Deletion of eIF2beta suppresses testicular cancer incidence and causes recessive lethality in agouti-yellow mice

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The agouti-yellow (Ay) deletion is the only genetic modifier known to suppress testicular germ cell tumor (TGCT) susceptibility in mice or humans. The Ay mutation deletes Raly and Eif2s2, and induces the ectopic expression of agouti, all of which are potential TGCT-modifying mutations. Here we report that the reduced TGCT incidence of heterozygous Ay males and the recessive embryonic lethality of Ay are caused by the deletion of Eif2s2, the beta subunit of translation initiation factor eIF2. We found that the incidence of affected males was reduced 2-fold in mice that were partially deficient for Eif2s2 and that embryonic lethality occurred near the time of implantation in mice that were fully deficient for Eif2s2. In contrast, neither reduced expression of Raly in gene-trap mice nor ectopic expression of agouti in transgenic or viable-yellow (Avy) mutants affected TGCT incidence or embryonic viability. In addition, we provide evidence that partial deficiency of Eif2s2 attenuated germ cell proliferation and differentiation, both of which are important to TGCT formation. These results show that germ cell development and TGCT pathogenesis are sensitive to the availability of the eIF2 translation initiation complex and to changes in the rate of translation.

INTRODUCTION

The agouti-yellow (Ay) mutation is one of the original mutants inherited from mouse fanciers at the beginning of genetics a century ago. Ay achieved notoriety because its inheritance was an apparent violation of Mendel’s law of segregation (1,2). The Ay mutation deletes Raly (a member of the hnRNP RNA-binding protein family) and Eif2s2 (the beta subunit of translation initiation factor eIF2), and also causes the ectopic expression of agouti (a melanocortin receptor antagonist) (Fig. 1A) (3,4). Ay produces remarkably diverse phenotypes. Ectopic expression of agouti and the resulting chronic inhibition of melanocortin receptor signaling causes dominantly inherited yellow fur, obesity, type II diabetes and susceptibility to somatic tumors (4–8). Loss of Raly expression has been implicated in the embryonic lethality of homozygous Ay mice (3,4,9). Interestingly, deletion of Eif2s2 in agouti-yellow mice has not been associated with an Ay phenotype, which is surprising considering the central role of eIF2 in regulating the global rate of translation initiation (10), the link between translation efficiency and tumorigenesis (11–13), and the lethal phenotype of eIF2 subunit mutations in yeast (14–16).

The Ay mutation is also the only known suppressor of susceptibility to spontaneous testicular germ cell tumors (TGCTs) when congenic on the 129 inbred background (17,18). TGCTs occur at an appreciable frequency only in the 129 family of inbred strains of mice (19). These TGCTs are strikingly similar to human pediatric testicular teratomas and teratocarcinomas, and arise during embryogenesis from primordial germ cells (PGCs), the precursors of sperm and eggs (20–24). As in humans, the genetic component of TGCT susceptibility in mice is strong but highly complex (18,19,25–28). In humans, the gr/gr deletion on the Y chromosome is the only variant that reproducibly associates with TGCT (27,28). In mice, targeted deficiency of Pten is the only mutation that increases susceptibility on all genetic backgrounds that have been tested (29). In addition, several single-gene mutations that modify TGCT susceptibility when congenic on the 129
Figure 1. The A^w and A^v loci. Genomic structure of the (A), white belly (A^w) and agouti-yellow (A^v) and (B), wild-type (A) and viable-yellow (A^v) agouti alleles. A ventral-specific promoter (upstream of exon 1A) and hair-cycle-specific promoters (upstream of exons 1B and 1C) regulate agouti transcription in A^w mice. For the A^w allele, transcription is regulated by the hair-cycle-specific promoters and the Raly promoter. For the A^v allele, the agouti gene is under the transcriptional regulation of an IAP inserted in pseudo-exon 1A (ps1A) and the hair-cycle-specific promoters. The agouti exon/intron structure is shown with untranslated and protein-coding sequences indicated by open and closed boxes, respectively.

The results of experiments involving gene expression in one of several cell lineages during the critical developmental window between embryonic days 11.5 and 13.5 (E11.5 to E13.5), and become evident microscopically as foci of embryonal carcinoma tumor stem cells at E15.5 to E16.5 (21,36). Currently, it is unknown whether TGCT susceptibility results from dysfunctions in PGCs, suppresses TGCT susceptibility means that large numbers of mice must be screened to test for reduced susceptibility. To enable a more statistically powerful test, A^v was bred onto the 129-Chr19MOLF (M19) chromosome substitution strain background, in which both copies of chromosome 19 are derived from the wild-derived MOLF/Ei inbred strain (35). Because homosomic M19 has a TGCT incidence of ~80%, tests for reduced susceptibility are relatively easy in a modest number of mice. A previous survey of M19-A^v/+ congenic consomic males showed that the A^v mutation significantly reduces the frequency of spontaneous TGCTs in M19 males (31). We increased the sample size of this survey, which now demonstrates that the A^v mutation reduces TGCT incidence in M19 males by 24% (Table 1). Therefore, because of these statistical advantages, subsequent studies to identify the TGCT-suppressing mutation in A^v mice were carried out using the M19 genetic background.

**RESULTS**

The A^v mutation reduces TGCT susceptibility in 129-Chr19MOLF consomic mice

We first sought to verify previous studies that showed TGCT incidence is significantly reduced in 129-A^v/+ males (17,19). However, the low incidence of TGCTs in wild-type 129 males

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of males</th>
<th>No. of affected males</th>
<th>Affected males (%)</th>
<th>Test score (χ², P-value)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>207</td>
<td>136</td>
<td>66</td>
<td>9.8, &lt;0.005</td>
</tr>
<tr>
<td>A^v/+</td>
<td>215</td>
<td>109</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>106</td>
<td>77</td>
<td>73</td>
<td>28.8, &lt;0.0001</td>
</tr>
<tr>
<td>Eif2s2^trap^/+</td>
<td>108</td>
<td>39</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>105</td>
<td>82</td>
<td>78</td>
<td>0.2, NS</td>
</tr>
<tr>
<td>Raly^trap^/+</td>
<td>112</td>
<td>85</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>121</td>
<td>94</td>
<td>78</td>
<td>0.1, NS</td>
</tr>
<tr>
<td>A^v/+</td>
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<tr>
<td>Wild-type</td>
<td>93</td>
<td>72</td>
<td>77</td>
<td>0.9, NS</td>
</tr>
</tbody>
</table>

NS, not significant.

*agouti^Tr*, agouti transgenic.

**Eif2s2, Raly and agouti are expressed in the fetal testis**

As a first step towards identifying the TGCT-suppressing mutation in A^v mice, we determined whether the three candidate genes are expressed in the testis during the correct developmental period. In mice, TGCTs arise from PGCs during a critical developmental window between embryonic days 11.5 and 13.5 (E11.5 to E13.5), and become evident microscopically as foci of embryonal carcinoma tumor stem cells at E15.5 to E16.5 (21,36). Currently, it is unknown whether TGCT susceptibility results from dysfunctions in PGCs, supporting somatic cells, or both. Thus, changes in candidate gene expression in one of several cell lineages during the period of susceptibility may influence TGCT incidence.

Eif2s2 and Raly were strongly expressed in the M19 embryonic testis at the time of TGCT initiation (Fig. 2). In contrast, agouti was expressed at minimally detectable levels in the M19 embryonic testis, as was observed in the adult M19 testis (Fig. 2C and Supplementary Material, Fig. S4B) (3). Analysis of wild-type embryonic testes that were FACS-sorted with a germ cell-specific Oct4ΔPE-GFP transgene (37) detected Raly and Eif2s2 in both PGCs and somatic cells (Supplementary Material, Fig. S1). In contrast, agouti, including a testis-specific splice variant (3), was detectable at minimal levels only in the somatic cells of the M19 embryo.
nic testis (Supplementary Material, Fig. S2). We then tested whether the \( A^v \) mutation altered the expression of the candidate genes. The \( A^v \) deletion of \( Eif2s2 \) and \( Raly \) significantly reduced expression of both genes in the embryonic testis at the time of TGCT initiation (Fig. 2A and B). Furthermore, the \( A^v \) mutation significantly increased \( agouti \) expression in both PGCs and somatic cells (Fig. 2C and Supplementary Material, Fig. S2). Because \( Eif2s2 \), \( Raly \) and \( agouti \) are expressed in the fetal testis and because \( A^v \) alters their expression, all three genes qualify as candidate TGCT modifiers.

**Deletion of \( Eif2s2 \), but not \( Raly \), reduces TGCT susceptibility**

The next test sought to determine whether partial deficiency of \( Eif2s2 \) or \( Raly \), in the absence of ectopic \( agouti \) expression, suppresses TGCT incidence. 129/Sv embryonic stem cell lines harboring 5′ untranslated region (5′-UTR) gene-trap alleles of \( Eif2s2 \) (\( Eif2s2^{Trap} \)) or \( Raly \) (\( Raly^{Trap} \)) were identified, and were independently bred onto the M19 genetic background (Supplementary Material, Fig. S3A–D). M19 mice heterozygous for \( Eif2s2^{Trap} \) (\( Eif2s2^{Trap/+} \)) or \( Raly^{Trap} \) (\( Raly^{Trap/+} \)) were viable, fertile and showed no gross abnormal phenotypes other than TGCTs. In both \( Eif2s2^{Trap/+} \) and \( Raly^{Trap/+} \) embryonic testes and adult tissues, expression of the targeted gene was reduced to the levels detected in \( A^v/+ \) embryonic and adult tissues (Fig. 2A and B and Supplementary Material, Fig. S3E and F).

The TGCT surveys of M19-\( Eif2s2^{Trap/+} \) and M19-\( Raly^{Trap/+} \) mice produced interesting results. The TGCT incidence in M19-\( Eif2s2^{Trap/+} \) mice was significantly reduced 2-fold compared with wild-type M19 siblings (Table 1). This decrease in TGCT susceptibility was similar to the previously reported reduction in TGCT incidence in M19-\( A^v/+ \) mice (31). In contrast, a survey of M19-\( Raly^{Trap/+} \) mice and M19 siblings showed that reduced expression of \( Raly \) did not reduce TGCT incidence (Table 1). Therefore, reduced expression of \( Eif2s2 \), but not \( Raly \), acts as a TGCT suppressor in agouti-yellow mice.

**Ectopic expression of \( agouti \) does not modify TGCT susceptibility**

To test whether the ectopic expression of \( agouti \) also influences TGCT susceptibility, we bred a ubiquitously expressed \( agouti \) transgene onto the M19 background (Supplementary Material, Fig. S4A). Compared with expression in wild-type M19 embryonic testes, \( agouti \) was strongly expressed in hemizygous transgenic embryonic testes, in both somatic cells and PGCs, at the time of TGCT initiation (Fig. 2C and Supplementary Material, Fig. S2). Furthermore, the \( agouti \) transgene was expressed in all adult tissues assayed (including the testis) and resulted in yellowing of the fur and a significant increase in body weight compared with wild-type siblings (Supplementary Material, Fig. S4B–D). Importantly, despite reproducing these dominant \( A^v \) phenotypes and inducing \( agouti \) expression in the fetal and adult testis, the transgene failed to reduce TGCT susceptibility in M19 mice (Table 1).

As a second test of the influence of \( agouti \) on TGCT susceptibility, we transferred the viable yellow (\( A^v \)) mutation onto the M19 background. \( A^v \) results from an intracisternal A particle (IAP) insertion in pseudo-exon 1A of \( agouti \) (Fig. 1B) (38). Cryptic promoters or enhancers in the long terminal repeats of the IAP drive ubiquitous expression of \( agouti \) without affecting \( Raly \) or \( Eif2s2 \), and thereby produce phenotypes similar to those of \( A^v/+ \) mice (8). Expression of \( agouti \) was significantly increased in \( A^v/+ \) embryonic testes, in both somatic cells and PGCs, at the time of TGCT initiation (Fig. 2C and Supplementary Material, Fig. S2). Additionally, the \( A^v \) mutation did not alter expression of \( Raly \) and \( Eif2s2 \) in the embryonic testis (Fig. 2A and B). As with the \( agouti \) transgene, the \( A^v \) mutation failed to reduce TGCT susceptibility in M19-\( A^v/+ \) or M19-\( A^v/+ \) males (Table 1 and Supplementary Material, Table S1).

We next tested whether variable levels of \( agouti \) expression in \( A^v/+ \) mice influence TGCT susceptibility. Promoter
activity of the A⁺⁺ IAP varies due to incomplete erasure of epigenetic modifications in the maternal germ line (39,40), resulting in a continuum of agouti expression and variable phenotype expressivity in isogenic A⁺⁺ mice (38). Therefore, if agouti influences TGCT susceptibility, tumor incidence may be correlated with promoter activity and agouti expression levels. Coat color can be used as a proxy for promoter activity of the A⁺⁺ IAP. Yellow mice have IAP activity in all cells, mottled-yellow mice are mosaics of cells with or without IAP activity, and agouti-like mice have an inactive IAP promoter in most cells (41). TGCT incidence was similar in yellow, mottled-yellow and agouti-like M19-A⁺⁺/+ and 129-A⁺⁺/+ mice (Supplementary Material, Table S2). The effect of IAP silencing extended to the A⁺⁺/+ embryonic testis, with expression varying over a 5-fold range and with one sample having almost undetectable levels of agouti expression (Fig. 2C). These results demonstrate that agouti expressivity did not affect TGCT susceptibility. Together, the results from the TGCT surveys of Eif2s2 gene-trap, Raly gene-trap, agouti transgenic and A⁺⁺ mice support the conclusion that reduced expression of Eif2s2 acts as the TGCT suppressor in agouti-yellow mice.

Homozgyosity for the Eif2s2 gene-trap causes embryonic death

For many TGCT modifiers, tumor susceptibility is elevated in heterozygotes and higher still in homozygous mutant males, but for other modifiers homozgyosity causes embryonic lethality (19,33–35,42). Previous in vitro and genomic-based studies suggested that deletion of Raly causes the embryonic lethality of A⁺⁺/A⁺⁺ mice (4,9). However, at the time of those studies, presence of Eif2s2 within the A⁺⁺ deletion interval was not known and the contribution of Eif2s2 deficiency to embryonic development was not evaluated. Therefore, to test whether homozgyosity for Eif2s2 Trap further reduces TGCT susceptibility or causes lethality, Eif2s2 Trap/+ mice were intercrossed to produce Eif2s2 Trap/Eif2s2 Trap/+ offspring. Interestingly, these intercrosses failed to produce homozygous progeny, demonstrating that the loss of Eif2s2 expression was lethal (Table 2). In contrast, homozgyous Raly gene-trap offspring were viable and present at the expected Mendelian frequency (Table 2).

Homzygous A⁺⁺ mice die near the time of implantation, which is in part determined by genetic background (43). Therefore, we monitored Eif2s2 gene-trap intercross progeny from birth to weaning and did not observe lethality. Additionally, a small decrease in intercrosses litter size was observed when compared with backcross litters (Supplementary Material, Table S3). These observations are consistent with an embryonic lethal phenotype. To determine whether Eif2s2 Trap/Eif2s2 Trap embryos are lost near the time of implantation, E8.5 embryos from heterozygous intercrosses were genotyped. At E8.5, wild-type and heterozygous embryos were present at the expected Mendelian frequency for an intercross, but no homozygotes were found (Table 2). Additionally, approximately one-quarter of decidual swellings were empty, the majority of these probably corresponded to implantations of Eif2s2 Trap/Eif2s2 Trap embryos that induced a decidual response but failed to develop into gastrulated embryos.

Table 2. Progeny numbers from gene-trap intercrosses

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>No. of observed</th>
<th>No. of expected</th>
<th>Test score (χ², P-value)</th>
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<tbody>
<tr>
<td>3 weeks</td>
<td>Wild-type</td>
<td>11</td>
<td>11</td>
<td>14.7, &lt;0.005</td>
</tr>
<tr>
<td></td>
<td>Eif2s2 Trap/+</td>
<td>31</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eif2s2 Trap/Eif2s2 Trap</td>
<td>0</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>E8.5</td>
<td>Wild-type</td>
<td>8</td>
<td>8</td>
<td>8.1, &lt;0.025</td>
</tr>
<tr>
<td></td>
<td>Eif2s2 Trap/+</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eif2s2 Trap/Eif2s2 Trap</td>
<td>0 (11)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3 weeks</td>
<td>Wild-type</td>
<td>9</td>
<td>9</td>
<td>1.0, NS</td>
</tr>
<tr>
<td></td>
<td>Raly Trap/+</td>
<td>14</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raly Trap/Raly Trap</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant.

*The number of empty residua are provided in parentheses.

Assumes that M19 (wild-type) mice were observed at the expected frequency and a 1:2:1 segregation ratio.

Eif2s2-deficiency affects embryonic germ cell proliferation

Eif2s2 functions as one of the three subunits of eIF2, which is involved in one of the most tightly regulated steps in translation initiation (10). In response to stress, the availability of active eIF2 for translation initiation is reduced, which in turn decreases the global rate of protein synthesis (46). Modulating eIF2 availability controls cell division and survival, and increased eIF2 activity induces neoplastic transformation in vitro and is associated with several cancers in vivo (11,12). Conversely, several putative anti-cancer agents, which target the various regulatory steps of translation, including the availability of eIF2, decrease the rate of translation and inhibit tumor formation by inducing apoptosis or blocking cell cycle progression (47–50). Similarly, deficiency of eIF2 subunits likely decreases the availability of active eIF2 complexes and reduces the rate of translation initiation, which could act as a suppressor of tumorigenic potential.

Stevens and others proposed that TGCTs in 129/Sv mice arise from PGCs that continue to divide instead of entering mitotic arrest, which normally occurs around E13.5 (17). We therefore tested whether partial deficiency of Eif2s2 reduces TGCT incidence by decreasing proliferation, inducing apoptosis, or inhibiting the differentiation of germ cells after E13.5. To test whether the Eif2s2 gene-trap influences PGC proliferative capacity, we immunostained embryonic testis sections for phospho-Histone H3, a nuclear marker of mitotic cells (51). Interestingly, no significant difference in the percentage of proliferating PGCs was detected between M19 and M19-Eif2s2 Trap/+ testes from E13.5 to E15.5 (Fig. 3A). However, at E16.5 the percentage of proliferating PGCs was significantly reduced in M19-Eif2s2 Trap/+ testes compared
with those from M19 control embryos (Fig. 3A–C). These findings are similar to a previous study of 129-A'y++ embryo-nic testes, which demonstrated that the Ay mutation does not reduce the number of aberrantly proliferating PGCs until E16.5 (17). Thus, partial deficiency of Eif2s2 accounts for the reduced TGCT incidence and aberrant PGC proliferation in Ay males.

Chronic suppression of eIF2 activity induces apoptosis under certain cellular conditions (52). Therefore, we immuno-stained embryonic testis sections for cleaved caspase 3, a key mediator of programmed cell death (53), to test whether Eif2s2-deficiency influences the survival of aberrantly proliferating PGCs. Interestingly, apoptotic PGCs were rare in both M19-Eif2s2Trap+/+ and M19 fetal testes, and a difference in the percentage of apoptotic PGCs was not observed from E13.5 to E16.5 (Fig. 3D). Taken together, these proliferation and apoptosis assays demonstrate that partial deficiency of Eif2s2 influences the mitotic activity but not the survival of PGCs.

Interestingly, the effect of the Eif2s2 gene-trap on PGC proliferation was restricted to a subset of cells. PGCs actively proliferate during development, expanding from a population of a few dozen cells at E7.5 to 25 000 cells at E13.5 (54). If reduced Eif2s2 expression influenced the proliferative capacity of the entire PGC population, then the total number of PGCs in the Eif2s2Trap+/+ embryonic testis should be reduced compared with wild-type controls by E13.5. However, total germ cell numbers were similar between M19 and M19-Eif2s2Trap+/+ embryonic testes at E14.5 and E16.5 (Fig. 3E). Therefore, the effect of reduced Eif2s2 expression on PGC proliferation appears to be restricted to the small population of aberrantly proliferating PGCs at a specific developmental time-point.

Eif2s2-deficiency affects adult germ cell development

Reduced expression of Eif2s2 also affected adult germ cell development. Adult testis weight is often used as an indirect measure of alterations in germ cell numbers (55) and reduced fertility is an established TGCT risk factor (56,57). Interestingly, we observed a small but significant decrease in M19-Eif2s2Trap+/+ testis weight compared with M19 controls at 6 weeks of age (Fig. 4A). Reductions in the spermatogonial stem cell pool can often lead to reduced germ cell numbers in the adult testis. However, comparison of testes from 1-day-old (P1) M19 and M19-Eif2s2Trap+/+ neonates showed that the gene-trap did not reduce the initial spermatogonial stem cell population (Fig. 3E). Additionally, we did not observe significant defects in spermatogenesis in the adult gene-trap testes when compared with M19 controls (Fig. 4B and C). These results suggest that reduced Eif2s2 expression impeded but did not repress adult germ cell maturation.

An adult germ cell phenotype similar to that of mice partially deficient for Eif2s2 was reported for mice partially deficient for Eif2s3 (eIF2-gamma) (58). In mice, Eif2s3 is encoded on the short arm of the Y chromosome (Eif2s3y)
and the X chromosome (Eif2s3x) (59). The Sxr<sup>y</sup> Y chromosome deletion of Eif2s3y causes a defect in spermatogonial proliferation and differentiation at several important stages of spermatogenesis, resulting in reduced numbers of meiotic spermatocytes and infertility (55,58). Additionally, Eif2s3y is one of several genes known to escape X inactivation, indicating that Eif2s3 gene dosage is critical in both sexes (59). Together with our results, these observations indicate that eIF2 subunit gene dosage is vital for proper maintenance of the germ cell lineage.

**DISCUSSION**

A systematic series of tests including gene expression analyses and tumor surveys revealed that reduced expression of Eif2s2 is the TGCT suppressor in agouti-yellow mice. Reduced Eif2s2 expression in gene-trap mice decreased TGCT incidence by 2-fold. Furthermore, reduced Eif2s2 expression decreased the number of aberrantly proliferating PGCs at E16.5 in TGCT susceptible testes and impaired adult spermatogenesis. In contrast, neither reduced expression of Raly in gene-trap mice nor ectopic expression of agouti in A<sup>Y</sup> or agouti transgenic mice suppressed TGCT susceptibility. Importantly, this is the first dominant A<sup>Y</sup> phenotype to be associated with the deletion of Eif2s2 and the only TGCT suppressing mutation to be characterized in humans and mice. Surprisingly, homozygosity for the Eif2s2 but not the Raly gene-trap resulted in embryonic lethality. As with A<sup>Y</sup>/A<sup>Y</sup> mice, death of Eif2s2 homozygous gene-trap mice occurred around the time of implantation, demonstrating that the loss of Eif2s2 expression accounts for the recessive embryonic lethality of A<sup>Y</sup>

It remains to be determined how reduced expression of Eif2s2 suppresses TGCT susceptibility. However, changes in translation efficiency are a common feature of tumorigenesis (11,60). Several oncogenes and cell cycle regulators, including c-Myc, p27 and cyclin D1, have complex 5'-UTR secondary structures and require highly efficient translation initiation to maintain their expression (11,12,61). Activation of translation also suppresses expression of pro-apoptotic proteins (11). Not surprisingly, it has been proposed that increased translation rates promote tumor progression by maintaining proliferation, suppressing apoptosis and promoting pluripotency (12,13). In vitro studies support these hypotheses and implicate a role for increased eIF2 availability in proliferation, survival and neoplastic transformation (11,12). Conversely, decreased eIF2 availability likely prevents these processes. In the TGCT susceptible tests, our results suggest that reduced Eif2s2 expression suppresses proliferation and ultimately transformation.

The defects in adult male germ cell development in mice partially deficient for Eif2s2 and Eif2s3 may provide clues to the effect of reduced Eif2s2 expression on PGC development and TGCT pathogenesis. Differentiation from a spermatogonial stem cell to a meiotically committed Type B spermatagonia requires several concomitant mitotic divisions, which are intricately associated with germ cell differentiation (62). These division and differentiation steps appear to be sensitive to gene dosage effects of eIF2 subunits. In a similar manner, a reduction in the availability of eIF2 may attenuate the ability of aberrantly proliferating PGCs to obtain the self-renewal capacity and pluripotency of TGCT stem cells (63). In fact, we observed an effect on self-renewal in our proliferation assays. Eif2s2-deficiency did not reduce the number of aberrantly proliferating PGCs until E16.5, a developmental time point at which TGCT foci have already begun to form (21,36). It is intriguing to speculate that the reduced number of proliferating PGCs observed in Eif2s2-deficient, E16.5 gonads resulted from blocking the acquisition of self-renewal capacity in the aberrantly proliferating PGC population. As observed in TGCT-resistant germ cells, the few remaining aberrantly proliferating PGCs in the majority of Eif2s2 gene-trap testes likely entered mitotic arrest (63).

Interestingly, Eif2s2 is not the only TGCT modifier that regulates translation. We previously identified a nonsense point mutation in Dead end 1 (Dnd1) as the cause for germ cell loss and increased TGCT susceptibility in 129/Sv-Ter mutant mice (33). Dnd1 has significant sequence similarity to the RNA-binding subunit (Acf) of the RNA editing complex and has been shown to block miRNA access to 3'-UTRs of transcripts involved in PGC development (Nanos1) and cell cycle regulation (Cdkn1b) (33,64). Argo- naute or Piwi proteins associated with miRNAs in the RNA-induced silencing complex (miRISC) bind to 3'-UTRs of target transcripts (65). This complex inhibits translation...
by associating with the 5′ cap of mRNAs and blocking assembly of the cap-binding complex (65). Interestingly, the Dnd1<sup>Ter</sup> mutation and the 3′ deletion of Eif2s2 interact to modulate TGCT susceptibility (32), which implies that 5′ cap and 3′-UTR regulation of translation are both important to the development of the PGC lineage and the pathogenesis of TGCTs.

**MATERIAL AND METHODS**

**Mice**

129S1/SvImJ (129/Sv) (002448), C57BL/6J-<sup>A</sup>/+ (000021) and C57BL/6J-<sup>A</sup>/+ (000017) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). 129S1/SvImJ mice homozygous for the Oct4<sup>ΔPE.GFP</sup> transgene (33) and homozygous for the Chr19 MOLF chromosome substitution (35) were obtained from our research colony. The <sup>A</sup> and <sup>3′</sup>-UTR mutations as well as the Oct4<sup>ΔPE.GFP</sup> transgene were back-crossed for at least 10 generations onto 129/Sv to establish congenic strains and were then transferred with crosses to the M19 genetic background (See Supplementary Material, Table S4 for genotyping primer sequences). All protocols were approved by the CWRU Institutional Animal Care and Use Committee.

**Transgenes and gene-traps**

An agouti cDNA transgene driven by the human beta-actin promoter (pBAP-a) was modified to contain the loxP-flanked neomycin resistance cassette from pL452 (Supplementary Material, Fig. S4A) (6,66). The transgenic construct was introduced by electroporation into R1 ES cells, a hybrid of 129X1/SvJ and 129S1/SvJ. G418 selection and quantitative real-time PCR (Q-PCR) were used to identify transgenic clones. BayGenomics ES cell clones RST374 and XH413 (derived from the 129P2/OlaHsd ES cell line E14Tg2a.4) were identified to have insertions in the second intron of Raly and the first intron of Eif2s2, respectively. Both inserted 5′ of the open reading frames and sequencing verified proper splicing of gene-trap sequences to 5′-UTR exons. The reverse transcriptase–PCR (RT–PCR) analysis of ES cell RNA verified proper splicing of the gene-traps to exon 2 of Raly and exon 1 of Eif2s2, respectively (Supplementary Material, Fig. S3A–C). ES cells were injected into C57BL/6J blastocysts using standard procedures to produce chimeras and the agouti transgene and gene-traps were transferred with crosses onto M19 background (see Supplementary Material, Table S5 for RT–PCR primer sequences).

**Genotyping**

Yellow fur and light pigmentation of the tail were used to distinguish <sup>A</sup>+/+, <sup>3′</sup>-UTR/+ and agouti transgenic mice from their wild-type siblings (56). PCR genotyping was used to identify <sup>A</sup>+, agouti transgenic and gene-trap mice and embryos. RT–PCR genotyping of liver tissue was used to identify <sup>A</sup>− embryos (See Supplementary Material, Table S4 for genotyping primer sequences).

**Timed-matings and embryonic gonad dissections**

Embryonic day 0.5 (E0.5) was assumed to be noon of the day the vaginal plug was observed. On the morning of the desired embryonic day, pregnant females were euthanized by cervical dislocation and embryos removed from the uterus in ice-cold 1xPBS. Embryos older than E14.5 were decapitated prior to dissection and gonads were removed in ice-cold 1xPBS. Tissues were collected and processed for specific assays and genotyping. PCR genotyping for Sry identified the sex of E12.5 embryos (Supplementary Material, Table S4). Gonad morphology identified the sex of E13.5–E16.5 embryos.

**Q-PCR expression analysis**

Closes between M19 and M19-agouti mutant, transgenic or gene-trap mice, in both parental directions, were used to produce embryos for gonad dissections. Embryonic testes were lysed and RNA was prepared using the RNeasy Micro Kit (Qiagen). Adult tissues were collected and stored at −20°C in RNAlater (Ambion). RNA from adult tissues was collected by TRIReagent extraction. RNA, 500 ng, was reverse transcribed with the SuperScript First-Strand Synthesis System. Q-PCR for agouti, Raly and Eif2s2 expression was performed with the Chromo4 real-time PCR system (MJ Research) and the PerfeCTa SYBR Green Supermix kit (VWR Scientific) using manufacturer-suggested protocols. Expression of agouti, Raly and Eif2s2 was normalized to the ubiquitously expressed housekeeping gene Rpl7 (67). Serial dilutions of wild-type (Raly and Eif2s2 expression analysis) or <sup>3′</sup>-UTR (agouti expression analysis) adult testis cDNA were used to generate standard curves for each primer set. Significant differences in expression were tested by one-way ANOVA with the Bonferroni post-test for pair-wise comparisons (see Supplementary Material, Table S5 for Q-PCR primer sequences).

**FACS-sorted cells**

M19-Oct4<sup>ΔPE.GFP</sup> females were bred to M19-agouti mutant, transgenic or gene-trap males to produce embryos for gonad dissections. Single-cell suspensions of embryonic testes were generated as previously described (68). Gonads were digested in 0.25% trypsin (Invitrogen) for 15 min at 37°C. Tissues were triturated into single-cell suspensions and filtered through a 40 mm nylon mesh cell strainer (BD Falcon). The mesh was washed with 2% BSA in 1xPBS and the cells were kept on ice until FACS with the BD Biosciences FACSAria system. The Oct4<sup>ΔPE.GFP</sup> transgene was used to sort GFP-positive PGCs from GFP-negative somatic cells, which typically yielded 1500 to 3000 GFP-positive germ cells (98% purity) from both gonads of a single embryo (Supplementary Material, Fig. S2A) (68). Sorted cells were lysed and then RNA was prepared and reverse transcribed using the protocols described for embryonic testes. cDNA samples were PCR-amplified for 35 cycles for the appropriate genes (see Supplementary Material, Table S5 for RT–PCR primer sequences).
Tumor surveys

Crosses between wild-type and A<sup>av</sup>, agouti<sup>Tg</sup>−/−, Raly<sup>Trap/+</sup> or Eif2s<sup>−/−</sup><sup>Trap/+</sup>, in both parental directions, were used for the TGCT surveys. Male offspring were necropsied at 4–6 weeks of age and testes were visually examined for tumors, which are readily detected at these ages (31,35). Chi-square contingency tests were used to test for statistical differences between the number of affected control and experimental mutant or transgenic progeny. Finally, A<sup>av</sup> males were subcategorized by coat color, and chi-square contingency tests were used to test for statistical differences in TGCT incidence between coat color categories.

Immunohistochemistry and histology

M19-Eif2s<sup>−/−</sup><sup>Trap/+</sup> females were bred to M19-Oct4<sup>ΔPE;GFP</sup> males for timed-matings. On the appropriate day, embryonic or neonatal testes were dissected and fixed overnight at 4°C in 4% paraformaldehyde. Samples were cryopreserved in 30% sucrose in 1xPBS and embedded in O.C.T. compound (Tissue Tek). Samples were sectioned (10 μm) and blocked in 5% goat serum and 3% BSA with PBS. Sections were incubated overnight at 4°C in blocking solution containing 1:200 diluted rabbit polyclonal anti-phospho-Histone H3 (Ser 10) (06-570, Upstate) or 1:200 diluted rabbit polyclonal anti-cleaved Caspase-3 (Asp175) (9661, Cell Signaling), anti-cleaved PARP (9662, Cell Signaling), anti-cleaved PARP (9662, Cell Signaling), diluted rabbit polyclonal anti-phospho-Histone H3 (Ser 10) (06-570, Upstate) or 1:200 diluted rabbit polyclonal anti-cleaved Caspase-3 (Asp175) (9661, Cell Signaling), with PBS, and incubated for 3 h at room temperature with goat anti-rabbit AlexaFluor 633 secondary antibody (A21701, Invitrogen) diluted 1:400 in blocking solution. Nuclei were counter-stained with DAPI. GFP-positive germ cells were counted using the Volocity (Version 4) software suite; antibody stained germ cells were counted blind to embryo genotype in three to four sections spaced 20–40 μm apart. Germ cells per tubule cross-section were calculated from 4 sections, 5–20 tubules/section, spaced 40 μm apart. Significant differences in antibody labeled cells and germ cells per tubule were tested by unpaired t-tests. For histology sections, adult tissues were fixed in 10% buffered formalin, sectioned (5 μm) and stained with hematoxylin and eosin.

Weight gain and testis weight

Body weight of M19, M19-agouti<sup>Tg</sup>−/− and M19-A<sup>av</sup>/+ male mice was measured every 2–4 weeks (± 3 days). From weaning until 12 weeks of age, mice were fed Lab Diet 5010 <i>ad libitum</i>. Following weaning at 12 weeks, M19 and M19-agouti<sup>Tg</sup>−/− mice were fed a high fat, high sucrose diet (Research Diets D12331) <i>ad libitum</i> to accelerate differences in weight gain (6). M19 and M19-Eif2s<sup>−/−</sup><sup>Trap/+</sup> testis, without TGCTs, were weighed at 6-weeks of age. Significant differences in body and testis weight were tested using unpaired t-tests and P-values. Body weight P-values were corrected for multiple testing (five tests).

Eif2s2 gene-trap integration site mapping

To identify the integration site of the XH413 gene-trap, a primer walking and sequencing strategy was utilized (34). Forward primers were designed to anneal to sequences at 500 bp intervals across Eif2s2 intron 1 and individually paired in PCR reactions with a reverse primer, 5′-GTTATCGATCTCGATCTGC-3′, which anneals to sequences ~1500 bp within the pGTL1PLx gene-trap vector. PCR of Eif2s<sup>−/−</sup><sup>Trap/+</sup> DNA produced a 2 Kb product using forward primer 5′-CAGCTGTGGTTCGACTGTGGT-3′. Sequencing of this PCR product with primer 5′-TCAACAGGCGTTTATGTGCTC-3′ (within the amplified Eif2s2 sequence) identified the gene-trap insertion site of ES cell line XH413 (Supplementary Material, Fig. S3D).

Homozygous lethality

M19-Raly<sup>Trap/+</sup> or M19-Eif2s<sup>−/−</sup><sup>Trap/+</sup> were intercrossed and weaning-age mice or E8.5 embryos were genotyped for gene-trap copy number. A Taqman Q-PCR assay for the gene-trap β-galactosidase/neomycin resistance (β-geo) cassette was used to identify heterozygous and homozygous animals. DNA (5 ng) was assayed using the Chromo4 real-time PCR system (MJ Research) and the Taqman Universal PCR Master Mix (Applied Biosystems) using manufacturer suggested protocols. Primers 5′-CCTGCTGATGAAGCA GAACA-3′ and 5′-TTGGCTTCTACCCACACATA-3′ and probe 5′-FAM CGTGTGCGATATCGACGACATTCA TAMRA-3′ were used to amplify and detect β-geo. Primers 5′-TGCTACAAGTTATGCTGTCG-3′ and 5′-CTCC TCTGGGACTTTGCTATC-3′ and probe 5′-VIC AC GGTCTGCGTCCTAAGGCGATCA TAMRA-3′ were used to amplify and detect Ngf, which normalized for DNA concentrations. Eif2s<sup>−/−</sup><sup>Trap</sup> offspring genotype was verified by PCR genotyping for the wild-type allele with primers F: 5′-AGGAACATAATTACATGTGGCATAA-3′ (upstream of the gene-trap integration) and R: 5′-TCAAGAAG GGGGAAATCCT-3′ (downstream of the gene-trap integration), and the Eif2s<sup>−/−</sup><sup>Trap</sup> allele with primers F: 5′- TAA GGTTGCTGGATTTCTCG-3′ (upstream of the gene-trap integration) and R: 5′- GGCTACGGGCTAAACTGTGAA-3′ (within the gene-trap). Chi-square goodness-of-fit tests were used to test for statistical differences between the observed and expected number of progeny.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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