Supplementary discussion

Directly targeting KRAS-oncoproteins has been a longstanding objective in precision oncology. $KRAS^{G12C}$ inhibitors are now in clinical testing and early data from a Phase I clinical trial show a nearly 50% response rate in lung cancer patients[30]. Most of the tumor responses, however, are partial; with approximately ~50% regression of target lesions by RECIST criteria. In an effort to explain how cancer cells bypass the effect of G12Ci-treatment, we identified an adaptive fitness mechanism that allows groups of cancer cells within a population to rapidly escape $KRAS^{G12C}$ inhibition.

Profiling the effect of G12Ci-treatment in bulk lung cancer cell populations revealed an initial inhibition followed by a reactivation within 72h. This pattern is most consistent with what has been described as adaptation (or adaptive resistance). The alternative possibility that the reactivation occurs because of the selection of 'de-novo' resistant subpopulations is less likely, given the treatment times used in this study. Selection requires that a subclone resists treatment from the beginning. However, the majority of cells were initially inhibited by the drug, giving rise to almost undetectable levels of KRAS-GTP by ~6-12h of treatment and induction of quiescence by 24h of treatment. At 72h, approximately 20% of the inhibited cells reactivated KRAS signaling and escaped quiescence. The doubling time of the cells used in this study is approximately 36h, and one to two population doublings are not sufficient for a sparse subclone to be selected and expand to the degree required to explain the reactivation. Selection of rare resistant subclones, however, may play a role in the emergence of resistance at longer treatment intervals.

By studying the response of lung cancer cells to the G12Ci-treatment at the single-cell level, we found that the treatment initially sequesters the cancer cell population in a quiescent state with low KRAS activity. Cells in this state produce new $KRAS^{G12C}$, which is not bound to the drug. Then, depending on the presence of upstream-acting adaptive signals, in some cells, new $KRAS^{G12C}$ is converted to its active/drug-insensitive state. These cells adapt to the drug and resume proliferation. Cells where the adapting signals are suppressed remain sensitive to drug treatment, because new $KRAS^{G12C}$ is either not available, or it exists predominantly in its inactive/drug-sensitive state. These processes together give rise to a divergent phenotype shortly after treatment.

Our model suggests that the adaptive re-accumulation of KRAS-GTP is non-uniform and multifactorial, dependent on new $KRAS^{G12C}$ synthesis, EGFR and AURK signaling. The

transcriptional activation of KRAS is inversely proportional to KRAS/RAF/MEK/ERK signaling activity and peaked in quiescent cells with the lowest KRAS output score. New $KRAS^{G12C}$ is sufficient for the KRAS-GTP rebound and the bimodal distribution following treatment. Combining a G12C-specific siRNA with dox-inducible siRNA-resistant $KRAS^{G12C}$ expression phenocopied the effect of the drug. Moreover, siRNAs targeting HRAS, NRAS or NRAS and HRAS did not enhance the antiproliferative effect of G12Ci at 72h (data not shown). Thus, the bimodal effect of G12Ci-treatment on the induction of quiescence cannot be explained solely by parallel pathway activation independent of $KRAS^{G12C}$ (i.e. through WT RAS, PI3K signaling etc.). It may be that $KRAS^{G12C}$-independent processes modulate G12Ci-treatment at longer treatment exposure and these effects are not apparent in our study because of the short drug-treatment times. Also, our model does not exclude the contribution of acquired or pre-existing genetic alterations, nor the contribution of the tumor microenvironment, both of which may enhance and/or consolidate the cell-intrinsic behaviors identified in this study.

Newly synthesized $KRAS^{G12C}$ is maintained in its active/drug-insensitive state by the EGFR and AURK signaling pathways. EGFR signaling stimulates nucleotide exchange to reactivate new $KRAS^{G12C}$. The downstream intermediate PTPN11/SHP2 mediates this effect by recruiting the nucleotide-exchange factor SOS1, as described recently[53-56]. In our system, EGFR signaling was activated by the transcriptional activation of HBEGF in a subset of cells and potentiated by the loss of ERK-dependent negative feedback, as a consequence of G12Ci-treatment. These agree with previous work showing that RAF inhibitors induce growth-factor expression in BRAF mutant models[57,58] and that such treatment results in the loss of ERK-mediated suppression of RTK/SOS1/RAS signaling[50]. It is possible that activation of EGFR signaling during G12Ci-treatment stimulates other signaling pathways, in addition to KRAS/RAF/MEK/ERK. Of these, PI3K/mTOR signaling, which can be activated in a growth-factor dependent manner, has been shown to limit the effect of G12Ci-treatment[59].

We also find that AURKA interacts with $KRAS^{G12C}$ to enhance its active state and effector signaling. This is complementary to a recent study in non-malignant cells showing that AURKA interacts with HRAS to stabilize its active state and its interaction with CRAF[24]. Expanding on this study, we found that combined inhibition of AURKA and $KRAS^{G12C}$ was required for both AURKA and CRAF to be displaced from $KRAS^{G12C}$ and for maximal suppression of CRAF/MEK/ERK signaling. AURKA or its intermediates phosphorylate and activate MAPK and RAL signaling intermediates downstream of KRAS[23,60,61]. In addition, AURKA regulates cell cycle progression

and apoptosis, and attenuates EGFR inhibitor-treatment in lung cancer[27]. Because suppressing AURKA prevents entry into G1, it is also possible that the transcription of new KRAS mRNA, and the pool of new KRAS protein available for adaptation, may be diminished indirectly. While these downstream or parallel effects may also contribute to the adaptive phenotype, they do not fully explain the effect of AURKA during the adaptive phase of G12Ci-treatment, particularly at the level of KRAS interaction/activation.

The re-challenge and dox-induced HA-KRAS^{G12C} experiments suggest that the bimodal distribution during G12Ci-treatment occurs because new KRAS^{G12C} assumes both its active (drug-insensitive) and inactive (drug-sensitive) conformations. The mode by which KRAS is transcriptionally activated during G12Ci-treatment provides a clue into why newly-synthesized and baseline KRAS^{G12C} have different susceptibilities to drug-treatment. Unlike at baseline, when KRAS^{G12C} is expressed in the setting of high ERK activity, new KRAS^{G12C} is produced in response to inhibited ERK output. ERK negatively regulates the nucleotide-exchange factor SOS1[62,63]. Thus, because new KRAS^{G12C} is synthesized in the absence of such ERK-mediated negative feedback, it may be more prone to undergo activation by nucleotide-exchange than baseline KRAS^{G12C}. In turn, this would extend the residency time of new KRAS^{G12C} in an active state, which decreases the probability of drug-binding, while restoring effector signaling.

The adaptive fitness mechanism described in this study limits the response to G12Ci-treatment and must be suppressed for complete and durable responses to be achieved in patients. Our model provides a blueprint for optimizing the therapeutic responses to these first-in-class inhibitors. Improving the affinity of G12Ci for the inactive state will enhance the probability that new KRAS^{G12C} is bound by the drug before it can undergo nucleotide exchange (to its drug-insensitive state). Combination therapies that suppress the induction of KRAS^{G12C} protein, or those that prevent its conversion to the active state, will also enhance the potency and duration of inhibition. The EGFR/G12Ci, SHP2/G12Ci and AURK/G12Ci combinations are three such potential therapies that can be immediately translated to the clinic.

Supplementary References:

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