INTRODUCTION

The accumulation of molecular damage to adult stem cells over time results in reduced capacity for self-renewal and tissue regeneration, and is thought to play a causative role in age-related frailty and disease. Mitochondrial dysfunction is a molecular hallmark of aging (Lopez-Otin et al., 2013) and the generation of a progeria mouse model with an error-prone version of the mitochondrial DNA (mtDNA) polymerase (PolgA mut/mut mice) provided evidence that mtDNA mutations (Kujoth et al., 2005; Trifunovic et al., 2004) and resulting defects in oxidative phosphorylation (OXPHOS) (Baines et al., 2014; Vermulst et al., 2007) could cause premature aging. Subsequent analysis of the underlying mechanisms showed that stem cell dysregulation played a major role (Ahlqvist et al., 2012; Chen et al., 2009; Fox et al., 2012). mtDNA mutations resulting in OXPHOS defects are observed in aging human stem cell populations (Fellous et al., 2009; McDonald et al., 2008; Taylor et al., 2003), including the colonic epithelium (~15% of crypts are OXPHOS-deficient by age 70
(Greaves et al., 2010; Taylor et al., 2003). We have recently shown that age-associated defects in OXPHOS complexes I (CI) and IV (CIV) can increase tumour cell proliferation rates in a mouse model of intestinal tumorigenesis, accelerating colonic adenoma growth (Smith et al., 2020). However, the effects of CI and CIV deficiency on normal colonic stem cell proliferation rates are unknown.

We investigated this by crossing the PolgA^{mut/mut} mice with Lgr5-EGFP-ires-creERT2 mice, which have an EGFP under the Lgr5 (a well-accepted stem cell marker) promoter (Barker et al., 2007). For ease, mice are referred to by their PolgA genotype only. Levels of NDUFB8 (CI) and MTCO1 (CIV) were quantified in PolgA^{+/mut} and PolgA^{mut/mut} mice at 12 months of age and compared with age-matched PolgA^{+/+} controls using a validated immunofluorescent protocol (Rocha et al., 2015; Smith et al., 2020) (Figure 1a). Mean intensities were normalized to the mitochondrial mass marker TOMM20, and z-scores relative to the PolgA^{+/+} mice were generated (Figure 1b,c). Crypts with z-scores of <-4.5 were defined as deficient. In the PolgA^{mut/mut} mice, 31.88% of crypts were CI-deficient, 44.24% CIV-deficient and 11.06% CI + CIV-deficient. In the PolgA^{mut/mut} mice, 53.1% of crypts were CI-deficient and 46.9% CI + CIV-deficient (Figure 1d). No crypts were classified as deficient in TOMM20 (Figure 1e), indicating no differences in mitochondrial mass. This was confirmed using a second mass marker, VDAC1 (Figure 1f,g).

Total crypt cell and LGR5^{High} stem cell proliferation indices were analysed using multiple thymidine analogue labelling (Smith et al., 2020; Stoll et al., 2011). Mice were injected daily with CldU for 4 days, and a final injection of IdU was given 15 h before death. These times were based on the reported murine colonic crypt turnover time of 4–5 days and a 24-h stem cell cycle (Barker et al., 2007). CldU+ cells had divided at least once within the previous 4 days, IdU+ cells had divided within the previous 15 h, and those that were CldU+/IdU+ had divided at least twice within the 4-day period (Figure 2a). All data were analysed using a Poisson (CldU, IdU, CldU + IdU and Ki-67) model allowing variation between individual mice to be accommodated for as a random effect. First, we compared cell proliferation indices between PolgA^{+/+} and PolgA^{mut/mut} mice. PolgA^{mut/mut} mice had a significantly higher number of cells per longitudinl cross-sectional area (p < 0.0001) and increased rates of CldU, IdU and CldU + IdU incorporation compared with PolgA^{+/+} mice (Figure 2b–e p < 0.001 for all). The proportion of Ki-67+ cells was significantly lower in PolgA^{mut/mut} mice (p < 0.001, Figure 2f) suggesting a smaller proliferative zone relative to the mitochondrial mass marker TOMM20, and z-scores

**FIGURE 1** Mitochondrial OXPHOS labelling in colonic crypts of 12-month-old PolgA^{+/+}, PolgA^{mut/mut} and PolgA^{mut/mut} mice. (a) Immunofluorescent panel showing levels of mitochondrial NDUFB8 (CI, Alexa Fluor 647), MTCO1 (CIV, Alexa Fluor 546) and TOMM20 (Alexa Fluor 488). Yellow arrows: OXPHOS normal; red arrows: CIV deficiency; green arrows: CI deficiency; and white arrows: CI + CIV deficiency. Cell nuclei are labelled with DAPI (blue). Scale bars = 50 µm (b–c) Dot plots showing z-scores of NDUFB8 and MTCO1 relative to TOMM20. Crypts with z-score <-4.5 (dashed line) were defined as “deficient.” Error bars show mean ± SD. Numbers of crypts quantified per mouse were as follows: PolgA^{+/+}: (I–r) n = 223, 259, 306 and 332; PolgA^{mut/mut}: (I–r) n = 201, 200, 200 and 200; PolgA^{mut/mut}: (I–r) n = 253, 191, 257 and 222. (d) The percentage of crypts per genotype with the 4 OXPHOS categories, bars show mean, and error bars are SEM. (f) Immunofluorescent images showing VDAC1 (Alexa Fluor 488) levels. Scale bars = 50 µm (g) Dot plots showing z-scores of VDAC1 levels. n = 150 crypts were quantified per mouse. Error bars show mean ± SD.
of defects in these proteins in stem cells. Previous work looking at cell cycle progression in the small intestine of PolgA<sup>mut/mut</sup> mice at 3 months of age showed a decrease in proliferation rates, suggesting there are either differential effects of OXPHOS deficiency between the colon and small intestine, or adaptive changes with age. Our previous studies have shown an increasing upregulation of de novo serine synthesis and one-carbon metabolic pathway enzymes in the PolgA<sup>mut/mut</sup> colon from 6 months onwards (Smith et al., 2020). These pathways are associated with biomass production in rapidly dividing cells and may influence stem cell division rates (Maddocks et al., 2017). We do not know the molecular effects of CI deficiency on LGR5<sup>High</sup> stem cell function beyond the changes in proliferation rates shown by this study; however, given the importance of control of the stem cell compartment to maintain epithelial crypt homeostasis, further work is required to determine the underlying molecular mechanisms.

## EXPERIMENTAL PROCEDURES

### Animals

Female mice were group-housed in individually ventilated cages at 25°C with a 12-h light/dark cycle. All mice were on a C57BL6/J background. Animal experiments were conducted in compliance with the UK Home Office (PPL P3052AD70) and the Newcastle University Animal Welfare Ethical Review Board (AWERB 425).

### Thymidine analogue labelling

Mice (n = 4 per group, age: 49–51 weeks) were injected with 50 mg/kg of CldU at 10am and 6 pm on days 1–3. On day 4, they were injected with 50 mg/kg of CldU at 10am followed by 50 mg/kg IdU at 6 pm (Smith et al., 2020). Mice were humanely killed by cervical dislocation 15 h later. The intestines were removed and fixed in 10% neutral buffered formalin for 24 h before standard dehydration and paraffin embedding.

### Immunofluorescence

OXPHOS immunofluorescence was performed on 3-µm serial sections of colon using the same antibodies as previously described (Rocha et al., 2015), and immunofluorescence was performed to detect the thymidine analogues as described (Smith et al., 2020). Antibody specificity was determined using mice singly injected with either CldU or IdU, and no cross-reactivity was observed. Sections were imaged using a Zeiss Axio Imager M1 fluorescent microscope and labelled proteins quantified as previously described (Rocha et al., 2015). For the thymidine analogue labelling analysis, crypts were selected where a full longitudinal section was visible from crypt base to apex. The total number of cells per crypt, and the number of LGR5<sup>High</sup>, Ki-67+, CldU+ and IdU+ cells per crypt were counted. GLMMs were performed using R programming, code is available on request.

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## CONFLICT OF INTEREST

The authors declare no competing financial interests.

## AUTHOR CONTRIBUTIONS

CS and CB bred and maintained the mice. CS, JCW, DH ALMS and EAS performed experimental work and collected data. JCW and APB performed statistical analysis. DMT and LCG designed the study, supervised the project and wrote the manuscript.

## DATA AVAILABILITY STATEMENT

Source data for this study are available from the corresponding author upon request.

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## REFERENCES

Ahlgqvist, K. J., Hämäläinen, R. H., Yatsuga, S., Uutela, M., Terzioglu, M., Götz, A., Forsström, S., Salwen, P., Angers-Loustau, A., Kopra, O. H., Tynismaa, H., Larsson, N.-G., Wartiovaara, K., Prolla, T., Trifunovic, A., & Suomalainen, A. (2012). Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in polg mutator mice. Cell Metabolism, 15(1), 100–109. https://doi.org/10.1016/j.cmet.2011.11.012
