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Rho inhibition promotes vemurafenib resistance in BRAF mutant melanoma

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RHO INHIBITION PROMOTES VEMURAFENIB RESISTANCE IN BRAF MUTANT MELANOMA

by

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Abstract

Current treatments for patients with BRAF mutated melanoma show limited success utilizing the drug vemurafenib by only temporarily stopping cancer cell growth. Eventually BRAF mutated cells come back completely resistant to vemurafenib. The mechanisms behind emerging resistance are not well understood, yet studies suggest that actin polymerization through Rho small family GTPase signaling have an important role. In an attempt to address this working hypothesis we created an in vitro model using the A375 cell line and treated the cells with vemurafenib combined with RNAi knockdowns of Rho or using a drug named fasudil, which inhibits ROCK kinase a downstream target of Rho. Our data showed that through either direct or indirect Rho pathway inhibition there was a promotion of acquired resistance in the A375 cell line. Additionally we see an up regulation of transcription factors Yap/Taz and increased protein expression of pPaxillin Y31, pMEK S217, and pMEK S298. All of these proteins have been associated with Rac1 activity or BRAF activity within the cell. The increase in Rac1 activity may help give us a better explanation of what cellular pathways BRAF mutated cells utilize to promote resistance.

Introduction

Identification of a major mutation, *BRAFV*600E, in cutaneous melanoma patients was a major breakthrough in the search for effective clinical treatments for this deadly disease. This non-conservative mutation of valine to glutamic acid at the 600 position of the *BRAF* protein is present in approximately 50% of all skin melanomas.[1-4] Current treatments for BRAF mutant melanoma utilize the drug vemurafenib, which acts as a *BRAF* kinase inhibitor. Vemurafenib treatment significantly prolongs survival times in patients with the *BRAFV*600E mutation; however, resistant cells eventually emerge in patients and tumors typically come back within ~6 months. [5-7] There is evidence that Rho family small GTPases may play significant roles in acquired resistance to vemurafenib.[8] When Rho is in it's active, GTP-bound form it signals to a downstream effector called ROCK kinase. ROCK kinase acts as a key regulator for actin polymerization in the cell and plays a role in cell migration.[9] Previously published research suggests that inhibition of actin remodeling can potentially suppress acquired resistance to vemurafenib.[10] In preliminary experiments we developed an vitro model of acquired resistance using *BRAFV*600E mutant A375 melanoma cells. In A375 cells, Rho GTPase signaling was then either directly inhibited through RNAi knockdowns of RhoA/C or indirectly inhibited through ROCK kinase utilizing the drug fasudil. Resulting data illustrated that vemurafenib and inhibition of Rho signaling caused acquired resistance to emerge in a shorter time than vemurafenib-only treated cells. This recently obtained data suggests that suppressing RhoA/C signaling promotes acquired resistance, possibly by up regulating the activity of other Rho family small GTPases. Exploring other small GTPases such as Rac1 could reveal a possible explanation. Rac1 also plays a role in actin polymerization and signals to a downstream effector PAK1 that plays a similar role to ROCK kinase, acting as a key regulator of actin polymerization.[11] Further investigation of which Rho family GTPases may be the major contributors to acquired resistance could potentially fill the gap in previous knowledge towards what causes the emerging resistance in vemurafenib treated cells.

Methods

Cell Culture

A375 cells were thawed from liquid nitrogen then passaged in standard DMEM growth medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% glutamine, 1% pen strep. Cells were then incubated at 37°C for propagation.

In vitro growth assays

RhoA/RhoCsh1 and RhoA/RhoCsh2 knockdown assay

RhoAsh1, RhoCsh1, RhoAsh2, and RhoCsh2 were treated with DMSO or 3 mM vemurafenib. A standard DMEM growth medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% glutamine, 1% pen strep was added to make volume 2 ml total. The cells were re-fed with their respective drug concentrations and re-seeded to 40,000 every four days. The cells were harvested, counted, and recorded accordingly at every four-day time point as well. The cells were left growing until 60 days.

A375 fasudil assay

A total of 40,000 A375 cells per well were plated in a six well plate and were treated with DMSO, 3 mM vemurafenib, 10 mM fasudil, or 3 mM vemurafenib and 10 mM fasudil. A standard DMEM growth medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% glutamine, 1% pen strep was added to make volume 2 ml total. The cells were re-fed with their respective drug concentrations and re-seeded to 40,000 every four days. The cells were harvested, counted, and recorded accordingly at every four-day time point as well. The cells were left growing until 65 days.

A375 fasudil assay lysate setup

A375 cells from a 100% confluent T75 ml flask were harvested with 10% being placed into a 60mm dish with DMSO or 10 mM fasudil and 40% being placed into a 60mm dish with 3 mM vemurafenib or 3 mM vemurafenib + 10 mM fasudil. Two extra 60mm dishes were made for vemurafenib and vemurafenib + fasudil conditions. A standard DMEM growth medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% glutamine, 1% pen strep was added to make volume 4 ml total. Cells were re-feed every four days and cells were lysed at 4 days, 14 days, or 30 days to be immunoblotted.

Immunobloting

Cells were rinsed with 20 mm HEPES, pH 7.5, 150 mm NaCl, 5 mm $MgCl_2$ (HBSM) and lysed in 1X SDS laemmli buffer. The lysates were scraped, boiled, and sheared using 27G1/2 needle. The protein concentration was evaluated using Red 660 Protein Assay (G Biosciences #786-676). Normalized protein lysates were resolved in SDS-PAGE and transferred to Nitrocellulose membrane. Blots were blocked using AquaBlock buffer (EastCoast Bio #PP82) and probed with the primary antibodies in blocking buffer + 0.1% Tween-20. Additional antibodies binding to Paxillin, pPaxillin Y31, Tubulin, Yap/Taz, Fak, pFak, Akt, pAkt, pErk, c-Met, pc-Met, Rac1, PAK, phospho-PAK, phosphor-MEK, and MEK were also utilized. Blots were developed by using Alexa Fluor 680 (Invitrogen A21058) and Alexa Fluor 790 (Invitrogen A11369) diluted in 1:20000 blocking buffer + 0.1 % Tween 20. Blots were observed using a LI-COR Odyssey blot imager (LI-COR

Biosciences). The licor blot imager quantified each blot and differences in the relative expression level specific signaling proteins were tested for significance using ANOVA.

Results
Loss of Rho signaling in the A375 cell line promotes resistant colony formation when treated with vemurafenib.

To understand the effective role of Rho signaling with vemurafenib we utilized A375 cells that were RNAi knockdowns of RhoAsh1 and RhoCsh2 and we compared their growth rate to NT control cells. At 60 days there is significant difference in population doubling between RhoA/RhoC Sh1&Sh2 group and NT groups, every Rho knockdown A375 line has at least double the NT group population (Fig.1A). A dose response growth inhibition assay was utilized in order to find a concentration of fasudil to use for the experimental process that would not outright destroy A375 from a lethal dosage. $10_{\mu M}$ was our selected concentration of choice (Fig.1B). Treating cells with fasudil yields very similar results where we see emerging resistant colonies emerging faster than our only vemurafenib treated cells (Fig. 1C). At 18 days we can see that the vemurafenib + fasudil treated cells are already growing back while the vemurafenib only treated cells start growing back around the 33 day mark. Additionally there is a major difference at the 65 day mark, where the vemurafenib + fasudil treated cells have more than two times the population doubling than the vemurafenib alone treated cells (Fig 1C.). Interestingly we can see that the direct inhibition of Rho kinase or inhibition of downstream target such as ROCK kinase hastens the emergence of resistant cells compared to cells treated with vemurafenib alone.
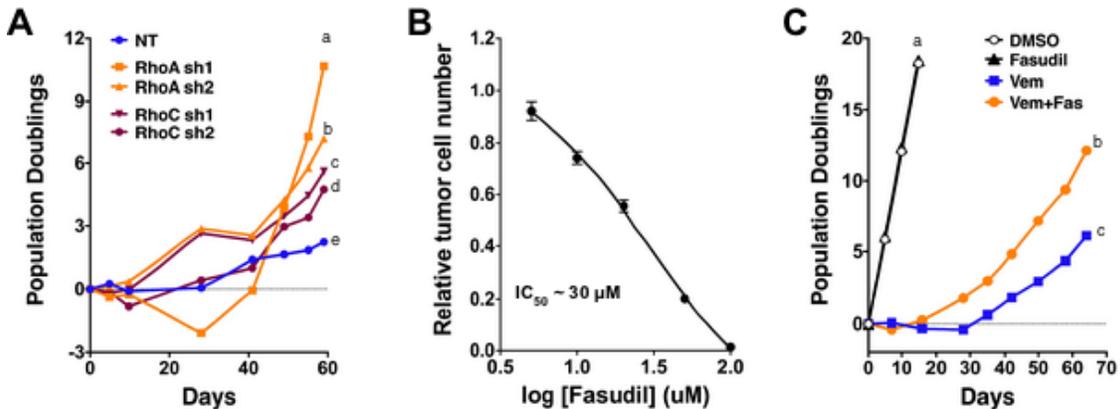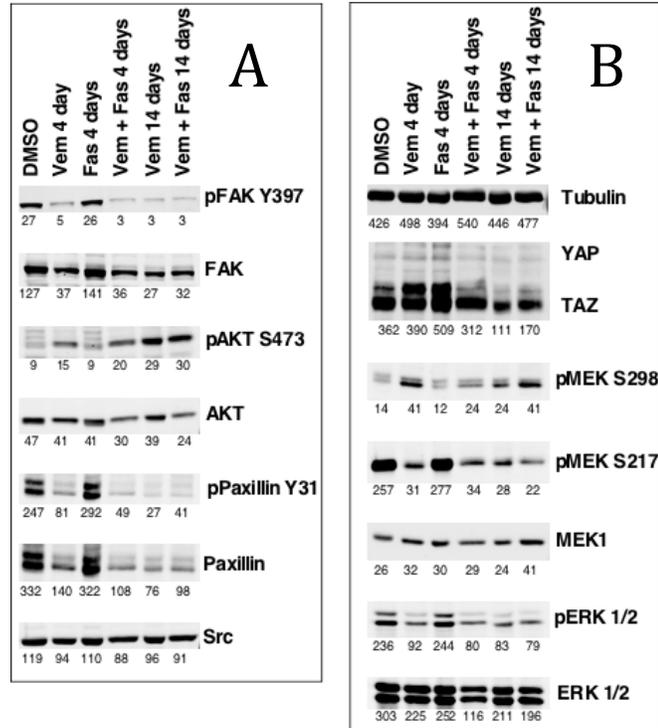

Figure 1: *Loss of Rho signaling in A375 cell line promotes a decreasing resistant colony formation time when treated with vemurafenib.* **A**, Population doubling versus time. **a**, RhoA sh1 knockdown A375 cell line. **b**, RhoA sh2 knockdown cell line. **c**, RhoC sh1 cell line **d**, RhoC sh2 cell line **e**, NT cell line. All five line's were grown in $3_{\mu M}$ of vemurafenib. The cell populations were terminated at 60 days. **B**, IC_{50} showing tumor cell number versus fasudil concentration **C**, Population doubling versus time. **a**, DMSO and Fasudil treated condition's * they are stacked directly on top of each other. **b**, Vemurafenib + Fasudil treated conditions. **c**, Vemurafenib treated only condition. The cell populations were terminated at 65 days.

Short-term fasudil treatment leads to subtle differences in key Rho, BRAF, and Rac proteins.

 In order to understand how fasudil or Rho inhibition promotes resistance to vemurafenib in A375 cells we blotted for various Rho, BRAF, and Rac1 proteins. We selected pPaxillin Y31 because it may possibly be associated with an increase in Rac1 activity.[17] Working very close with paxillin is another protein we blotted for named FAK. There is evidence that they cross talk and interact directly with each other.[13] FAK's relation to Rac1 activity is that FAK activates Rac1.[14] Interestingly we only see a very small increase in FAK with vemurafenib + fasudil compared to vemurafenib alone at the 14 day mark but not the 4 day mark (Fig. 2A). At day 4 there appears to be no significant differences among vemurafenib only and vemurafenib + fasudil conditions (Fig 2A). Yap and Taz are transcription factors

Figure 2: *Title* **A**, Western blot comparing the differences among pFAK Y397, FAK, pAKT S473, AKT, pPaxillin Y31, Paxillin, and Src protein expression. Protein was loaded from cell lysates grown under DMSO, Fasudil, Vemurafenib, and Vemurafenib + Fasudil conditions. There are two time points one at 4 days and another at 14 days. We did not carry a DMSO or Fasudil plate to the 14 day mark. **B,** Is part of the same Western blot containing Tubulin, YAP/TAZ, pMEK S298, pMEK S217, MEK1, pERK ½, and ERK ½ protein expression. Lysates were from exact same cells as in figure 2A.

that were selected because they are thought to regulate the Hippo pathway and increased expression patterns have been associated with increased cell proliferation and epithelial–mesenchymal transition.[15] Yap and Taz have some interesting differences among vemurafenib + fasudil and only vemurafenib treated cells. We can see that within the first 4 days there is no significant difference among the vemurafenib and the vemurafenib + fasudil treated cells. Then at 14 days out we can see an intriguing result vemurafenib + fasudil treated cells have almost double the Yap/Taz expression than the vemurafenib only cells (Fig. 2B). We utilized pMEK S298 because S298 is where PAK kinase phosphorylates MEK and PAK kinase functions downstream of Rac1.[16] Our blot showed that early expression at Day 4 had no real significant differences in pMEK S298 expression levels. As time progressed to 14 days we saw that pMEK S298 has just about two times the protein expression in vemurafenib + fasudil compared to vemurafenib only (Fig. 2B). Similar to pMEK S298 another pMEK phosphorylation site was selected called pMEK S217, which is a protein that is related to BRAF activity.[21] Our results displayed that over the first 14 days there is no substantial change in pMEK S217 activity from vemurafenib only and vemurafenib + fasudil (Fig. 2B). Another protein we stained for that is linked to BRAF activity is ERK. [22] We found the same results as we did in pMEK 217 where there were no major differences in expression during the first 14 days (Fig. 2B). Other proteins we stained like AKT and Src are linked to Rac1 expression. AKT level activation has shown to inhibit Rac1-GTP binding.[12] On the other hand, increased Src levels have been associated with increased Rac1 activity.[23] Looking

at our blot there really is no large differences in either AKT or Src expression during the first 14 days (Fig. 2A). We do see a little increase in total AKT at day 14, however since the active form of AKT, pAKT S473, has a virtually the same expression, this result gives us no supporting evidence that active AKT levels are increased significantly (Fig. 2A). Finally the protein tubulin was also stained for and we see very similar expression throughout all 14 days for all cell treated conditions (Fig. 2B).

Long-term fasudil treatment may promote resistance through the up regulation of Yap/Taz, pPaxillinY31, Paxillin, pMEK S298, and pMEK S217.

While searching for clues during the early stages of treatment using fasudil, we might gain more insight to how resistant mechanisms emerge through analyzing the cells protein expression levels at a later time point. There are many pieces within figure 3 that help us solve this puzzle. Starting off we see Yap/Taz, pPaxillin Y31, and paxillin have crucial differences comparing vemurafenib only- and vemurafenib + fasudil-treated cells. We can see that there is about a four times fold increase in each of these proteins in the cells treated with vemurafenib + fasudil (Fig. 3A). Referring back to Fig. 2 A and B we can see that at day 4 there is no real significant difference and towards day 14 we can see that the vemurafenib + fasudil treated cells start producing higher expression levels of Yap/Taz, pPaxillin Y31, and paxillin. A more obvious difference can be seen now at day 30 where expression of these proteins increased substantially. Other interesting information from our blot can be seen in AKT and pAKT S473 (Fig. 3A). We see

Figure 3: *Title* **A,** Western blot comparing the differences among pAKT S473, AKT, YAP/TAZ, pPaxillin Y31, and Paxillin protein expression. Protein was loaded from cell lysates grown under Vemurafenib or Vemurafenib + Fasudil conditions for 30 days. **B,** Is part of the same Western blot containing pMEK S217, pMEK S298, MEK1, pERK ½, and tubulin protein expression. Lysates were from exact same cells as in figure 2A.

that the trend is identical to Fig. 2A where the expression is not showing any serious differences in vemurafenib only and vemurafenib + fasudil. . Further information can be obtained by analzying pMEK S298, which now has a four times fold increase when comparing vemurafenib only to vemurafenib + fasudil. The other MEK protein, pMEK S217, displayed an increase as well when comparing the same vemurafenib and vemurafenib + fasudil groups to each other (Fig. 3B). Other clues can be found from analyzing MEK1 and pERK 1/2. At 30 days out we see no real evidence of there being any substantial difference in MEK1 or pERK1/2 expression between vemurafenib only and vemurafenib + fasudil (Fig. 3B). Additionally tubulin elicited protein expression levels that were almost identical in both of our conditions supporting that no drastic changes in tubulin occurred either (Fig 3B).
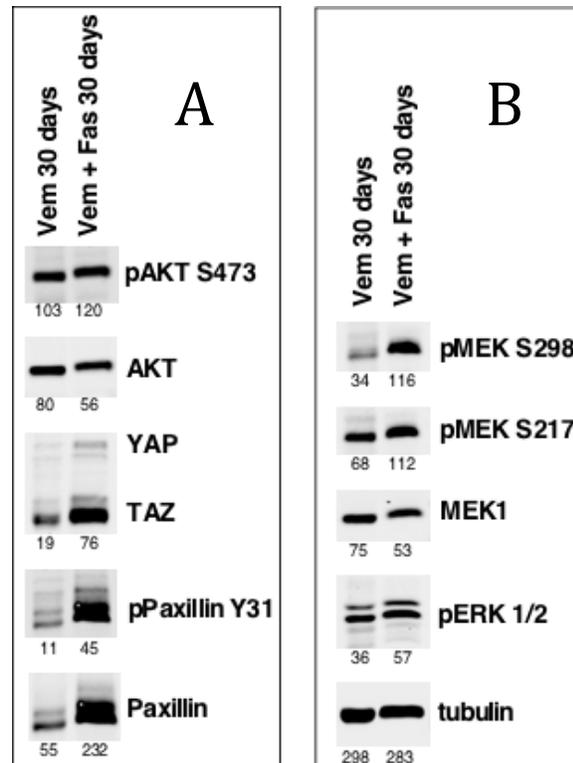
Fasudil overtime promotes increased Yap/Taz, pMEK S298, and pMEK S217.

In order obtain a clear comparison of significant changes in protein expression from day 4 all the way to day 30, we placed all time points on a single blot The most critical findings can be seen within Yap/Taz and pMEK S298 protein expression levels. Specifically looking at

Figure 4: Title **A,** Western blot comparing the differences Yap/Taz, pMEK S298, Tubulin, and MEK1 protein expression. Protein was loaded from cell lysates grown under DMSO, Fasudil, Vemurafenib or Vemurafenib + Fasudil conditions from 4 to 30 days **B,** Is part of the same Western blot containing pMEK S217, Anti-Src, and MEK1 protein expression. Lysates were from exact same cells as in figure 2A.

Yap/Taz we can see that over time fasudil up regulates these proteins. At day 30 there is a very clear distinction when we see a four fold increase from vemurafenib alone to vemurafenib + fasudil (Fig. 4A). Tying in pMEK S298 we see that exact same trend where at day 30 we have a four fold increase from vemurafenib only to vemurafenib + fasudil (Fig. 4A). These results are directly in line with the data we received from our other blots (Fig. 2A/B & 3A/B). This may explain the long-term effects fasudil has on A375 cells heavily promoting Yap/Taz and pMEK S298 protein levels. Looking at pMEK S217 we can see that there is a small increase from vemurafenib alone to vemurafenib + fasudil, however the change in no where near as dramatic compared to pMEK S298 and Yap/Taz expression levels (Fig. 4A/B). Other proteins tubulin, Anti-Src, and MEK1 all gave very similar expression levels throughout our 30 day time line, thus their role may not be as significant as Yap/Tas or our pMEK proteins (Fig. 4A/B).

Discussion

While vemurafenib treatment initially causes BRAF mutated tumor cell drop out, it is unclear which cellular mechanism the resisting cells utilize in order to promote resistantance. Some studies have pointed towards the small family Rho GTPases as a key modulator of resistance.[8] We now have evidence that illustrates knocking down Rho or downstream effectors such as ROCK promote resistance in the BRAF mutated A375 cell line. We saw that as early as 18 days potential resistant cell colonies emerge, which would cut the normal resistance time of vemurafenib in half. This result gave us the opposite of what we initially thought and prompted us to investigate further into what protein expression may look like in these cells.

Through analyzing relative protein concentrations of common BRAF, Rho, and Rac1 pathway targets there is supporting data that Rho knockdown cells or inhibition of Rho kinase targets such as ROCK kinase, may promote resistance through increasing
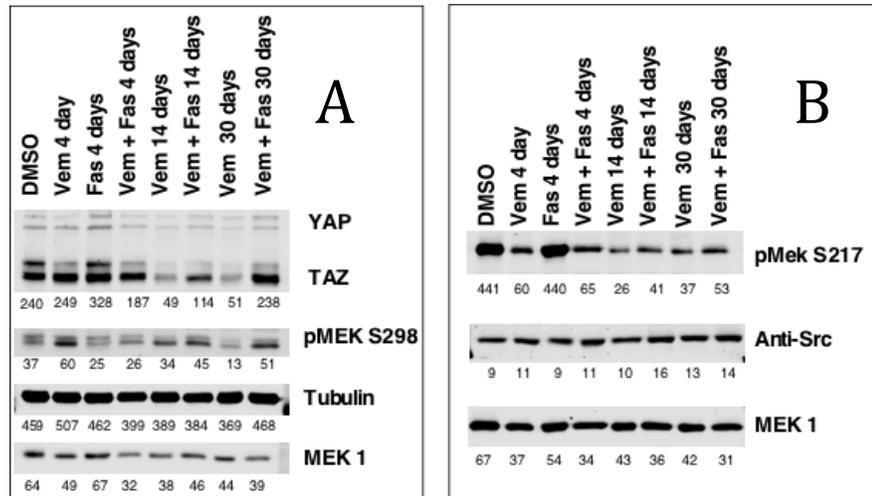
Yap/Taz, pMEK S298, pPaxillin Y31, and pMEK S217 expression. Having increased regulation of Yap/Taz is known to directly promote cell resistance within BRAF mutated cells through actin remodeling.[10] Yap and Taz have also been linked to the regulation of the Hippo pathway and increased expression patterns have also been associated with increased cell proliferation and epithelial–mesenchymal transition.[15] How pPaxillin Y31 and pMEK S298 fit in is unclear at this point, however their association with increased Rac1 signaling expression could possibly lead to an up regulation of cancer cell motility.[24] Delving further into this discussion, pMEK S298 displayed similar results as pPaxillin Y31 and acts as downstream target of PAK kinase.[16] PAK is downstream of Rac1, which illustrates possible Rac1 increased activity. Yet another clue can be seen through the slight increase in pMEK S217 expression, which is associated with increased BRAF activity.[21] The increase in BRAF activity may also help explain why inhibition of Rho promotes resistance. On the other hand, since the activation of proteins downstream of Rac1 is much stronger, there is supporting evidence that Rac1 plays a larger role in promotion of resistance in our model. Strikingly pPaxillin Y31 and pMEK S298 both had about a four fold increase in protein expression at 30 days. Both can be associated with Rac1 signaling, possibly contributing to our observed increase in resistance. Some reports show that Rac and Rho small family GTPases have reciprocal actions on one another.[18, 20] Therefore the down regulation of Rho can may drive increased activity of Rac1 in the cell and possibly explain what is happening within our in vitro model. In conclusion a possible explanation of our results that inhibiting Rho signaling promotes vemurafenib-resistant BRAF mutated cells is that inhibiting Rho may have increased Rac1 activity and increased Yap/Taz expression.

Our working hypothesis of Rho inhibition promoting resistance in BRAF mutated cells is becoming clearer. Understanding signaling proteins that are upregulated in response to Rho inhibition has given us insight into what just may be going on mechanistically in our cells. Yap/Taz increased expression provided our first clue. Being linked in many other studies to promoting resistance we have data and results that would be directly in line with these other studies.[10,15] Other clues to our mechanism are the increased expression of pPaxillin Y31 and pMEK S298. Both can be correlated with an increase in Rac1 expression possibly showing an increase in Rac1 could relate to promotion of vemurafenib resistance in the BRAF mutation. This may describe how the inhibition of Rho directly or indirectly leads to an up regulation of Rac1 activity. Analyzing Rac1 activation in BRAF mutated cells may be a good investigation for future research. Other projects report that Rac1 is a great target to inhibit in BRAF melanomas but similarly the exact knowledge of the mechanism it utilizes to promote resistance was unclear.[19] It could be very beneficial to knockdown Rac1 or it's downstream targets. We could see what this would do to resistance within BRAF mutated cells and also see what would happen with Rho activity. Another approach would be to duplicate our exact model, instead we could create more cell cultures using different cell lines in order to obtain cell lysates from multiple vemurafenib + fasudil treated cells. Then we could analyze multiple blots stemming from multiple cell populations. This would give more significant numbers and give us better statistical data to support our findings. It would be striking to find that 4-5 cell populations all reinforce the idea of having increased Yap/Taz, pPaxillin, pMEK S298, and pMEK S217 expression and really help solidify exactly what proteins Rho inhibition up regulates. We are also interested in the possibility of creating a model with patient derived xenografts to see if Rho inhibition acts similarly in xenograft tissues or possibly extending our current model to other BRAF mutated cell lines.

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