Eukaryotic Elongation Factor 2 Kinase Activity Is Required for the Phenotypes of the Rpl24<sup>Bst</sup> Mouse

TO THE EDITOR

The variant murine Rpl24<sup>Bst</sup> allele reduces gene expression by 40% and results in a white belly spot and kinked tail of variable severity and penetrance (Oliver et al., 2004). White belly spots lack melanocytes owing to defects in their motility during development, whereas kinked tails result from fused or wedge-shaped vertebral elements (Oliver et al., 2008; Watkins-Chow et al., 2013). Despite this genetic evidence of a link between ribosomal protein genes—Rps7, Rps21, Rps19, Rps20, and RACK1—result in belly spots (Dinh et al., 2021; McGowan et al., 2008; Volta et al., 2013; Watkins-Chow et al., 2013). Despite this genetic evidence of a link between ribosomal proteins and belly spots, the mechanism(s) behind the phenotype are unknown. The Rpl24<sup>Bst</sup> mutation reduces protein synthesis in embryonic neural tube cells (Kondrashov et al., 2011) and models of cancer (e.g., as reported in Knight et al. [2021]), suggesting that reduced protein synthesis underpins the developmental defects. In agreement, reduced RACK1 limited protein synthesis in murine embryonic fibroblasts (Volta et al., 2013). However, the other belly-spot variants do not affect protein synthesis (Kondrashov et al., 2011; Watkins-Chow et al., 2013). Thus, to understand the mechanistic link between protein synthesis and belly spots, experiments are required to directly alter protein synthesis rates in variant mice.

We previously showed that Rpl24<sup>Bst</sup> mutation suppresses protein synthesis through the activation of eukaryotic elongation factor 2 kinase (eEF2K) (Knight et al., 2021). Suppression of protein synthesis in Rpl24<sup>Bst</sup> variants is completely reversed by eEF2K inactivation, providing a tool to reverse the protein synthesis defects in Rpl24<sup>Bst</sup> variants. The Eef2<sup>K<sub>D273A</sub></sup> allele is a germline knockin that dramatically reduces eEF2K catalytic activity throughout the whole mouse from conception (Gildish et al., 2012). We therefore used the Eef2<sup>K<sub>D273A</sub></sup> allele to assess the influence of eEF2K inactivation on Rpl24<sup>Bst</sup> phenotypes. To achieve this, belly spot and tail severity were scored using a scale from 0 (normal) to 4 (severe) for mice generated in our previous study (Supplementary Figure S1a). The combined belly-spot and tail score of Rpl24<sup>Bst<sup>+/−</sup></sup> mice has a median of 3 of 8 (Figure 1a). In comparison, the belly-spot and tail score of Rpl24<sup>Bst<sup>+/+</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice was significantly lower at only 1.5. Furthermore, there is a lower incidence of severe phenotypes, with only 7% of Rpl24<sup>Bst<sup>+/−</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice scoring ≥5 compared with 34% of Rpl24<sup>Bst<sup>+/+</sup></sup> mice. Therefore, inactivation of eEF2K suppresses the observable skin and tail phenotypes of the Rpl24<sup>Bst</sup> mutation. The main contributing factor to this difference is the tail score, with 35% of tails scoring ≥2 in Rpl24<sup>Bst<sup>+/−</sup></sup> mice compared with 3.6% in Rpl24<sup>Bst<sup>+/+</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice (Supplementary Figure S1b).

Next, we took a complementary approach measuring the effect of the Rpl24<sup>Bst</sup> and Eef2<sup>K<sub>D273A</sub></sup> mutations on melanocytes using the Dct-lacZ system (Mackenzie et al., 1997). Genetically engineered Dct-lacZ mice express β-galactosidase from the melanocytes-specific dopachrome tautomerase (Dct) promoter, allowing whole-mount visualization and quantification of melanocyte location. On embryonic day 13.5, melanocytes are transiting the forelimbs of embryos, with the fraction of melanocyte-positive forelimb a metric for changes in melanocyte migration. We observe a significant reduction in melanocyte migration in Rpl24<sup>Bst<sup>+/+</sup></sup> embryos, consistent with their belly spots in adulthood, whereas the inactivation of eEF2K has no effect (Figure 1b). Compared with that in Rpl24<sup>Bst<sup>+/+</sup></sup> embryos, melanocyte migration is significantly reverted in Rpl24<sup>Bst<sup>+/−</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> embryos (Figure 1b). Thus, eEF2K activity is required for the perturbed melanocyte migration phenotype of Rpl24<sup>Bst</sup> embryos. Why the reverted migration in Rpl24<sup>Bst<sup>+/−</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> embryos (Figure 1b) does not correlate with a reversal of adult belly spot phenotype (Supplementary Figure S1b) is unclear. It is possible that migration is reversed at embryonic day 13.5 but subsequently slows to produce a belly spot. Unfortunately, staining of Dct-lacZ is not possible at later embryonic stages owing to reduced skin permeabilization (Mackenzie et al., 1997). We noted that Rpl24<sup>Bst<sup>+/−</sup></sup> and Rpl24<sup>Bst<sup>+/+</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice were weaned at lower than Mendelian frequencies: Rpl24<sup>Bst<sup>+/−</sup></sup> mice at 1 in 3 (32.1%) when expected at 1 in 2 and Rpl24<sup>Bst<sup>/−</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice even less frequently at 1 in 5 (20.7%) (Figure 2a). Unexpectedly, when we compared the frequency of Rpl24<sup>Bst<sup>+/−</sup></sup> Eef2<sup>K<sub>D273A</sub></sup> mice with the experimentally determined frequency of Rpl24<sup>Bst<sup>+/−</sup></sup> mice (32.1%), we found this to be significantly different (Figure 2a). Thus, the viability of Rpl24<sup>Bst<sup>+/−</sup></sup> mice is at least in part dependent on eEF2K activity. To analyze this effect further, we calculated the frequencies of the same genotypes in embryonic day 13.5 embryos, finding that the incidence of the Rpl24<sup>Bst</sup> mutation was close to Mendelian with active or inactive eEF2K.
This work extends the relationship between RPL24 and eEF2K to include melanocyte migration, tail deformation, and organism survival. RPL24Bst/+ mice have been used in cancer studies, to model retinal degeneration, and to study brain development (Herlinger et al., 2019; Riazifar et al., 2015). This work shows that eEF2K may play a role across these diverse biological settings, with further work merited to investigate its importance.

Declaration for animal use

Studies were carried out under license from the UK Home Office (60/4183 and 70/8646). Mice were maintained in open-top cages with a 12-hour light/dark cycle and free access to water and diet. Experiments were initiated on inbred C57BL/6j male and female mice aged between 6 and 12 weeks, without blinding or randomization.

Data availability statement

No datasets were generated or analyzed during this study.

ORCIDs

John R. P. Knight: http://orcid.org/0000-0002-8771-5484
Christopher G. Proud: http://orcid.org/0000-0003-0704-6412
Giovanna Mallucci: http://orcid.org/0000-0001-8504-1191
Tobias von der Haar: http://orcid.org/0000-0002-6011-9254
C. Mark Smales: http://orcid.org/0000-0002-2762-4724
Anne E. Willis: http://orcid.org/0000-0002-1470-8531
Owen J. Sansom: http://orcid.org/0000-0001-9540-3010

CONFLICT OF INTEREST

OJS reports funding unrelated to work in this project from Astra Zeneca, Novartis, RedX, and Cancer Research Horizons. The remaining authors state no conflict of interest.

Disclaimer

The funders had no role in study design, data collection, and interpretation or in the decision to submit the work for publication.

AUTHOR CONTRIBUTIONS

Conceptualization: JRPK, OJS; Formal Analysis: Funding Acquisition: GM, TvdH, CMS, AEW, OJS; JRPK; Project Administration: JRPK, OJS; Resources: CGP, Supervision: OJS; Writing - Original Draft Preparation: JRPK; Writing - Review and Editing: JRPK, CGP, GM, TvdH, CMS, AEW, OJS

ACKNOWLEDGMENTS

Funding was provided by Cancer Research UK (A17196, A24388, A21139, A31287) and H2020 European Research Council (311301) to OJS; by the Wellcome Trust (201487) to GRM, TvdH, CMS, OJS, and AEW; and by the National Health and Medical Research Council to CGP.

Figure 1. Inactivation of eEF2K reverses the phenotypes of the Rpl24Bst mutation. (a) Quantification of Bst score from mice with Rpl24Bst mutation alone (n = 115 mice) or in combination with Eef2kD273A/D273A (n = 28 mice). Significance was determined by Kolmogorov–Smirnov test. Right: representative (median Bst score) image of Rpl24Bst/+ and Rpl24Bst/+ eEF2K0273A/0273A mice. (b) Images of transgenic Dct-lacZ mice at E13.5 mice stained for β-galactosidase (blue) within melanocytes. The upper image shows whole embryos, and the lower image shows an expanded view of one forelimb. Bars = 500 μm. Right: quantification of melanocyte migration scored as the distance from torso to furthest melanocyte/length of forelimb. Significance was determined by one-way ANOVA (Tukey’s multiple comparison). Left to right n = 14, 15, 16, and 13. For both graphs, boxes mark the 25th and 75th percentiles, and the central lines mark the median. Each point represents an individual mouse/embryo. Bst, belly-spot and tail; E13.5, embryonic day 13.5; eEF2K, eukaryotic elongation factor 2 kinase.
**Figure 2.** eEF2K is required for preweaning survival of Rpl24<sup>Bst</sup>/+ mice. (a) The percentage of Rpl24<sup>Bst</sup>/+ mice weaned per litter is plotted in black, with each point representing an individual litter. The box marks the 25th and 75th percentiles, and the central line marks the median incidence of mice weaned per litter. The box marks as the expected frequency compared to the actual frequency of Rpl24<sup>Bst</sup>/+ Eef2k<sup>D273A/D273A</sup> embryos (n = 57 litters). (b) Analysis as in (a) but for embryos at E13.5. For Rpl24<sup>Bst</sup>/+ embryos n = 5 litters and for Rpl24<sup>Bst</sup>/+ Eef2k<sup>D273A/D273A</sup> embryos n = 6. E13.5, embryonic day 13.5; eEF2K, eukaryotic elongation factor 2 kinase.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2022.06.019

**REFERENCES**


This work is licensed under a Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/
SUPPLEMENTARY MATERIALS AND METHODS

Materials availability
All materials are freely available, where legally permitted, on acceptance of a material transfer agreement.

Belly-spot and tail scoring
Genotyping was carried out by Transnetyx (Memphis, TN). Scoring was carried out at the endpoint of published experiments that determined sample size, with no power analysis performed for this study. Scoring was performed regardless of the presence of other alleles such as Villin\textsuperscript{CreER}, Apc\textsuperscript{D}, Apc\textsuperscript{Min}, or Kras\textsuperscript{G12D} because these alleles do not manifest a belly-spot or tail phenotype.

\textbf{Dct-lacZ}
Overnight vaginal plugs were designated embryonic day 0.5, and embryos were collected on embryonic day 13.5. Embryo studies were performed blind. Tails were used for genotyping, and the remainder were fixed in ice-cold 0.25% glutaraldehyde (G6257, Sigma-Aldrich, St. Louis, MO) in PBS, on a rotational mixer for 30 minutes at 4°C. Embryos were washed in cold PBS and permeabilized in two rounds of 2 mM magnesium chloride, 0.1% sodium deoxycholate (D6750, Sigma-Aldrich), and 0.02% NP40 (74385, Sigma-Aldrich) in PBS for a total of 40 minutes at room temperature. Embryos were stained in 2 mM magnesium chloride, 0.1% sodium deoxycholate, 0.02% NP40, 4.7 mM potassium hexacyanoferrate (II) trihydrate (P9387, Sigma-Aldrich), 4.8 mM potassium hexacyanoferrate (III) (P8131, Sigma-Aldrich), and 0.5 mg/ml X-Gal (V394A, Promega, Madison, WI) in PBS at 4°C on a rotational mixer protected from light for 36 hours. Embryos were washed in PBS, visualized by light microscopy, and stored in formalin. The sample size required for significance was estimated on the basis of pilot studies using G*Power (Faul et al., 2009).

**SUPPLEMENTARY REFERENCE**

**Supplementary Figure S1. The spectrum of belly-spot and tail scores.** (a) Examples of mice exhibiting each score on the belly-spot and tail scale. These mice show equivalent scores for each parameter for ease of representation, but equivalent scores between both metrics were not always the case. (b) Graphs showing the belly-spot and the tail scores that comprise the overall belly-spot and tail score shown in Figure 1a. \textit{Rp\textsuperscript{24Bst+/+}} is shown in green; \(n=115\) mice. \textit{Rp\textsuperscript{24Bst+/+} Eef2k\textsuperscript{D273A/D273A}} is shown in blue; \(n=28\) mice. The box marks the 25th and 75th percentiles, and the central line marks the median. Each point represents an individual mouse. Significance was tested by Kolmogorov–Smirnov test.