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Supplemental Information

Reprogramming of endolysosomes for melanogenesis in BLOC-1-deficient melanocytes

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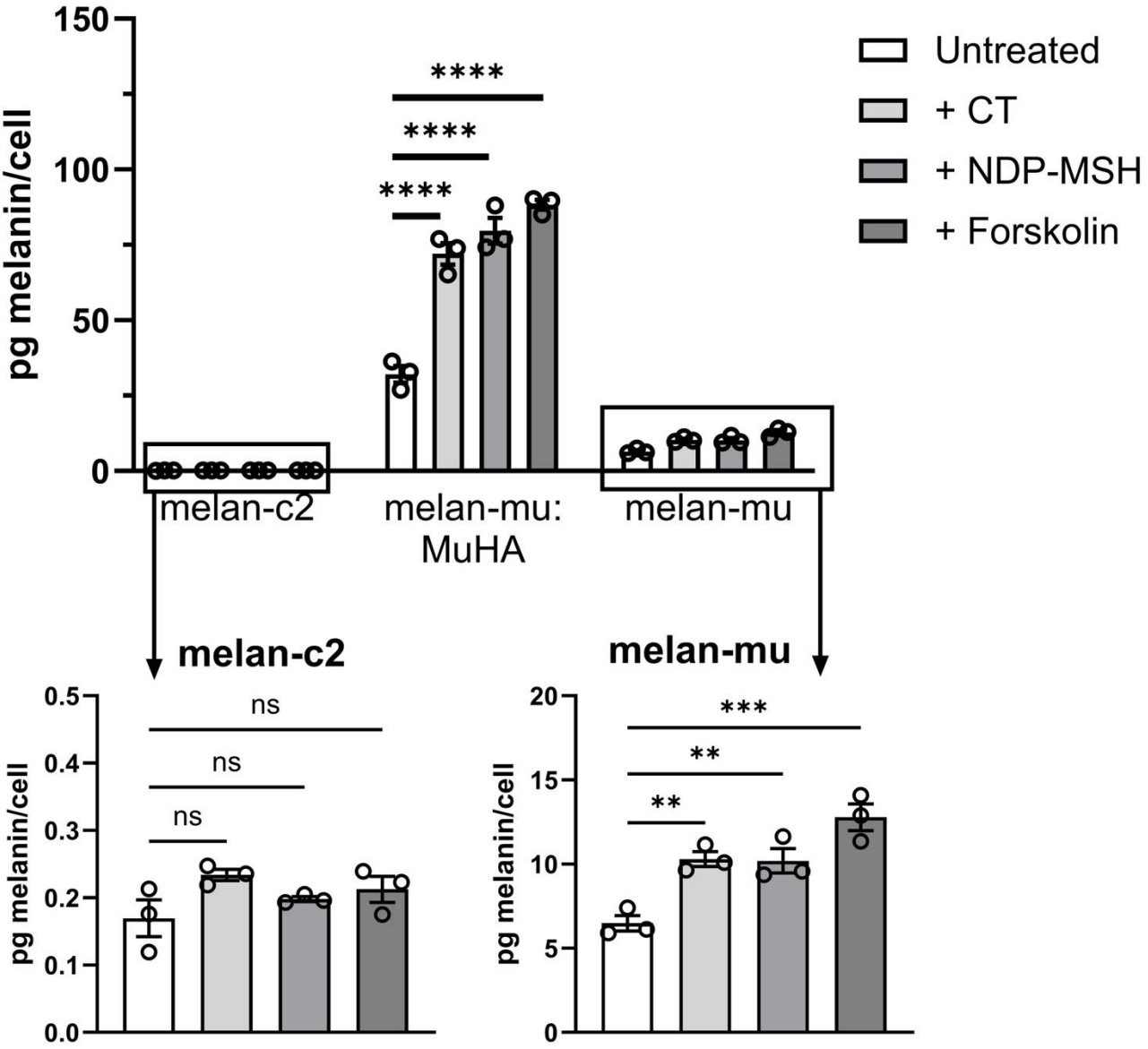

FIGURE S1. cAMP elevating agents increase melanin content of BLOC-1^{R} and BLOC-1-deficient melanocytes but not tyrosinase-deficient melan-c2. Related to Figure 1. Quantification of melanin content of melan-c2, melan-mu:MuHA and melan-mu cultured for 7 days in the presence or absence of either 200 nM CT, 100 pM NDP-MSH or 20 μM forskolin. Data are from 3 independent experiments expressed as mean pg melanin/cell ± SEM. Statistical significance relative to the untreated control was determined by one-way ANOVA with Dunnett's multiple comparison test. ns, not significant; **, p<0.01; ***, p<0.001; ****, p<0.0001. Bottom panels show enlarged insets from top panels.

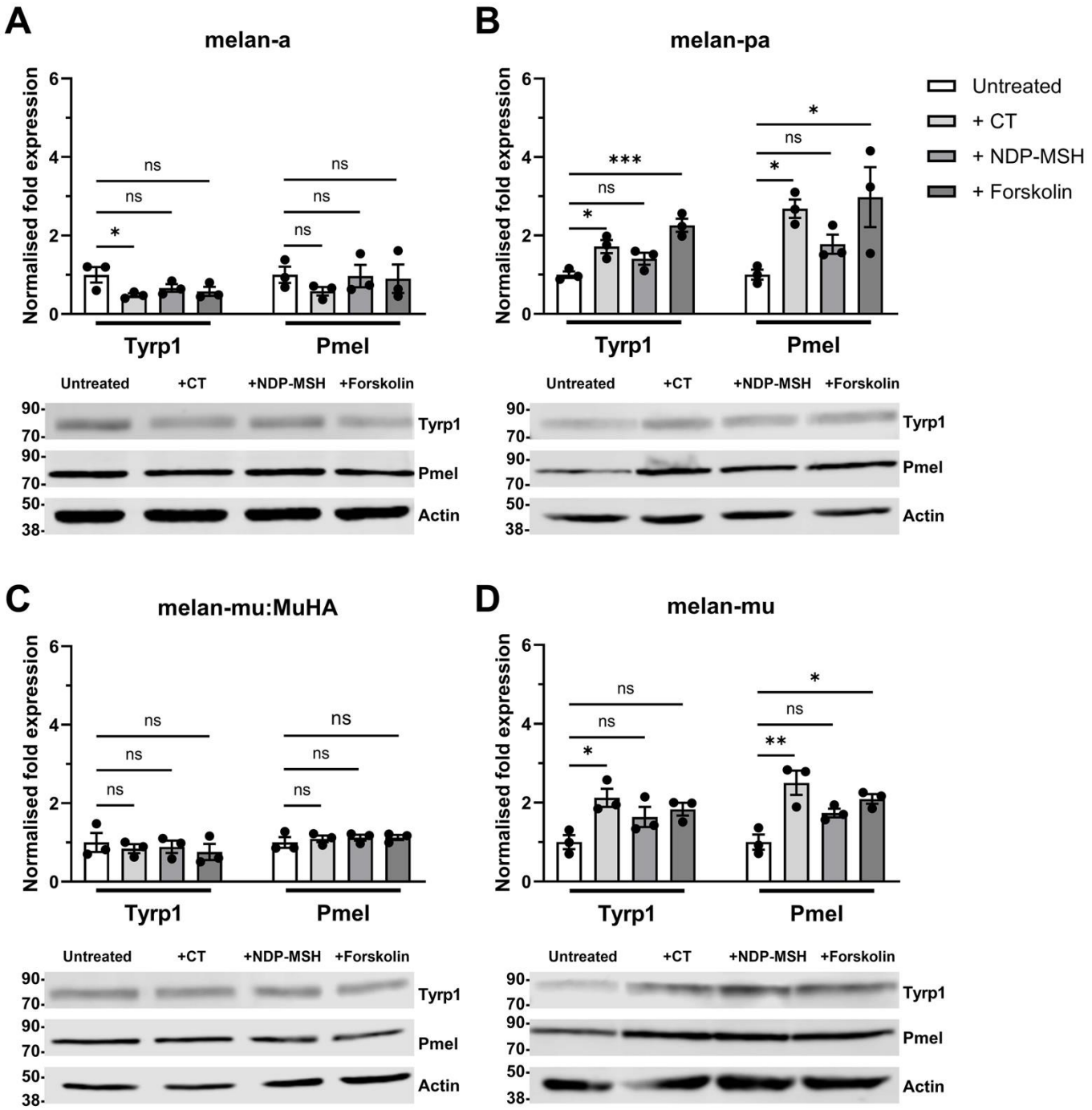

FIGURE S2. TYRP1 and PMEL protein expression is enhanced upon treatment with cAMP elevating agents in BLOC-1-deficient melanocytes. Related to Figure 2. BLOC-1 competent (wild-type melan-a and BLOC-1^{R} melan-mu:MuHA) and BLOC-1-deficient (melan-pa and melan-mu) melanocytes were cultured in the absence or presence of the indicated cAMP-elevating agents for 7 days, and then cell lysates were analysed by western blotting for TYRP1, PMEL, and actin as a loading control. Representative blots are shown at bottom, with positions of MW standards (in kDa) shown at the left (for PMEL, the unprocessed P1 form is shown), and quantifications from 3 independent biological replicates are shown at top. Quantitative data are the mean normalised fold expression of TYRP1 or PMEL protein relative to untreated control ± SEM. Statistical significance relative to untreated control was determined by one-way ANOVA with Dunnett's multiple comparison test. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001.

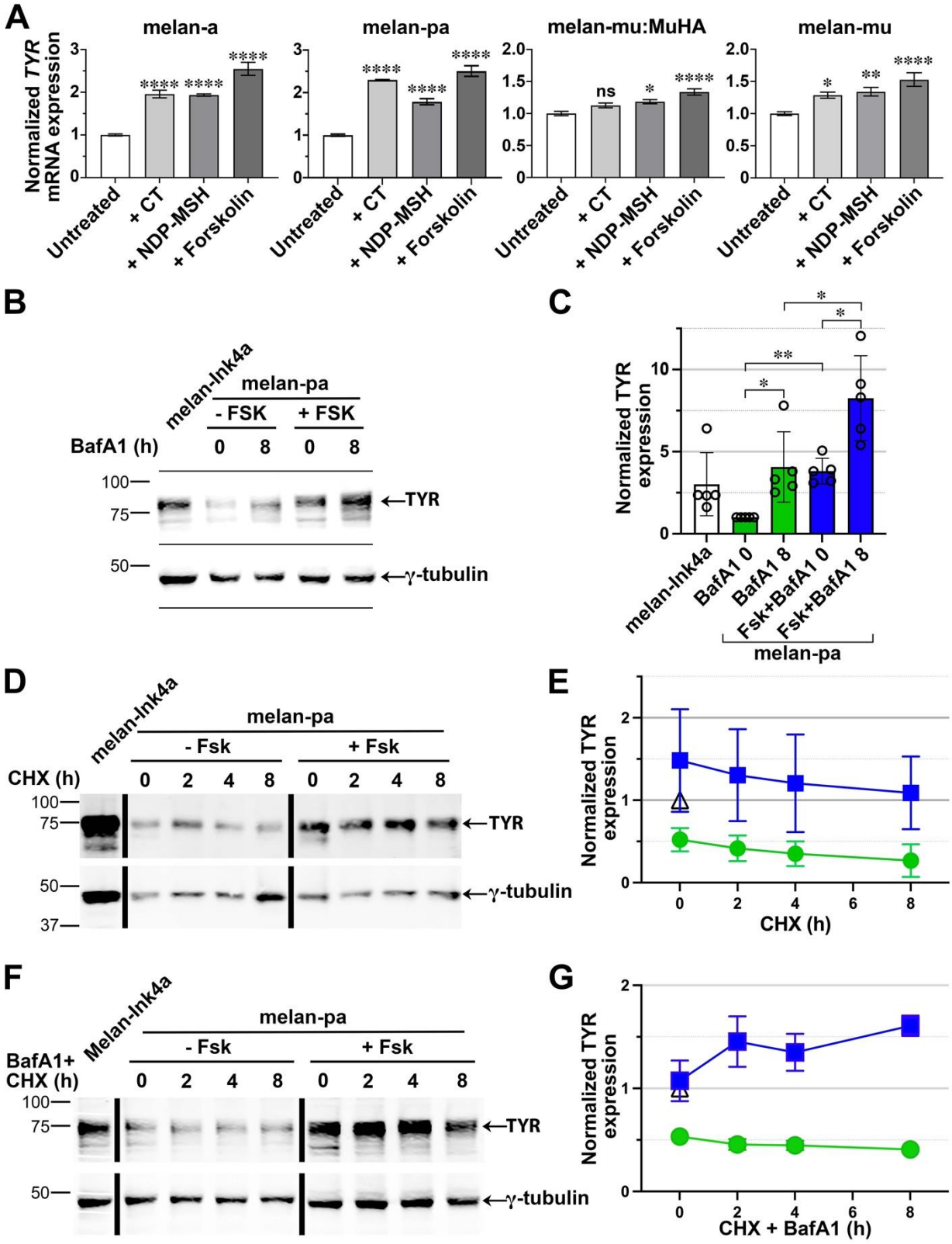

FIGURE S3. Forskolin both enhances TYR synthesis and reduces TYR degradation in BLOC-1-deficient melanocytes. Related to Figure 2. A. TYR mRNA expression of melanocytes treated with cAMP elevating agents. Melan-a, melan-pa, melan-mu:MuHA and melan-mu cells were treated for 24 hours in the presence or absence of either 200 nM CT, 100 pM NDP-MSH or 20 μM forskolin, RNA was isolated, reverse-transcribed into cDNA and analysed by RT-qPCR. Data presented are the geometric means of 3 biological replicates ± SEM normalised to the untreated control. All statistical analyses were performed on log-transformed normalised expression per sample. Statistical significance relative to the untreated control was determined by one-way ANOVA with Dunnett's multiple comparison test. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. **B-G.** Forskolin treatment slows acid-dependent TYR degradation in late endosomes/ lysosomes. BLOC-1$^{-/-}$ melan-pa cells were untreated (green in **C**, **E**, **G**) or treated with 20 μM forskolin (FSK; blue in **C**, **E**, **G**) for at least 7 d. Cells were then treated with 20 nM bafilomycin A1 (BAF) for 0 or 8 hours (**B**, **C**; n=5), with cycloheximide (CHX; 25 μg/ml) for 0-8 hours to assess TYR half-life (**D**, **E**;

n=4), or with both CHX and BAF for 0-8 hours to assess half-life sensitivity to pH neutralisation (**F**, **G**; n=3). Vehicle (DMSO) was added to untreated cells. Cell lysates were fractionated by SDS-PAGE and immunoblotted for TYR and for γ-tubulin as a loading control. Lysates from untreated melan-Ink4a cells (white; similar to melan-a) in all experiments were included for comparison. **B**, **D**, and **F**; representative blots. **C**, **E**, and **G**; TYR and γ-tubulin band intensities in all experiments were measured, TYR levels were first normalised to γ-tubulin levels in each lane and then normalised values were further normalised to values for melan-pa cells **(C)** or melan-Ink4a cells **(E, G)** within each experiment. Shown are mean ± standard deviation, with individual experimental values additionally shown in **C**. In **C**, data were analysed by one-way ANOVA and Tukey's multiple comparisons test; *, $p<0.05$; **, $p<0.01$.

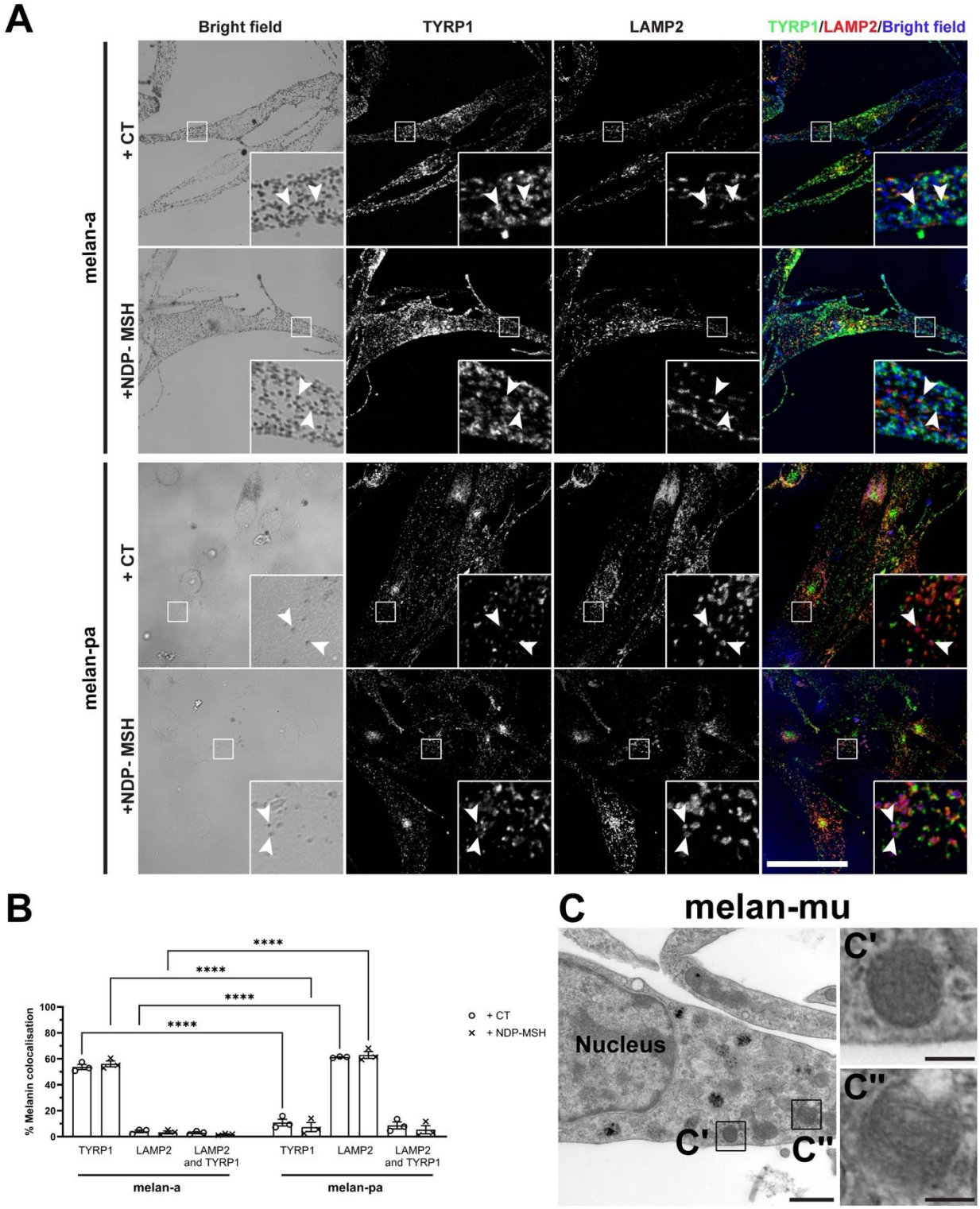

FIGURE S4. Melanin in BLOC-1-deficient melanocytes resides in LAMP2-positive organelles. Related to Figure 3 and Figure 4. A. Wild-type melan-a or BLOC-1-deficient melan-pa cells were treated with 200 nM CT or 100 pM NDP-MSH for 7 days, then fixed, immunolabelled, and analysed by confocal IFM and bright field microscopy to determine the localisation of melanin (visible in bright-field images) in relation to melanosomes (TYRP1, green) and late endosomes/lysosomes (LAMP2, red). The bright-field images were inverted and pseudo-coloured blue in the merged image at the right. Insets, boxed regions enlarged 4.5x. Arrowheads show overlap of melanin with TYRP1 but not LAMP2 in melan-a or with LAMP2 but not TYRP1 in melan-pa. Scale bar, 50 μm. **B.** The percentage of total melanin that colocalised with either TYRP1, LAMP2 or both markers in melan-a and melan-pa treated with 200 nM CT or 100 pM NDP-MSH for 7 days. Data represent mean ± SEM from 3 independent experiments (minimum 10 cells analysed per experiment) with the following total number of cells (CT-treated melan-a 30, NDP-MSH-treated melan-a 31, CT-treated melan-pa 32, NDP-MSH-treated melan-pa 30). Statistical significance was determined by one-way ANOVA with post-hoc Tukey test with only significant equivalent comparisons between cell lines shown. ****, $p<0.0001$. **C.** Transmission electron microscopy image of untreated BLOC-1-deficient melan-mu cells. Insets (C' and C") show 5x enlarged images of boxed regions in main panels representing lysosomes without melanin. Scale bar in main panel, 600 nm. Scale bar in insets, 150 nm.

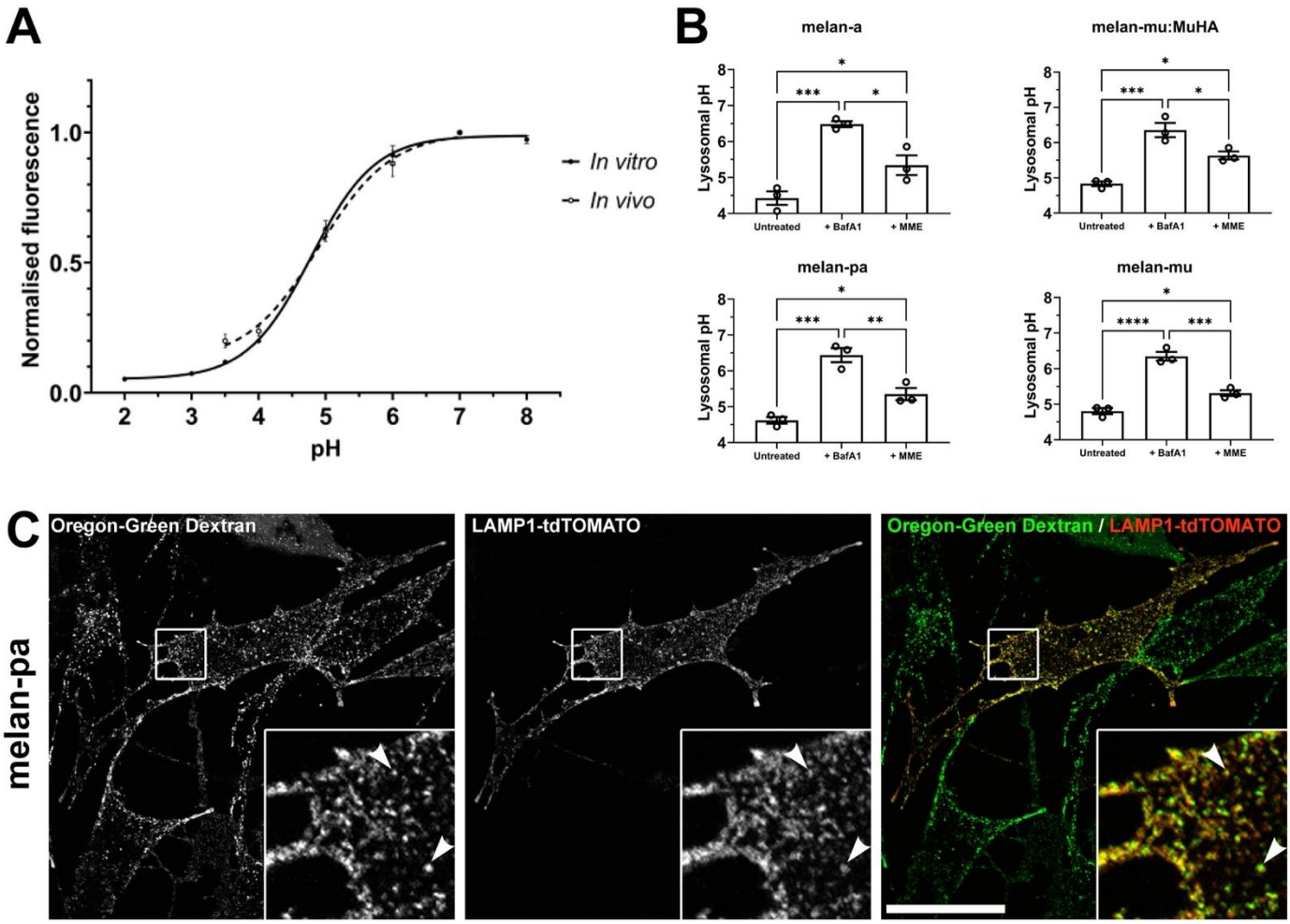

FIGURE S5. Oregon Green-Dextran is pH sensitive and labels late endosomes and lysosomes. Related to Figure 5. A. *In vitro* measurements were performed by diluting 50 µg/ml Oregon Green-Dextran in calibration buffers titrated to known pH values (pH 2.0-8.0) and measuring fluorescent intensity using a fluorometer. *In vivo* measurements were obtained using the wild-type melan-a melanocytes that had internalised Oregon Green-Dextran for 16 hours followed by a 2-hour chase period. Cells were then treated with 5 µM monensin and 5 µM nigericin in calibration buffers titrated to known pH values (pH 3.0-8.0) before measuring fluorescent intensity. Both *in vitro* and *in vivo* fluorescence data were normalised to the maximum fluorescence values at pH 7.0 (Oregon Green). A four-parameter dose response curve is shown for each condition. Data represent mean ± SEM of 3 independent experiments. **B.** Validation of fluorometric Oregon Green-Dextran assay. BLOC-1 competent (melan-a and melan-mu:MuHA) and BLOC-1-deficient (melan-mu and melan-pa) melanocytes were cultured for 7 days before treatment with either 20 nM BafA1 (for final 6 hours) or 10 mM MME (for final 24 hours), and then exposed to Oregon Green-Dextran overnight and chased for two hours to label late endosomes and lysosomes. Organellar pH was determined by fluorometry based on resting fluorescence relative to maximal fluorescence observed after exposure to ammonium chloride. Data represent mean ± SEM from 3 independent experiments. For each cell line, statistical analysis was performed by one-way ANOVA with post-hoc Tukey test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. **C.** BLOC-1-deficient melan-pa melanocytes were transiently transfected to express LAMP1-tdTomato and then exposed to Oregon Green-Dextran for 16 hours followed by a 2-hour chase period. Cells were then fixed and imaged by confocal microscopy. Shown is a representative image for each individual label and a merged image at right. Arrowheads point to examples in which labelling for Oregon Green and LAMP1-tdTomato overlap. Scale bar, 50 µm.

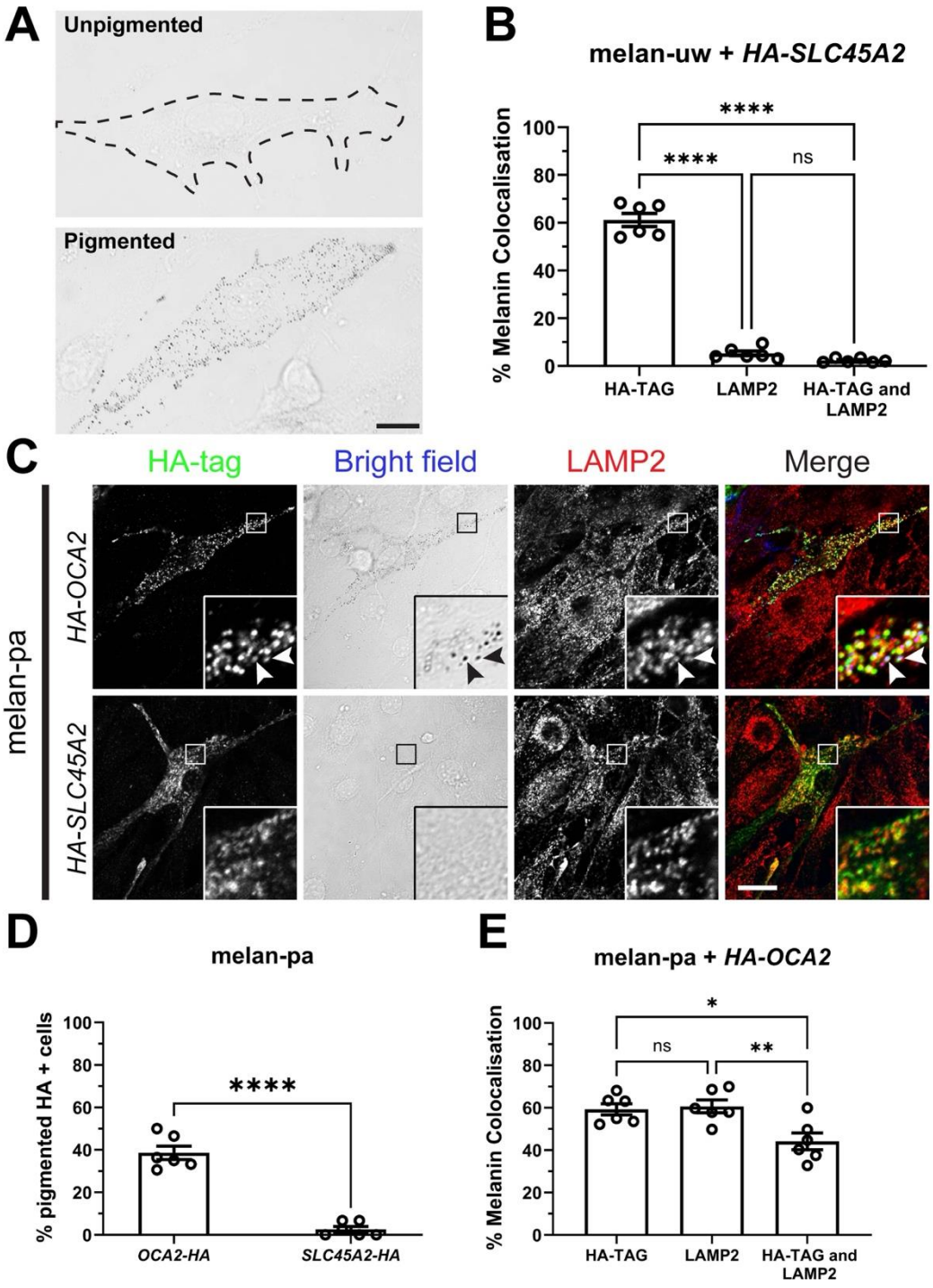

FIGURE S6. Overexpression of OCA2 in BLOC-1-deficient melanocytes partially restores pigmentation. Related to Figure 6. A. Examples of unpigmented and pigmented BLOC-1-deficient (melan-mu) cells. Scale bar, 10 μm. **B.** Quantification of the percentage of total melanin in melan-uw expressing HA-SLC45A2 that colocalised with HA-tag, LAMP2 or both markers. Data represent mean ± SEM from 6 independent experiments with a total number of 32 cells analysed. Statistical significance was determined by one-way ANOVA with post-hoc Tukey test. Ns, not significant; ****, p<0.0001. **C-E.** BLOC-1-deficient melan-pa melanocytes were transiently transfected with HA-OCA2 or HA-SLC45A2, fixed, immunolabelled for HA and LAMP2 and analysed by confocal IFM and bright-field microscopy to visualise melanin. **C.** Shown are each individual label and a merged image at right which the bright field image was inverted and pseudo-coloured blue. Arrowheads indicate overlap between HA tag, LAMP2 and melanin in cells overexpressing HA-OCA2. Insets of boxed regions are enlarged 4.5x. Scale bar, 20 μm. **D.** Quantification of the percentage of HA positive melan-pa cells that were pigmented. Data represent mean ± SEM from 6 independent experiments. Statistical significance relative to the % pigmented SLC45A2 HA positive cells was determined by two-tailed Student's t-test. ****, p<0.0001. **E.** Quantification of the percentage of total melanin in melan-pa expressing HA-OCA2 that colocalised with HA-tag, LAMP2 or both markers. Data represent mean ± SEM from 6 independent experiments with a total number of 30 cells analysed. Statistical significance was determined by one-way ANOVA with post-hoc Tukey test. ns, not significant; *, p<0.05; **, p<0.01.

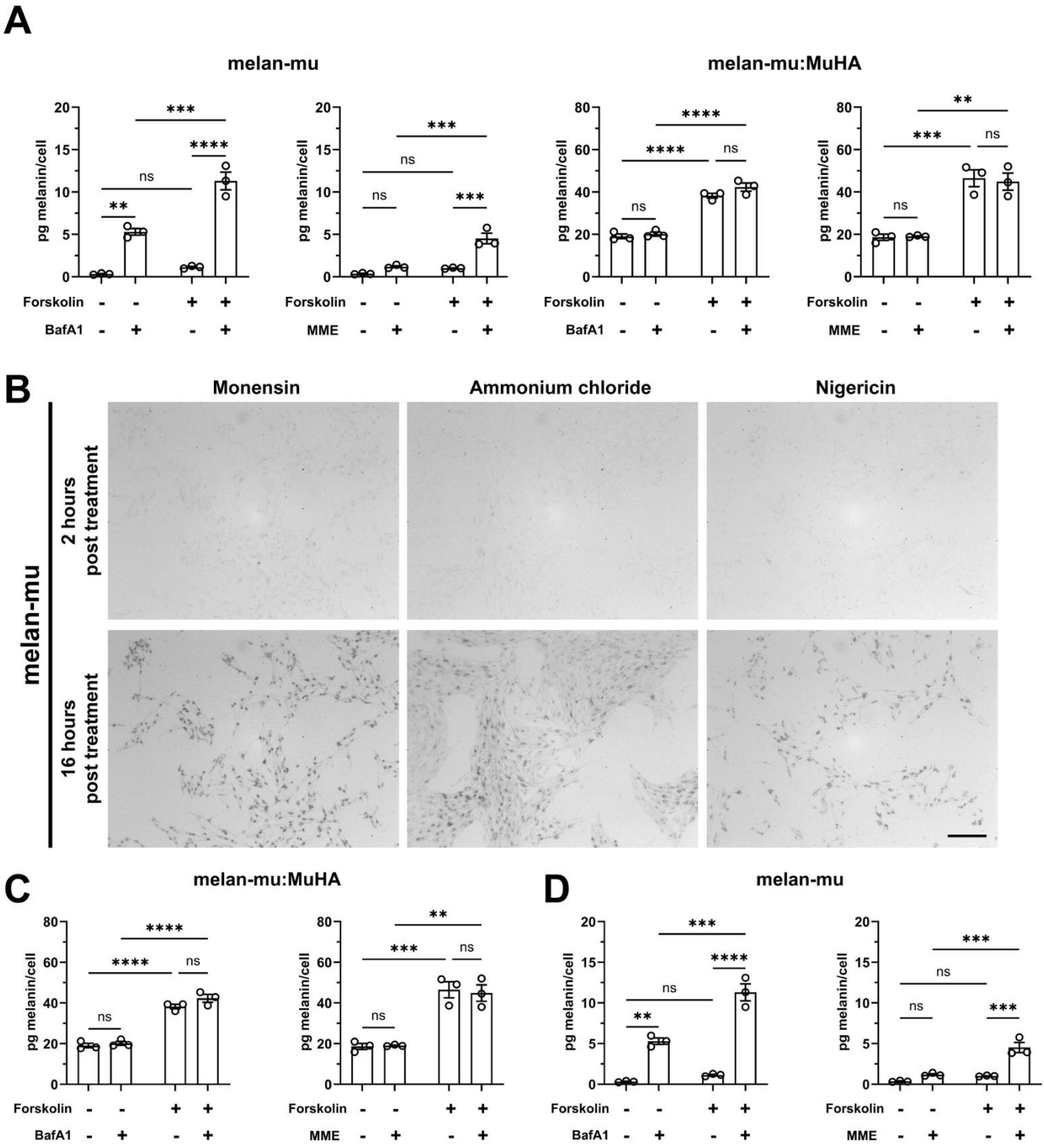

FIGURE S7. Deacidification agents significantly restore melanin to BLOC-1-deficient melanocytes. Related to Figure 7. A. Bright field microscopy image of BLOC-1-deficient melan-mu melanocytes treated for 24 hours with 10 mM MME. Inset of boxed region is enlarged 4.5x. Scale bar, 50 μm. **B.** Bright field microscopy images of BLOC-1-deficient melan-mu melanocytes treated with either 20 mM ammonium chloride, 2 μM monensin, or 2 μM nigericin. Images were taken at 2 and 16 hours post treatment. Scale bar, 200 μm. **C, D.** Quantification of melanin content of BLOC-1^{R} (melan-mu:MuHA) **(C)** or BLOC-1-deficient (melan-mu) **(D)** melanocytes cultured for 7 days in the absence or presence of 20 μM forskolin and either 20 nM BafA1 (for final 6 hours) or 10 mM MME (for final 24 hours). Data are from 3 independent experiments and are expressed as mean pg melanin/cell ± SEM. Statistical significance was determined by two-way ANOVA with a post-hoc Tukey test. ns, not significant; **, p<0.01; ***, p<0.001; ****, p<0.0001.