expression of an Rpl22e N-terminal deletion (lacking SUMO motifs) shows that truncated Rpl22e co-sediments with polysomes, implying that Rpl22e SUMOylation is dispensable for ribosome biogenesis and function. In mitotic germ cells, both paralogues localize within the cytoplasm and nucleolus. However, within meiotic cells, both paralogues localize within the cytoplasm and nucleolus. However, within meiotic cells, phase contrast microscopy and co-immunohistochemical analysis with nucleolar markers nucleostemin1 and fibrillarin reveals diffuse nucleoplasmic and nucleolar localization that transitions to a punctate pattern as meiotic cells mature, suggesting an Rpl22e role outside of translation. Germline-specific knockdown of SUMO shows that Rpl22e nucleoplasmic distribution is sensitive to SUMO levels, as immunostaining becomes more dispersed. Overall, these data suggest distinct male germline roles for Rpl22e and Rpl22e-like-PA.

Duplicated ribosomal protein (Rp) gene families often encode highly similar or identical proteins with redundant or unique roles. Eukaryotic-specific paralogues Rpl22e and Rpl22e-like-PA are structurally divergent within the N terminus and differentially expressed, suggesting tissue-specific functions. We previously identified Rpl22e-like-PA as a testis Rp. Strikingly, Rpl22e is detected in immunoblots at its expected molecular mass (m) of 33 kD and at increasing m of ~43–55 kD, suggesting Rpl22e post-translational modification (PTM). Numerous PTMs, including N-terminal SUMOylation, are predicted computationally. Based on S2 cell co-immunoprecipitations, bacterial-based SUMOylation assays and in vivo germline-specific RNAi depletion of SUMO, we conclude that Rpl22e is a SUMO substrate. Testis-specific PTMs are evident, including a phosphorylated version of SUMOylated Rpl22e identified by in vitro phosphatase experiments. In ribosomal profiles from S2 cells, only unconjugated Rpl22e co-sediments with active ribosomes, supporting an extra-translational role for SUMOylated Rpl22e. Ectopic expression of an Rpl22e N-terminal deletion (lacking SUMO motifs) shows that truncated Rpl22e co-sediments with polysomes, implying that Rpl22e SUMOylation is dispensable for ribosome biogenesis and function. In mitotic germ cells, both paralogues localize within the cytoplasm and nucleolus. However, within meiotic cells, both paralogues localize within the cytoplasm and nucleolus. However, within meiotic cells, phase contrast microscopy and co-immunohistochemical analysis with nucleolar markers nucleostemin1 and fibrillarin reveals diffuse nucleoplasmic and nucleolar localization that transitions to a punctate pattern as meiotic cells mature, suggesting an Rpl22e role outside of translation. Germline-specific knockdown of SUMO shows that Rpl22e nucleoplasmic distribution is sensitive to SUMO levels, as immunostaining becomes more dispersed. Overall, these data suggest distinct male germline roles for Rpl22e and Rpl22e-like-PA.

Ribosome heterogeneity and may broaden the translational regulatory spectrum in cells under certain physiological conditions. Noteworthy is the fact that some Rps perform extra-ribosomal functions in addition to their roles in translation (for a review, see ref. 5). Certainly through the course of evolution, a duplicated Rp paralogue may have acquired a new role distinct from its presumed original canonical role as a structural component of the ribosome. Notwithstanding the acquisition of a new function, some degree of functional redundancy between Rp paralogues might also have been retained. Given that different functional roles have often been attributed to structurally similar paralogues, it is reasonable to propose that disparate functions could be ascribed to structurally dissimilar paralogues, particularly in instances where

*Correspondence to: Vassie C. Ware; Email: vcw0@lehigh.edu
Submitted: 03/02/13; Revised: 05/20/13; Accepted: 06/03/13
http://dx.doi.org/10.4161/nucl.25261

www.landesbioscience.com
Nucleus 241–258; May/June 2013; © 2013 Landes Bioscience.
tissue-specific expression patterns accompany parologue structural divergence. The conserved eukaryotic-specific RpL22e family in Drosophila melanogaster represents a model protein family whose structurally divergent members may have evolved disparate functions.

The fly RpL22e family includes two genes, rpL22 and rpL22-like, hereafter included with an “e” designation to signify the gene and products as “eukaryotic-specific” and not homologous to bacterial rpL22. A single protein product was previously annotated for human RpL22e.14 Both proteins share a Rp signature with rRNA binding motifs (as defined for human RpL22e) at the C-terminal end.15 Our previous ribosomal profile analyses confirm as well that within the testis, RpL22e-like-PA is found in ribosomes and in polysomes, though other possible functions cannot be excluded at this time. A fly-specific N-terminal extension (of unknown function) with homology to the C-terminal end of histone H1 (previously described only for Rpl23a and Rpl22e by Koyama et al.) is clearly the most divergent structural feature between the two proteins.15 Therefore, any potential functional differences between these proteins might be mediated through interactions in the N-terminal domain.

In the male reproductive system of the fly, RpL22e is expressed in embryonic and adult gonads and germ cell lines (gonads, primordial germ cells [PGCs], adult ovary germ cells stem cells [GSCs]) and in adult testes, but not adult ovary from microarray analyses.1617 On the other hand, Rpl22e is ubiquitously expressed in embryos and adults.18 With paralogue-specific antibodies (Abs), we determined that RpL22e-like-PA is expressed in a tissue-specific manner, found only in germ cells in adult testes and in heads of both sexes.15 Thus the gonadal protein expression pattern aligned well with previously reported mRNA expression patterns.

Well established as a 60S ribosomal subunit protein, RpL22e is only 37% identical in amino acid (aa) sequence to Rpl22e-like-PA.19 Both proteins share a Rp signature with rRNA binding motifs (as defined for human RpL22e) at the C-terminal end.15 Our previous ribosomal profile analyses confirm as well that within the testis, RpL22e-like-PA is found in ribosomes and in polysomes, though other possible functions cannot be excluded at this time. A fly-specific N-terminal extension (of unknown function) with homology to the C-terminal end of histone H1 (previously described only for Rpl23a and Rpl22e by Koyama et al.) is clearly the most divergent structural feature between the two proteins. Therefore, any potential functional differences between these proteins might be mediated through interactions in the N-terminal domain.

In the male reproductive system of the fly, RpL22e is expressed in the testis, accessory gland, seminal vesicle and the ejaculatory duct. RpL22e-like-PA is only expressed within germ cells in embryonic and adult gonads and germ cell lines (gonads, primordial germ cells [PGCs], adult ovary germ cells stem cells [GSCs]) and in adult testes, but not adult ovary from microarray analyses.1617 On the other hand, Rpl22e is ubiquitously expressed in embryos and adults.18 With paralogue-specific antibodies (Abs), we determined that RpL22e-like-PA is expressed in a tissue-specific manner, found only in germ cells in adult testes and in heads of both sexes.15 Thus the gonadal protein expression pattern aligned well with previously reported mRNA expression patterns.

Well established as a 60S ribosomal subunit protein, RpL22e is only 37% identical in amino acid (aa) sequence to Rpl22e-like-PA.19 Both proteins share a Rp signature with rRNA binding motifs (as defined for human RpL22e) at the C-terminal end.15 Our previous ribosomal profile analyses confirm as well that within the testis, RpL22e-like-PA is found in ribosomes and in polysomes, though other possible functions cannot be excluded at this time. A fly-specific N-terminal extension (of unknown function) with homology to the C-terminal end of histone H1 (previously described only for Rpl23a and Rpl22e by Koyama et al.) is clearly the most divergent structural feature between the two proteins. Therefore, any potential functional differences between these proteins might be mediated through interactions in the N-terminal domain.

In the male reproductive system of the fly, RpL22e is expressed in the testis, accessory gland, seminal vesicle and the ejaculatory duct. RpL22e-like-PA is only expressed within germ cells in embryonic and adult gonads and germ cell lines (gonads, primordial germ cells [PGCs], adult ovary germ cells stem cells [GSCs]) and in adult testes, but not adult ovary from microarray analyses.1617 On the other hand, Rpl22e is ubiquitously expressed in embryos and adults.18 With paralogue-specific antibodies (Abs), we determined that RpL22e-like-PA is expressed in a tissue-specific manner, found only in germ cells in adult testes and in heads of both sexes.15 Thus the gonadal protein expression pattern aligned well with previously reported mRNA expression patterns.

Well established as a 60S ribosomal subunit protein, RpL22e is only 37% identical in amino acid (aa) sequence to Rpl22e-like-PA.19 Both proteins share a Rp signature with rRNA binding motifs (as defined for human RpL22e) at the C-terminal end.15 Our previous ribosomal profile analyses confirm as well that within the testis, RpL22e-like-PA is found in ribosomes and in polysomes, though other possible functions cannot be excluded at this time. A fly-specific N-terminal extension (of unknown function) with homology to the C-terminal end of histone H1 (previously described only for Rpl23a and Rpl22e by Koyama et al.) is clearly the most divergent structural feature between the two proteins. Therefore, any potential functional differences between these proteins might be mediated through interactions in the N-terminal domain.

In the male reproductive system of the fly, RpL22e is expressed in the testis, accessory gland, seminal vesicle and the ejaculatory duct. RpL22e-like-PA is only expressed within germ cells in embryonic and adult gonads and germ cell lines (gonads, primordial germ cells [PGCs], adult ovary germ cells stem cells [GSCs]) and in adult testes, but not adult ovary from microarray analyses.1617 On the other hand, Rpl22e is ubiquitously expressed in embryos and adults.18 With paralogue-specific antibodies (Abs), we determined that RpL22e-like-PA is expressed in a tissue-specific manner, found only in germ cells in adult testes and in heads of both sexes.15 Thus the gonadal protein expression pattern aligned well with previously reported mRNA expression patterns.
mechanistic processes that specify RpL22e paralogue functions within the testis.

Results

RpL22e is differentially post-translationally modified in different tissues. We have previously reported the tissue- and sex-specific expression of the duplicated member of the RpL22e family, RpL22e-like-PA.6 In the adult testis, RpL22e-like-PA protein is detected at its predicted m of 34 kD and has an electrophoretic pattern identical to recombinant protein. However, we noted that the ubiquitously expressed RpL22e was detected predominantly at an m of ~50 kD, greater than its predicted m of 33 kD. Here we further refine this observation by characterizing RpL22e in various tissues and show evidence for RpL22e PTM.

Comparing RpL22e electrophoretic patterns between Drosophila S2 tissue culture cells and adult gonads, m variation in accumulating proteins is seen in the ~33–55 kD range (Fig. 1A). To facilitate protein comparative analysis, we established a protein nomenclature based on the approximate observed m. The predicted m of RpL22e based on the annotated coding sequence is 33 kD and is seen in all tissues. Two additional proteins, designated 43α and 43β, accumulate at varying amounts at the ~43 kD range in S2 cells, ovaries, as well as in testes. The

modified substrates by altering intra- or intermolecular protein interactions.32 Further, the SUMOylation pathway is critical at multiple stages of spermatogenesis in several species, including Drosophila (for a review, see ref. 33).

Together with the detection of higher m immunoreactive RpL22e species with paralogue-specific, peptide-derived Abs,6 computational predictions of a SUMO motif within the N-terminal region of RpL22e and proteomics evidence for association of RpL22e in complexes with other SUMO substrates,29 we propose that RpL22e is a SUMO substrate. To investigate this possibility, we use a combination of biochemical, molecular and genetic approaches that included co-immunoprecipitations from S2 cells using anti-SUMO and anti-RpL22e Abs, a bacterial-based SUMOylation assay and in vivo germline-specific RNAi depletions of SUMO. Another goal was to determine if high m immunoreactive RpL22e species associate with 60S subunits, 80S monosomes and/or polysomes in ribosome profiles from S2 cells. Such an association would support involvement in translation. On the other hand, lack of co-sedimentation with ribosomal components would favor involvement in extra-translational pathways. Further, using immunohistochemistry (IHC) we refine the cellular and subcellular localization patterns for both paralogues in the male reproductive tract, previously described by Kearse et al.6 Collectively, these investigations provide insights into
43 kD proteins accumulate at varying amounts not only in different tissues and cells (compare S2 cells with testis and ovary) but also within different samples from the same cell type (compare S2 cells in Fig. 1A–C; Fig. S1). The amounts of these particular species were consistently variable and may indicate that the 43 kD products are less stable modification pathway intermediates that fluctuate with metabolic state. Immuno-reactive proteins migrating in the ~55 kD range are also evident, with 55α present in all tissues examined. Interestingly, testis tissue contains two additional immuno-reactive proteins, designated 55β and 55γ. Western analysis of accessory glands (removed during testes dissections) suggests that 55α and 55γ are testis-specific Rpl22e species within the male reproductive tract (Fig. S1). Furthermore, comparison of Rpl22e immunodetection patterns between whole testis tissue and isolated apical tip tissue suggests that 55γ is restricted to mature post-mitotic spermatocytes (Fig. S1). Th APPLICATIONar absence of these species from ovary and S2 cells (this report) as well as from salivary glands and head tissue suggests that these species may indeed be unique to testis tissue.

Initially, to confirm higher m proteins as bona fide Rpl22e gene products, we first performed pre-immune (see ref. 6) and then peptide inhibition experiments (Fig. 1B). When anti-Rpl22e polyclonal Ab was pre-incubated with a blocking peptide (a C-terminal peptide originally used for Ab production), detection of all proteins was significantly reduced compared with protein detection in the absence of the blocking peptide. That the specific peptide acts to block detection of the higher m proteins as well as Rpl22e at ~33 kD favors the interpretation that the Ab is detecting Rpl22e proteins. To provide additional support that high m products detected with the Rpl22e polyclonal Ab are Rpl22e proteins, we next attempted to express FLAG-tagged Rpl22e in S2 cells. While the addition of the FLAG-tag did not hinder protein stability, only minimal amounts of protein that migrated slower than the predicted m of 33 kD accumulated in

some experiments (Fig. S8). Production of higher m Rpl22e proteins within S2 cells may have been curtailed by (1) steric hindrance of the FLAG tag on the function of the protein modification machinery or (2) an imbalance in protein modification machinery relative to the abundance of FL-FLAG-tagged Rpl22e in overexpression experiments. We later explored the possibility that overexpression of FL-FLAG-tagged Rpl22e within S2 cells overwhelms the modification machinery and determine that higher m products are produced when S2 cells overexpress components of the Drosophila SUMOylation pathway. More definitive evidence to confirm the identity of higher m species as Rpl22e proteins was provided by RNAi knockdown of Rpl22e in S2 cells. By treating cells with dsRNA targeting codons 1–100 of Rpl22e, immuno-reactive proteins of the predicted m (33 kD) as well as all other higher m proteins are significantly reduced over time compared with dsRNA GFP controls (Fig. 1C). Taken together, we conclude that immunoreactive proteins in the 33, 43 and 55 kD range are true Rpl22e proteins.

By RT-PCR, we eliminated the possibility that alternative splicing of the rpl22e gene could produce transcripts that would encode higher m proteins, as amplicons larger than those that would be predicted from the coding sequence are not evident (data not shown). We therefore hypothesize that the accumulation of higher m Rpl22e proteins is a result of PTM. Initial investigation of PTMs by in silico probing (via Eukaryotic Linear Motif scanner) for conserved modification motifs predicts multiple modifications for Rpl22e (Fig. 2, Table S1). Seven putative phosphorylation targets are predicted in Rpl22e, however, the small m of such a modification (95Da per phosphate group), even if combined, would not account for the observed ~10 and ~20 kD increase in m (Fig. 1). However, a conserved SUMOylation motif was predicted for Rpl22e and if covalent attachment of the 10 kD SUMO protein does occur, this could account for the observed electrophoretic shift seen by immunodetection.

Figure 2. Computational predictions of post-translational modifications within the Rpl22e family. Eukaryotic Linear Motif (ELM) scanner predicts multiple modifications in both Rpl22e (Figp00015286; Figp00015348) and Rpl22e-like-PA (Figp00014857; Figp00017958) as consensus sequences were conserved for various phosphorylation and SUMOylation motifs. The black domains represent the conserved region between the fly paralogues and other eukaryotic Rpl22e proteins. Dark and light gray domains represent the fly-specific histone H1-like N-terminal extension that has less homology between the paralogues. Consensus sequences and motifs within Rpl22e family members are found in Table S1.
Given that initial expression of FLAG-tagged RpL22e in S2 cells readily produce an abundance of high m products, we surmised that if RpL22e is SUMOylated, then its overexpression in S2 cells would produce unequal stoichiometry between the target protein and SUMO, likely resulting in relatively little SUMO-modified FLAG-tagged RpL22e. To rectify this imbalance, it is common to overexpress elements of the SUMOylation pathway, including the E2 conjugating enzymes (Ubc9 in Drosophila) and SUMO itself. Therefore, we used the previously developed S295U S2 stable cell line, which harbors expression vectors for FLAG-SUMO and HA-Ubc9, both under the control of the Ca2+-inducible metallothionein promoter for FLAG-tagged RpL22e expression experiments. Western analysis of FLAG-RpL22e transfections shows accumulation of FLAG proteins with m of 33 kD, as well as (although at lower quantities) ~43 kD and ~55 kD (Fig. 3A). High levels of endogenous RpL22e may hinder FLAG-RpL22e modification in S2 cell-based assays. Although SUMO is FLAG-tagged in the cell line and we would expect any FLAG-SUMO conjugate to be detected with anti-FLAG Ab, we note that FLAG-SUMO conjugates are present at ~43–55 kD, consistent with known higher m RpL22e species. These FLAG-SUMO conjugates are only detected when RpL22e, but not GFP, is transiently expressed. These data are consistent with the hypothesis that high m species could be SUMOylated RpL22e proteins or alternatively yet less likely, RpL22e proteins are either not stable or do not accumulate to detectable levels.

We next assessed RpL22e SUMOylation by co-immunoprecipitation (co-IP) experiments. Using S2 cell lysates, SUMOylated proteins were immunoprecipitated using anti-Drosophila SUMO. The 55a RpL22e species was captured in IP reactions containing anti-Drosophila SUMO, but not in control reactions with beads alone (Fig. 3B). The amount of 43 kD species in this particular S2 sample is effectively undetectable compared with quantities in other samples (e.g., Figure 1); thus, it is unknown if the 43 kD species would be captured in co-IP experiments as effectively as the 55a species. Additionally, a SUMO immunoreactive protein of 55 kD (and not 43 kD) is captured from S2 cell lysates in IP reactions with anti-RpL22e, but not in control reactions (Fig. S4).

To further test if RpL22e is a SUMOylation substrate, we used the previously developed bacterial-based SUMOylation assay utilizing the Drosophila SUMOylation pathway enzymes and SUMO protein. E. coli were co-transformed with plasmids encoding FLAG-RpL22e and either an incompetent (Q Δα) or competent (Qαβγ) form of SUMO. Western analysis shows a 20 kD electrophoretic shift of FLAG-RpL22e when co-transformed with a competent form of SUMO (Qαβγ), but not with an incompetent form (QΔα) (Fig. 4A). Based on the observed m, we conclude the modification is due to the addition of two SUMO moieties (55a).

In an attempt to gain evidence for functional diversification between the RpL22e paralogues, we extended the in silico investigation of predicted PTMs of RpL22e-like-PA. We have not detected RpL22e-like-PA above its predicted m in testis protein lysates. Therefore, if modified, proteins are either not stable or do not accumulate to detectable levels. Nevertheless, we tested if RpL22e-like-PA is a SUMO substrate.
assessed the phosphorylation state of RpL22e in S2 cells and testis. Western analysis of extracts treated with phosphatase shows a significant reduction of the testis-specific 55 γ species exclusively compared with control reactions (Fig. 5a). Thus, the 55 γ species is a phosphorylated form of SUMOylated RpL22e. Whether this 55 γ contains a single or multiple phosphate moieties is not addressed here. Additionally, other lower m RpL22e species (e.g., 43 β) may also be phosphorylated but not accessible to the phosphatase in vitro.

Multiple phosphorylation targets are predicted in both RpL22e and RpL22e-like-PA (Fig. 2; Table S1). No evidence for significant modification of germline-expressed RpL22e-like-PA has been observed (this work included). Phosphatase treatments of testis extracts did not result in any discernible electrophoretic shifts in Western analysis of RpL22e-like-PA (Fig. 5b), supporting the conclusion that RpL22e-like-PA is not phosphorylated at accumulating levels in the testis.

Further evidence of RpL22e SUMOylation in testis and the male germline is provided by SUMO knockdown. Using the UAS-GAL4 binary system and the pVALIUM20 RNAi vector to express a miR1 cassette for knockdown, smt3 (encodes the single Drosophila SUMO protein) was specifically targeted in the male germline using the bam-GAL4-VP16 driver. Confirmation of a smt3 knockdown effect is shown by an altered pattern of SUMOylated proteins detected by anti-Drosophila SUMO in the smt3 knockdown compared with controls (Fig. 5C). Western analysis of testis tissue from F1 males when compared with using the bacterial-based SUMOylation assay. Consistent with in vitro testis results, SUMOylation of RpL22e-like-PA is not evident in this bacterial SUMO assay (Fig. 4B). Positioning of the predicted motif and/or its structural context may render this SUMO motif inaccessible or nonfunctional not only in the bacterial assay, but in the testis environment as well. Alternatively, additional essential factor(s) and/or conditions may be required for RpL22-like-PA SUMOylation that are neither present in the bacterial system or by inference, in the testis germline environment as well.

In many instances SUMOylation is known to impact target protein stability (for a review, see ref. 32). RpS3 stability is enhanced by SUMOylation. In order to determine if a similar effect could be demonstrated for RpL22e, we used an in vitro proteolytic assay described by Jang et al., previously used to assess SUMOylated RpS3 stability. We did not observe an impact of SUMOylation on the stability of RpL22e, previously expressed and SUMOylated in a bacterial assay. The proteolytic sensitivity of unmodified and SUMOylated RplL22e was equivalent in this assay (Fig. 5). We do conclude, however, that SUMOylated RplL22e from S2 cells and testis is highly stable, as revealed by western blot analysis (Fig. 1C).

Male germline-specific modifications include specific phosphorylation of SUMOylated RplL22e. To characterize the testis-specific RplL22e modifications (Fig. 1), we proceeded to investigate possible phosphorylation and additional SUMOylation events. Using in vitro calf-intestinal alkaline phosphatase treatments, we assessed the phosphorylation state of RplL22e in S2 cells and testis. Western analysis of extracts treated with phosphatase shows a significant reduction of the testis-specific 55 γ species exclusively compared with control reactions (Fig. 5a). Thus, the 55 γ species is a phosphorylated form of SUMOylated RplL22e. Whether this 55 γ contains a single or multiple phosphate moieties is not addressed here. Additionally, other lower m RplL22e species (e.g., 43 β) may also be phosphorylated but not accessible to the phosphatase in vitro.

Multiple phosphorylation targets are predicted in both RplL22e and RplL22e-like-PA (Fig. 2; Table S1). No evidence for significant modification of germline-expressed RplL22e-like-PA has been observed (this work included). Whether this 55 γ contains a single or multiple phosphate moieties is not addressed here. Additionally, other lower m RplL22e species (e.g., 43 β) may also be phosphorylated but not accessible to the phosphatase in vitro.

Further evidence of RplL22e SUMOylation in testis and the male germline is provided by SUMO knockdown. Using the UAS-GAL4 binary system and the pVALIUM20 RNAi vector to express a miR1 cassette for knockdown, smt3 (encodes the single Drosophila SUMO protein) was specifically targeted in the male germline using the bam-GAL4-VP16 driver. Confirmation of a smt3 knockdown effect is shown by an altered pattern of SUMOylated proteins detected by anti-Drosophila SUMO in the smt3 knockdown compared with controls (Fig. 5C). Western analysis of testis tissue from F1 males when compared with
control tissue shows that both 43 kD species increase in amount after SUMO knockdown, consistent with the hypothesis that these species represent an intermediate with one SUMO group. Additionally, a strong reduction in the amount of testis-specific 55β and 55γ RpL22e protein species is displayed (Fig. 5C). Depletion of the 55β species in response to SUMO knockdown suggests that this species is also a SUMOylated protein that may arise from an additional SUMO residue added to SUMOylated 55α RpL22e, although specific data to address this possibility are currently unavailable. We note, however, that the “~55 kD” designation in our nomenclature provides only an approximate m for these slower migrating proteins, as 55β and 55γ are progressively increasing in m compared with 55 kD (and 55α) and may fall within the ~63–65 kD range to account for an additional SUMO moiety added. Depletion of phosphorylated 55γ would be expected if derived from phosphorylation of the 55β species. Collectively, quantitative changes in modified RpL22e proteins in the 43 kD range further support the conclusion that RpL22e is a SUMO substrate.

In vivo knockdown of SUMO also provides evidence that the phosphorylated 55γ species is found in germ cells, as the bam-GAL4-VP16 driver (UAS-GAL4-VP16, UAS-Dicer2) altered SUMOylated protein levels in the testis, determined by Western analysis, confirm smt3 knockdown. The testis RpL22e immunodetection pattern is significantly altered upon smt3 knockdown compared with control tissue. No change in RpL22e-like-PA accumulating levels is seen. In an attempt to produce a stronger smt3 knockdown, we used the early germline-specific GAL4 driver, nos-GAL4; however, smt3 depletion in this case results in complete loss of the germline (data not shown).

**Figure 5.** Testis RpL22e, but not FLAG-RpL22e-like-PA, is susceptible to phosphatase in vitro and smt3 (SUMO) knockdown in vivo. (A) Incubation of S2 cell and testis tissue extracts with calf intestinal alkaline phosphatase in vitro significantly reduces immunodetection of the testis-specific 55γ RpL22e species (arrow). (B) Phosphatase treatment has no effect on the RpL22e-like-PA immunodetection pattern in testis. (C) In vivo knockdown of smt3 (via UAS-GAL4 binary system) was achieved by expressing a miR1 cassette targeting smt3 using a germline-specific GAL4 driver (bam-GAL4-VP16, UAS-Dicer2). Altered SUMOylated protein levels in the testis, determined by Western analysis, confirm smt3 knockdown. The testis RpL22e immunodetection pattern is significantly altered upon smt3 knockdown compared with control tissue. No change in RpL22e-like-PA accumulating levels is seen.
or modified Rp within a particular type of ribosomal particle provides insight into a putative role in assembly or active translation. Conversely, the lack of accumulation in ribosomal particles would provide evidence for an extra-ribosomal function.

To assess a role for Rpl.22e in translation, we performed polyome analysis in S2 cells, using distribution of a large ribosomal subunit protein (Rpl.23a) for comparison. In cycloheximide-treated cells, unmodified Rpl.22e (seen at 33 kD) co-sediments with the 60S large subunit, 80S monosomes, and polysomes. Treatment with the chain terminator puromycin (causing a disruption of polysomes and accumulation of 80S monosomes; gray line) shifts the immunodetection pattern of unmodified Rpl.22e from polysomes to monosomes. Endogenous Rpl.23a was used as a positive control.

Figure 6. Modified Rpl.22e does not co-sediment with the translation machinery. (A) S2 cell extracts were separated in a 10–50% buffered sucrose gradient for polyosome analysis to assess Rpl.22e co-sedimentation with ribosomal subunits, 80S monosomes, and polysomes. In cells treated with the elongation inhibitor cycloheximide (black line), all modified Rpl.22e (43k, β, and 55k) accumulates at the top of the gradient and only the unmodified 33 kD Rpl.22e co-sediments with the 60S large subunit, 80S monosomes, and polysomes. Treatment with the χ blocker puromycin (causing a disruption of polysomes and accumulation of 80S monosomes; gray line) shifts the immunodetection pattern of unmodified Rpl.22e from polysomes to monosomes. Endogenous Rpl.23a was used as a positive control. (B) Deletion of fly-specific histone H1-like domain that harbors five putative SUMOylation motifs (ΔH1; residues 176–299) results in stable FLAG-tagged Rpl.22e protein (C terminally tagged) in S2 cells. Full length (residues 1–299) is represented as WT. Tubulin was used as a loading control. (C) Polyome analysis of S2 cells expressing Rpl.22e-FLAG (full length) or Rpl.22eΔH1-FLAG shows an equal distribution pattern, as both were found to co-sediment with the translation machinery. The presence of FLAG-Rpl.22 in less dense "top" fractions may be attributable to high overexpression levels. Endogenous Rpl.23a was used as a positive control.
representing the majority of this protein in S2 cells (43a, 43b and 55a) accumulates at the top of the gradient, well segregated from ribosomal subunits and active translation machinery, strongly indicative of a role apart from translation. Unlike modified RpL22e, no RpL23a accumulated at the top of the gradient.

Further, to confirm that unmodified RpL22e (33 kD) is associated with the translation machinery, cells were treated with the chain terminator puromycin, resulting in dissociation of poly-somes and accumulation of 80S monosomes. Similar to what is seen for RibL22e, no RpL23a accumulated at the top of the gradient. However, deletion of the histone H1-like domain of the domain in efficient ribosome incorporation (Fig. S6).

To determine if SUMOylation of RpL22e has a role in ribosome assembly, we investigated a mutant that lacks all predicted SUMO motifs, consisting primarily of the C-terminal domain that harbors the rRNA-binding signature. In the in vitro bacterial assay, SUMOylation of a K39R mutant was not completely abolished (data not shown), suggesting that a lysine(s) other than the predicted major acceptor lysine was critical for SUMO modification. Therefore, we deleted the fly-specific N-terminal histone H1-like domain (defined by Koyama et al.) that harbors all predicted SUMO motifs (Fig. 2). Deleting the N-terminal domain (residues 1–175) results in a sequence that is highly conserved between all metazoans and closely resembles orthologs from yeast, C. elegans and human. Noteworthy is the fact that no SUMO modification motifs are predicted by ELM (data not shown) in these RpL22e orthologs. The resulting coding sequence, RpL22eΔH1-FLAG (residues 176–299), can be expressed in S2 cells at similar levels as the full length protein, suggesting that deletion of the domain does not hinder stability (Fig. 6B). Polysome analysis of full length (RpL22e-FLAG) and truncated RpL22e (RpL22eΔH1-FLAG) shows that both have a similar distribution pattern as endogenous RpL23a and both co-sediment with the active translation machinery (Fig. 6C). The significance of a slightly different distribution of truncated RpL22eΔH1-FLAG in 40S subunits compared with RpL22e in 40S subunits is unknown, but may only reflect slight gradient variations since the distribution pattern for RpL23a is similar in each case. As seen in Figure 3, the FLAG tag decreases the amount of modified RpL22e and the unmodified form of RpL22e is expected to migrate with the polysomes and not be free at the top of the gradient. Therefore, we postulate that stable modification of RpL22e, most notably SUMOylation, is not required for RpL22e assembly into the 60S ribosomal subunit or for ribosome function. Instead, SUMOylation of RpL22e likely shunts the protein into an extra-ribosomal pathway.

We have previously shown that RpL22e-like-PA co-sediments with polysomes and polysomes in sucrose gradients. Though no evidence for PTM of RpL22e-like-PA in testis is apparent in immunoblots (ref. 6 and this study), we explored the possibility that the histone H1-like domain is a necessary element for RpL22e-like-PA incorporation into ribosomes in S2 cells. Gradient analysis shows that the majority of truncated RpL22e-like-PA (ΔH1) co-sediments with ribosomes and polysomes, suggesting that the histone H1-like domain is dispensable for RpL22e-like-PA assembly into ribosomes and for association with the active translation machinery (Fig. S6). However, deletion of the histone H1-like in RpL22e-like (ΔH1) does result in an excess of free and unincorporated protein, distributed across the top of the gradient unlike the distribution for full-length RpL22e-like-PA, suggesting a role of the domain in efficient ribosome incorporation (Fig. S6).

RpL22e paralogues are differently localized in the male germ line. In yeast, localization of 54 out of the 59 pairs of duplicated ribosomal proteins, typically encoding identical or nearly identical proteins, has been studied using a GFP fusion approach. Of the 54 pairs investigated, only five pairs (RpL22e is not included) had paralogues with unique, separate localization patterns, suggesting possible non-redundant roles. We have previously reported that within the male reproductive system, RpL22e-like-PA is specifically localized to the male germine and RpL22e-like-PA ubiquitously localized throughout the male reproductive tract. Insights into redundant or novel functions of both paralogues may be provided by comparing subcellular localization. Using paralogue-specific Abs and confocal microscopy, we assessed the distribution pattern of the RpL22e family in the male germline.

Drosophila male germline development is a coordinated event requiring somatic and germline stem cell signaling cascades that steer germ cells toward sperm cell differentiation, as well as testis-specific gene expression and dramatic changes in nuclear and cytoplasmic morphology in germ cells (for a review, see ref. 47). Briefly, germ cells originate at the apical tip of the testis from asymmetric mitotic divisions of germline stem cells (GSCs) to produce a spermatogonial cell and a GSC. Surrounded by two somatic cyst cells, each spermatogonial cell undergoes four rounds of mitosis with incomplete cytokinesis to generate a 16-cell cyst containing post-mitotic, primary spermatocytes primed to enter meiosis. After a prolonged G2 phase of extensive cell volume growth (~30×) and gene expression, early primary spermatocytes enter meiosis, resulting ultimately in 64 round spermatids per cyst. Spermiad elongation ensues during spermiogenesis and the individualization process is completed to form mature, separated spermatids. The gradient of germ cell maturation (with the most immature cysts at the apical tip and mature spermatids positioned distally to move into the seminal vesicle) allows for easy staging of germline development. Notably, nuclei are very distinct in phase contrast microscopy (and when immunostaining cytosolic proteins) can be counted to identify the cyst stage. Additionally, maturing spermatoctyes are distinguishable as their nuclei enlarge during the prolonged G2 phase before meiosis I.

IHC reveals distinct and separate subcellular localization patterns for RpL22e paralogues in the mitotic and meiotic germ line. Indicative of a ribosomal protein, nuclear and strong cytoplasmic localization is evident for RpL22e-like-PA in mitotic spermatogonia (Fig. 7A). In early primary spermatocytes (before first meiotic division), nuclear accumulation of RpL22e-like-PA is still evident although less pronounced; abundant cytoplasmic
There is, however, a clear difference in the nuclear distribution of RpL22e within mitotic cells compared with meiotic germ cells. Nucleolar localization is seen in mitotic spermatogonia, partially co-localizing with RpL22e-like, with partial immunostaining in the cytoplasm (Fig. 7a). Nu
cleolar localization was confirmed by co-localizing RpL22e with the nucleolar protein nucleostemin1 (Fig. 8), shown to be enriched in the granular component of the nucleolus in Drosophila.50 GFP-tagged nucleostemin1 was expressed specially in the early male germline (limited to mitotic spermatogonia and early post-mitotic primary spermatocytes)

Rpl22e family members are differently localized in the male germline. (A) The mitotic and post-mitotic germline are separated by a dashed line. Mitotic spermatogonia are proximal to the apical tip (asterisk), where germline stem cells are located and germline development begins. Post-
miotic primary spermatocytes (will further develop and enter meiosis I) are found distal to the apical tip. Immunofluorescence (used to localize Rpl22e family members) reveals distinct localization patterns in the male germline. Rpl22e-like-PA (green) is primarily cytoplasmic, with some subnuclear accumulation (presumably in the nucleus) in mitotic germ cells. Strong cytoplasmic localization is seen meiotic spermatocytes. Rpl22e (red) is primarily distributed in the nucleus. A punctate Rpl22e localization is seen in the mitotic germline (thin arrow), but becomes more nucleoplas-

There is, however, a clear difference in the nuclear distribution of Rpl22e within mitotic cells compared with meiotic germ cells. Nucleolar localization is seen in mitotic spermatogonia, partially co-localizing with Rpl22e-like, with partial immunostaining in the cytoplasm (Fig. 7A). Nucleolar localization was confirmed by co-localizing Rpl22e with the nucleolar protein nucleostemin1 (Fig. 8), shown to be enriched in the granular component of the nucleolus in Drosophila.50 GFP-tagged nucleostemin1 was expressed specially in the early male germline (limited to mitotic spermatogonia and early post-mitotic primary spermatocytes).
smoothened-GAL4-VP16 driver. Although immunostaining shows that Rpl22e co-localizes with the nucleolus primarily in mitotic cells (Fig. 7A and B; Fig. 8), limits in the amount of the co-localization signal with GFP-NS1 can be accounted for by the expression pattern for NS1, driven by a germline driver that is most active in early stage mitotic spermatogonia and early primary spermatocytes.

The punctate nuclear Rpl22e pattern seen in the mitotic germ line dissipates and becomes increasingly nucleoplasmic in maturing post-mitotic spermatocytes (Fig. 7A and B; Fig. 9A). Using phase contrast microscopy of tissue immunolabeled for Rpl22e, we observed distinct segregation (although still close proximity) of Rpl22e from the nucleolus within post-mitotic primary spermatocytes (Fig. 9B). We further confirmed the Rpl22e nucleoplasmic pattern by co-staining for fibrillarin, a marker for the dense fibrillar center of the nucleolus. Confocal microscopy confirms the close proximity, but separate localization of Rpl22e and fibrillarin, as co-localization is not evident (Fig. 9C; Fig. 5A). In more mature meiotic spermatocytes, the Rpl22e staining pattern is less uniformly diffuse in the nucleoplasm and includes focused, punctate staining in the nuclear periphery, creating a cage-like lattice pattern in the nucleoplasm, visualized by confocal microscopy (Fig. 5B). As spermiogenesis continues, sperm cell nuclei become increasingly compact. It is unclear how the Rpl22e staining pattern is modified in this maturing population of cells (as images of this population were difficult to capture), but it is notable that the absence of individual sperm cells show strong Rpl22e and/or Rpl22e-like-I9 staining. For Rpl22e, it is unclear if the diffuse staining results from extrusion of Rpl22e from the nucleoplasm into the cytoplasm as the sperm nucleus undergoes compaction or if new Rpl22e protein synthesis in the cytoplasm provides the explanation.

Co-localization of Rpl22e and GFP-nucleostemin1, a protein localized to the granular component of the nucleolus as reported by Rosby et al., and a high level of cytoplasmic staining may collectively support a ribosomal role for Rpl22e during mitotic stages of spermatogenesis.6 The presence of Rpl22e within nuclei may, however, suggest other non-ribosomal roles as well. The function of nucleoplasmic Rpl22e in meiotic spermatocytes remains unknown, but nucleoplasmic immunolocalization favors the hypothesis that in the meiotic germ line, the bulk of Rpl22e does not have a role in ribosome biogenesis or in active translation. The predominant nucleoplasmic immunostaining pattern for Rpl22e in the absence of strong cytoplasmic staining in meiotic germ cells correlates well with results from S2 cells showing that modified Rpl22e does not co-sediment with polyosomes, but instead sediments at the top of ribosomal profile gradients (Fig. 6). These data favor the proposal for an extra-ribosomal role for modified Rpl22e in the testis as well. Immunohistochemistry of S2 cells shows strong cytoplasmic staining as well as some subnuclear staining for Rpl22e (Fig. 5C). The subcellular distribution of modified and unmodified Rpl22e within S2 cells is unknown, but based on ribosome profiles from S2 cells, it is reasonable to attribute at least some cytoplasmic staining to the small pool of actively translating ribosomes containing unmodified Rpl22e (as shown in Fig. 6).

SUMO knockdown perturbs Rpl22e localization in the meiotic germ line. We next determined the impact of SUMO knockdown on Rpl22e localization. As previously seen in Figure 5C, knockdown of SUMO causes a drastic change in Rpl22e modification. Immunostaining shows that Rpl22e becomes more widely nucleoplasmic, as compared with the control (Fig. 10). The characteristic focused, cage-like staining near the nuclear periphery is not observed, but staining remains more diffuse within the nucleoplasm. Whether or not this change in nucleoplasmic localization is a direct effect of the altered Rpl22e modification pattern or of subsequent nuclear defects from SUMO knockdown is unclear. Could the change in localization pattern (Fig. 10) reflect the loss of the 55kDa (phosphorylated) molecular species as seen in Figure 5C? Nevertheless, Rpl22e nucleoplasmic localization is sensitive to SUMO protein levels.
Figure 9. RpL22e does not co-localize with the nucleolus in mature meiotic spermatocytes. (A) Schematic representation of male reproductive tract as shown in Kearse et al. (2011). Insert shows region of testis distal from the apical tip where populations of meiotic spermatocytes are represented. Immunohistochemistry of the RpL22e paralogues shows distinct punctate nuclear localization of RpL22e (red) in maturing primary spermatocytes, whereas RpL22e-like (green) remains primarily cytoplasmic (open arrows). The developmental gradient of germine maturation (from less mature to more mature) is represented by the long closed arrow. SV: seminal vesicle. (B) Phase contrast and immunohistochemistry of mature meiotic primary spermatocytes shows juxtaposition, but not co-localization, of the phase dark nucleoli and RpL22e (red; arrows). DAPI staining (green) was used to confirm nuclear co-localization. (C) Co-staining of the nucleolar marker fibrillarin (green) and RpL22e (red) in mature meiotic primary spermatocytes confirms RpL22e is nucleoplasmic, not nucleolar (arrows). DAPI staining (blue) was used to confirm nuclear co-localization.
Discussion

Rpl22e is differentially post-translationally modified in different tissues. Evidence from S2 cell-based expression assays and in vivo expression analyses in several tissues shows that Rpl22e is expressed not only at its predicted m of 33 kD, but also at a higher m of ~43–55 kD. Collectively, co-IPs and bacterial SUMOylation assays favor the conclusion that higher m Rpl22e is attributable to PTMs that include SUMOylation, with the amount of SUMO-conjugated Rpl22e accumulating in varying amounts in different tissues. These results extend the proteomics report of Nie et al. and show that Rpl22e is a direct SUMO target.29

How Rpl22e function changes in response to SUMOylation is unknown, but in S2 cells, SUMOylated Rpl22e is not found in association with ribosomal subunits or translating ribosomes nor is SUMOylation required for Rpl22e incorporation into ribosomes or for ribosome function, the latter based on our analysis in S2 cells of the expression and gradient sedimentation of an N-terminal Rpl22e truncation in which all predicted SUMO motifs were deleted (Rpl22eΔH1-FLAG). Thus, we conclude that Rpl22e has no less than a dual cellular

Figure 10. Rpl22e localization in mature spermatocytes is sensitive to SUMO levels. In vivo knockdown of SUMO (smt3) was achieved by expressing a miRNA cassette targeting smt3 (Fig. 5C). Nucleoplasmic Rpl22e immunolocalization (red) in mature meiotic spermatocytes is generally widespread (thin arrows) as a result of the smt3 knockdown, as compared with control tissue (wildtype) where the Rpl22e nucleoplasmic pattern is more punctate (arrows with filled-in arrowhead). DAPI staining is seen in blue.
role including an extra-ribosomal function(s); regulated by SUMOylation.

Testis expression of Rpl22e paralogues is further distinguished by a unique pattern of PTMs for Rpl22e but not for Rpl22e-like-PA. Additional testis-specific Rpl22e modifications include phosphorylation and may include conjugation of an additional SUMO moiety. Phosphorylation of the 55γ species of SUMOylated Rpl22e appears to give rise to the 55γ moiety. Based on its absence from testis apical tip extracts and from day 1 of (SUMO)-depleted testis extracts (that primarily accumulate primary spermatocytes), the 55γ species may be a unique component generated only in post-mitotic cells. More definitive evidence of phosphorylation timing requires more extensive biochemical investigation of cohorts of cells at different spermatogenesis stages.

Differential sub-compartmentalization of Rpl22e paralogues and its functional implications. Our IHC data show that both paralogues are co-expressed within germ cells, but paralogue localization changes as germ cells mature. We have previously determined that Rpl22e-like-PA is a bona fide testis Rp.3 Within mitotic germ cells closest to the apical tip, both paralogues are distributed within the nucleolus and cytoplasm. This localization pattern is consistent with a ribosomal function for both paralogues within mitotic germ cells. If so, then two different ribosomal populations based on Rpl22e paralogue content could be identified, and may constitute a class of "specialized ribosomes" (as recently proposed by Xue and Barna) with unique translational roles at mitotic stages of spermatogenesis. In addition, the nucleolus to nucleoplasmic transition for Rpl22e in the germine may reflect a non-ribosomal function(s) for this paralogue from early through late stages of germ cell maturation.

In post-mitotic primary spermatocytes, the cytoplasmic localization pattern for Rpl22e-like-PA is retained; however, the Rpl22e pattern is primarily nuclear; relatively little cytoplasmic staining is noted at this stage, but may still signal that a fraction of the Rpl22e pool is incorporated into cytoplasmic ribosomes. Rpl22e nuclear staining appears uniformly diffuse at this stage except that staining is generally excluded from nucleoli. Primary spermatocytes undergo tremendous cellular growth and increases in protein synthesis as they enter meiosis. It is unclear what novel interactions for newly synthesized Rpl22e or previously synthesized Rpl22e account for the observed change in nuclear distribution in maturing primary spermatocytes.

SUMOylation has been linked to regulating localization of nuclear proteins and formation of nuclear bodies. The small GTPase-activating protein RanGAP was the first SUMO substrate identified and its localization is regulated by SUMOylation. Unmodified RanGAP is cytoplasmic, but SUMOylated RanGAP localizes to nuclear pores. The formation of PML nuclear bodies is dependent on SUMOylation of the PML protein. We show that the nucleoplasmic localization of Rpl22e in the meiotic germine is sensitive to SUMO levels. Whether this redistribution is a direct effect from interfering with Rpl22e SUMOylation or the result of a change in nuclear architecture due to SUMO knockdown remains to be investigated.

Many studies have found mechanisms that rapidly degrade excess Rps (for review, see refs. 54 and 55). Therefore, it is likely that Rpl22e accumulates within meiotic cell nuclei for a specific, functional role. Ni et al. reported a chromatin-Rpl22e association in a transcriptional repressor complex with histone H3 in Drosophila Kc cells, suggesting that Rpl22e has an alternate function in transcriptional regulation aside from its function as an Rp.6 We note that no higher m Rpl22e species were identified in immunoblots in the Ni et al. report and thus it is unclear if SUMOylated Rpl22e is a contributing effector in these studies. The lack of detection of modified Rpl22e proteins in immunoblots in the Ni et al. report may be attributable to differences in polyclonal Ab specificities used in the two studies. Recent studies in Schizosaccharomyces pombe reported that other Rp-chromatin complexes are enriched at tRNA genes and centromeres, implicating Rps as effectors in tRNA biogenesis and centromere functions.7

Mounting evidence therefore positions numerous Rps in nuclear locations that suggest alternate Rp roles. We speculate that in a variety of cell types, SUMOylated Rpl22e may associate with chromatin as cells undergo nuclear architectural changes, chromatin remodeling and/or transcriptional silencing. In the male germ line additional PTMs would further regulate and expand the role of Rpl22e beyond functions found in other cells and tissues. Within post-mitotic cells undergoing extensive nuclear remodeling, SUMO accumulates in chromatin during histone removal, but its role and targets are unknown. Whether or not testis-specific modification of SUMOylated Rpl22e is part of the mechanism to promote chromatin condensation and/or transcriptional repression in maturing germ cells awaits determination.

Overall, this study finds additional evidence to support the proposal that Rpl22e paralogues have evolved disparate functions within the male germ line. That these paralogues are partitioned into different biochemical pathways leading to differential PTM and different subcellular accumulation within germ cells makes a compelling argument for the pursuit of Rpl22e function within the male germine.

Materials and Methods

*Drosophila* stocks. Unless noted, all flies used were wildtype Oregon R from Carolina. The bam-GAL4-VP16 driver was a kind gift from Marina Wolfsner (Cornell), but originally developed by Dennis McKearin. The GFP-Nucleostemin1 stock was a kind gift from Pat DiMario. The smt3 (SUMO) RNAi line, originally developed by the TRiP, uses the pVALIUM20 vector to generate a hairpin and was obtained from Bloomington Stock Center (43625). We thank the TRiP at Harvard Medical School (NIH/NIGMS R01-GM084947) for providing transgenic RNAi fly stocks and/or plasmid vectors used in this study. All stocks were kept at room temperature on standard cornmeal medium.

Fly in vivo RNAi and ectopic expression. SUMO (smt3) knockdown was performed using the UAS-GAL4 binary system. UAS-VALIUM20-smt3 females were crossed with...
Approximately 15 pairs were lysed in 30 μl of PBS and immediately frozen on dry ice. Approximately 15 pairs were dissected from wildtype adults in 1 × PBS and immediately SDS (supplemented with 1 mM PMSF) for 10 min on ice. Gonads were then electrotransfered onto a 0.2 μm Westra-S PVDF membrane (Whatman, #10413096) for 1 h in chilled transfer buffer. Upon blocking in 5% non-fat dry milk (NFDM) for 1 h, primary antibodies were incubated overnight at 4°C in 3% NFDM. HRP conjugated secondary antibodies were incubated for 2 h at 4°C in 3% NFDM. ECL Plus (GE Healthcare) was used for chemiluminescent detection on Kodak Bio-Max film as directed by the manufacturer.

Peptide inhibition experiments were completed by pre-incubating Ab with 5-fold excess (by weight) of peptide used for antibody production in 500 μl 1 x PBS at room temperature for 2 h before using for Western analysis. Addition of PBS in lieu of blocking peptide was used for negative control samples.

S2 cell RNAi. Knockdown of Rpl22e by RNAi was achieved by serotonin-starvation induced uptake of dsRNA (final 3’ m) as described.28 dsRNA was generated using PCR amplicons with 5’ recognition sites at 5’ and 3’ ends with the MEGAscript T7 in vitro transcription kit (Invitrogen/Ambion, #AM1333) and purified as described by manufacturer’s recommendations. Annealing of dsRNA was achieved by incubation 30–60 min at 65°C and allowed to cool slowly at room temperature. Samples were taken at designated time points, pelleted and frozen for subsequent analysis. Samples were lysed and quantitated as described above.

Cloning and mutagenesis. Previously cloned cDNAs were used as templates for PCR. A FLAG tag was added to the N-terminus by PCR for Rpl22e and Rpl22e-like-PA and cloned into pMT/V5-His-KO-TOPO (Invitrogen, #K81250) for expression studies in S2 cells were constructed by standard PCR methods using previously cloned RpL22e cDNA.6 A FLAG-tag was incorporated by inserting the FLAG coding sequence into the reverse primer sequence (5’-TACGAAATTC TTACTTGTCA TCGTCATCCT TGTAGTCGCC -3’) following manufacturer’s recommendations. Subsequently, to create Rpl22eH1-FLAG, a methionine codon (ATG) was added upstream of the coding sequence starting at amino acid 176 in the forward primer (5’-GAATTCATGA AGAACGCTGCT CGGTGGCCAGG GCCAGGCAGAA GGAGAGAGAG AGAGAGAGAG 3’) and cloned into pMT/V5-His-TOPO. Proper FLAG-tag fusion and construct sequence was confirmed at each cloning step by Sanger sequencing.

Bacterial SUMOylation assay. Assays were performed essentially as previously reported.26 Q304Arg and Q304Cys plasmids were generated with the Vegan tool (UCLA). For Rpl22e, BL21 (DE3) cells were transformed with pMT/V5-His-TOPO plasmid and induced with 1 mM IPTG at 37°C for 4 h. Bacterial cells were collected by centrifugation and resuspended in 1× PBS (pH 7.2) containing 20 μM E64d and 1 mM PMSF. lysates were prepared by sonication in the presence of 20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. Lysates were then clarified by centrifugation at 20,000 g for 10 min at 4°C. Supernatants were then subjected to molecular weight cut-off centrifugation at 30,000 g for 15 min at 4°C. Pellets were discarded and supernatants were subjected to SUMO pull-down.

Preparation of recombinant SUMO fusion proteins. Recombinant SUMO fusion proteins were induced in BL21 (DE3) cells and purified as described above. SUMO fusion proteins were then used as substrates for SUMOylation assays.

Western analysis. SDS-PAGE was conducted under reducing (βME) conditions. Proteins were then electrotransferred onto a 0.2 μm Westra-S PVDF membrane (Whatman, #10413096) for 1 h in chilled transfer buffer. Upon blocking in 5% non-fat dry milk (NFDM) for 1 h, primary antibodies were incubated overnight at 4°C in 3% NFDM. HRP conjugated secondary antibodies were incubated for 2 h at 4°C in 3% NFDM. ECL Plus (GE Healthcare) was used for chemiluminescent detection on Kodak Bio-Max film as directed by the manufacturer.

Protein expression and purification. Protein expression and purification were performed as described.7 RpL22e, RpL22e-like-PA, and RpL22e-PA were expressed in E. coli BL21 (DE3) cells and purified by nickel affinity chromatography as described.8 RpL22e, RpL22e-like-PA, and RpL22e-PA were analyzed by SDSPAGE and Western blot analysis.
Star (DE3) E. coli cells (Invitrogen) were co-transformed with pExp5-Flag-L22e-like-PA or pExp5-Flag-L22e K39R along with QSUMO or QΔ, and plated on to double selective media (LB agar with 100 μg/mL Ampicillin and 50 μg/mL Kanamycin) at 37°C. Resistant transformants were selected to inoculate overnight seed cultures in LB broth with Ampicillin and Kanamycin at 37°C. 100 μL of seed culture was used to inoculate 50 mL auto-inducing media as described by Studier with antibiotics in 500 mL baffled flasks at 200 rpm. Cultures were incubated in a 26°C water bath until 0.0 OD600. For protein prep, 10 mL of culture was pelleted and resuspended in 1 mL sonication buffer (1X PBS, 0.1% Triton X-100, 1 mM PMSF). Samples were lysed with three 10 sec sonication cycles with 1 min intervals resting ice followed by a 10 min centrifugation step (16,000 x g at 4°C) to clear debris. Lysates were quantified and used for SDS-PAGE and western analysis as described above. For Rpl22e-like-PA, the assay was performed identically with pExp5-Flag-L22e-like-PA.

In vitro proteolysis. Proteolysis assays of bacterial SUMOylation assay lysates (see above) using purified trypsin (Sigma) was performed as previously described. Reactions were stopped by the addition of an equal volume of reducing sample buffer and boiled for 5 min. Subsequently, 5 μg of lysate was used for SDS-PAGE and Western analysis.

Immunoprecipitation. Indirect immunoprecipitation (IP) protocols were adapted from Sanz et al. for S2 cells. 10 mL of S2 cells were seeded at 1.0 × 106 cells/mL in T25 flasks and incubated for 3 d at 26°C. Cells were pelleted at 100 xg for 5 min, washed with PBS and lysed in 1 mL of IP lysis buffer [10 mM Tris-HCL (pH 7), 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 100 μg/mL cycloheximide] for 10 min on ice. A post-nitochondrial fraction was created by centrifugation at 16,000 x g for 10 min in a microcentrifuge at 4°C. 20 μg anti-Drosophila SUMO antibody (Abgent, #AP1287b) incubated with 400–500 μL of lysate overnight at 4°C with constant agitation. Antibody-antigen complexes were captured by the addition of 40 μL of prepared magnetic protein A beads (Millipore, #LSKMAGA02) as recommended by the manufacturer with constant agitation for 20 min at RT. Upon three washes with high salt IP wash buffer (300 mM KCl), captured proteins were eluted by incubation of excess free peptide at 4°C for 50 min (two rounds of 200 μL free peptide at 100 μg/mL with constant agitation). Eluates were pooled, TCA precipitated as described by Houmani and Ruf, resuspended in reducing SDS-sample buffer, separated by SDS-PAGE and probed for Rpl22e in western analysis.

Rpl22e IP reactions were performed as described above, but capture used 20 μg anti-Rpl22e. Eluates were subjected to Western analysis and probed with anti-Drosophila SUMO.

Phosphatase treatment. 10 μL reactions using CaF Intestinal Alkaline Phosphatase (New England Biolabs, M0290S) were set up as suggested by New England Biolabs. Protein samples were diluted to 1–2 μg/μL in 1X NEBuffer 2. Upon the addition of 1 unit of alkaline phosphatase, reactions were incubated at 37°C for 60 min. PBS was added to negative control samples in lieu of phosphatase. Reactions were either frozen on dry ice and stored at −80°C or directly used for SDS-PAGE by adding equal volume of reducing SDS-sample buffer and boiled.

Sucrose gradient ultracentrifugation. S2 cells were seeded at 1 × 106 cells/mL (9 mLs per drug treatment) and allowed to grow for 3 d at 26°C. Cells were then treated with 100 μg/mL cycloheximide or 300 μg/mL puromycin for 10 min on ice, pelleted and washed with ice-cold 1X PBS containing cycloheximide or puromycin. Cells were then lysed in 1mL of ribosome lysate buffer and layered on top of a 10–50% buffered sucrose gradient. Gradients were centrifuged at 39,000 rpm for 3 h at 26°C. Gradient preparation, centrifugation conditions and subsequent protein extraction by TCA precipitation was performed as previously described by Houmani and Ruf. 11 Gradients were fractionated using a Brandel syringe pump and Foxy Jr. R1 gradient fractionators along with an Eppendorf 5415C for continuous absorbance reading at 254 nm. Fractions were collected in 0.5 mL volumes with 40 sec fraction collection times set with 0.75 mL/min pump speed.

For analysis of Rpl22e-FLAG and Rpl22eΔH1-FLAG, three wells of S2 cells were transfected as described above. 48 h post-induction, wells were pooled, treated with cycloheximide, lysed and subjected to sucrose gradient ultracentrifugation as described above. 62,63

Immunohistochemistry. Testis squashes and immunostaining was performed as previously described for all analyses. 3 S2 cell immunostaining was performed as previously described. 9 Anti-Rpl22e was used at 1:100 in blocking solution for 1 h incubation at room temperature. Cells were mounted in Fluoromount-G (Southern Biotech, #0100–01) and imaged with a Nikon Eclipse TE2000U.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

Acknowledgments
Financial support was provided in part from Lehigh University Faculty Research Grants (607233 and 607277) to V.C.W. The work described here is in partial fulfillment of the requirements for the Ph.D degree for M.G.K., who was partially supported by a Nemes Fellowship. J.A.I., S.M.P. and A.S.C. were supported in part as a Lehigh University-Howard Hughes Medical Institute (HHMI) student from a grant to Lehigh University from the HHMI through the Precollege and Undergraduate Science Education Program. We thank members of the fly community (noted in Materials and Methods) for their generosity for sharing fly stocks, plasmids, antibodies and cell lines. We also extend our gratitude to Maria Brace for figure assistance and members of the Wase lab for discussions. Members of M.G.K.’s dissertation committee are acknowledged for stimulating discussions about the work reported here.

Supplemental Materials
Supplemental materials can be found here: www.landesbioscience.com/journals/nucleus/article/25261
15. Ashburner M, Millburn GH, et al. The ribosomal protein L22e family members in Drosophila melanogaster. FEBS Lett 2005; 577:2306-12; PMID:16466393; http://dx.doi.org/10.1016/j.febslet.2005.04.029.

16. Kai T, Kaneko T, et al. The expression pattern of the active 60 S ribosomal subunit. FEBS Lett 2006; 577:486-502; PMID:16950879; http://dx.doi.org/10.1016/j.febslet.2006.02.048.

17. Ruvinsky I, Sharshar D, et al. Methylation of ribosomal protein S10 by protein-arginine methyltransferase 3 (PRMT3) regulates gene expression by activating the expression of the LAG1 silencer in the bam gene. J Biol Chem 2011; 286:11391-400; PMID:21278366; http://dx.doi.org/10.1074/jbc.M111.252887.

18. D’Souza-Sутар SP, Bargiello TA. The expression of extraneous leader sequences in ribosomal protein synthesis. Proc Natl Acad Sci U S A 2002; 99:5942-7; PMID:11983894; http://dx.doi.org/10.1073/pnas.112000299.

19. Ruvinsky I, Kravin, S, Dils M, et al. SUMO in cell proliferation. Nat Rev Mol Cell Biol 2007; 3:715-20; PMID:17534367; http://dx.doi.org/10.1038/nrg2049.

20. Williams D, Spradling AC. The expression program of the germline stem cells. Dev Biol 2003; 262:173-91; PMID:12800176; http://dx.doi.org/10.1016/j.ydbio.2003.06.006.

21. Williams D, Spradling AC. Mitotic recombination of the Drosophila genome by homologous recombination. EMBO J 1996; 15:2306-12; PMID:8641296.

22. Hossain MA, Liu X, Hanai S, Uchida K, Miwa K. How common are extraribosomal genes in Drosophila S2 cells? Gene 2006; 374:173-80; PMID:16957970; http://dx.doi.org/10.1016/j.gene.2006.01.013.

23. Williams D, Spradling AC. The expression program of the germline stem cells. Dev Biol 2003; 262:173-91; PMID:12800176; http://dx.doi.org/10.1016/j.ydbio.2003.06.006.

24. Williams D, Spradling AC. Mitotic recombination of the Drosophila genome by homologous recombination. EMBO J 1996; 15:2306-12; PMID:8641296.

25. Hossain MA, Liu X, Hanai S, Uchida K, Miwa K. How common are extraribosomal genes in Drosophila S2 cells? Gene 2006; 374:173-80; PMID:16957970; http://dx.doi.org/10.1016/j.gene.2006.01.013.

26. Williams D, Spradling AC. The expression program of the germline stem cells. Dev Biol 2003; 262:173-91; PMID:12800176; http://dx.doi.org/10.1016/j.ydbio.2003.06.006.

27. Williams D, Spradling AC. Mitotic recombination of the Drosophila genome by homologous recombination. EMBO J 1996; 15:2306-12; PMID:8641296.
45. Paneraro OO, Guillot MA. Presence of Not5 and ubiquitinated p70S6K in polyribosome fractions derived from the Drosophila embryo. Mol Membr Biol 2012; 1, 1460-5; PMID:22925939; http://dx.doi.org/10.1007/s10121-011-0075-7.

46. Hall WK, Evil JV, Goett CR, Caroll AS, Howson BW, Weitman JS, et al. Global analysis of protein localization in budding yeast. Nature 2003; 1, 906-11; PMID:14566209; http://dx.doi.org/10.1038/nature03205.

47. de Groen M, Mattei EL. The main cell cycle lesson from the Drosophila testis. Development 2011; 1, 280-8; PMID:21963909; http://dx.doi.org/10.1242/dev.064822.

48. White-Cooper H. Studying how block factor suppresses autoregulation in Drosophila testes. Mol Cell Endocrinol 2010; 1, 350-6; PMID:19565966; http://dx.doi.org/10.1016/j.mce.2008.11.026.

49. White-Cooper H. Molecular mechanisms of gene regulation during Drosophila spermiogenesis. Reproduction 2010; 1, 131-21; PMID:17755486; http://dx.doi.org/10.1530/REP-09-0684.

50. Buttry R, Cai Z, Rogers E, deLamirade MA, Robinson VL, Olschner PJ. Knockdown of the Drosophila GTPase Ran-1 impairs large ribosomal subunit biogenesis, cell growth, and midgut precursor cell maintenance. Mol Biol Cell 2009; 1, 2042-34; PMID:19570436; http://dx.doi.org/10.1091/mbc.E08-06-0192.

51. Mannion MJ, Gassmann E, Mohle R. A novel ubiquitin-like modification mediates the partitioning of the RasGTPase-activating protein RabGAP1 between the cytosol and the nuclear pore complex. J Cell Biol 1996; 1, 1945-57; PMID:8679803; http://dx.doi.org/10.1083/jcb.139.4.1457.

52. Mahajan R, Delpitt C, Gian T, Giusa T, Melkonian E. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex proteins. PLoS Cell 1997; 1, 89-97; PMID:9019411; http://dx.doi.org/10.1371/journal.pone.0041840.

53. Zhang S, Miller S, Rosenthal H, Svoboda P, Dujon B, Pandolfi PP. Role of SUMO-2-modified PML in nuclear body formation. Blood 2008; 1, 35-748-52; PMID:18157146.

54. Perry RP. Balanced production of ribosomal protein. Gene 2007; 1, 661-7; PMID:17808089; http://dx.doi.org/10.1016/j.cell.2007.07.047.

55. Sant YV, Lemonon M, Maitre M, Anderson JF. Analysis of nucleolar protein dynamics reveals the nucleus location of ribosomal proteins. Genes Dev 2007; 1, 1779-90; PMID:17466578; http://dx.doi.org/10.1101/gad.280841.

56. Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

57. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

58. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

59. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

60. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

61. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

62. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

63. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.

64. Shi QJ, Shi QJ, Liu Z, Jiang D, Rindfleisch J, Sai H. Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription. Genes Dev 2006; 1, 1039-50; PMID:16401601; http://dx.doi.org/10.1101/gad.280841.