Abrogation of RAB27A expression transiently affects melanoma cell proliferation

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Abstract

The role of the small GTPase RAB27A as an essential melanosome trafficking regulator in melanocytes is well-accepted. Based on copy number and gene expression data in early passage melanoma cell lines, RAB27A was identified as a driver gene that promotes melanoma progression. RAB27A has also been linked to another propeller of cancer progression: exosome secretion. We have recently demonstrated that RAB27A is overexpressed in a subset of melanomas. High RAB27A gene and protein expression correlates with poor prognosis in melanoma patients. Mechanistic investigations revealed that the generation of pro-invasive exosomes was RAB27A-dependent and, therefore, silencing RAB27A reduced melanoma cell invasion *in vitro* and *in vivo*. However, previous studies have implicated RAB27A to be involved in both proliferation and invasion of melanoma cells. In this study, we demonstrate that the effects of abrogating RAB27A expression on proliferation are temporary, while the effects on tumor invasion and metastasis are persistent. Herein, we dissect the short-term versus long-term effects of RAB27A knockdown on melanoma cell proliferation, invasion, and metastasis. We believe that our findings provide novel insights into the effects of RAB27A blockade.

Significance

RAB27A is known to serve as an essential regulator for melanosome trafficking. However, to date its role in melanoma biology has not been completely deciphered. While there are consistent independent reports on the pro-invasive effects of RAB27A, there are conflicting data on its impact on cell proliferation. Here we show that indeed abrogation of RAB27A reduces both proliferation and invasion. However, while the effect on proliferation is only transient, the impact on invasion is persistent. This finding offers an explanation for an apparent contradiction in the literature and provides a deeper understanding of RAB27A function in melanoma cell biology.

Main Text

The small GTPase RAB27A is a well-recognized regulator of intracellular signaling and mediator of melanosome trafficking in melanocytes (Strom et al., 2002). Past studies have linked RAB27A to promoting melanoma progression. Employing copy number and gene expression analysis, RAB27A has been identified as a driver gene in a subset of melanomas that express high levels of RAB27A (Akavia et al., 2010). Furthermore, Peinado and colleagues have demonstrated that abrogation of RAB27A expression leads to reduced exosome secretion in melanoma cells and thereby suppresses melanoma metastasis. They further established that the pro-tumoral function of RAB27A is mediated by tumor cell-derived exosomes that home to the bone marrow and educate the bone marrow progenitor cells towards a more vasculogenic phenotype, thus ultimately assisting in the creation of a pre-metastatic niche (Peinado et al., 2012). We have recently dissected the role of RAB27A in melanoma progression and metastasis (Guo et al., 2019a, Guo et al., 2019b). Our study revealed that both *RAB27A* RNA and protein expression levels correlated with poor survival in melanoma patients (Guo et al., 2019a). Moreover, the abrogation of RAB27A expression in melanoma cells led to a marked reduction in tumor invasion and metastasis both *in vitro* and *in vivo* (Guo et al., 2019a). We found that this was caused by the reduction in the secretion of pro-invasive exosomes, even though the total number of secreted exosomes remained the same (Guo et al., 2019a). These findings identified RAB27A as a key cancer regulator, as well as a potential prognostic marker and plausible therapeutic target for the treatment of advanced melanoma.

Various studies to date have implicated RAB27A in promoting cell proliferation and tumor growth in many types of cancers, including melanoma (Akavia et al., 2010, Peinado et al., 2012), bladder (Liu et al., 2017), breast (Bobrie et al., 2012) and pancreatic cancer (Li et al., 2017). A recent study

demonstrated that abrogation of *RAB27A* expression caused a reduction in RAB27A-mediated exosome secretion, which resulted in a cell proliferation defect (Li et al., 2019). In concordance with these studies, we have also found a proliferation defect post abrogation of *RAB27A* expression in melanoma cells. However, we show that this defect in proliferation is transient (this study), while the defects in cell invasion and metastasis were persistent (Guo et al., 2019a). This suggests that the effects of *RAB27A*-knockdown on melanoma cell proliferation may be more complex than initially anticipated.

To understand the role of RAB27A in melanoma cell proliferation, we abrogated *RAB27A* expression in RAB27A-high melanoma cell lines, namely WM164 and WM983C, using shRNA-expressing lentiviral particles. *RAB27A*-knockdown (KD) with two different shRNAs initially caused modest cell death and more rounded cell morphology (Figure 1A). This was not a transduction artifact as cells transduced with the non-targeting shRNA showed no such effect. However, after three passages (approximately 4 weeks), the surviving WM164 and WM983C *RAB27A*-KD cells started to regain normal morphology (Figure 1B) and F-actin structure (Figure 1C). Immunoblotting showed that the surviving *RAB27A*-KD cells had maintained RAB27A abrogation (Figure 1D, E).

Early passage (passages 3-5 after transduction) WM164 and WM983C *RAB27A*-KD cells showed proliferation and colony-forming defects (Figure 2A, B, C) that were gradually lost at high passage numbers after transduction (≥7 passages; Figure 2D, E, F). This was not due to the reverting of RAB27A expression, as RAB27A protein levels remained low in the high passage shRNA expressing cells (Figure 1D, E).

Because the normalization of proliferation and colony-forming defects could not be attributed to the recovery of *RAB27A* expression, we anticipated compensation by other members of the RAB GTPase family to be responsible for these observations. Since it has been reported that RAB27B can compensate for the loss of RAB27A (Barral et al., 2002), we analyzed the expression of *RAB27B* mRNA via quantitative real-time PCR. We found that the expression of *RAB27B* in late passage *RAB27A*-KD cells was not significantly elevated compared to the control cells (Figure 3A). Thus, compensation for the loss of RAB27A in late passage cells could not be conclusively attributed to an increase in *RAB27B* expression.

To determine the effect of the loss of RAB27A on survival and proliferation in a melanoma cell line endogenously expressing low levels of RAB27A protein, we performed shRNA-KD in C8161 cells. Western blotting confirmed that *RAB27A* shRNA-expressing cells had substantially reduced RAB27A (Figure 3B). *RAB27A*-KD did not affect cell morphology, cell death, or proliferation in C8161 cells (Figure 3C, D). This indicates that RAB27A-low melanoma cells do not rely on RAB27A for proliferation or survival. *RAB27A*-KD (Figure 3B) in a melanoma cell line that has intermediate levels of RAB27A (1205Lu) did not cause cell death (Figure 3C). However, similar to WM983C and WM164, *RAB27A*-KD in 1205Lu cells led to a reduced proliferation phenotype (Figure 3D).

To further validate that the effect of abrogating RAB27A expression on proliferation is only transient, we carried out a complete knockout of *Rab27A* in B16-F10 melanoma cells employing CRISPR-Cas9 technology (Figure 3E). Single cell clones of *Rab27A* knockout B16-F10 were generated and assessed for proliferation. Similar to WM164 and WM983C, no significant differences in proliferation post *Rab27A* KO were observed (Figure 3E). However, the capacity of

B16-F10 cells to metastasize had significantly reduced post *Rab27A* KO *in vivo* as reported in our previous study (Guo et al., 2019a).

Recent reports have linked RAB27A with the regulation of cell cycle genes in other cancer types (Feng et al., 2017, Liu et al., 2017). Therefore, we carried out *RAB27A*-KD in FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) expressing WM164 cells (Haass et al., 2014, Spoerri et al., 2017) and examined the proportion of cells within each phase of the cell cycle. Interestingly, we found no statistically significant difference in the cell cycle profile between control and *RAB27A*-knockdown cells (Figure 3F), which suggested that the transient proliferation defect caused by *RAB27A*-knockdown was not related to skewing of the cell cycle.

In this study, we demonstrate that *RAB27A*-knockdown reduced cell proliferation and colony formation potential and had a modest effect on survival in metastatic melanoma cell lines that express high levels of RAB27A. However, in contrast to the persistent reduction in cell invasion and metastasis post abrogating *RAB27A* expression as reported previously (Guo et al., 2019a), we find that all melanoma cell lines tested regained proliferative potential over time. Here we offer an explanation for the apparent contradiction to previous studies by others demonstrating that abrogating *RAB27A* expression in melanoma and other cancer cells reduces cell proliferation and colony formation (Akavia et al., 2010, Bobrie et al., 2012, Li et al., 2019, Li et al., 2017, Liu et al., 2017). The possible reason for these differences could be due to the methodologies employed. Two studies used siRNA-based transient transfection to demonstrate that knocking down RAB27A induced modest increase of apoptosis, enhanced sensitivity to cisplatin and reduced the invasive and proliferative capacity of pancreatic (Li et al., 2017) or bladder cancer cells (Liu et al., 2017). An elegant study demonstrated that shRNA-expressing lentiviral mediated RAB27A blockade in

breast cancer cells (mammary carcinoma 4T1) led to a reduced secretion of exosomes and reduced the primary tumor growth as well as metastasis to lungs. However, the authors clearly state that they used these cells within a one-month period post-lentiviral transduction (Bobrie et al., 2012). The seminal study, which identified RAB27A as a driver gene in melanoma, also demonstrated reduced proliferation post shRNA mediated knockdown of RAB27A in RAB27A-high cells (Akavia et al., 2010). However, they also looked at earlier time-points as they measured growth till day 6 post-knockdown. In addition, they found changes in cell cycle genes after *RAB27A*-knockdown (Akavia et al., 2010). Notably, we also observed *CDK2* as a gene associated with RAB27A-high melanoma clinical samples in our recent study (Guo et al., 2019a). However, here we discovered that the cell cycle profile did not change significantly after *RAB27A*-knockdown. In summary, most of these studies use *RAB27A*-abrogated cells before the compensation that we describe here has fully taken effect. It is reasonable to use freshly transduced/transfected cells to ensure efficient knockdown of *RAB27A*, but in our study, we confirmed the persistent *RAB27A* knockdown after culturing the cells for multiple passages (more than a month) and discovered loss of the proliferation defect. Moreover, we confirmed these findings by CRISPR-mediated knockout of *Rab27a* in murine melanoma cells. This proves the existence of a compensation mechanism specifically for the proliferation defect rather than simply loss of RAB27A knockdown over time. We hypothesized that the proliferation defect post RAB27A downregulation is mediated through the upregulation of another RAB-family member. However, in our study, the closely related RAB27B did not appear to be responsible for the rescue of the proliferation defect. Importantly, the defects in cell invasion and metastasis post-abrogating RAB27A expression, also seen in the studies discussed above, were found to be persistent (Guo et al., 2019a).

In conclusion, although RAB27A is an important promoter of melanoma progression, our study demonstrates that its effects on cell proliferation and survival, in contrast to migration, invasion and metastasis (Guo et al., 2019a), are not persistent. This finding provides a deeper understanding of RAB27A function in melanoma cell biology.

Materials and Methods:

Cell lines. The human melanoma cell lines C8161, 1205Lu, WM164 and WM983C were genotypically characterized (Davies et al., 2009, Smalley et al., 2007a, Smalley et al., 2007b) and grown as described (Spoerri et al., 2017). Cell line authentication was accomplished by STR fingerprinting (Molecular Genetics facility, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia). Stable expression of Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI) in WM164 cells was performed as previously described (Haass et al., 2014, Spoerri et al., 2017). Mycoplasma contamination in all cell lines was ruled out via PCR profiling at the Garvan Institute of Medical Research.

Lentiviral transductions were performed as previously described (Guo et al., 2019a). Briefly, *RAB27A* shRNA or non-target shRNA lentivirus particles (MISSION, Sigma/Merck) were produced using the calcium phosphate precipitation method in HEK293T cells. Melanoma cell lines were transduced with lentivirus and 4µg/mL polybrene followed by selection with puromycin (2µg/mL).

Proliferation assays. Cells were seeded at a density of 20,000 cells/well in 12-well plates (triplicate wells per condition), trypsinized and counted by hemocytometer on days 1-4 after seeding.

Rab27A CRISPR. Rab27A CRISPR knockout was performed as previously described (Guo et al., 2019a). Briefly, guide RNAs targeting *Rab27A* were designed as described (Ran et al., 2013) and cloned into the pX330-IRES-GFP vector. Melanoma cell lines were transfected with the vector alone and plasmids encoding *Rab27A* gRNA. Post-transfection, GFP positive cells were FACS sorted. Single cell cloning was then achieved by serial dilution. The clones were expanded and *Rab27A* knockout was confirmed by western blotting.

Colony-formation assays. 2000 cells (duplicate wells per condition) were seeded in 12-well plates, with culture medium changed twice weekly. After 2 weeks of growth, colonies were gently washed with PBS, fixed on ice with methanol for 15 min, and then stained with 0.5% crystal violet/methanol for 15 min. Excess stain was removed by carefully washing with distilled water. Plates were left to dry at RT for 1h and then scanned using a color scanner (Konica Minolta Bizhub C360). Quantification of colony formation was determined by percentages of stained area/total area per well using ImageJ software.

Western blotting was performed as previously described (Guo et al., 2019a). The following antibodies were used: mouse anti-human RAB27A (WH0005873M2, Sigma-Aldrich, Merck KGaA), rat anti-tubulin (Ab6160, Abcam, Cambridge, MA, USA). Membranes were washed with TBS-T for 30min, then probed with the appropriate secondary HRP conjugated antibody (Sigma) for 1-2h at room temperature. Proteins were visualized with SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific) and the ChemiDoc MP System (BioRad).

Conflict of interest

The authors declare no conflict of interest.

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Figures

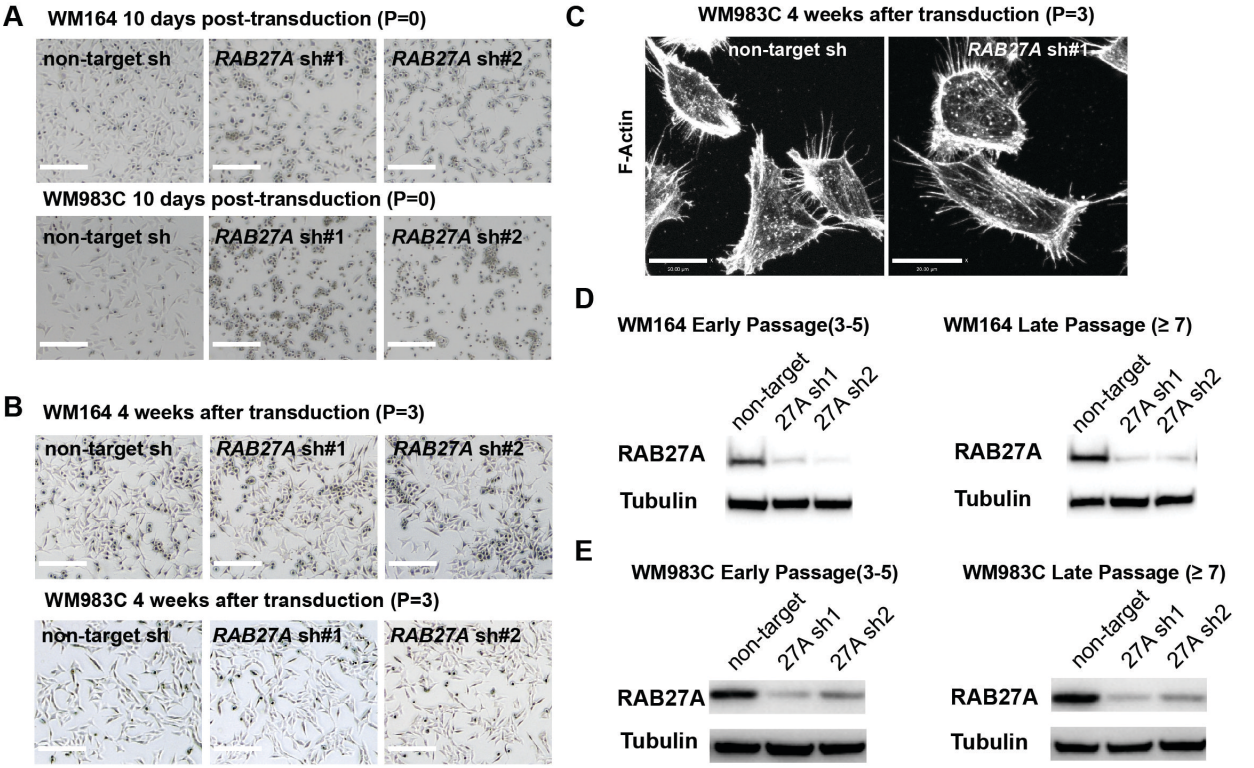

Figure 1. *RAB27A*-knockdown in RAB27A-high melanoma cell lines causes cell death and morphology alteration only in early passages. (A) Phase contrast images of WM164 and WM983C melanoma cells approximately 10 days after transduction with *RAB27A* shRNA or control lentivirus and 8 days after selection with puromycin. *RAB27A* shRNA KD cells are at Passage 0 after transduction. Note that all untransduced cells were dead after 2-3 days of puromycin selection. Scale bars = 100µm. Representative of at least 3 independent experiments. **(B)** Phase contrast images of WM164 and WM983C melanoma cells approximately 4 weeks (3 passages) after transduction with *RAB27A* shRNA or control lentivirus Scale bars = 100µm. Representative of at least 3 independent experiments. **(C)** Confocal extended focus image of F-actin staining in WM983C cells transduced with *RAB27A* shRNA or non-targeting shRNA (passage 3 after transduction). Representative of 3 independent experiments. **(D, E)** Western blots showing the level of RAB27A protein in WM164 (**D**) and WM983C cells (**E**), non-targeting shRNA control (non-target), *RAB27A* shRNA1 (27A sh1) and *RAB27A* shRNA#2 (27A sh2) (early = passage 3-5 after transduction, late ≥ passage 7). Representative of at least 3 independent experiments.

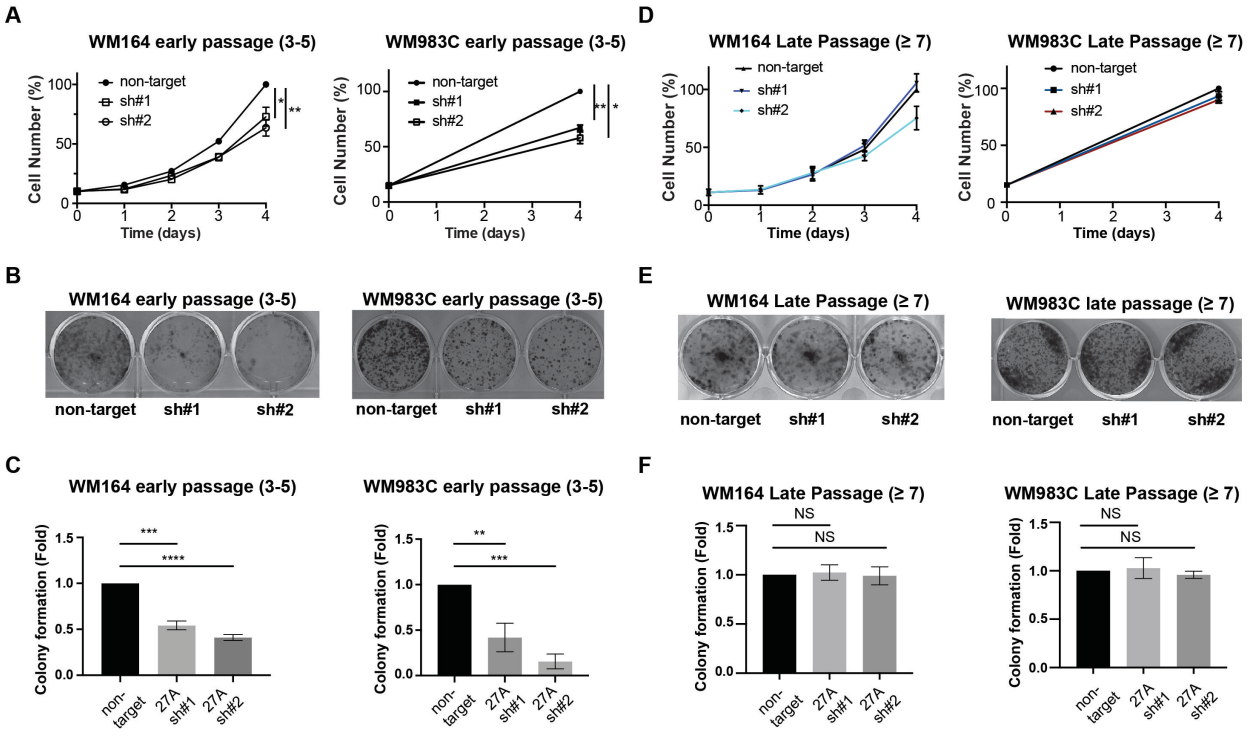

Figure 2. *RAB27A*-knockdown in RAB27A-high melanoma cell lines reduces proliferation and colony formation potential only in early passages. (A) Normalized cell counts over the indicated days in early passage (p3-5) WM164 or WM983C cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test at day 4. **(B)** Colony-forming assay of early passage (p3-5) WM164 or WM983C cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Representative of 3 independent experiments. **(C)** Quantification of colony formation of early passage (p3-5) WM164 or WM983C cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test. **(D)** Normalized cell counts over the indicated days in late passage (passage ≥ 7) WM164 cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test and no significance was detected. **(E)** Colony-forming assay of late passage (≥ 7 passage) WM164 and WM983C cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Representative of 3 independent experiments. (**F**) Quantification of colony formation of late passage (≥ 7 passage) WM164 or WM983C cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test.

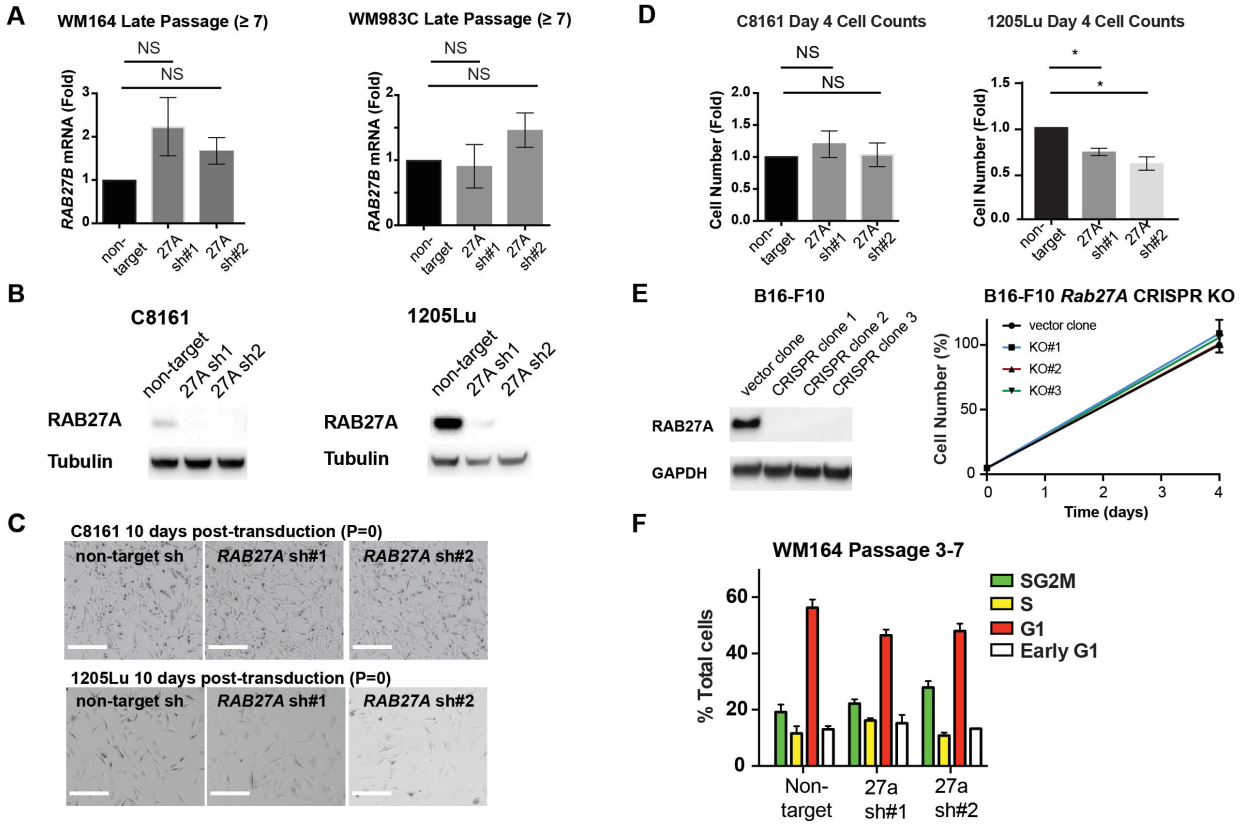

Figure 3. *RAB27A*-knockdown does not alter *RAB27B* expression, cell cycle profile as well as proliferation and colony formation in RAB27A-low melanoma cell lines. (A) Real time PCR TaqMan analysis of *RAB27B* mRNA expression in WM164 and WM983C cells (≥ 7 passage) transduced with the indicated *RAB27A* shRNA or control constructs. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test and no significance was detected. NS, not significant. **(B)** Western blots showing the level of RAB27A protein in early passage (passage 3-5 after transduction) C8161 and 1205Lu, non-targeting shRNA control, *RAB27A* shRNA#1 (27A sh1) and *RAB27A* shRNA#2 (27A sh2) cells. Representative of at least 3 independent experiments. **(C)** Phase contrast images of C8161 and 1205Lu melanoma cells approximately 10 days after transduction with *RAB27A* shRNA or control lentivirus and 8 days after selection with puromycin. Scale bars = 100µm. Representative of at least 3 independent experiments. **(D)** Normalized cell counts over the indicated days in early passage (passage 3-5 after transduction) C8161 and 1205Lu cell lines expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test. No significance was detected in C8161 cell line comparisons. (**E**) Western blots showing the levels of Rab27A (left) and proliferation assay (right) of B16-F10 empty vector control, *Rab27A* CRISPR-knockout cells: clones 1, 2 and 3 (KO#1, KO#2 and KO#3). Data was generated from 3 independent experiments. **(F)** Percentages of cells within each stage of the cell cycle of WM164 cells expressing the non-targeting shRNA control, *RAB27A* shRNA#1 or *RAB27A* shRNA #2. Error bars represent the

mean ± SEM of at least n=3 independent experiments. Non-targeting control was compared to the other samples via ANOVA and a Dunnett's test and no significance was detected.