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Letter

RAF1 kinase activity is dispensable for KRAS/p53 mutant lung tumor progression

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KRAS mutant tumors account for at least one-fourth of all human cancers. Yet, almost 40 years after their identification in human tumors, no effective therapies have been developed, with the possible exception of those tumors carrying G12C mutation (Ostrem et al., 2013). Ironically, KRAS oncogenes primarily signal via the MAPK pathway made up of druggable kinases. Unfortunately, none of the kinase inhibitors developed during the last decade have shown anti-tumor efficacy (Drosten and Barbacid, 2020). Previous studies using genetically engineered mice have revealed that complete inactivation of MAPK signaling is incompatible with adult life (Blasco et al., 2011). Moreover, genetic interrogation of the putative therapeutic properties of each of the kinases has revealed that only ablation of RAF1 results in significant levels of tumor regression of advanced lung adenocarcinomas induced by *Kras* and *Trp53* mutations (Sanclemente et al., 2018). RAF1 ablation also resulted in complete regression of a subset of pancreatic ductal adenocarcinomas, in combination with the EGF receptor (Blasco et al., 2019). Although these therapeutic responses have been achieved by ablating RAF1 expression, it appears that the most direct way to translate these observations to a clinical setting would be to develop selective kinase inhibitors against RAF1.

To interrogate the suitability of this therapeutic strategy, we have generated mouse strains that express conditional knockin alleles that encode two independent RAF1 kinase dead isoforms, $RAF1^{D468A}$ and $RAF1^{K375M}$ (Figure S1A). Both of these mutations completely abolish RAF1 kinase activity, as determined by the lack of phosphorylation of their natural substrate MEK1 (Figure S1B). Surprisingly, systemic expression of these kinase isoforms under the control of the endogenous *Raf1* locus in mice bearing advanced *Kras*/*Trp53* tumors (Sanclemente et al., 2018) failed to induce significant levels of tumor regression (Figure S1C). Indeed, tumor-bearing mice in which RAF1 expression was replaced by expression of the $RAF1^{D468A}$ isoform upon Cre-mediated recombination behaved exactly the same as mice that retained expression of the wild-type protein (Figure S1C). Cre-mediated recombination efficacy of this conditional *Raf1* kinase-dead allele was over 90% in all cases analyzed, indicating that most tumor cells expressed the $RAF1^{D468A}$ isoform rather than the wild-type protein. In the case of the $RAF1^{K375M}$ isoform, tumor progression was more moderate than in control animals, and we observed limited levels of tumor regression in a small percentage (15%) of animals (Figure S1C). Cre-mediated recombination efficacy of this conditional *Raf1* kinase-dead allele was over 80%, indicating that most tumor cells expressed the $RAF1^{K375M}$ isoform (Figure S1C). The slightly differential results obtained with these two isoforms is due to the fact that $RAF1^{K375M}$ is partially unstable, resulting in overall expression levels significantly lower than those of either $RAF1^{D468A}$ or wild-type RAF1 proteins (Figure S1C). These results, taken together, conclusively show that the contribution of RAF1 to *Kras*/*Trp53*-driven tumors is not mediated by its kinase activity.

Further analysis of these kinase-dead isoforms revealed that the partially unstable $RAF1^{K375M}$ isoform is not phosphorylated in S338 and S621, an event thought to be mediated in *cis* and required for proper protein stability (Figure S1D) (Noble et al., 2008). However, both residues are phosphorylated in $RAF1^{D468A}$-expressing cell lines, indicating that their phosphorylation must be mediated by other kinases, since this isoform does not have kinase activity. Therefore, the lack of S338 and S621 phosphorylation in $RAF1^{K375M}$ might be a consequence of conformational changes rather than of the absence of kinase activity. Finally, all tumors expressing $RAF1^{D468A}$ or $RAF1^{K375M}$ retained significant levels of MEK1 phosphorylation regardless of the differential properties of these RAF1 kinase-dead isoforms (Figures S1C and S1D), thus indicating that elimination of RAF1 kinase activity has little effect, if any, on MAPK signaling, an activity likely to be sustained by BRAF.

Previous studies have indicated that, in addition to its role in MAPK signaling, RAF1 has anti-apoptotic activity. This effect is mediated, at least in part, by its ability to inhibit the pro-apoptotic kinases ASK1 and MST2. Moreover, *in vitro* studies suggested that this anti-apoptotic activity is likely to be kinase independent (Chen et al., 2001; O'Neill et al., 2004). To interrogate whether the

anti-proliferative effect of ablating RAF1 is mediated by these kinases, we blocked proliferation of human A549 lung adenocarcinoma cells as well as of cells obtained from a patient-derived xenograft (PDX) model, PDX-dc1, with lentiviral vectors expressing two independent shRNAs against *RAF1* (Sanclemente et al., 2018). Co-infection of these *RAF1* shRNAs with two independent shRNAs against *ASK1* or *MST2* that efficiently blocked expression of these pro-apoptotic kinases (Figure S1E) restored the proliferative properties of these human lung tumor cells (Figure S1F). Re-expression of a cDNA encoding the murine RAF1 protein, whose sequences could not be recognized by the human *RAF1* shRNAs, restored proliferation of the A549 and PDX-dc1 cells to levels similar to those observed upon co-infection with *ASK1* or *MST2* shRNAs. Similar results were obtained expressing the murine $RAF1^{K375M}$ and $RAF1^{D468A}$ kinase-dead isoforms in PDX-dc1 cells, thus adding additional evidence to support the concept that the antitumor effect observed upon RAF1 ablation is independent of its kinase activity. These results demonstrate that the anti-proliferative effect of silencing RAF1 expression in human lung adenocarcinoma cells is mediated by the pro-apoptotic properties of ASK1 and MST2.

As illustrated here, $RAF1^{K375M}$ retained anti-apoptotic activity similar to that of the wild-type RAF1 protein, as determined by its inability to induce cleaved caspase-3 in *Kras/Trp53* mouse tumor cells. Technical difficulties derived from the limited recombination efficiency exhibited by the endogenous Cre recombinase prevented us from interrogating whether the $RAF1^{D468A}$ isoform also retained anti-apoptotic activity. More importantly, the anti-proliferative effect of ablating RAF1 in two independent lung human tumor cell lines, A549 and PDX-dc1, could be efficiently reverted by downregulating the expression of the pro-apoptotic ASK1 and MST2 kinases. These results, taken together, illustrate that the therapeutic effect observed upon RAF1 ablation in *Kras/Trp53* mutant lung tumors is mediated by loss of its anti-apoptotic properties rather than of its kinase catalytic activity.

These observations have important implications for the design of effective therapeutic strategies to block progression of *KRAS* mutant human cancers. They also help to explain, at least in part, the poor results obtained so far in the clinic with RAF inhibitors that block either their kinase activity or other features that regulate their involvement in MAPK signaling. Indeed, recent studies from our laboratory using some of these RAF kinase inhibitors, including the panRAF inhibitors MLN2480 and LSN3074753; a RAF1-selective inhibitor, GW5074; and the paradox breaker PLX8394, have also failed to show significant therapeutic activity on *Kras/Trp53* mutant lung tumors (Esteban-Burgos et al., 2020).

In summary, these studies strongly suggest that current therapeutic strategies based on inhibition of RAF1 kinase activity are unlikely to produce anti-tumor results in the clinic. Instead, pharmacological targeting of RAF1 will require novel strategies that prevent the anti-apoptotic activity of RAF1, either by blocking its interaction with the ASK1 or MST2 kinases (Chen et al., 2001; O'Neill et al., 2004) or, more directly, by inhibiting RAF1 expression with selective RAF1 degraders. The limited toxicities resulting from systemic ablation of RAF1 expression in adult mice (Sanclemente et al., 2018) suggest that the increased apoptotic activity that may result from loss of RAF1 expression has limited effect on normal tissues. Yet, toxicities in mice and humans may not be the same.

The last decade has witnessed a tremendous surge in the use of degron chemistry to inactivate targets whose contribution to tumor development is not mediated by classical enzymatic activities (Lai and Crews, 2017). Indeed, a significant number of cancer genes encode undruggable proteins such as KRAS, with the possible exception of those carrying G12C mutations (Ostrem et al., 2013). The development of pharmacologically viable proteolysis-targeting chimeras (PROTACs) might be a suitable therapeutic option to block RAF1 expression via proteasome-mediated degradation. These bifunctional chemotypes do not require binding to functionally relevant domains, thus offering wider options than catalytic inhibitors or inhibitors of protein-protein interactions. Unveiling the complete structure of RAF1 should provide critical information not only to design PROTACs capable of inducing its degradation but also to identify potential vulnerabilities that may result in RAF1 degradation. Hopefully, pharmacological phenocopying of the results obtained in genetically engineered mouse tumor models will provide therapeutic benefit to patients suffering from KRAS mutant cancers in the not-too-distant future.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ccell.2021.01.008.

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