The inhibition of three distinct signaling pathways eliminates the oncogenic Tribbles homolog 2 from melanoma cells

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ABSTRACT

Tribbles homolog 2 (TRIB2) is one of three members of the Tribbles family of pseudo serine/threonine kinases. It acts as an oncogene whose expression is correlated with tumor stage and therapy response in melanoma. While metastatic melanoma, the most aggressive form of skin cancer now has two effective treatment options, namely MAPK pathway and immune checkpoint inhibitors, the majority of patients primarily fail to respond or acquire secondary resistance. Hence, innovative approaches to identify disease-relevant, druggable targets to remove the roadblock of therapy resistance are urgently needed. TRIB2 has been implicated in conferring resistance to various anti-cancer therapies suggesting TRIB2 as a therapeutic target for resistant tumors. This study explores the pharmacological targeting of TRIB2, revealing several independent routes of pharmacological manipulations. TRIB2 is a short-lived protein stabilized by inhibition of the PI3K/AKT pathway. Conversely, inhibitors of BRAF, MEK and ERK significantly decrease TRIB2 expression by a mechanism that involves transcription. Strikingly, increasing concentrations of the kinase inhibitor PIK75 effectively eliminate TRIB2 in melanoma cells surpassing its PI3K inhibitory activity. Additionally, Polo-Like Kinases (PLKs) inhibitors significantly reduce TRIB2 protein expression and stability. We demonstrate that inhibition or silencing of PLK2 leads to a decrease in TRIB2 levels. Overall, we identify three distinct classes of compounds that efficiently eliminate the oncogenic TRIB2 protein from melanoma cells based on different molecular mechanisms and exhibiting 40 to 200 times greater potency than the previously reported afatinib.

INTRODUCTION

Tribbles homolog 2 (TRIB2) is the most ancestral member of the tribbles protein family, from which the other mammalian homologues TRIB1 and TRIB3 have evolved [1]. Similar to other tribbles family members, TRIB2 possesses a conserved C-terminal motif that interacts with the ubiquitin E3 ligase machinery, along with a unique pseudocatalytic domain that lacks canonical metal-binding amino acids [2]. Although the kinase activity of TRIB2 remains controversial, its role in promoting target protein degradation and regulating various signal transduction pathways has been well-established [3]. Consequently, cells exhibit high sensitivity to changes in TRIB2 expression levels. Mechanistically, TRIB2 acts as an adaptor protein for substrate ubiquitination combining a kinase-like domain with a domain that facilitates interaction with ubiquitin ligases. While the pseudokinase domain is involved in substrate binding, the C-terminus of these proteins recruits the E3 ubiquitin-protein ligase COP1 leading to proteasomal degradation of the bZIP transcription factor C/EBPa [4]. In melanoma, we have identified TRIB2 as a repressor protein of the FOXO3 transcription factor [5]. The oncogenic role of TRIB2 stems from its function as a versatile scaffold protein that mediates the degradation of tumor suppressor proteins and the activation of tumor drivers. Overexpression of TRIB2 has been associated with malignant and/or chemoresistant phenotype in various tumors, including in acute myeloid leukaemia [6], chronic myelogenous leukemia [7], melanoma [5,8,9], small cell lung cancer [10], glioblastoma [11], colorectal cancer [12,13], and laryngeal squamous cell carcinoma [14]. Recent findings also suggest that TRIB2 plays an important role in cancer immunotherapy, potentially protecting cold tumors from anti-cancer immunity [15]. Taken together, the accumulating evidence supports TRIB2 as a therapeutic target for therapy-resistant tumors including melanoma [16]. Additionally, TRIB2 has emerged as a druggable target [17] capable of binding ATP and undergoing autophosphorylation [2]. Covalent inhibitors of the EGFR family such as the approved drug afatinib have been shown to bind to specific cysteine residues within the TRIB2 protein and induce its degradation [18]. However, high concentrations of afatinib are required for effective TRIB2 degradation. Recently, Monga et al reported the direct binding of the antiviral drug daclatasvir to TRIB2 promoting its degradation through the proteasome [13]. Daclatasvir was found to re-sensitizes enzalutamide-resistant prostate cancer cells to androgen receptor antagonist treatment. Moreover, a recent study identified nanobodies that bind the TRIB2 pseudokinase domain with low nanomolar affinity [19]. The authors presented the first high-resolution structure of TRIB2 bound to a nanobody providing insights into its activated conformation. Advances in understanding TRIB2 regulation, structure and function lay the foundation for exploring various therapeutic strategies including structure-based drug design, ligand-based drug discovery as well as targeting TRIB2 expression and protein stability. In this study, we investigate TRIB2 stability and modulation of its expression using pharmacological approaches. Our findings demonstrate that TRIB2 protein is stabilized through the PI3K/AKT pathway activity, while its expression is downregulated by inhibition of components of the Mitogen-activated protein kinase (MAPK) pathway. Inhibition or silencing of Polo-Like Kinase 2 (PLKs) reduces the TRIB2 protein levels.

RESULTS

TRIB2 is rapidly degraded independent of the proteasome

To investigate the potential for targeting TRIB2 through pharmaceutical means, we initially conducted an analysis of TRIB2 stability and the mechanism of its turnover. Given that melanoma exhibits the highest expression of TRIB2 compared to other cancer types ([5] and Supplementary Fig. 1A) and considering the significant role TRIB2 plays in the clinical outcome of melanoma, we specifically focused on measuring the half-life of TRIB2 in UACC-62 melanoma cells. To block protein synthesis in these cells, we employed cycloheximide (CHX) treatment for varying durations of 10 to 60 minutes. Following each 10-minute interval, we analysed the remaining amount of TRIB2 through western blotting. Fig. 1A demonstrates that TRIB2 undergoes rapid degradation, with less than half of the protein remaining after 30 minutes of inhibiting new protein synthesis. Furthermore, after one hour of CHX treatment, almost no TRIB2 protein is detectable. Prolonged incubation with CHX for 2 to 8 hours renders TRIB2 undetectable (Supplementary Fig. 1B). Intriguingly, we observed that the inhibition of proteasomal degradation using the proteasome inhibitor MG132 led to increased TRIB2 protein suggesting that proteasomal degradation is involved in the process (Fig. 1B).

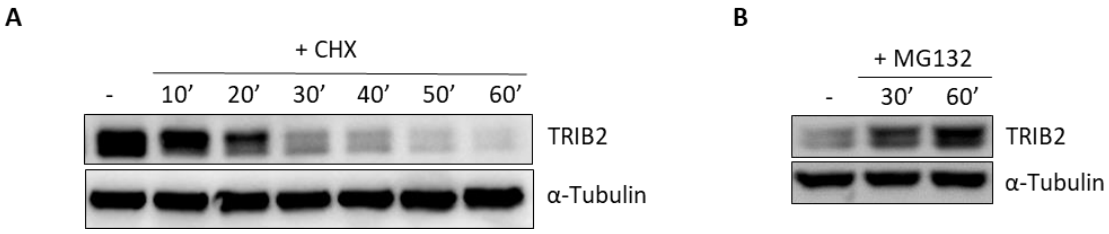

Fig. 1. *TRIB2 protein is rapidly degraded.* ***A)*** *Protein synthesis was blocked by Cycloheximide (CHX, 50 µg/mL) for the indicated time. The half-life of TRIB2 was measured by Western blot.* ***B)*** *Effect of proteasomal inhibition by 10µM MG132 treatment for the indicated time on TRIB2 protein levels measured by Western blot. α-Tubulin was used as loading control.*

Induction of TRIB2 protein level upon PI3K/AKT pathway inhibition

To examine the impact of small molecule compounds that interfere with specific signal transduction pathways on TRIB2 protein amount, we used a collection of small molecule compounds with known mode of action. As afatinib was previously shown to bind to and promote the degradation of the TRIB2 protein in HeLa cells [18], we aimed to determine its effect in UACC-62 melanoma cells. While the treatment of UACC-62 cells with 5 and 10µM afatinib for 4 hours rather increased the TRIB2 protein level, treatment with 20µM afatinib slightly decreased the amount of TRIB2 in these cells (Supplementary Fig. 2). These data indicate that covalent inhibitors of EGFR require optimization of their selectivity and potency for potential clinical exploitation of their anti-TRIB2 activity. Given our previous findings that TRIB2 influences PI3K/AKT signalling [20], we employed PI3K and MTOR inhibitors to treat UACC-62 cells and assessed their impact on the amount of TRIB2 . As depicted in Fig. 2, inhibition of the PI3K/AKT pathway led to an increase in TRIB2 protein levels. Treatment with the PI3K inhibitors PI-103 and LY294002, the dual PI3K/MTOR inhibitor dactolisib (BEZ-235), and the mTORC1 inhibitor rapamycin

significantly increased TRIB2 expression suggesting a reciprocal regulation between TRIB2 and AKT (Fig. 2). Furthermore, we confirmed the inhibition of P70S6K phosphorylation in UACC-62 cells upon the exposure to these compounds (Fig. 2). We also noticed the disappearance of the double band upon the exposure to PI3K or MTOR inhibitors suggesting that direct phosphorylation of TRIB2 is involved in its destabilization. These data also could provide a mechanistic explanation of the paradoxical induction of TRIB2 upon the treatment with afatinib at 5 and 10µM known to inhibit components of the PI3K/AKT signalling pathway.

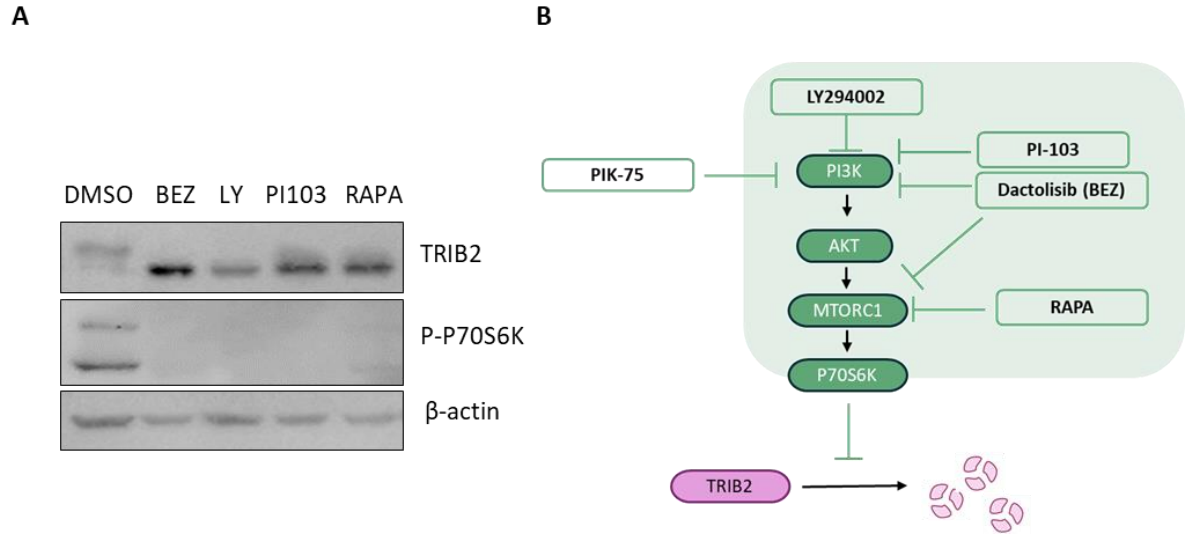

Fig. 2. *TRIB2 protein is regulated by the PI3K/AKT pathway.* ***A)*** *The treatment with dactolisib 500nM (BEZ), LY294002 25µM (LY), PI103 500nM or Rapamycin 5µM (RAPA) increases TRIB2 protein levels and decreases the level of P70S6K phosphorylation as determined by Western Blot. β-actin was used as loading control.* ***B)*** *Schematic model of the effect of different drugs on the PI3K pathway.*

PIK-75 eliminates TRIB2 acting at the protein level

Interestingly, unlike other PI3K inhibitors, increasing nanomolar concentrations of PIK-75 resulted in a reduction of TRIB2 proteins levels after 4 hours of treatment. Notably, 200nM of PIK-75 was sufficient to override the stabilizing effect, leading to a significant decrease in the amount of TRIB2 protein. Treatment with 500nM PIK-75 virtually eliminates TRIB2 from UACC-62 cells (Fig. 3A). The decline in TRIB2 expression began as early as one hour into PIK-75 treatment and reached complete elimination after 2 hours (Fig. 3B). To investigate whether the decrease in TRIB2 protein was due to suppressed TRIB2 transcription, we performed qPCR analysis. Interestingly, treatment with 500nM PIK-75 only resulted in a minor reduction of TRIB2 transcripts (Fig. 3C). To further confirm that PIK-75-mediated elimination of TRIB2 in melanoma cells primarily occurred at the protein level, we ectopically expressed Myc-tagged TRIB2 transcribed from a constitutively active promoter. Fig. 3D demonstrates that PIK-75 affected the stability of the ectopically expressed TRIB2 protein suggesting that its action is independent of transcription. In order to examine whether drug-induced TRIB2 destabilization is a cell-specific phenomenon, we treated different cell lines with small molecule compounds. In particular, we used A375 melanoma cells and osteosarcoma

U2OS cells stably expressing TRIB2-GFP to PIK-75. As shown in Supplementary Fig. 4 endogenous TRIB2 in A375 cells decreased upon PIK-75 exposure. Similarly, PIK-75 also affected TRIB2 level when ectopically expressed from a constitutively active gene promoter. These data indicate that PIK-75 treatment exerts its effect independent of the cell type and affects TRIB2 independently of transcription at the protein level. Interestingly, co-treatment of UACC-62 cells with MG132, a proteasome inhibitor, and 500nM PIK-75 only modestly rescued TRIB2 levels compared to cells treated with DMSO vehicle control indicating that decrease in TRIB2 induced by PIK-75 is partly independent of proteasomal degradation (Fig. 3E). To determine whether the negative regulation induced by 500nM PIK-75 dominates over the positive effect of PI3K inhibition, we combined PIK-75 treatment with the exposure to the potent panPI3K/MTOR inhibitor PI-103. As shown in Fig. 3E, in the presence of 500nM PIK-75, PI-103 is unable to counteract the PIK-75-mediated decrease of TRIB2 protein. We hypothesized that as the concentration of PIK-75 increases, its specificity for its primary target PI3K, may diminish. Previously, PIK-75 was reported to block both PI3K lipid and 21 additional serine/threonine kinases [21]. We tested commercially available inhibitors for several of these kinases, but none of them significantly affected TRIB2 (data not shown). Alternatively, PIK-75 might directly bind to TRIB2 and destabilize it. To investigate this possibility, we performed Cellular Thermal Shift Assays (CETSAs) which involve subjecting cell lysates to increasing temperatures and quantifying the presence of the protein of interest in the soluble fraction through immunoblotting. Interestingly, TRIB2 exhibited faster solubility loss upon heating compared to the control proteins PI3K and α-tubulin (Fig. 3F). While PI3K displayed stabilization in the presence of PIK-75 at 55°C, no detectable difference was observed in TRIB2 with or without PIK-75. These findings suggest that PIK-75 does not directly bind to TRIB2.

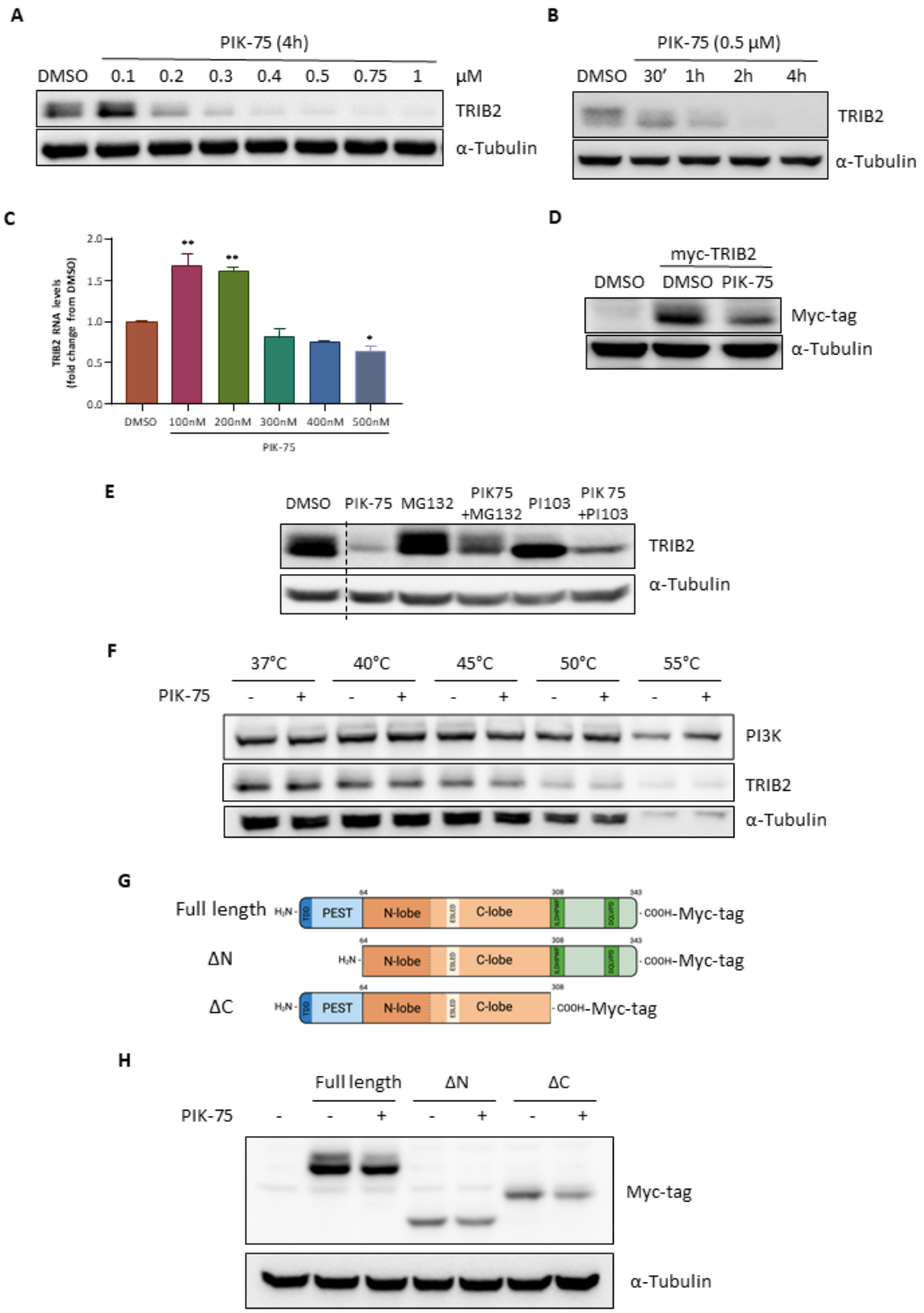

Fig. 3: *TRIB2 protein is downregulated by PIK-75.* ***A)*** *Time-course of the effect of PIK-75 (500nM) treatment on TRIB2 protein levels at the indicated times measured by Western Blot.* ***B)*** *Dose-response of PIK-75 treatment at 4h on TRIB2 protein levels measured by Western Blot.* ***C)*** *Effect of PIK-75 treatment at 4h on TRIB2 RNA levels measured by qPCR.* ***D)*** *Transfected myc-TRIB2 is also downregulated by PIK-75 treatment at 500nM and 4h.* ***E)*** *Effect of the combination of PIK-75 with the cell-*

permeable proteasome inhibitor MG132 or the PI3K inhibitor PI-103 on TRIB2 levels determined by Western Blot. ***F)*** *Effect of 30 minutes PIK-75 (100µM) treatment at the indicated temperatures on TRIB2 levels. PI3K has been used as a positive control of the stabilizing effect of PIK-75.* ***G)*** *Representation of the full leght, and the deletion mutants ΔN and ΔC of TRIB2.* ***H)*** *Effect of PIK-75 on TRIB2 deletions mutants. α-Tubulin has been used as loading control.*

The N-terminus of TRIB2 is necessary for PIK-75-mediated TRIB2 destabilization

To identify the specific region of the TRIB2 protein responsible for the destabilizing effect of PIK-75, we employed deletion mutants of TRIB2 genetically fused to a Myc-tag as previously described [22]. We transfected the full-length construct and the derived deletion mutants (Fig. 3G) into UACC-62 cells. Subsequently, we analyzed the ratio of TRIB2 protein levels between vehicle-treated cells and cells exposed to 500nM PIK-75 for each deletion mutant using western blotting. Interestingly, only the N-terminal deletion protected TRIB2 from PIK-75-mediated degradation while the C terminal deletion remained susceptible to the effects of PIK-75 (Fig. 3H). These results suggest that the N-terminus of TRIB2 contains critical amino acids that may undergo posttranslational modifications which contribute to the stabilization of TRIB2 protein.

MAPK pathway inhibition decreases TRIB2 expression

We also investigated the impact of inhibitors targeting the MAPK pathway, which plays a crucial role in melanoma and is frequently mutated in this type of cancer [23]. Furthermore, TRIB2 has been shown to regulate MAPK signaling and bind MEK [24]. Notably, treatment with vemurafenib, trametinib, and SCH-772984, inhibitors of BRAF, MEK and ERK respectively resulted in a decrease the level of endogenous TRIB2 protein (Fig. 4A). To analyze the kinetics of this effect, we treated UACC-62 cells with trametinib for different durations: 30 minutes, 1 hour, 2 hours, and 4 hours. While no noticeable effect was observed after 30 minutes or 1 hour of exposure, the TRIB2 protein level started to decline at 2 hours and after 4 hours almost no TRIB2 protein remained (Fig. 4B).

To investigate whether the inhibition of the MAPK pathway affectsTRIB2 at the protein level, we performed western blot analysis using cell lysates from cells ectopically expressing TRIB2 under the control of a constitutively active promoter. Fig. 4C demonstrates that SCH-772984 did not affect the protein level of ectopically expressed TRIB2. Furthermore, we examined whether MAPK pathway inhibition could interfere with TRIB2 transcription. The results revealed that vemurafenib, trametinib and SCH-772984 downregulated the expression of TRIB2 transcripts (Fig. 4D). In agreement, these MAPK inhibitors also decreased TRIB2 levels in A375 cells, indicating that the effect is not specific to UACC-62 cells. On the other hand, UACC-62 cells were co-treated with MAPK pathway inhibitors and the proteasome inhibitor MG132 for 4 hours. As shown in Fig. 4E, In the presence of MG132 and MAPK pathway inhibitors, TRIB2 levels were higher than in the DMSO vehicle treatment. These findings suggest that inhibition of MAPK pathway components can act at both the RNA and the protein levels.

In order to investigate whether the effect of MAPK pathway inhibitors on TRIB2 expression could be mediated through the regulation of downstream components of the pathway by ERK, we validated whether vemurafenib, trametinib and SCH-772984 affect the enzymatic activity of ERK. To this end we monitored the phosphorylation status RSK,

a well-established substrate of ERK [25]. Supplementary Fig. 3 demonstrates effective and potent inhibition of ERK and RSK phosphorylation upon exposure to the MAPK inhibitors. Additionally, we found that trametinib and vemurafenib failed to affect P70S6K phosphorylation and trametinib was unable to counteract P70S6K inhibition mediated by PI-103 (Supplementary Fig. 3). These data indicate that MAPK inhibitors effectively inhibit the enzymatic activity of ERK and act on TRIB2 levels independently of P70S6K. Conversely, we examined whether PIK-75 could affect MAPK signaling by monitoring the phosphorylation status of ERK and RSK. We showed that treatment of UACC-62 cells with PIK-75 failed to affect the phosphorylation of these proteins (Supplementary 3). Taken together, our results demonstrate that PIK-75 and MAPK inhibitors affect TRIB2 levels through two independent mechanisms.

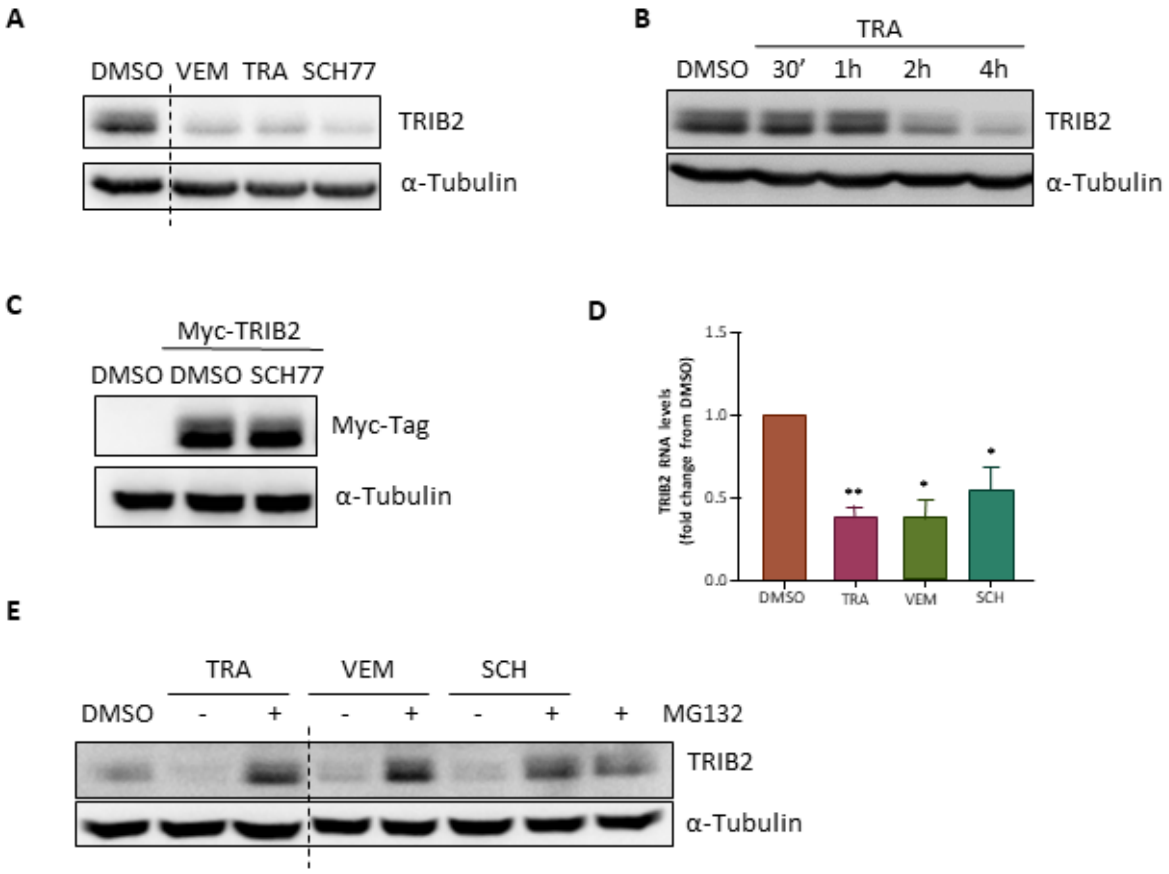

Fig. 4: *TRIB2 protein is downregulated by MAPK pathway inhibitors. A) Treatment with 500nM of vemurafenib (VEM), trametinib (TRA) or SCH-772984 (SCH77) reduces TRIB2 protein levels determined by Western Blot. B) Time-course of the effect of trametinib (500nM) treatment on TRIB2 protein levels at the indicated times measured by Western Blot. C) Transfected myc-TRIB2 is also downregulated by SCH-772984 (SCH77) treatment at 500nM 4 h D) Effect of Vemurafenib (VEM), Trametinib (TRA) or SCH-772984 (SCH) treatment for 4 h on TRIB2 RNA levels measured by qPCR. E) Treatment with the proteasomal inhibitor MG132 (10µM) revert the downregulation of TRIB2 protein levels mediated by 500nM of tramentinib (TRA), vemurafenib (VEM) and SCH-772984 (SCH) treatment for 4 h. α-Tubulin has been used as loading control.*

PLK inhibition abolishes TRIB2 expression destabilizing the protein

Intriguingly, the PLK1 inhibitor volasertib exhibited a time-dependent effect on TRIB2 expression. After just one hour of treatment, TRIB2 expression was significantly reduced, and it was eliminated after two hours. (Fig. 5A). We used a chemically very similar derivative of volasertib, BI-2536 to test if we could confirm the effect of PLK1 inhibition on TRIB2 protein level. Similarly, to volasertib, BI-2536 significantly reduced TRIB2 protein level after 4 hours of treatment in a dose-dependent manner (Fig. 5B). Notably, cells treated with 100nM BI-2536 exhibited significantly lower detectable levels of TRIB2 compared to cells treated with DMSO alone. Furthermore, TRIB2 levels continued to decrease at higher concentrations of BI-2536, namely 500nM and 1µM**.** Similar to MAPK pathway inhibitors, volasertib did not modify the protein levels of TRIB2 whether its expression was driven by the endogenous or a constitutively active promoter (Fig. 5C). An also these PLK inhibitors led to a decrease in TRIB2 RNA levels (Fig. 5D) indicating that TRIB2 inhibition occurs at the transcriptional level**.** This mechanism of TRIB2 inhibition underscores the effectiveness of PLK inhibitors in regulating TRIB2 expression and highlights their potential as therapeutic agents for targeting TRIB2-mediated processes. To determine whether the effect of PLK inhibition on TRIB2 is dominant over PI3K inhibition we co-treated cells with volasertib or BI-2536 in combination with the potent panPI3K/MTOR inhibitor PI-103. As depicted in Fig. 5E, the combined treatment also effectively reduced TRIB2 levels indicating that inhibition of PLKs completely overwrites the PI3K-mediated stabilization of TRIB2. To exclude the possibility that the effect of volasertib and BI-2536 is mediated through inhibition of the MAPK pathway, we measured the effect of these agents on the phosphorylation status of the ERK substrate RSK. As demonstrated in Supplementary Fig. 3, both inhibitors failed to affect RSK phosphorylation confirming that volasertib and BI2536 act independent of MAPK pathway inhibition.

As volasertib and BI-2536 potently inhibit not only PLK1 but also PLK2 and PLK3, albeit with slightly lower potency but still in the lower nanomolar range, we aimed to identify which PLK isoform is primarily responsible for the effect on TRIB2. We investigated the specific PLK1 inhibitor GSK-461364, which exhibits more than 1000-fold selectivity against PLK2/3. Interestingly, GSK-461364 failed to affect TRIB2 levels (Fig. 5F) suggesting that TRIB2 stability is regulated by PKL2 or 3 rather than PLK1. Additionally, wortmannin known to potently inhibit PLK1 and PLK3 [26] actually increased TRIB2 protein levels due to its PI3K inhibitory activity (data not shown). Collectively, these findings suggest that PLK2 is likely the key enzyme responsible for mediating the effect of volasertib and BI-2536 on TRIB2. To verify our hypothesis, we silenced PLK2 and measure its effect on TRIB2 protein levels. As shown in Fig. 5G, transient PLK2 silencing reduced TRIB2 levels indicating that the inhibition of PLK2 promotes TRIB2 downregulation and thus, supporting that the effect of volasertib and BI-2536 on TRIB2 is mediated by PLK2.

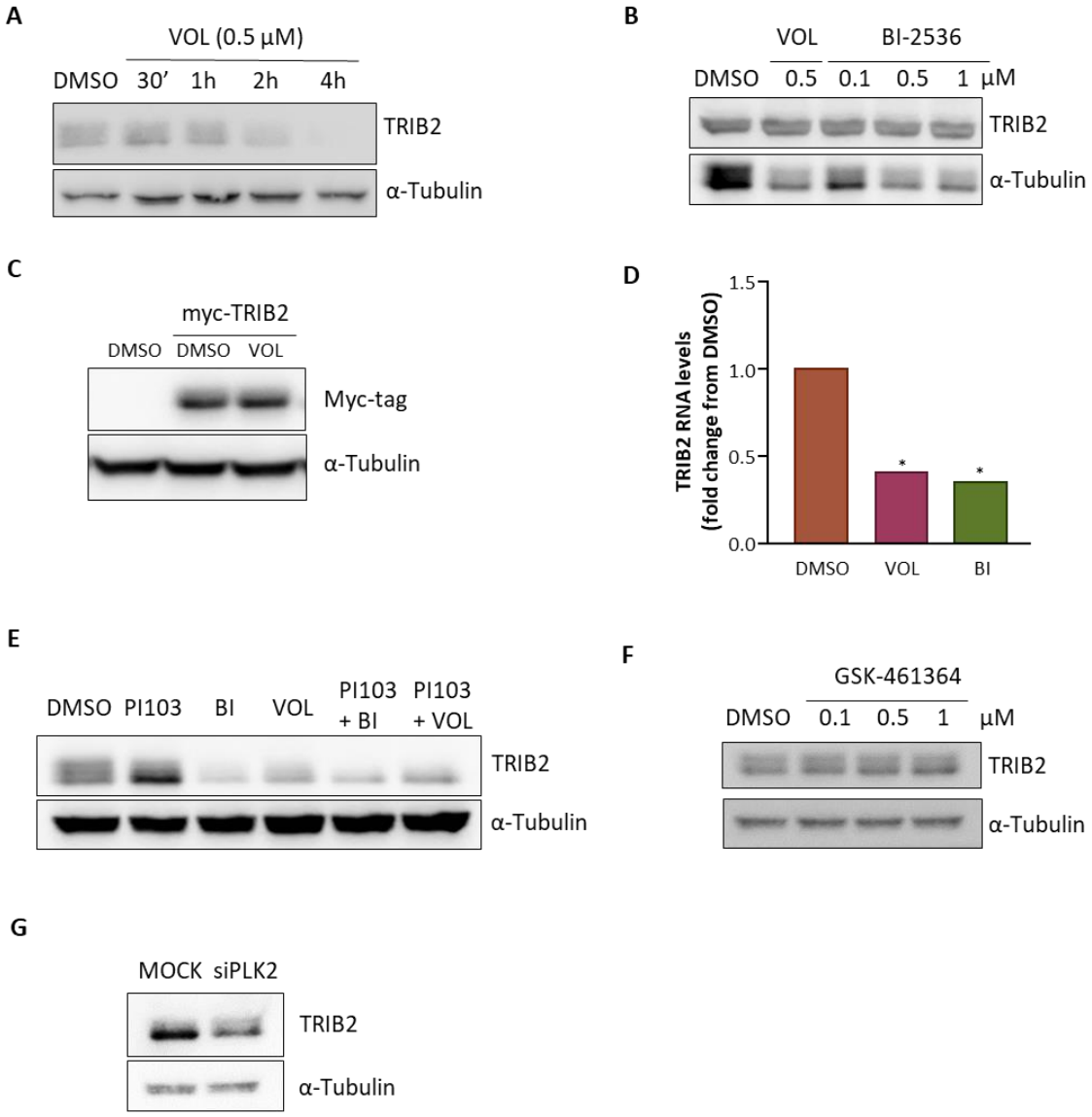

Fig. 5: *TRIB2 protein is downregulated by the PLK1 inhibitors Volasertib and BI-2536. A) Time-course of the effect of volasertib (VOL; 500nM) treatment on TRIB2 protein levels at the indicated times measured by Western Blot. B) Dose-response of BI-2536 treatment at 4h on TRIB2 protein levels measured by Western Blot. C) Transfected myc-TRIB2 is not downregulated by volasertib (VOL) at 500nM 4h. D) Effect of volasertib and BI-2536 treatment for 6h on TRIB2 RNA levels measured by qPCR. E) Effect of the combination of volasertib (VOL) and BI-2536 (BI) with the PI3K inhibitor (PI-103) on TRIB2 levels determined by Western Blot. F) Dose-response of GSK-461364 treatment at 4h on TRIB2 protein levels measured by Western Blot. I) Effect of PLK2 downregulation using esiRNA technology on TRIB2 protein levels determined by Western Blot.*

Isoform-specific effect of TRIB2 inhibitors on Tribbles proteins

We proceeded to examine the impact of the identified TRIB2 inactivating compound on other members of the Tribbles protein family, TRIB1 and TRIB3, in melanoma cells. The cells were treated with the previously used inhibitors: PI-103 (PI3K/MTOR), SCH-772984 (ERK), PIK-75 (multi-kinase), BI-2536, GSK-461364 and volasertib (PLKs) for a duration of 4 hours. Fig. 6 illustrates that none of the compounds produce a decrease in the quantity of TRIB1. However, in the case of TRIB3, SCH-772984, BI-2536 and volasertib downregulate its amount in the treated cells. Intriguingly, PIK-75 failed to exert an effect on TRIB1 or TRIB3 indicating its specificity towards TRIB2.

To determine whether the influence of ERK or PLK inhibition on TRIB3 levels occurs at the protein level, we treated cells that produced TRIB3 from a constitutive promoter. Fig. 6 demonstrates that the quantity of ectopic TRIB3 remained unchanged following any of the treatments. This suggests that the agents affect TRIB3 through a mechanism that involves transcription, implying that the posttranslational regulation observed for TRIB2 is specific to this particular isoform.

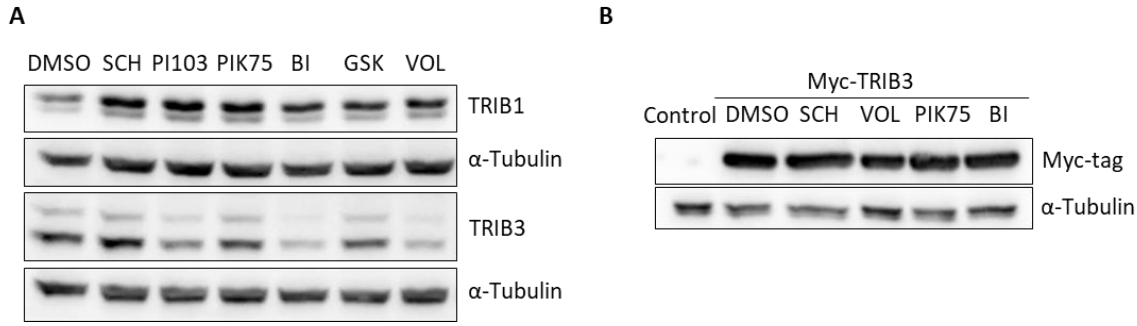

Fig. 6: *Effect of a panel of kinase inhibitors in TRIB1 and TRIB3 levels. A) Effect of PI-103, SCH-772984 (SCH), PIK-75, BI-2536 (BI), GSK-461364 (GSK) and volasertib (VOL) on TRIB1 and TRIB3 levels. B) Effect of SCH-772984 (SCH), PIK-75, BI-2536 (BI) and volasertib (VOL) on myc-TRIB3 ectopically expressed. Non-transfected cells were used as control and α-tubulin was used as loading control.*

Our study revealed three different, independent inhibitory mechanisms capable of eradicating TRIB2 from melanoma cells. Fig. 7 provides a comprehensive model that summarizes the results.

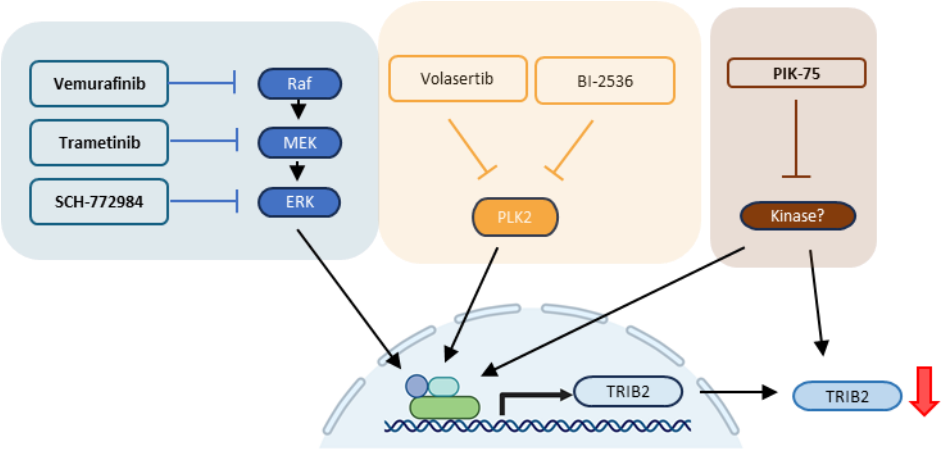

Fig. 7. Model for the mode of action of the chemical probes/drugs on TRIB2. *PIK-75 stabilizes TRIB2 protein at lower nanomolar concentrations. However, at higher nanomolar concentrations, PIK-75 loses its specificity for PI3K and induces TRIB2 destabilization independent of transcription, overriding its stabilizing effect. PIK-75-mediated TRIB2 destabilization is likely due to the inhibition of a kinase. On the other hand, MAPK inhibitors like vemurafenib, trametinib, and SCH-772984 inhibit ERK-mediated phosphorylation of a substrate, which eventually interferes with the transcription of TRIB2. Volasertib and BI-2536 act through PLK2 to interfere at both the transcriptional and post-translational levels, leading to decreased TRIB2 levels.*

DISCUSSION

TRIB2 has emerged as a promising therapeutic target in various tumor types, particularly in melanoma, to overcome therapy resistance [3,17,27]. In this study, we present the identification of pharmacological strategies to modulate TRIB2 expression at both the transcriptional and protein levels, shedding light on the underlying molecular mechanisms. We observed four distinct pharmacological effects on TRIB2 mediated by different molecular mechanisms. Firstly, inhibitors of the PI3K/AKT signaling pathway led to increased TRIB2 protein levels. Secondly, inhibitors of the MAPK pathway downregulated TRIB2 at the transcriptional level. Thirdly, PIK-75 destabilized TRIB2 at the protein level independently of transcription, overriding its inhibitory effect on PI3K. Lastly, PLK inhibition downregulates TRIB2 RNA expression and destabilized TRIB2 protein. Notably, the observation that chemical agents can upregulate TRIB2 protein levels suggests their potential to drive drug resistance mechanisms and potentially elicit a feedback loop.

Compounds targeting components of the insulin pathway, such as PI3K, AKT, or MTOR, reduce phosphorylation and enzymatic activity of AKT [28]. Paradoxically, they simultaneously increase the expression of TRIB2, known to activate AKT. This could represent a potent mechanism of acquired drug resistance following treatment with these inhibitors. Further exploration of the clinical impact of this mechanism, correlating TRIB2 expression with response to these agents, is warranted. This task will become more achievable as more of these compounds enter clinical practice and patient samples become more accessible. Validation of the relationship between TRIB2 levels and resistance to these drugs would provide a strong rationale for clinical trials investigating combination therapies incorporating TRIB-targeting agents.

The stabilizing effect of PI3K/AKT signalling on TRIB2 might be mediated by P70S6K which has been reported to phosphorylate TRIB2 at its N-terminus [29]. Impaired P70S6K-mediated TRIB2 phosphorylation has been shown to promote TRIB2 stabilization. Regarding the bimodal effect of the PI3K inhibitor PIK-75 on TRIB2, two hypotheses are considered. At higher concentrations, PIK-75 may inhibit other kinases or enzymes that stabilize TRIB2, or it may directly bind to TRIB2 and lead to its destabilization. However, our focused candidate approach, based on published data on PIK-75 targets and commercially available inhibitors, did not identify any agent capable of phenocopying the effect of PIK-75 on TRIB2. Additionally, based on CETSA experiments, PIK-75 did not directly bind to TRIB2. These findings suggest that the identity of the enzyme, likely a kinase, directly modifying or indirectly contributing to the stabilizing posttranslational modification within the first 64 amino acids of the TRIB2 protein, remains to be identified. Target identification will lay the groundwork for developing drug-like compounds capable of mimicking the effect of PIK-75 on TRIB2, which can be further optimized through medicinal chemistry. Notably, our discovery that vemurafenib and trametinib, two targeted drugs approved for treating BRAF-mutated melanomas, downregulate TRIB2 - a significant driver of therapy resistance is highly intriguing. We hypothesize that the drug-induced downregulation of TRIB2 contributes to the clinical efficacy of these agents. Investigating the relationship between TRIB2 expression and response to vemurafenib and trametinib treatment is necessary to test this hypothesis. If clinically relevant, TRIB2 could serve as a useful marker for stratifying patients who would benefit from treatment with these inhibitors. Additionally, our data linking PLK inhibition to TRIB2 downregulation establishes a connection between these kinases involved in eukaryotic cell division and TRIB2. Two chemically similar derivatives, BI-2536 and volasertib, were found to effectively mediate TRIB2 downregulation with high potency. Importantly, also both compounds led to a slight downregulation of TRIB3 transcripts, protein degradation remained unaffected on TRIB1 and TRIB3.
In summary, our study highlights TRIB2 as an attractive therapeutic target in melanoma and potentially other tumor types. We have identified pharmacological approaches to manipulate TRIB2 expression at both the transcriptional and protein levels, uncovering various independent, molecular mechanisms involved. The findings suggest the potential of these compounds to influence drug resistance mechanisms and indicate the need for further investigation regarding the impact of TRIB2 on treatment response. The destabilizing effect of PI3K/AKT signaling on TRIB2 and the link between PLK inhibition and TRIB2 downregulation provide valuable insights into TRIB2 regulation and its potential as a therapeutic target.

MATERIALS AND METHODS

Chemical compounds

Table 1 lists compounds used to analyze TRIB2 regulation. All compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solutions and stored at -80°C.

Table 1: *List of compounds used to modulate TRIB2 levels.*

Name	Target	Source
afatinib	EGFR	Selleckchem
BI-2536	PLK1,2,3	Selleckchem
cyclohexamide	60S ribosomal subunit	Sigma Aldrich

dactolisib	panPI3K/MTOR/ATR	Selleckchem
GSK-461364	PLK1	Selleckchem
LY294002	PI3K (p110α, β, δ)	Selleckchem
MG132	Proteasome	Selleckchem
PI-103	panPI3K/MTOR	Selleckchem
PIK-75	PI3K (p110α, δ, γ), DNA-PK, multikinase	Selleckchem
rapamycin	mTORC1	LC Laboratories
SCH-772984	ERK1/2	Sigma Aldrich
trametinib	MEK	Selleckchem
vemurafinib	BRAF	Selleckchem
volasertib	PLK1,2,3	Selleckchem
wortmannin	PI3K, PLK1,3	LC Laboratories

Cell Culture

The human melanoma cell lines A375 and UACC-62, and the osteosarcoma cell line U2OS were purchased from ATCC, maintained in DMEM supplemented with 10% FBS (Sigma) and antibiotics (Gibco). Cell cultures were maintained in a humified incubator at 37°C with 5% CO2 and passaged when confluent using trypsin/EDTA.

Plasmids and cell transfection

Expression vectors were transiently transfected into UACC-62 parental or TRIB2 KO cells with turbofect transfection reagent (Thermo Fisher Scientific) following manufacturers protocol and analyzed 48 hours after transfection. We used two different plasmids with the complete TRIB2 sequence preceded by a constitutive promoter and tagged with myc or flag at the N terminal end, two myc-TRIB2 deletion mutants: for the N terminal (aminoacids from 64 to 353) or the C terminal (aminoacids from 1 to 308), and the phospho-mimetic flag-TRIB2 S94D mutant.

siRNA-mediated silencing

A heterogeneous validated pool of siRNA against PLK2 mRNA (esiRNA, MERCK) was transiently transfected using TurboFect transfection reagent (Thermo Fisher Scientific) for 48 hours following the manufacturer's protocol. The effective downregulation of PLK2 and its effect on TRIB2 levels were analyzed by Western blot.

Quantitative Real-Time PCR

To determine the transcription levels of genes affected by PIK-75, trametinib, volasertib, SCH772984, volasertib or BI-2536 treatments, cells were treated with each drug alone, 24 hours after plating, at a cell confluency of 70-90% on 6cm plates. After 6 hours of treatment, RNA was extracted (DNA, RNA and protein purification Kit, #74098 Macherey-Nagel) and then converted to cDNA (High Capacity cDNA Reverse Transcription, Applied Biosystems). Quantitative PCR was performed on 7900HT Fast Real Time PCR (Applied Biosystems) using SYBR GREEN PCR Master Mix (Applied Biosystems). Relative quantification of gene expression was determined by the 2-ΔΔCt method [72].

Cycloheximide (CHX) chase assays

Protein stability was assessed by using CHX chase assays. Protein biosynthesis was blocked by the inhibition of translational elongation using 50 µg/mL CHX. Subsequently, protein lysis was carried out at the indicated time points, followed by Western Blot analysis to determine TRIB2 protein degradation over time.

Cellular Thermal Shift Assays (CETSAs)

To investigate the direct binding of small molecule compounds to their target protein, we employed the Cellular Thermal Shift Assay (CETSA), following a previously published protocol [30]. Briefly, cells were cultured for 24 hours, washed with PBS, and protein extraction was performed using PBS supplemented with protease and phosphatase inhibitors (1mM PMSF, 1mM benzamidine, 1mM iodoacetamide, 1mM phenanthroline, 0.1mM sodium orthovanadate). Subsequently, the extracted proteins were divided into separate tubes and treated with either DMSO, 100µM afatinib, or 100µM PIK-75 for 30 minutes to facilitate compound-protein binding. Each treatment group was then subjected to incremental temperature increases (37, 40, 45, 50, 55°C). Following incubation, the samples were centrifuged at 17,000 g for 30 minutes, and the resulting supernatant was collected. Finally, 1/5 volume of Laemmli 5X buffer containing 5% β-mercaptoethanol was added to the supernatant, followed by boiling at 95°C for 5 minutes. The prepared samples were then frozen at -20°C for subsequent analysis via immunoblotting.

Western Blot

For the preparation of whole-cell lysate, cells were harvested and lysed in lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1% Triton X-100, 50mM NaF, 1mM EDTA, 1mM EGTA, 2.5mM sodium pyrophosphate, 1mM b-glycerophosphate, 10nM Calyculan A, and EDTA-free complete protease inhibitor cocktail (PIC) (Sigma). Sample buffer was added to 1X final, and samples were boiled at 95°C for 5 minutes. Samples were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted according to the antibody manufacturer's instructions. The membranes were then probed with the following primary antibodies: TRIB2 (#13533 cell signaling), α-tubulin (T9026 sigma), β-actin (A5441 sigma), PI3K (#06-195 upstate), TRIB1 (09-126 millipore), TRIB3 (Ab75846 Abcam), myc.tag (16286-1-AP proteintech), phospho-Serine (#AB1603 millipore), phospho-P70S6K (#9234 cell signaling), phosphor-RSK (ABS1849 millipore) and RSK (610225 BD Biosciences). Horseradish peroxidase (HRP)-conjugated secondary antibodies were added (Santa Cruz Biotechnology) at 1:5000 dilution for 1 hour at room temperature. Visualization of signal was achieved using a FUSION Solo S (VILBER) and analyzed with Evolution-Capt software.

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Conflicts of Interest

The authors declare no conflict of interest. Wolfgang Link is the scientific co-founder of Refoxy Pharmaceuticals GmbH, Berlin and is required by his institution to state so in his publications. The funders had no role in the design and writing of the manuscript.

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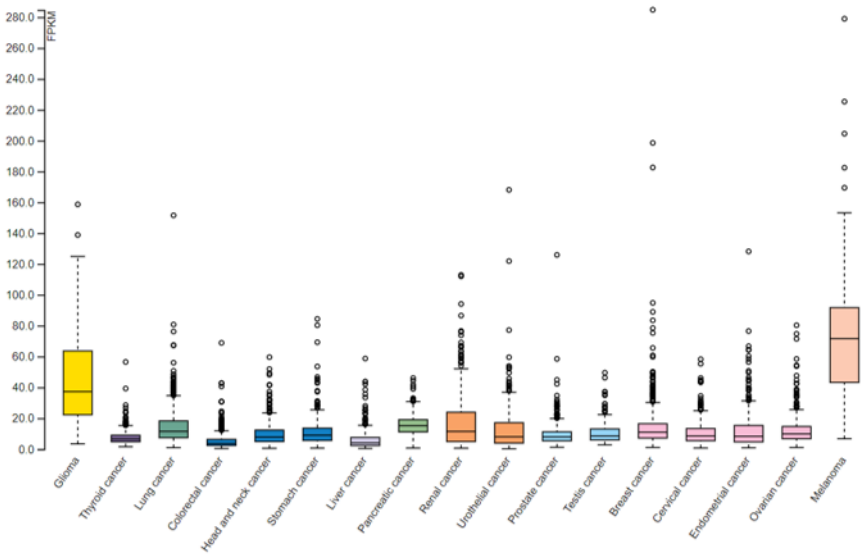
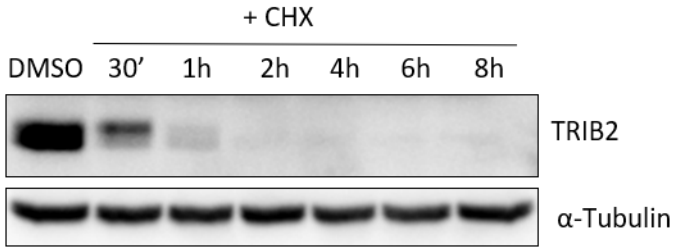
Supplementary Figures

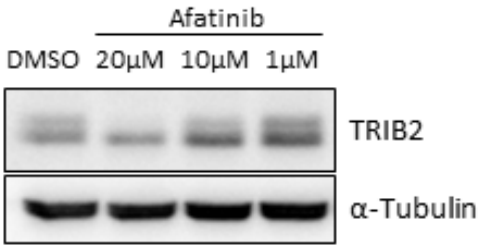
A



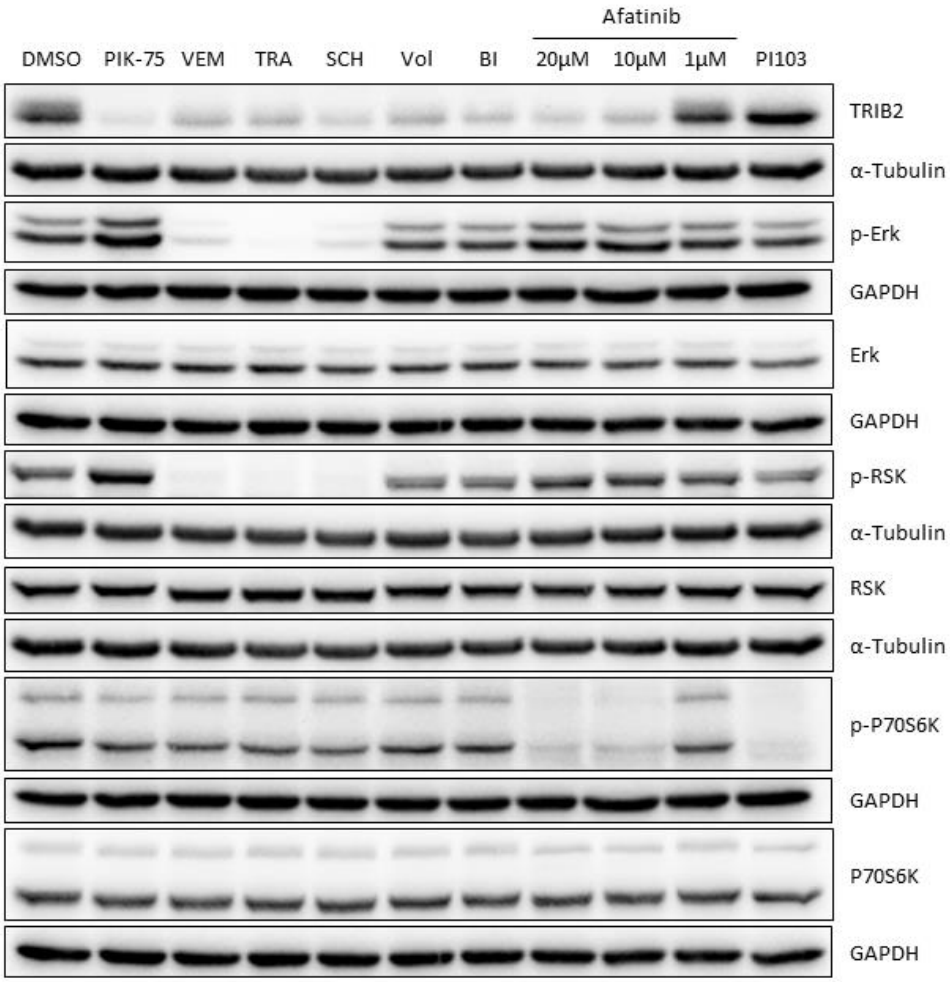
B



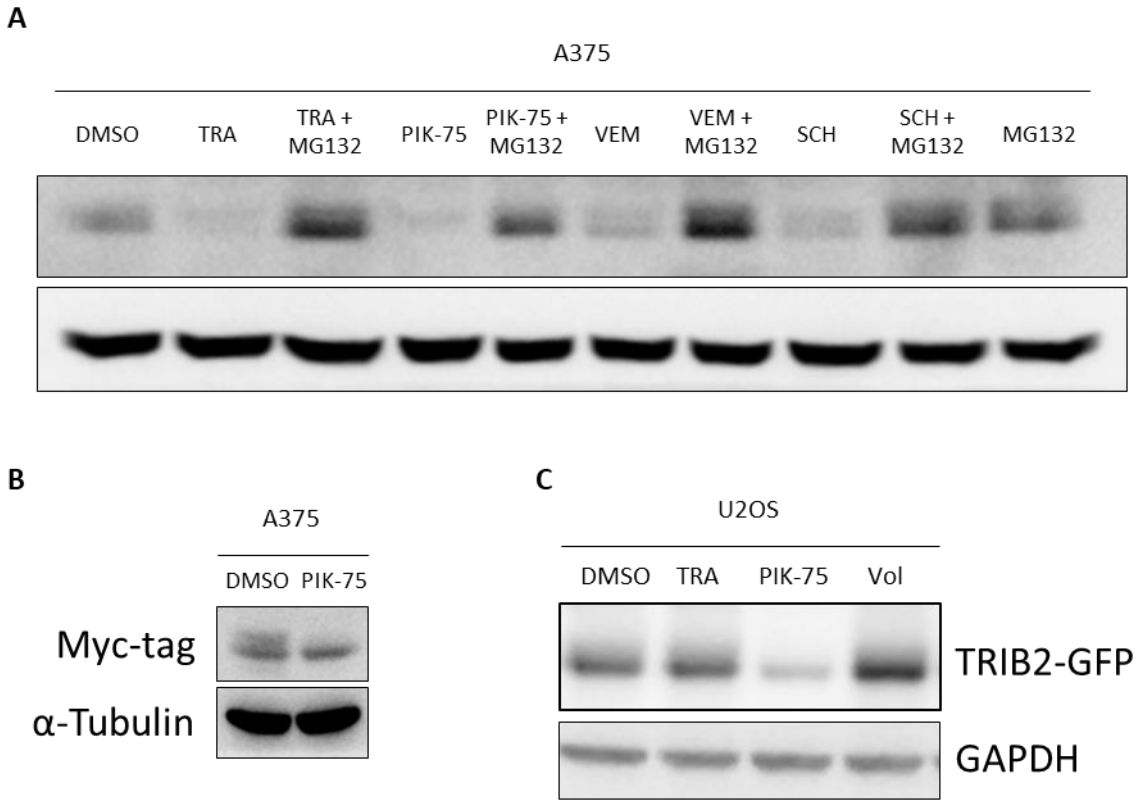
Supp. Figure 1: *A) Expression of TRIB2 based on RNA-seq data from 17 cancer types from TCGA data. The TCGA RNA-seq data was mapped using the Ensembl gene id available from TCGA, and the FPKMs (number Fragments Per Kilobase of exon per Million reads) for each gene were subsequently used for quantification of expression with a detection threshold of 1 FPKM. B) Protein synthesis was blocked by Cycloheximide (CHX, 50 ug/ml) for the indicated time. The half-life of TRIB2 was measured by Western blot. α-tubulin was used as loading control.*



Supp. Figure 2: *Effect of afatinib treatment in TRIB2 levels. TRIB2 protein levels were determined by western blot after 4h treatment with afatinib at the indicated concentrations. α-tubulin was used as loading control.*



Supp. Figure 3: *Effect of compounds that effect TRIB2 expression related to the MAPK pathway and PI3K pathway. p-Erk and p-RSK were used as target of the MAPK pathway and p-P70S6K as target of the PI3K pathway. Protein levels were determined by western blot after 4h of treatment with 500nM of all compounds except afatinib which was used at the indicated concentrations. α-tubulin and GAPDH were used as loading control.*



Supp. Figure 4: *A) The treatment with Trametinib 500nM (TRA), PIK-75 500nM, vemurafenib 500nM (VEM), or SCH-772984 500nM (SCH) decreases the level of TRIB2 as determined by Western Blot in the A375 melanoma cell line. B) Transfected myc-TRIB2 is also downregulated by PIK-75 treatment at 500nM and 4h in the A375 cell line. C) Also, PIK-75 treatment but not Trametinib (TRA) or Volasertib (Vol) at 4h in U2OS sarcoma cell line with a constitutive expression of TRIB2 GFP reduce the TRIB2 levels. α-tubulin was used as loading control.*