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Revealing the role of RAB27 in HER receptor family expression and signaling in melanoma cells

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Abstract

Background Alterations in signalling pathways fuel the growth and progression of melanoma. Therefore, understanding these processes is essential for developing effective treatment strategies. RAB27A and RAB27B are known to possess oncogenic effects by modulating cancer cell proliferation, invasion and drug resistance in various types of cancer, including melanoma. These proteins are mostly acknowledged as coordinators of the vesicular trafficking, however, their function in cellular signaling is less recognized. Therefore we aimed to investigate the relationship between RAB27 and oncogenic or signalling proteins in melanoma cells.

Methods We generated RAB27A knockout (KO) in SkMel28, A375, and patient-derived DMBC12 melanoma cell lines. Additionally, a double RAB27A/B knockout (dKO) A375 cell line was created. Firstly, we applied the Proteome Profiler array to identify proteins differentially expressed upon RAB27A/B loss. Subsequently, we picked selected specific proteins for a further in-depth analysis using RT-PCR, Western blot, and flow cytometry.

Results We found that silencing RAB27 markedly decreased the levels of various intracellular proteins linked with proliferation, invasion, angiogenesis, adhesion, or EMT at a cell-line dependent level. Among others, we observed a link between the expression of RAB27 and EGFR, HER2 and HER3. Altered levels of HER receptors disturbed the downstream signaling pathways by reducing the phosphorylation of AKT and ERK1/2 proteins.

Conclusions Our findings present novel, previously unpublished data on the relationship between HER family receptor expression and potential activity, and the involvement of RAB27 in melanoma cells.

Keywords Melanoma, RAB27, Signaling, HER receptors

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Background

Melanoma is the most aggressive type of skin cancer that originates from the malignant transformation of melanocytes. Early-stage melanoma can often be successfully treated by surgical intervention, however, the prognosis becomes poorer as the disease progresses to metastatic stages [1, 2].

Two isoforms of a small GTPase - RAB27A and RAB27B were reported to contribute to progression and metastasis of various cancers, including melanoma [3, 4]. Its increased expression was associated with the occurrence of different types of cancer and worse patient survival [3]. RAB27A and RAB27B are mostly acknowledged as vesicle trafficking regulators, that coordinate the release of small extracellular vesicles (exosomes) in almost all cell types, as well as more cell-specific organelles such as melanosomes in melanocytes, dense granules in platelets or synaptic vesicles in neuronal cells [5]. Additionally, RAB27A and RAB27B control the secretion of prooncogenic soluble factors (i.e. cytokines, metalloproteinases) and modulate the functioning of cancer cells and the tumor microenvironment [6]. By silencing one or both isoforms, RAB27 was demonstrated to promote the growth of various types of cancer, including melanoma. Silencing of *RAB27A* suppressed the proliferation [7] and invasion [8] of melanoma cell lines in vitro and tumor growth and metastasis in mice [8, 9]. Nevertheless, little is known about the interactions between RAB27 and oncogenic proteins in melanoma cells. *RAB27A* knockdown in melanoma cell lines upregulated genes annotated to the extracellular signal-regulated kinases (ERK) pathway, such as *MYC*, *FOSL1*, and *DUSP6* [7], while decreased the secretion of PIGF-2, PDGF-AA, and osteopontin [9]. Silencing of *RAB27B* inhibited phosphatidylinositol 3-kinases (PI3K)/ serine/threonine-protein kinase (AKT) activation in hepatocellular carcinoma cells [10] and ERK1/2 activation in erythroblasts [11], however, it was not studied in melanoma cells. Therefore we decided to investigate how the knockout of *RAB27* affected the signalling pathways in two commercially available and one patient-derived melanoma cell lines.

In this study, we demonstrate the altered level of oncogenic and signaling proteins in *RAB27A* KO (KO) or *RAB27A/B* KO (dKO) melanoma cells. Our findings also show that RAB27 affects epidermal growth factor receptor (EGFR) and human epidermal growth factor receptors 2 and 3 (HER2, HER3) receptors, which resulted in the AKT and ERK1/2 signaling proteins reduced phosphorylation. However, we point out that RAB27-related changes in signaling proteins occurred in a cell line-dependent manner. In general, this study sheds light on an unknown relationship between RAB27 and HER family receptor functioning.

Methods

Cell culture

The human melanoma SkMel28 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). The human melanoma A375 and patient-derived DMBC12 cell lines were a kind gift from Prof. Malgorzata Czyz (Medical University, Lodz, Poland). The A375 cell line was authenticated by Eurofins Genomics (Europe Applied Genomics GmbH, Ebersberg, Germany). All cell lines were maintained in RPMI 1640 medium (Corning, NY, USA) containing 10% filtered fetal bovine serum (FBS) (EurX, Gdansk, Poland), 100 U/mL penicillin (Corning, NY, USA), 100 µg/mL streptomycin (Corning) and 2 mM L-glutamine (Corning). Cells were cultured at 37 °C and 5% CO_2.

Generation of CRISPR-Cas9 knockout cell lines

Cells were transfected with the ribonucleoprotein complexes containing Cas9 nuclease (Thermo Fisher Scientific, Waltham, MA, USA) and a single guide RNA (sgRNA) (Horizon Discovery, Lafayette, CO, USA) targeting *RAB27A* or *RAB27B* in a 1:1 molar ratio. Four sgRNAs were tested to provide the best efficiency. In addition, a positive control (sgRNA targeting human *PPIB* gene) and a negative control (sgRNA not targeting human genome) were used. Ribonucleoproteins were delivered with Lipofectamine CRISPRMAX Cas9 transfection reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, the reagents were added to the Opti-MEM medium (Gibco/Thermo Fisher Scientific) and incubated for 5–10 min at room temperature. Complexes were added to cells and cultured for 2–3 days at 37 °C. Transfected cells were seeded in 96-well plates at a density of 0.5 cells/well to obtain homozygous cell lines. After the expansion of single cell clones the loss of RAB27A or RAB27B protein was verified by Western blot. Mutations at the target site were confirmed by Sanger sequencing.

RNA isolation and quantitative real-time reverse transcriptase PCR (RT-PCR)

Total RNA was isolated using TriPure Isolation Reagent (Roche Diagnostics GmbH, Mannheim, Germany) and quantified using NanoDropTM ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using iTaq Universal SYBR Green One-Step Kit (BioRad, Hercules, CA, USA), according to the manufacturer's instructions. Cycle thresholds were normalized to the reference gene (*GAPDH*). Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ formula. The sequences of the primers used are listed in the supplementary Table 1.

Western blot

Cells were pelleted, washed with ice-cold phosphate-buffered saline (PBS) twice, and lysed in RIPA lysis buffer (Sigma Aldrich, St. Louis, MO, USA) or a buffer containing Tris (20 mM), NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), Triton X-100 (1%) and PMSF (1%) (for AKT and ERK1/2 protein analysis), supplemented with protease inhibitors (cOmplete Mini, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics GmbH) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics GmbH) for 30 min on ice. Lysates were centrifuged at 14,000 x g at 4 °C and the protein concentration was quantified using BCA™ Protein Assay Kit (Thermo Fisher Scientific). Lysates were denatured with 4X Laemmli buffer at 95 °C for 5 min, ran on 8% or 10% SDS-polyacrylamide gels, and then transferred onto PVDF membranes for 1.5 h at 180 mA. The membranes were blocked with EveryBlot Blocking Buffer (BioRad) for 1 h at RT and incubated with primary antibodies at 4 °C overnight and washed three times with TBST. Afterwards the membranes were incubated with HRP-conjugated secondary antibody for 1 h at RT and washed again three times with TBST. The list of used antibodies is provided in Supplementary Table 2. After adding the ECL substrate (Clarity Western ECL Substrate, Bio-Rad, Hercules, CA), chemiluminescence was visualized using the Gel Documentation System (Uvitec Ltd., Cambridge, United Kingdom). Densitometric analysis was performed using ImageJ software.

Flow cytometry

Cells were stained with specific antibodies or isotype control antibodies dissolved in a buffer containing PBS, 2% BSA and 0.1% sodium azide for 1 h on ice. Then the cells were centrifuged at 180 x g for 4 min and washed twice with buffer containing 5% BSA and 0.1% sodium azide. Samples were resuspended in the same buffer and analysed on FACSCalibur. Cells were gated based on SSC and FSC scatter to exclude debris and dead cells. A total of 50.000 cells/events within defined gate were acquired for each sample. Unstained cells were used as a control for autofluorescence. The list of used antibodies is provided in Supplementary Table 2. Data was analysed using CellQuest Pro software and the results are presented as mean fluorescence intensity (MFI).

Proteome profiler array

The expression of 84 oncoproteins was analyzed using The Proteome Profiler Human XL Oncology Array (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, membranes dotted with biotinylated antibodies in duplicates were blocked for 1 h at RT and incubated with cell lysates (containing equal amount of proteins) overnight at 4 °C. The membranes were washed (3 × 10 min) and incubated with a secondary antibody for 1 h at RT, followed by washing again (3 × 10 min). Next, the membrane was incubated with streptavidin-HRP for 30 min at RT. After adding the chemiluminescent substrate the proteins were detected using Gel Documentation System (Uvitec Ltd., Cambridge, United Kingdom). Densitometric analysis was performed using ImageJ software.

Results

Changes in the expression of oncogenic and signaling proteins in RAB27 KO cells

RAB27A knockout was induced in SkMel28, DMBC12, and A375 melanoma cell lines using the CRISPR/Cas9 technology. Additionally, we generated a double *RAB27A/B* knockout A375 cell line to investigate whether a potential compensating mechanism between two isoforms occurs. The decreased *RAB27A* mRNA expression was analyzed by RT-PCR, while the loss of protein was confirmed by Western blot [12].

We used the Proteome Profiler array to explore how *RAB27* knockout affects the expression of various proteins involved in carcinogenesis and/or tumor progression in melanoma cell lines (Suppl. Figures 1–2). Densitometric analysis of the spots on the membranes showed significant differences in protein levels between lysates derived from *RAB27A* KO or *RAB27A/B* KO cells vs. wild-type cells (Fig. 1). Proteins whose signal exceeded the optical density (OD) value of 3000 and the difference between wild type (WT) and KO/dKO came to at least 20% were selected for analysis and grouped according to their function (Fig. 1). Proteins undergoing increased or decreased expression possess different functions, ranging from adhesion molecules to regulators of angiogenesis, immune response, epithelial to mesenchymal transition (EMT), extracellular matrix (ECM) components, proteases and their inhibitors. Significant differences were also observed in the levels of signaling proteins involved in apoptosis, cell cycle regulation, or receptor kinases.

Alterations in protein expression differed among studied cell lines. Those changes were the most impactful in SkMel28 *RAB27A* KO cells, in which 62 proteins were downregulated and two were upregulated. In DMBC12 *RAB27A* KO cells, reduced levels of 42 proteins and increased levels of one protein were observed. The levels of 13 proteins were decreased and eight proteins were increased in A375 *RAB27A* KO cells. All three knockout melanoma cell lines showed downexpression of Decorin, FOXO1, Serpin 5, Tenascin C, and Urokinase. As for GM-CSF, HIF1α, HNF-3β, and MMP3, their amounts were reduced in SkMel28 and DMBC12 KO cells, while increased in A375 KO cells. In contrast, the level of Kallikrein 6 was higher only in SkMel28 KO cells, and lower in the other two lines. The opposite was true for Serpin E1, its expression was lower in SkMel28 KO cells and higher

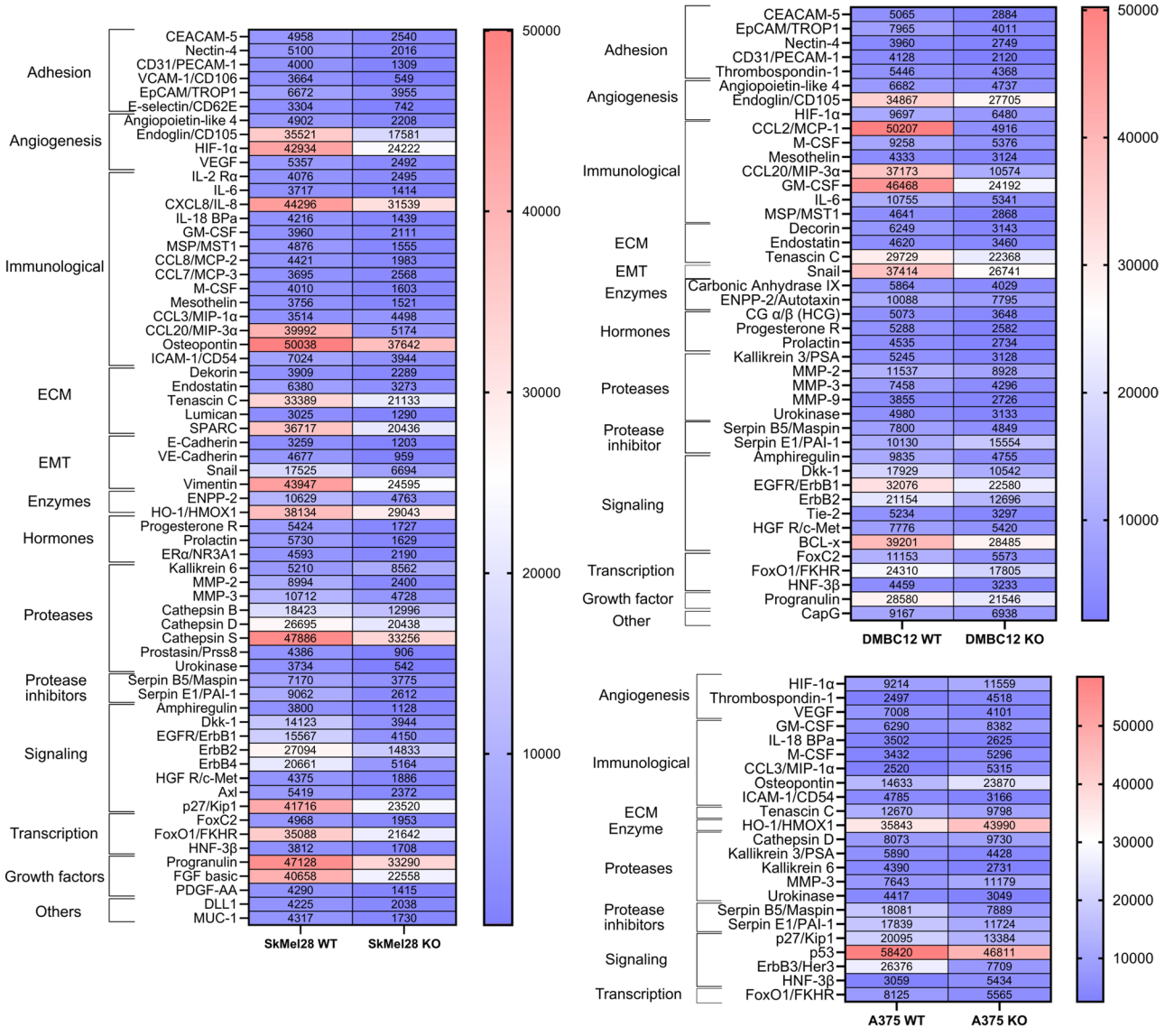

Fig. 1 Expression of cancer-related proteins in wild-type (WT) and *RAB27A* knockout (KO) SkMel28, DMBC12 and A375 cells. Signal intensity was quantified by densitometric analysis and data are presented as mean values of two technical replicates. Proteins whose signal exceeded the OD value of 3000 and the difference between WT and KO was at least 20% were grouped according to their function

in DMBC12 and A375 KO cells. Interesting alterations occurred in the levels of HER receptors. Even though they were not consistently up- or down-regulated in all cell lines, at least one of HER receptors was affected in each case (Figs. 2 and 3). EGFR and HER2 were downregulated in SkMel28 and DMBC12 KO cells, and HER3 was downregulated in A375 KO cells. Intriguing, only 13 proteins were downregulated as a result of RAB27A KO in A375 cell line, which is significantly fewer than in other cell lines. Despite this, HER3 expression was markedly reduced by 70% (Fig. 3). Considering the shared functions of HER proteins in signaling pathways, we hypothesized that a common mechanism might influence this receptor family.

Additionally, we analyzed the levels of oncoproteins in A375 *RAB27A/B* KO cells, in which 25 proteins were downregulated and 6 were upregulated. Interestingly, significant changes were observed between protein levels in A375 cells with single and double KO (Fig. 2). The amount of α-fetoprotein and Thrombospondin was higher in KO cells, compared to wild-type cells, and lower in dKO cells. The levels of Cathepsin D, GM-CSF, and HIF-1α, which were significantly higher in RAB27A KO cells, dropped to levels similar to those in wild-type cells in RAB27A/B KO cells. In contrast, Carbonic Anhydrase, EGFR, HO-1, MMP3, and p53 were overexpressed in dKO cells, compared to WT and KO cells. A significant decrease of Angiopoietin, Cathepsin S,

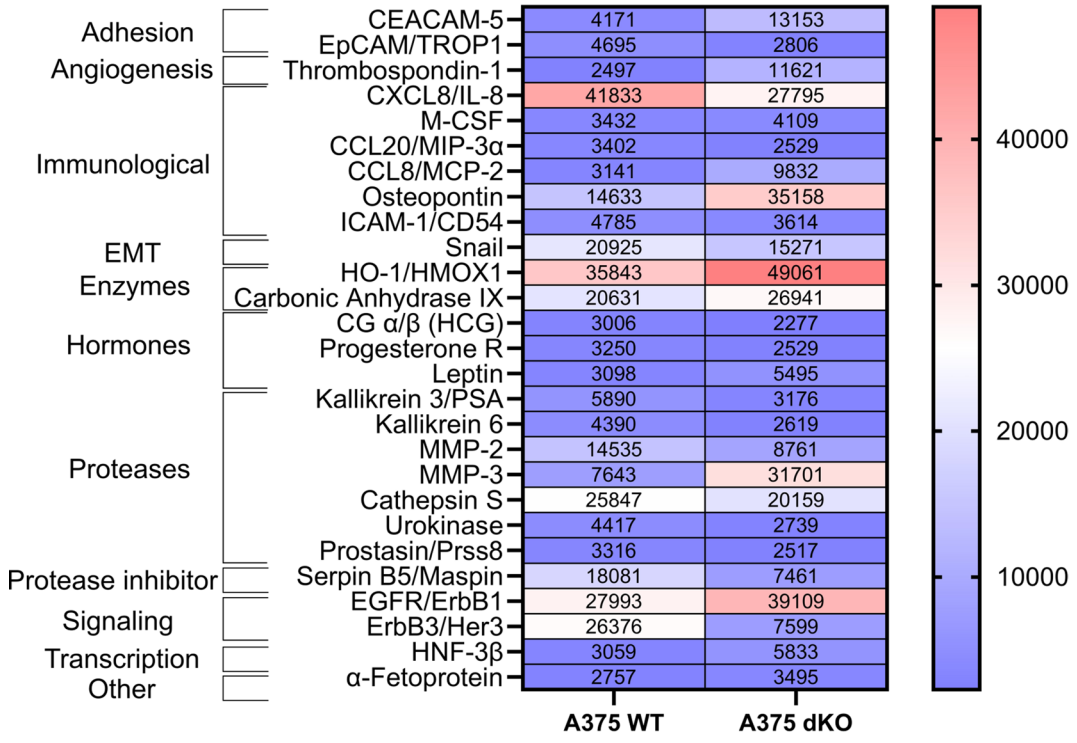

Fig. 2 Expression of cancer-related proteins in wild-type (WT) and *RAB27A/B* knockout (dKO) A375 cells. Signal intensity was quantified by densitometric analysis and data are presented as mean values of two technical replicates. Proteins whose signal exceeded the OD value of 3000 and the difference between WT and KO was at least 20% were grouped according to their function

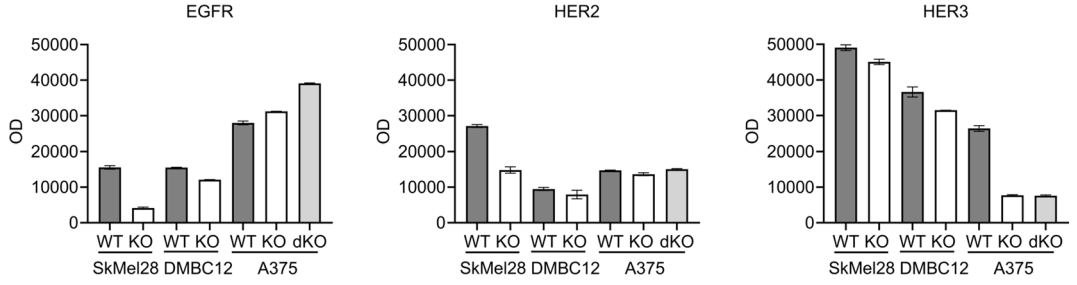

Fig. 3 Expression of HER receptors in wild-type (WT) and *RAB27A* knockout (KO) SkMel28, DMBC12 and A375 cells and *RAB27A/B* knockout (dKO) A375 cells measured using the Proteome Profiler array. Signal intensity was quantified by densitometric analysis and data are presented as mean OD values of two technical replicates

EpCam, CG α/β, CXCL8, Kallikrein 8, CCL20, MMP-2, MSP, PDGF-AA, CD31, Progesterone R, Prostasin, Snail and Tie2 was observed in dKO cells, in comparison to WT and KO cells. The levels of FOX01, p27, Serpin E1, and VEGF were higher than in KO cells, while still lower than in wild-type cells. Lastly, the increased expression of Osteopontin, observed in KO cells, was further increased in dKO cells. Moreover, EGFR was upregulated in dKO cells, despite its stable expression in KO cells. The level of HER2 in WT, KO and dKO cells remained constant while HER3 was downregulated in both KO and dKO cells (Figs. 2 and 3).

In conclusion, the knockout of *RAB27A* affected the expression of various proteins, however, the changes differed among the melanoma cell lines. The highest number of affected proteins occurred in the least aggressive cell line (SkMel28), while the proteome of the most aggressive one (A375) was modified to a lesser degree. Additionally, the proteome of A375 cells was further altered by silencing another RAB27 isoform, which indicates that those GTPases interact with proteins at least partially independently. We opted to investigate further the impact of RAB27 on HER family proteins due to their extensive function in cell signaling. To our best knowledge, the effect of RAB27 knockout on HER receptor expression has not been previously reported. Therefore, we focused our investigation on this novel observation.

HER receptors expression in RAB27 KO cells

The family of HER receptors plays a significant role in signaling pathways in cancer, therefore we decided to validate further the results obtained by Proteome Profiler Array. The expression of HER2, HER3, and EGFR receptors was examined by RT-PCR, flow cytometry, and Western blot. Due to the low protein level, we were unable to detect HER2 by Western blot in any of the studied cell lines.

The *RAB27A* knockout increased *HER2* mRNA expression in SkMel28 cells, while *EGFR* and *HER3* mRNA levels remained unchanged (Fig. 4A). A receptor occupancy evaluated by flow cytometry and Western blot showed that HER3 expression was reduced in SkMel28 KO cells, compared to WT cells, which is consistent with the Proteome Profiler result. In contrast, increased surface expression of HER2 was observed in *RAB27A* KO cells (Fig. 4B-D). EGFR was undetectable by the antibodies used in the abovementioned techniques.

DMBC12 *RAB27A* KO cells showed reduced *HER2* mRNA levels, compared to the wild-type cells (Fig. 4A). In addition, the HER3 protein expression was reduced, as seen on Western blot, although it did not reach statistical significance when analyzed by flow cytometry, possibly due to the wide dispersion of values between replicates (Fig. 4B-D). The marginal level of EGFR did not differ between KO and WT cells. Interestingly, these findings are slightly different from those obtained by the Proteome Profiler Array, which showed a decrease in EGFR and HER2 levels, while there was no change in HER3. However, considering, that the Proteome Profiler Array was performed only once and we regard it as a semi-quantitive method, this result might be less precise.

A375 *RAB27A* KO cells exhibited significantly lower HER3 levels than wild-type cells, detectable by all methods (Fig. 4A-D). The expression of *HER2* and *EGFR* mRNA was unchanged (Fig. 4A), while EGFR protein levels, measured by flow cytometry and Western blot, were somewhat increased, however not statistically significant (Fig. 4B-D). These results are not fully consistent with those obtained by the Proteome Profiler, where only HER3 was found to be downregulated, but again, we are aware of the limitations of this method.

We then investigated the expression of HER family receptors in A375 *RAB27A/B* KO cells (Fig. 5). No significant discrepancies were observed in the levels of *HER2* mRNA and protein, while the amount of *HER3* mRNA and protein was considerably lower in dKO cells, as confirmed by RT-PCR, WB, and flow cytometry. These findings are consistent with the results obtained by the Proteome Profiler array. The amount of *EGFR* mRNA in dKO cells was comparable to WT (Fig. 5A), while a notable overexpression of this receptor was observed on Western blot, similar to the Proteome Profiler Array. However, the increase of EGFR occupancy in dKO cells measured by flow cytometry did not reach statistical significance (Fig. 5B, C).

Our analysis revealed no consistent correlation between the basal level of RAB27A and the expression of EGFR, HER2, or HER3. The studied melanoma cell lines exhibited significant variations in the basal RAB27 expression [12]. SkMel28 cells, characterized by high RAB27A mRNA and protein expression, displayed undetectable surface occupancy or protein level of EGFR. On the other hand, A375 cells, which lower RAB27A expression, exhibited markedly higher EGFR level, compared to SkMel28 cells. Furthermore, DMBC12 and A375 cells, despite comparable RAB27A mRNA and protein levels, showed significant differences in HER3 expression. Therefore we assume that the impact of RAB27 on HER receptor expression is cell line-dependent, rather than solely dependent on its basal level.

To sum up, these results suggest that RAB27 potentially interacts with HER receptors, which could affect melanoma cell functioning.

AKT and ERK signaling protein expression and phosphorylation in *RAB27* KO cells

HER family receptors are involved in signal transduction and activate various signaling pathways, including PI3K/AKT and RAS/RAF/MEK/ERK. We examined the phosphorylation levels of AKT and ERK1/2 proteins to assess how RAB27-mediated changes in cellular levels of HER2, HER3, or EGFR potentially affected the signaling activity of these receptors. The results presented in Fig. 6 indicate that *RAB27A* knockout inhibited the phosphorylation of threonine and serine residues of AKT protein and tyrosine and threonine residues of ERK1/2 proteins only in cells of the DMBC12 *RAB27A* KO line. In contrast, no such changes were observed in cells of the SkMel28 and A375 lines. Interestingly, the double knockout A375 cells showed reduced phosphorylation of AKT and ERK1/2 proteins, which indicates that two RAB27 isoforms affect those processes separately (Fig. 7). However, considering that silencing both *RAB27A* and *RAB27B* may have a synergistic effect, we cannot fully assess the latter's impact on the phosphorylation of AKT and ERK1/2.

Discussion

In order to study whether *RAB27* knockout in SkMel28, DMBC12 and A375 melanoma cells affects the expression of cancer-related proteins, we performed a Proteomic Profiler array. We observed significant differences in the levels of proteins involved in processes such as EMT, apoptosis, immune modulation, angiogenesis, and signaling pathways. The proteins downregulated in all tested by us cell lines were Tenascin C and Urokinase. Tenascin C promotes oncogenesis, angiogenesis,

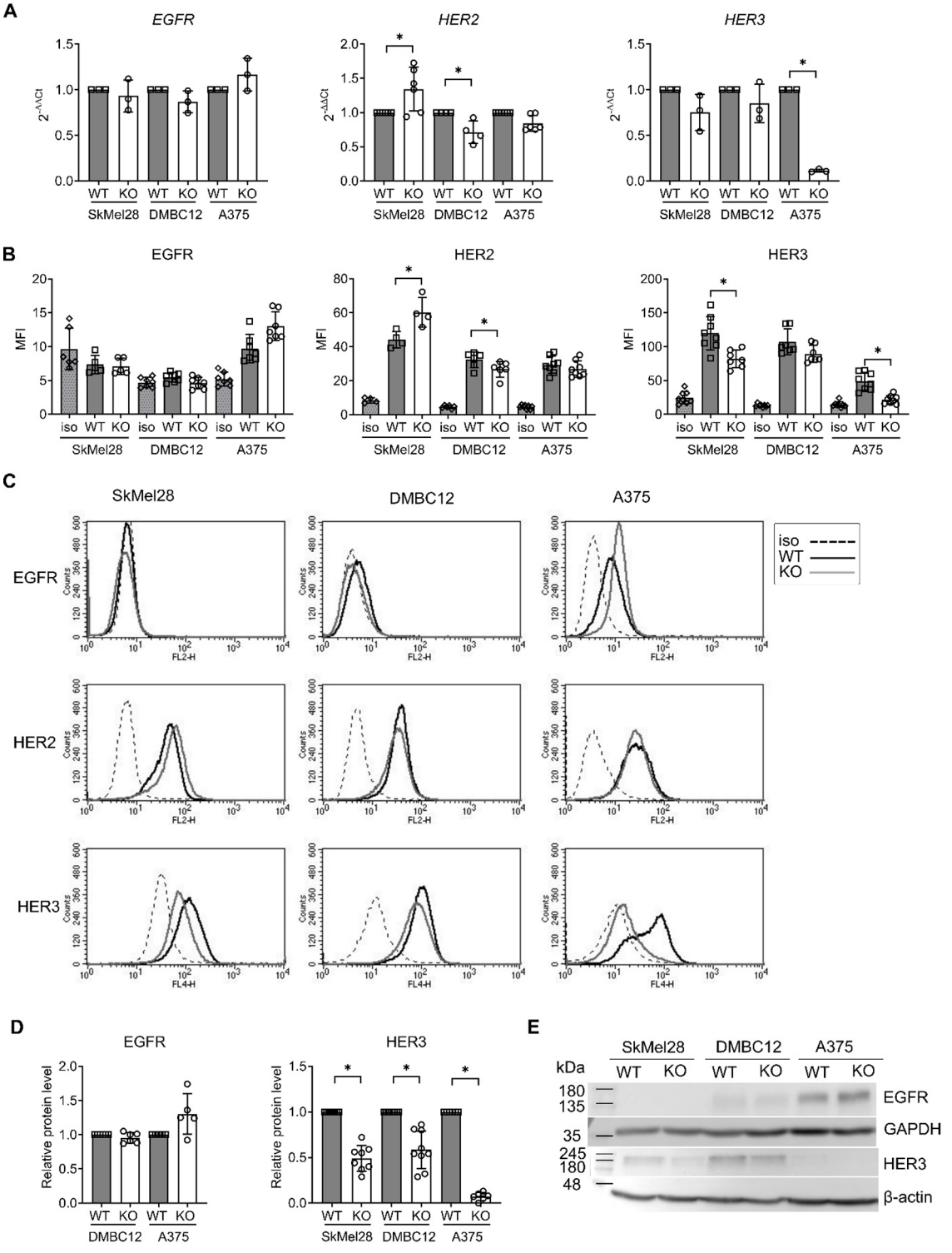

Fig. 4 RAB27A knockout altered the levels of EGFR, HER2 and HER3 in wild-type (WT) and *RAB27A* knockout (KO) SkMel28, DMBC12 and A375 cells. (**A**) mRNA expression analyzed by RT-PCR. Data presented as mean ± SD, $n \geq 3$, $p < 0.05$ (**B**) Receptor occupancy analyzed by flow cytometry. Data presented as mean ± SD, $n \geq 4$, $p < 0.05$ (**C**) Representative flow cytometry (**D**) Protein expression analysed by Western blot. The acquired bands were quantified by densitometric analysis. Data presented as mean ± SD, $n \geq 5$, $p < 0.05$ (**E**) Representative Western blot

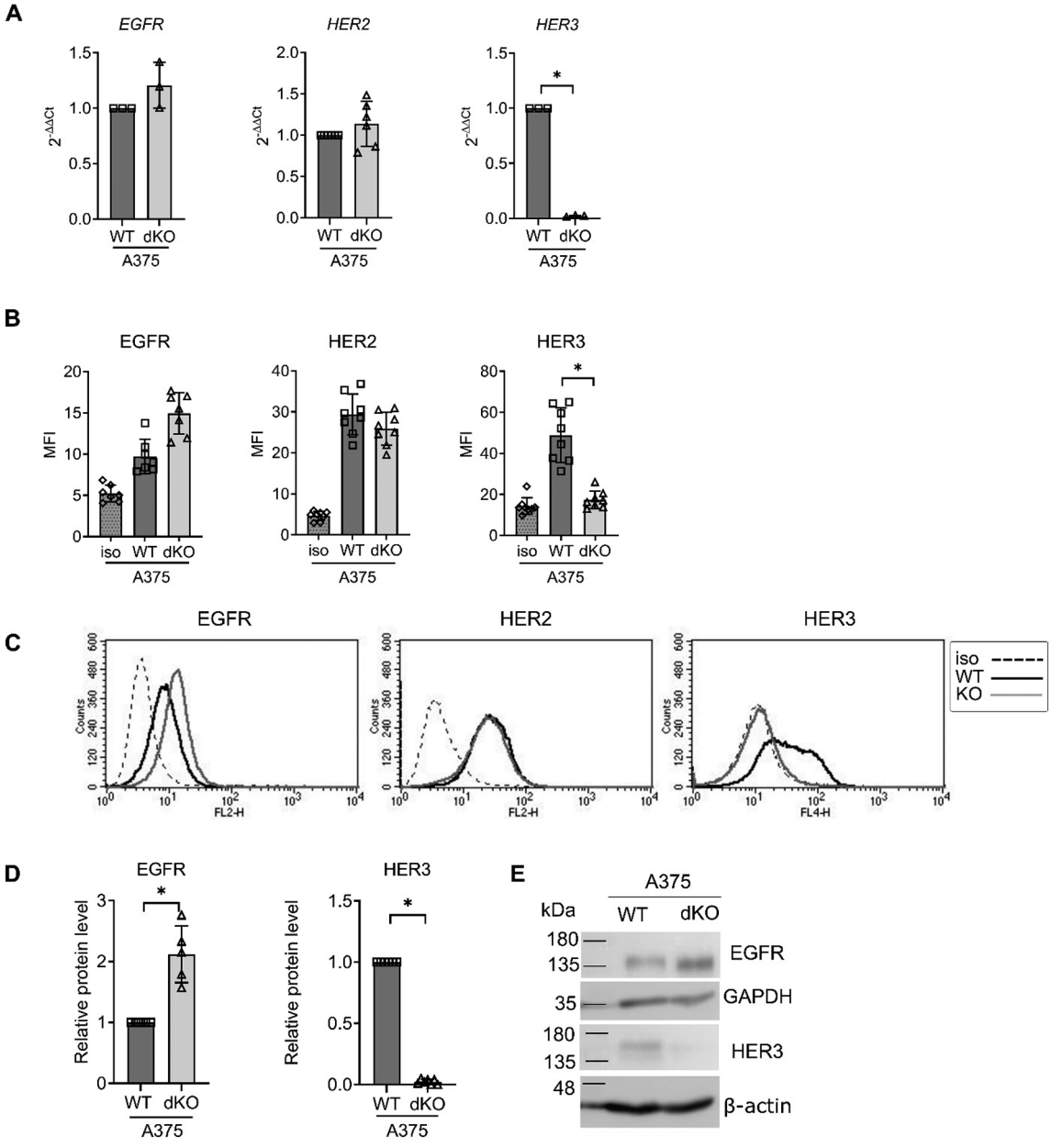

Fig. 5 RAB27A knockout altered the levels of EGFR, HER2 and HER3 in wild-type (WT) and *RAB27A/B* knockout (dKO) A375 cells. (**A**) mRNA expression analyzed by RT-PCR. Data presented as mean ± SD, $n \geq 3$, $p < 0.05$ (**B**) Receptor occupancy analyzed by flow cytometry. Data presented as mean ± SD, $n \geq 7$, $p < 0.05$ (**C**) Representative flow cytometry (**D**) Protein expression analysed by Western blot. The acquired bands were quantified by densitometric analysis. Data presented as mean ± SD, $n \geq 5$, $p < 0.05$ (**E**) Representative Western blot

metastasis or modulation of the immune system, and its overexpression is observed in cancer, chronic inflammation or bacterial and viral infections [13]. Urokinase is involved in ECM degradation, interacts with integrins and receptors leading to activation of signaling pathways, and promotes multidrug resistance [14]. However, we were particularly intrigued by alterations in the signaling protein expression, including HER receptors. While the majority of observed proteomic changes were cell-line specific, a consistent finding across all studied melanoma cell lines was the down- or upregulation of at least one HER receptor. This observation suggests a potential role for RAB27 in modulating cellular signalling pathways.

The loss of *RAB27* was previously shown to hinder the activation of various signaling proteins. For instance, downregulation of PI3K and decreased levels of pAKT (Thr308), pAKT (Ser473), pFOXO3A, and Cyclin D1 was observed in endothelial progenitor cells derived from *RAB27A* KO mice [15]. Furthermore, *RAB27B* knockdown inhibited phosphorylation of PI3K and

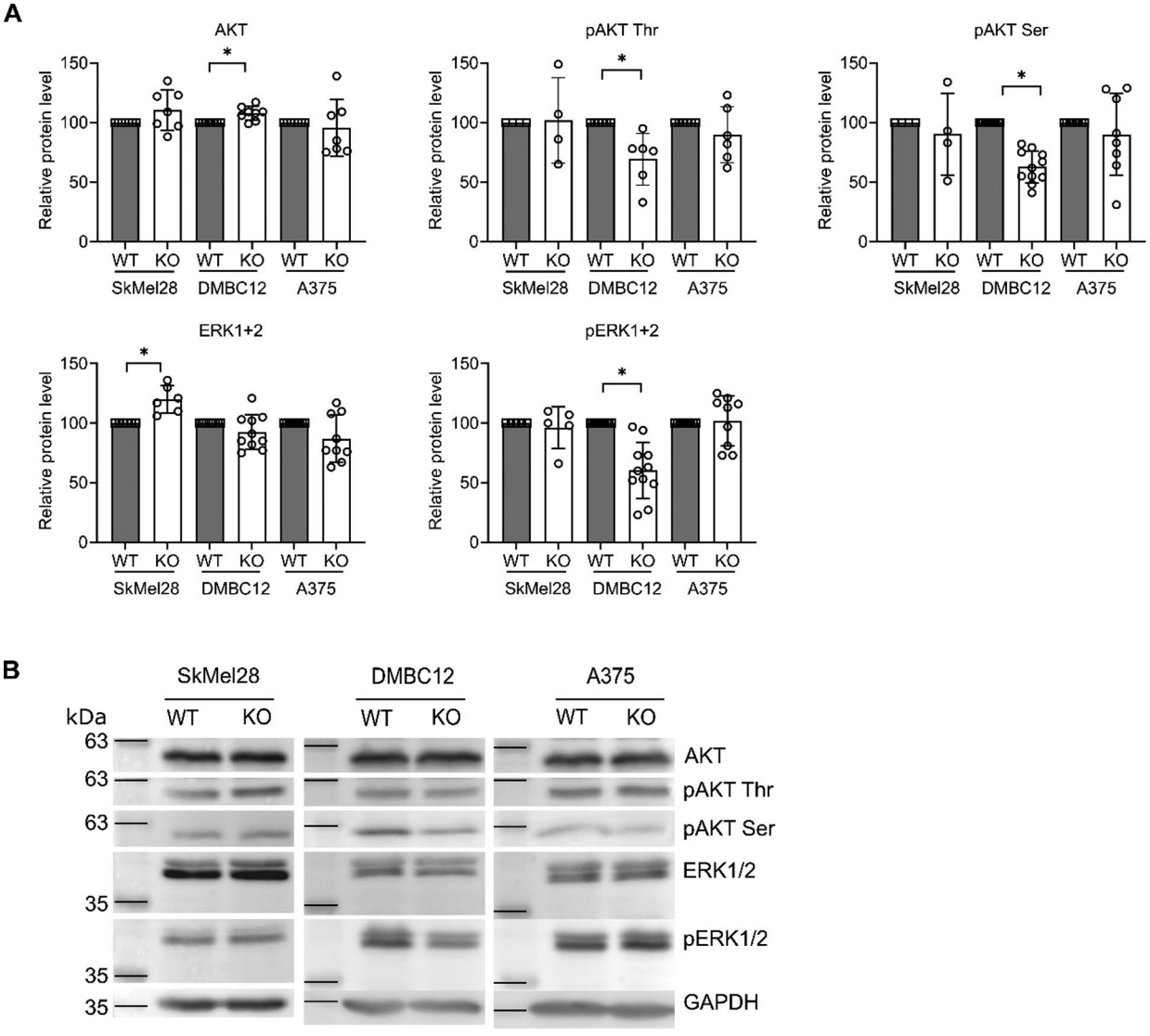

Fig. 6 *RAB27A* knockout affected the phosphorylation of AKT and ERK1/2 in some melanoma cell lines (**A**) Levels of AKT, phospho-AKT (Ser473, Thr308), ERK1/2 and phospho-ERK1/2 (Thr202, Tyr 204) analyzed by Western blot. The acquired bands were quantified by densitometric analysis. Data presented as mean ± SD, $n \geq 4$, $p < 0.05$ (**B**) Representative Western blots

AKT (Ser473) in hepatocellular carcinoma cells [10] and ERK1/2 in erythroleukemia cells [11]. However, little was known about the effect of RAB27 on the cellular processes occurring upstream of AKT or ERK1/2 executive proteins. HER receptors are acknowledged to be involved in an intricate network of signalling pathways, including RAS/RAF/MEK/ERK, PI3K/AKT, Src, PLCγ, or STAT, which govern fundamental cellular processes, such as proliferation, differentiation, migration, metabolism, and survival [16–18]. To our best knowledge, this is the first study to uncover the relationship between RAB27 and HER receptors in melanoma cell model. The knockout of *RAB27A* decreased HER3 expression in all tested by us melanoma cell lines, and the effect was even more prominent after silencing another isoform, *RAB27B* in A375 cells. Interestingly the level of HER2 was enhanced in SkMel28 *RAB27A* KO cells, while its decrease was observed in DMBC12 *RAB27A* KO. On the other hand, the expression of EGFR protein somewhat increased in A375 *RAB27A* KO cells and was significantly overexpressed in A375 *RAB27A/B* KO cells. These phenomena indicate, that the relationship between RAB27 and EGFR or HER2 is melanoma cell-line dependent.

The level of HER2 in melanoma tissues of patients is marginal [19–21], however, the expression and activation of HER3 were enhanced in melanoma cells, compared to normal melanocytes [17, 22]. Its upregulation was also detected in tissues of patients with this cancer, and a positive correlation has been shown between HER3 overexpression and disease progression, response to immune

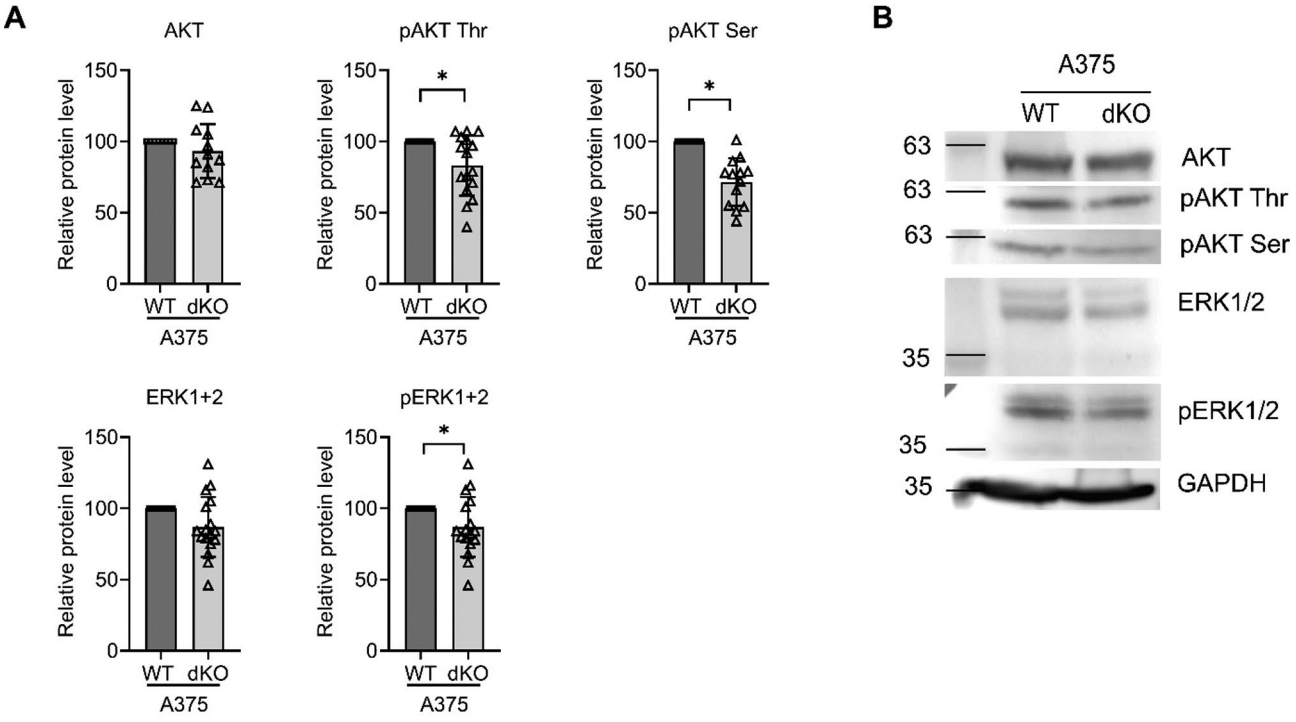

Fig. 7 *RAB27A/B* knockout reduced the phosphorylation of AKT and ERK1/2 in A375 melanoma cell line (**A**) Levels of AKT, phosphor-AKT (Ser473, Thr308), ERK1/2 and phospho-ERK1/2 (Thr202, Tyr 204) analyzed by Western blot. The acquired bands were quantified by densitometric analysis. Data presented as mean ± SD, $n \geq 12$, $p < 0.05$ (**B**) Representative Western blots

checkpoint blockade treatment, the occurrence of metastases, and worse patient survival [17, 23–25]. Pharmacological blockade of HER3 phosphorylation increased the sensitivity of melanoma cells to vemurafenib [26]. It was also observed that silencing *HER3* increased the sensitivity of melanoma cells to ferroptosis inducers [27] or dacarbazine [17]. Overall, suppressing HER receptor activity can have an anti-cancer effect on melanoma cells. Anti-HER3 antibody induced HER3 receptor internalization and lysosomal degradation, which decreased AKT phosphorylation and inhibited melanoma cell proliferation and migration [28].

The role of EGFR in melanoma is not fully defined. It is not present in normal melanocytes but is detected in melanoma cells [18, 29, 30]. Some studies show a lack of correlation between increased EGFR expression and the occurrence of metastases [31], while others found one [18]. In addition, increased amplification of EGFR gene copies was associated with melanoma progression and metastasis [29]. High levels of EGFR in metastatic tissues were also linked to disease recurrence after adjuvant treatment with immune checkpoint inhibitors [30].

To investigate how the fluctuations of HER receptor expression impacted the downstream signaling pathways we looked into the phosphorylation of AKT and ERK1/2 proteins. Those processes were again affected by silencing *RAB27* in a cell-line-dependent manner. There was a significant reduction in the phosphorylation of the threonine and serine residues of AKT and tyrosine and threonine of ERRK1/2 in DMBC12 *RAB27A* KO cells. Surprisingly, A375 and SkMel28 *RAB27A* KO cell lines did not show any changes in the phosphorylation of the aforementioned signaling proteins. This is particularly interesting given the almost complete inhibition of HER3 expression in A375 *RAB27A* KO cells, which could lead to the assumption, that the effect on signaling pathways regulated by this receptor would be the most significant. This phenomenon could theoretically be explained by the slightly increased (although not statistically significant) level of EGFR, which might compensate for the absence of HER3. However, although the expression of EGFR was even higher (statistically significant) in double knockout A375 cells, the phosphorylation of AKT and ERK1/2 was reduced. These results do not provide a unambiguous conclusion, however they open up a new avenue for further research. Above all, silencing *RAB27A* or *RAB27A/B* undoubtedly disturbed the HER receptor family expression, which could markedly disrupt cancer cell functioning. These findings support the suppression of proliferation, migration, and invasion that we previously observed in *RAB27A* KO melanoma cells [12].

Some studies have shown that the effect of *HER3* silencing on signaling pathways varies among melanoma cell lines. *HER3* knockdown in human melanoma

WM115 and SkMel24 cell lines increased ERK1/2 phosphorylation, while decreased pERK1/2 and pAKT levels were observed in human CHL-1 and Bowes [32] and murine B16-BL6 melanoma cell lines [33].

Nonetheless, it should be noted that apart from HER proteins, PI3K-AKT and RAS/RAF/MEK/ERK pathways are also activated by growth factor, insulin, cytokine, or chemokine receptors [34, 35]. Thus, despite the downregulation of HER3 expression in SkMel28 or A375 *RAB27A* KO cells, the phosphorylation of AKT or ERK1/2 may not be reduced, as this process is regulated by other receptors in those cells.

We hypothesize several possible mechanisms are happening between RAB27 and HER proteins. Internalized HER receptors undergo either recycling back to the plasma membrane or sorting into early and then late endosomes, from where they are transported for lysosomal degradation. One of the possibilities is the change of intercellular transport of receptors coordinated by RAB27. Such phenomenon was observed in the osteoclasts of *ashen* mice with RAB27A mutation, in which activation of the signaling cascade by macrophage colony-stimulating factor or receptor activator of nuclear factor κβ ligand was enhanced due to the altered transport of their receptors, c-fms, and RANK, respectively [36]. RAB27A and RAB27B govern docking and fusing the multivesicular endosomes to the plasma membrane [37]. Other RAB proteins, such as RAB4, RAB5, RAB7, RAB11, or RAB22 were shown to regulate EGFR endocytosis, recycling, and lysosomal degradation pathways [38]. For example, an active RAB7 mutation in HeLa cells caused the clustering of endosomes containing EGFR in the cell's perinuclear region, which delayed its degradation and enhanced the activation of ERK1/2 [39]. Therefore, if RAB27 affected the endosomal trafficking, either directly, or by causing disbalance in the RAB proteins network, it could provide some explanation for our observations in melanoma cells.

RAB27A and B are known to regulate the secretion of small extracellular vesicles (sEVs), which reflect the content and the physiological state of their donor cells [37]. To investigate the impact of RAB27 silencing on the loading of HER receptors into sEVs we analyzed the proteomic profile of vesicles derived from DMBC12 RAB27A KO and A375 RAB27A/B KO cells, which exhibited the most pronounced changes in cellular HER receptor expression (Suppl. Figure 3). The alteration in vesicular content of EGFR and HER2 mirrored those observed in DMBC12 KO cell lysates. HER3 level was also decreased in sEVs secreted by DMBC12 KO cells. Moreover, both A375 dKO cell lysates and sEVs exhibited increased level of EGFR and decreased level of HER3. These findings demonstrate that the protein content of small extracellular vesicles accurately reflects the HER receptor level in respective melanoma cell lines. This suggests that the observed changes in HER mRNA and protein expression in RAB27 KO cells are not directly caused by the altered RAB27-mediated vesicular transport pathway.

Moreover, RAB27 could control the stability of membrane-bound receptors. RAB27A was reported to regulate the palmitoylation of EGFR through zinc finger DHHC-type containing 13 (ZDHHC13), which promoted receptor retention in the membrane of oral squamous cell carcinoma cells [40]. RAB27A was also found to prevent vascular endothelial growth factor receptor-1 from lysosome-mediated degradation and stabilize it by regulating receptor palmitoylation by DHHC3 in endothelial cells [41].

Notably, a significant decrease in HER3 protein levels was observed in both A375 RAB27A KO and RAB27A/B KO cells, which correlated with decreased HER3 mRNA levels. Similarly, reduced HER2 protein levels in DMBC12 RAB27A KO cells mirrored a decrease in HER2 mRNA expression. In contrast, increased HER2 mRNA levels in SkMel28 KO cells corresponded to an increase in its surface expression. These findings suggest that RAB27 may up- or down-regulate the expression of HER receptor family genes, leading to cell line-specific fluctuations in protein level. Previous studies have demonstrated that RAB27 can regulate the various genes/proteins expression. For example, RAB27A overexpression increased the mRNA and protein levels of VEGF, cathepsin D, cyclin D1, uPA, and MMP-9 and decreased the level of p16 in breast cancer cells [42]. On the other hand, RAB27B knockdown downexpressed several genes, including TMEM30A, RPPH1, or PMCH, in renal cancer cells [43].

The presented studies were performed on a panel of melanoma cell lines, varying in basal RAB27A/B expression, as well as different proliferative/invasive potential, in order to investigate different cellular conditions. The SkMel28 cell line is considered less aggressive, while A375 cells are a particularly aggressive type of melanoma. In addition to commercially available cell lines, a patient-derived DMBC12 model of a nodular melanoma was also used. Based on our observations, this cell line is moderately invasive. All cell lines used in this study carry the *BRAF* V600E mutation. In addition, SkMel28 cells feature *CDK4* [44], *TP53*, *PTEN* [45] and *TERT* [46] mutations. Mutations of *CDKN2A* and *TERT* have been identified in A375 cells [45], and DMBC12 cells carry mutations in *CDKN2A*, *MRPS31*, *KNSTRN*, *FAM58A*, *E2F3*, *FBXW7*, *NOTCH2 and NOTCH3*, among others [47]. The studied cell lines also differ in their baseline levels of RAB27A and RAB27B, the expression of which is significantly lower in A375 and DMBC12 cells, compared to SkMel28. This could justify the highest number of dysregulated proteins in SkMel28 KO cells. On the other hand, the

phosphorylation of AKT and ERK1/2 was affected only in DMBC12 KO cells, which show a rather low level of RAB27A. Therefore, the role of RAB27A in melanoma signaling likely depends on a number of variables, such as proliferative/invasive potential or the genetic variant of a given lineage, rather than being directly due to the amount of this protein in the cells.

Our findings do not enable us to pinpoint the mechanisms underlying the interaction between RAB27 and HER receptors, therefore more in-depth research should be conducted in the future. Studying how the HER proteins respond to stimulation by their specific ligands in cells with or without *RAB27* knockout would shed more light on the functioning of those receptors. Monitoring cellular localization of HER receptors would allow to determine their fate upon internalization and figure out whether RAB27 affects the intercellular protein trafficking. In addition, other signaling pathways known to be activated by the HER family proteins should be investigated for a more extensive analysis.

Conclusions

In this study we present how the loss of RAB27A or both RAB27A and RAB27B changes the expression of cancer-related proteins and signalling proteins in melanoma cells. Our findings shed light on the relationship between RAB27, the levels of EGFR, HER2, and HER3 receptors, and the downstream phosphorylation of the AKT and ERK1/2 signaling proteins.

Abbreviations

AKT	serine/threonine-protein kinase
dKO	double knockout
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinases
HER	human epidermal growth factor receptor
KO	knockout
WT	wild-type

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Author contributions

Conceptualization - K.H., L.C., M.K.; Methodology - K.H.; Formal analysis - K.H., L.P.; Investigation -K.H., L.C., L.P.; Data Curation - K.H.; Writing– original draft preparation - K.H., M.K.; Writing– review and editing - K.H., M.K.; Visualization - K.H.; Supervision - M.K.; Funding acquisition - L.C. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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