BRAFi-induced RAS-mediated non-canonical nuclear β-catenin translocation induces a phenotypic switch in cancer-associated fibroblasts

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INTRODUCTION

Mutant BRAF (V600) is the most common oncogene in melanoma, promoting uncontrolled cell proliferation, cell survival, and tumor progression by hyper-activating the MEK/ERK signaling pathway[1]. The advent of FDA-approved BRAF mutation inhibitors (BRAFi), such as Vemurafenib, Dabrafenib, and Encorafenib, has marked a significant milestone in melanoma treatment. However long-term outcomes using BRAFi are still disappointing for many patients, primarily due to the occurrence of drug resistance[1].

A major contributing factor for tumor recurrence is the presence of a "plastic" microenvironment, which is comprised of heterogeneous stromal cell populations embedded in a dense and stiff extracellular matrix (ECM), including cancer-associated fibroblasts (CAFs)[2]. Nevertheless, how CAFs respond to targeted therapy drugs, especially BRAFi, and remodel the ECM microenvironment to influence tumor resistance remains undetermined. In this context, a deeper understanding of how CAFs respond to BRAFi could open new possibilities for developing strategies to increase drug efficacy and improve patient outcomes

BACKGROUND

1. BRAFi drives β-catenin nuclear translocation in CAFs

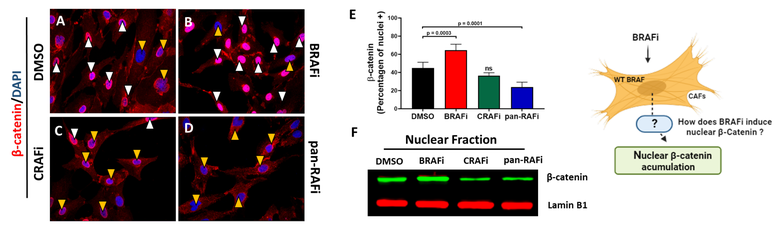

Figure 1. Representative images show β-catenin (red) immunostaining of CAFs treated with **(A)** DMSO (vehicle), **(B)** BRAFi (PLX4032), **(C)** CRAF inhibitor (GW5074), or **(D)** RAF709 (pan-RAF inhibitor). for 72hours. White triangles indicate cells with nuclear accumulation of β-catenin, and yellow triangles indicate cells without nuclear β-catenin. **(E)** Western blotting of β-catenin in the nuclear fraction of CAFs after 72h of RAF inhibitor treatment (1μM). Lamin B1 was used as nuclear loading control. **(F)** Bar chart shows the percentage of cells with nuclear β-catenin accumulation. One-Way ANOVA and Bonferroni post-hoc test was used. Statistical difference of *** p <0.001, when compared to the DMSO treated group.

RESULTS

2. BRAF and CRAF isoforms, but not ARAF, are associated with BRAFi-induced β-catenin nuclear translocation

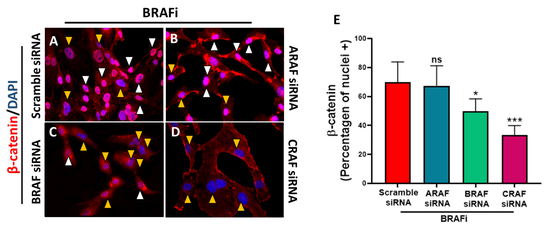

Figure 2. CAFs were transfected with siRNA to induce the knockdown of RAF isoforms (ARAF, BRAF or CRAF) and then treated with PLX4032 (1μM) for 72 hours. **A-D.** Representative images show β-catenin (red) immunostaining of BRAFi-treated CAFs with scramble siRNA or siRNA silencing the expression of ARAF, BRAF or CRAF. White triangles indicate cells with nuclear accumulation of β-catenin, and yellow triangles indicate cells without nuclear β-catenin. **E.** Bar chart shows the percentage of cells with nuclear β -catenin accumulation. One-Way ANOVA and Bonferroni post-hoc test was used. Statistical difference of *p <0.05, *** p <0.001.

3. BRAFi induces RAF dimerization and the recruitment of BRAF and CRAF to the plasma membrane, resulting in their phosphorylation and activation

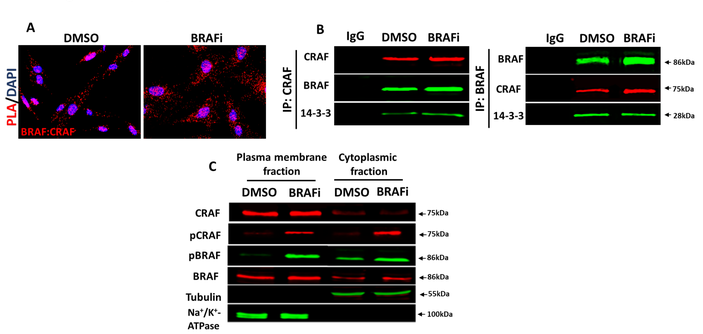

Figure 3. BRAF:CRAF dimerization in CAFs was assessed by **A.** Proximity Ligation Assay (PLA) and **B.** co-immunoprecipitation (co-IP). CAFs were treated by either DMSO or BRAFi (PLX4032) for 72 hours. PLA and co-IP experiment revealed that CRAF binding to BRAF was significantly enhanced in BRAFi-treated cells. **B.** 14-3-3 protein was co-precipitated with BRAF;CRAF complex, suggesting that the14-3-3 protein family plays an role in RAF dimerization. **(.** Western blotting of CRAF, phospho-CRAF(S338), BRAF and phospho-BRAF (T598) protein levels in the membrane or cytoplasmic fraction of PLX4032-treated CAFs. Tubulin and Na+/ K+ ATPase protein levels were used as cytoplasmic and plasma membrane loading control, respectively.

4. NRAS and KRAS are associated with BRAFi-induced hyperactivation of RAF signaling and nuclear β-catenin accumulation in CAFs

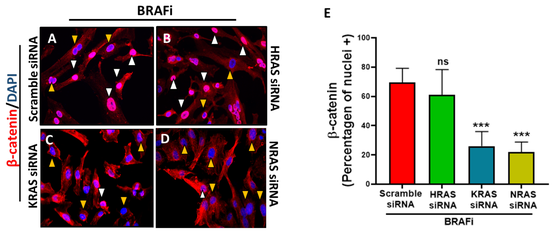

Figure 4. CAFs were transfected with siRNA to silence the expression of RAS isoforms (NRAS, HRAS or KRAS), then the cells were treated with BRAFi (PLX4032,1μM) for 72h. **A-D** Representative images show β-catenin (red) immunostaining of PLX4032-treated CAFs with scramble siRNA or silenced HRAS, KRAS or NRAS expression. White triangles indicate cells with nuclear accumulation of β-catenin, and yellow triangles indicate cells without nuclear β-catenin. **E.** Bar chart shows the percentage of cells with nuclear β-catenin accumulation One-Way ANOVA and Bonferroni post-hoc test was used. Statistical difference of *** p <0.001.

5. BRAFi induces paradoxical activation of MEK/ERK and ROCK downstream signaling in CAFs

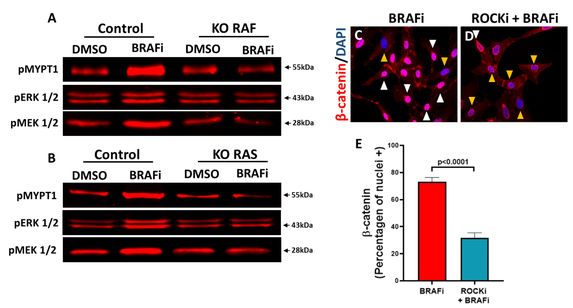

Figure 5. A-B. Western blotting of phospho-MYPT1, phospho-ERK 1/2, and phospho-MEK 1/2 in CAFs treated by DMSO or PLX4032 for 72 hours. **C-D**. Representative images show β-catenin (red) immunostaining of BRAFi-treated CAFs and ROCK inhibitor (HA-1077, 3μM) + BRAFi treated CAFs. White triangles indicate cells with nuclear accumulation of β-catenin, and yellow triangles indicate cells without nuclear β-catenin. **E.** Bar chart shows the percentage of cells with nuclear β-catenin accumulation. One-Way ANOVA and Bonferroni post-hoc test was used.

6. Upregulating β-catenin activity in CAFs promotes melanoma progression

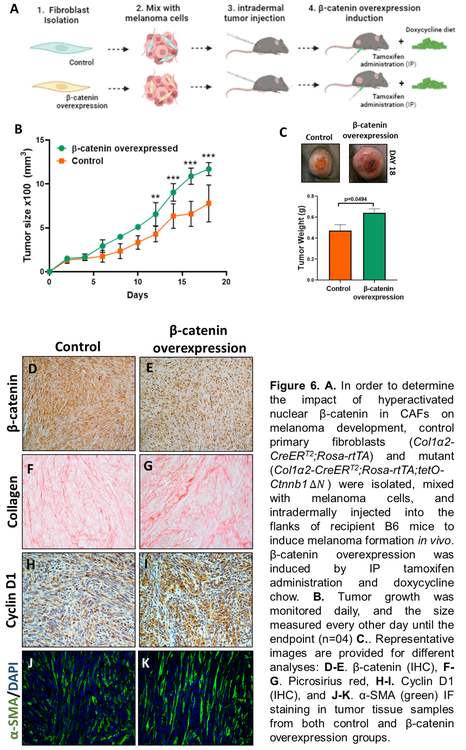

Figure 6. A. In order to determine the impact of hyperactivated nuclear β-catenin in CAFs on melanoma development, control primary fibroblasts (*Col1α2-CreER^{T2};Rosa-rtTA*) and mutant (*Col1α2-CreER^{T2};Rosa-rtTA;tetO-Ctnnb1 ΔN*) were isolated, mixed with melanoma cells, and intradermally injected into the flanks of recipient B6 mice to induce melanoma formation *in vivo*. β-catenin overexpression was induced by IP tamoxifen administration and doxycycline chow. **B.** Tumor growth was monitored daily, and the size measured every other day until the endpoint (n=04) **C.**. Representative images are provided for different analyses: **D-E**. β-catenin (IHC), **F-G**. Picrosirius red, **H-I.** Cyclin D1 (IHC), and **J-K**. α-SMA (green) IF staining in tumor tissue samples from both control and β-catenin overexpression groups.

CONCLUSION

Taken together, our results demonstrate that BRAFi robustly induces the formation of BRAF and CRAF heterodimers in CAFs, a crucial step for nuclear β-catenin translocation. Subsequently, RAF dimers are translocated to the cell membrane and phosphorylated for activation in a NRAS and KRAS-dependent process. Furthermore, BRAFi also promotes the activation of MEK/ERK and ROCK pathways, processes that have been shown to be, directly and/or indirectly, associated with the nuclear β-catenin accumulation. Additionally, our *in vivo* model demonstrates that elevated nuclear β-catenin levels in CAFs stimulate cell proliferation, remodel the tumor extracellular matrix, and thereby drive melanoma progression.

In summary, our data provide new insights into the molecular mechanisms underlying the paradoxical reprogramming of CAFs carrying wild-type BRAF by BRAFi in melanoma therapy through the non-canonical β-catenin pathway.

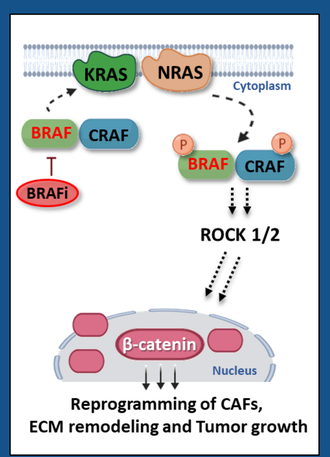

Figure 5. BRAFi binding induces BRAF and CRAF dimerization, a critical step in the NRAS and KRAS-dependent activation of the RAF/MEK/ERK pathway. Furthermore, BRAFi also triggers the paradoxical activation of the ROCK pathway, a process that plays a pivotal role in nuclear β-catenin accumulation.

REFERENCES:

1.Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. *Nature.* 2007;445:851–7
2. Liu T, Zhou L, Xiao Y, Andl T, Zhang Y. BRAF inhibitors reprogram cancer-associated fibroblasts to drive matrix remodeling and therapeutic escape in melanoma.