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## Multiplexed identification of RAS paralog imbalance as a driver of lung cancer growth

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29 **ABSTRACT**

30           Oncogenic *KRAS* mutations occur in approximately 30% of lung adenocarcinoma. Despite  
31 several decades of effort, oncogenic KRAS-driven lung cancer remains difficult to treat, and our  
32 understanding of the positive and negative regulators of RAS signaling is incomplete. To uncover  
33 the functional impact of diverse KRAS-interacting proteins on lung cancer growth *in vivo*, we used  
34 multiplexed somatic CRISPR/Cas9-based genome editing in genetically engineered mouse models  
35 with tumor barcoding and high-throughput barcode sequencing. Through a series of CRISPR/Cas9  
36 screens in autochthonous lung tumors, we identified HRAS and NRAS as key suppressors of  
37 KRAS<sup>G12D</sup>-driven tumor growth *in vivo* and confirmed these effects in oncogenic KRAS-driven  
38 human lung cancer cell lines. Mechanistically, RAS paralogs interact with oncogenic KRAS,  
39 suppress KRAS-KRAS interactions, and reduce downstream ERK signaling. HRAS mutations  
40 identified in KRAS-driven human tumors partially abolished this effect. Comparison of the tumor-  
41 suppressive effects of HRAS and NRAS in KRAS- and BRAF-driven lung cancer models  
42 confirmed that RAS paralogs are specific suppressors of oncogenic KRAS-driven lung cancer *in*  
43 *vivo*. Our study outlines a technological avenue to uncover positive and negative regulators of  
44 oncogenic KRAS-driven cancer in a multiplexed manner *in vivo* and highlights the role of RAS  
45 paralog imbalance in oncogenic KRAS-driven lung cancer.

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## 51 INTRODUCTION

52 The RAS family genes *KRAS*, *HRAS* and *NRAS* are frequently mutated across cancers, and  
53 *KRAS* mutations occur in approximately 30% of lung adenocarcinomas<sup>1-3</sup>. RAS proteins are small  
54 GTPases that switch between a GTP-bound active state and GDP-bound inactive state in response  
55 to upstream growth signaling<sup>4</sup>. RAS proteins regulate multiple downstream signaling pathways  
56 which control proliferation. Hotspot oncogenic mutations in codons 12, 13, and 61 reduce GTP  
57 hydrolysis and increase the fraction of RAS proteins in the GTP-bound state, which results in  
58 constitutive activation and widespread changes in RAS protein-protein interactions<sup>5, 6</sup>. These  
59 changes result in hyper-activation of RAS effector pathways, driving cellular transformation and  
60 tumorigenesis<sup>7, 8</sup>. Oncogenic *KRAS* therefore represents a key node in growth factor-induced  
61 signaling and a critical target for therapeutic intervention in lung adenocarcinoma. However,  
62 despite tremendous effort, the development of targeted therapies for oncogenic *KRAS*-driven  
63 tumors has proven challenging<sup>9</sup>.

64 Genetic and proteomic mapping has revealed that *KRAS* interacts with a large network of  
65 proteins<sup>10, 11</sup>. These *KRAS*-interacting proteins include canonical regulators and effectors, as well  
66 as many proteins that remain poorly understood in the context of oncogenic *KRAS*-driven lung  
67 cancer. Much of our understanding of RAS signaling has stemmed from diverse cellular and cell-  
68 free systems<sup>12-14</sup>. Thus, while recent studies have mapped *KRAS* protein-protein interaction  
69 networks and identified synthetic lethal interactions with oncogenic *KRAS* in human cell lines<sup>10,</sup>  
70 <sup>11, 15, 16</sup>, it remains difficult to assess the relevance of these biochemical and genetic interactions to  
71 cancer growth *in vivo*. Genetically engineered mouse models of oncogenic *KRAS*-driven cancer  
72 uniquely recapitulate autochthonous tumor growth and have contributed to our understanding of  
73 *KRAS* signaling<sup>17</sup>. However, the development and use of such models has traditionally been

74 insufficiently scalable to broadly assess modifiers of KRAS-driven tumor growth. The ability to  
75 uncover functional components of RAS signaling that affect lung cancer growth *in vivo* in a  
76 multiplexed manner would accelerate our understanding of RAS biology and could aid in the  
77 development of pharmacological strategies to counteract hyperactivated KRAS.

78 To enable the analysis of genetic modifiers of lung tumor growth *in vivo*, we recently  
79 integrated somatic CRISPR/Cas9-based genome editing with tumor barcoding and high-  
80 throughput barcode sequencing (Tuba-seq)<sup>18-20</sup>. This approach allows precise quantification of the  
81 effect of inactivating panels of genes of interest on lung tumor initiation and growth in a  
82 multiplexed manner. By employing Tuba-seq to assess the functions of KRAS-interacting proteins  
83 nominated by unbiased affinity purification/mass spectrometry (AP/MS), we show that wild-type  
84 HRAS and NRAS suppress the growth of oncogenic KRAS-driven lung adenocarcinoma.  
85 Competition between oncogenic KRAS and wild-type HRAS diminishes KRAS-KRAS interaction  
86 and suppresses downstream signaling. *In vivo* screening across multiple oncogenic contexts  
87 revealed that HRAS and NRAS specifically suppress the growth of tumors driven by oncogenic  
88 KRAS. Our study reveals that RAS paralog imbalance is a driver of oncogenic KRAS-driven lung  
89 cancer.

90

## 91 **RESULTS**

92

### 93 **Selection of candidate KRAS-interacting proteins to assess *in vivo***

94 To identify putative KRAS-interacting proteins that could affect oncogenic KRAS-driven  
95 lung tumor growth *in vivo*, we integrated previous proteomic data from AP/MS studies with gene  
96 expression data from cancer cells from autochthonous mouse models (**Figure 1a**)<sup>10, 21</sup>. We

97 prioritized a list of candidate genes according to the probability of their protein products interacting  
98 with KRAS, their mRNA expression in mouse models of oncogenic KRAS<sup>G12D</sup>-driven lung cancer,  
99 and the probability of their protein products interacting with other RAS GTPases (**Figure 1b-c,**  
100 **Figure S1a-d**)<sup>10, 21</sup>. We selected 13 proteins that represent diverse aspects of RAS biology,  
101 including RAS paralogs (HRAS, NRAS – which were supported by the identification of paralog-  
102 specific peptides), RAS regulators (RASGRF2, RAP1GDS1)<sup>22, 23</sup>, a RAS farnesyltransferase  
103 (FNTA)<sup>24, 25</sup>, and RAS effectors (RAF1, RGL2)<sup>26, 27</sup>, as well as several other proteins whose  
104 functions in RAS signaling are understudied. Analysis of human lung adenocarcinoma genomic  
105 data showed that while most of these candidate genes trend to be more often amplified in human  
106 adenocarcinoma, *NRAS*, *HRAS*, and *ALDH1A1* also have deep genomic deletions (**Figure S1e**)<sup>28</sup>.  
107 Interestingly, some of these proteins bound preferentially to either GTP- or GDP-bound KRAS,  
108 while others seemed to interact with KRAS independent of its nucleotide state (**Figure 1c**).

109

### 110 **Identification of KRAS-interacting proteins that impact lung tumor growth *in vivo***

111 Given that KRAS-interacting proteins could have either positive or negative effects on  
112 signaling and tumor growth, we first assessed whether Tuba-seq is capable of detecting gene-  
113 targeting events that have deleterious effects on tumor fitness. We initiated tumors in *Kras*<sup>LSL-</sup>  
114 *G12D/+; Rosa26*<sup>LSL-tdTomato</sup>; *H1I*<sup>LSL-Cas9</sup> (*KT; H1I*<sup>LSL-Cas9</sup>) and control *KT* mice with a pool of barcoded  
115 Lenti-sgRNA/Cre vectors encoding sgRNAs targeting two essential genes (*Pcna* and *Rps19*), a  
116 known tumor suppressor (*Apc*)<sup>20, 29</sup>, and several inert sgRNAs (Lenti-sgEssential/Cre; **Figure**  
117 **S2a**). After 12 weeks of tumor growth, we performed Tuba-seq on bulk tumor-bearing lungs and  
118 quantified the number and size of tumors initiated with each Lenti-sgRNA/Cre vector (**Figure**

119 **S2b)**. By incorporating measures of tumor number and size, we could confidently identify genetic  
120 deficiencies that reduced tumor fitness (**Figure S2c-g and Methods**).

121 To quantify the impact of inactivating our panel of KRAS-interacting proteins on  
122 oncogenic KRAS<sup>G12D</sup>-driven lung tumor growth *in vivo*, we generated a pool of barcoded Lenti-  
123 sgRNA/Cre vectors targeting the genes that encode these proteins, as well as sgInert control  
124 vectors (Lenti-sg*KrasIP/Cre*; **Figure 1d**). Given the importance of farnesylation in KRAS  
125 localization and signaling, sgRNA targeting *Fnta* served as a control for KRAS dependency<sup>30, 31</sup>.  
126 We initiated tumors with the Lenti-sg*KrasIP/Cre* pool in *KT;H11<sup>LSL-Cas9</sup>* and *KT* mice and  
127 calculated metrics of tumor size and number after 12 weeks of tumor growth (**Figure 1e**). To our  
128 surprise, inactivation of the *Kras* paralogs *Hras* and *Nras* had the most dramatic effect on tumor  
129 growth. Inactivation of *Cand1* also increased tumor size, while deletion of several genes including  
130 *Fnta*, *Nme2*, *Rap1gds1*, and *Aldh1a* decreased tumor size and/or number, suggesting reduced  
131 cancer cell fitness (**Figure 1f and S3a-d**).

132 Given the fundamental importance of the p53 tumor suppressor in oncogenic KRAS-driven  
133 lung cancer, as well as previous data suggesting crosstalk between RAS and p53 signaling<sup>19, 32, 33</sup>,  
134 we determined whether p53 deficiency changed the impact of inactivating KRAS-interacting  
135 proteins on tumor growth. We initiated tumors with the Lenti-sg*KrasIP/Cre* pool in *Kras<sup>LSL-</sup>*  
136 *G12D/+;Rosa26<sup>LSL-tdTom</sup>;Trp53<sup>fllox/fllox</sup>;H11<sup>LSL-tdTom</sup>* (*KT;Trp53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>*) mice and performed  
137 Tuba-seq after 12 weeks of tumor growth (**Figure 1e**). The effects of inactivating each gene  
138 encoding a KRAS-interacting protein on tumor size, tumor number, and overall tumor burden were  
139 generally consistent between the p53-proficient and -deficient settings (**Figure 1g, Figure S3e-h**).  
140 Notably, the inactivation of either *Hras* or *Nras* also significantly increased growth of p53-  
141 deficient tumors (**Figure 1g, Figure S3e**). Collectively, these results suggest that HRAS and

142 NRAS are tumor suppressors within *in vivo* models of oncogenic KRAS-driven lung cancer, while  
143 several other KRAS-interacting proteins, including CAND1, ALDH1A, and NME2, have less  
144 consistent effects on tumor growth between p53-proficient and -deficient backgrounds (**Figure**  
145 **S3e-h**).

146

### 147 **Validation of HRAS and NRAS as suppressors of oncogenic KRAS-driven lung tumor** 148 **growth**

149 To further validate the effect of inactivating six top candidate genes (*Hras*, *Nras*, *Cand1*,  
150 *Aldh1a*, *Fnta*, and *Nme2*) on oncogenic KRAS-driven tumor growth *in vivo* and confirm that these  
151 results are driven by on-target effects, we generated and barcoded three Lenti-sgRNA/Cre vectors  
152 targeting each gene. To contextualize the effect of *Hras* and *Nras* inactivation on lung tumor  
153 growth relative to established tumor suppressors we included vectors targeting three established  
154 tumor suppressors (*Lkb1*, *Rbm10*, and *Rb1*) in this pool (Lenti-sgValidation/Cre; **Figure 2a**)<sup>18, 20,</sup>  
155 <sup>34</sup>. We initiated tumors with the Lenti-sgValidation/Cre pool in *KT;H11<sup>LSL-Cas9</sup>* and *KT* mice and  
156 assessed metrics of tumor initiation and growth 12 weeks after tumor initiation (**Figure 2b-c**).  
157 Targeting *Fnta* with all three sgRNAs consistently reduced growth fitness, while the impact of  
158 inactivating *Aldh1a* and *Nme2* was more variable (**Figure 2d**, **Figure S4**). Most importantly, all  
159 three sgRNAs targeting *Hras* and all three sgRNAs targeting *Nras* significantly increased tumor  
160 growth (**Figure 2d-e**, **Figure S4b**). Notably, *Hras* inactivation increase tumor growth to a similar  
161 extent as inactivation of the *Rb1* and *Rbm10* tumor suppressors (**Figure 2d**, **Figure S4b**). These  
162 results suggest a potentially pivotal role for wild-type HRAS and NRAS in constraining oncogenic  
163 KRAS-driven lung tumor growth *in vivo*.

164 In addition, we validated the tumor-suppressive function of HRAS and NRAS in oncogenic  
165 KRAS-driven lung tumor growth by initiating tumors in *KT;H1I<sup>LSL-Cas9</sup>* mice with individual  
166 *sgInert-*, *sgHras-* and *sgNras-*containing Lenti-sgRNA/Cre vectors (**Figure 2f**). Inactivation of  
167 either *Hras* or *Nras* increased tumor growth as assessed by direct fluorescence and histological  
168 analyses (**Figure 2g-k**). Collectively, these results suggest that RAS paralogs constrain the growth  
169 of oncogenic KRAS<sup>G12D</sup>-driven lung cancer growth.

170

### 171 **HRAS and NRAS can be growth-suppressive in human lung cancer cells**

172 To assess the relevance of HRAS and NRAS as tumor suppressors in human lung cancer,  
173 we tested the function of HRAS and NRAS in oncogenic KRAS-driven human lung  
174 adenocarcinoma cell lines. Previous genome-scale CRISPR/Cas9 screens revealed that  
175 inactivating these genes was generally detrimental to cancer cell line growth under standard culture  
176 conditions (**Figure S5a**)<sup>10,35</sup>. Interestingly, HRAS and NRAS suppressed the growth of oncogenic  
177 KRAS<sup>G12S</sup>-driven A549 cells and of several oncogenic KRAS-driven lung cancer cell lines when  
178 grown in 3D culture conditions, suggesting that these genes can function as tumor suppressors in  
179 certain contexts (**Figure S5b-c**)<sup>10,15</sup>. To further assess the functions of HRAS and NRAS in  
180 oncogenic KRAS-driven human adenocarcinoma cell lines, we performed gain and loss of function  
181 studies on H23 (KRAS<sup>G12C/+</sup>) and H727 (KRAS<sup>G12V/+</sup>) cells under growth factor restricted growth  
182 conditions. We inactivated *HRAS* and *NRAS* using CRISPR/Cas9 and generated variants with  
183 doxycycline-inducible wild-type *HRAS* re-expression. Inactivation of *HRAS* or *NRAS* in oncogenic  
184 KRAS-driven cells increased cell growth when cells were grown with limited serum and increased  
185 clonal growth potential when cells were grown in anchorage-independent conditions (**Figure 3a,**  
186 **c, d**). Conversely, re-expression of HRAS in these *HRAS*-null cells impaired proliferation and



187 clonal growth (**Figure 3b, e, f**). H23 cells with inactivated *HRAS* or *NRAS* also formed larger and  
188 more proliferative tumors after intravenous and subcutaneous transplantation (**Figure 3g-k,**  
189 **Figure S6**). These results demonstrate that wild-type *HRAS* and *NRAS* can also function as tumor  
190 suppressors in oncogenic *KRAS*-driven human lung cancer cells *in vitro* and *in vivo*. This  
191 consistency between human cell culture and autochthonous mouse models further suggests that  
192 *HRAS* and *NRAS* are tumor suppressors in oncogenic *KRAS*-driven lung adenocarcinoma.

193

#### 194 **Inactivation of RAS paralogs increases signaling downstream of oncogenic KRAS**

195 Wild-type *KRAS* has been shown to be tumor-suppressive in multiple experimental models  
196 of oncogenic *KRAS*-driven cancer, likely due to its ability to interact with and antagonize  
197 oncogenic *KRAS*<sup>36-38</sup>. We have demonstrated that wild-type *HRAS* and *NRAS* suppress oncogenic  
198 *KRAS*<sup>G12D</sup>-driven lung cancer growth *in vivo*. Thus, to further explore the molecular mechanism  
199 driving this effect, we initially assessed whether *HRAS* and *NRAS* alter signaling downstream of  
200 oncogenic *KRAS*. We initially performed pERK immunohistochemistry on lung tumors initiated  
201 with Lenti-sgRNA/Cre vectors containing *sgInert*, *sgHras* or *sgNras* in *KT;H1<sup>LSL-Cas9</sup>* mice.  
202 Inactivation of *HRAS* or *NRAS* increased the number of pERK-positive cells in *KRAS*<sup>G12D</sup>-driven  
203 lung cancer (**Figure 4a, Figure S7a**). Subcutaneous tumors from transplanted H23 cells with  
204 inactivated *HRAS* or *NRAS* also contained more pERK-positive cells when compared to tumors  
205 from transplanted wildtype (*sgSAFE*) H23 cells (**Figure 4b, Figure S7b**). In addition, sorted  
206 cancer cells from *KT;H1<sup>LSL-Cas9</sup>* mice with lung tumors initiated with Lenti-*sgHras*/Cre also had  
207 greater pERK and pAKT compared to those from tumors initiated with Lenti-*sgInert*/Cre (**Figure**  
208 **4c, Figure S7c**). Inactivation of either *Hras* or *Nras* in mouse (HC494) or human (H23 and  
209 HOP62) oncogenic *KRAS*-driven cell lines increased ERK phosphorylation, while their effects on

210 AKT phosphorylation was more cell context dependent (**Figure 4d-e, Figure S7d-e**). Conversely,  
211 re-expression of wild-type HRAS in HRAS-null H23 and HOP62 human lung cancer cells reduced  
212 ERK phosphorylation again with cell context dependent effect on AKT phosphorylation (**Figure**  
213 **4f, Figure S7f**). Previous publications have shown that inactivating wild-type KRAS increases  
214 sensitivity to MEK inhibitors<sup>37, 39</sup>. Consistent with these studies, we found that inactivation of  
215 *HRAS* in H23 cells increased sensitivity to the MEK inhibitor trametinib while re-expression of  
216 HRAS made cells more resistant (**Figure 4g, h**). These data suggest that inactivation of *HRAS* or  
217 *NRAS* hyper-activates MAPK-ERK signaling in KRAS mutant cancer cells<sup>40-42</sup>.

218

### 219 **RAS paralogs suppress oncogenic KRAS-KRAS interaction**

220 RAS proteins interact and form functional clusters on membranes to efficiently recruit  
221 downstream effectors<sup>43-45</sup>. Whether RAS proteins form dimers or oligomers through direct  
222 interactions or through close physical proximity is debated within the field<sup>16, 46-48</sup>. We next  
223 assessed whether HRAS and NRAS “interact” with KRAS without attempting to distinguish direct  
224 from proximity-driven interactions. AP/MS data suggest that all three RAS proteins are able to  
225 interact with their paralogs, supporting the existence of heterotypic RAS-RAS interactions (**Figure**  
226 **5a**). To assess the ability of RAS paralogs to interact with oncogenic KRAS<sup>G12D</sup>, we adapted a  
227 luminescent reporter system (ReBiL2.0 system), which relies on luciferase complementation to  
228 quantify RAS-RAS interactions in living cells<sup>16</sup> (**Figure 5b**). Through expression of wild-type  
229 KRAS, HRAS, or NRAS in KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interaction reporter cells and control reporter  
230 cells, we found that all wild-type RAS paralogs are able to disrupt KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup>  
231 interactions (**Figure 5c, Figure S10a**).

232

233 **Patient-derived HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> mutations impair interaction of HRAS with**  
234 **oncogenic KRAS and abrogate its tumor suppressive function**

235 Our findings suggest that the tumor-suppressive function of wild-type HRAS is mediated  
236 by competitive interactions with oncogenic KRAS, therefore we hypothesized that there could be  
237 *HRAS* mutations in human tumors with oncogenic *KRAS* that impair this interaction. To evaluate  
238 this possibility, we analyzed data from AACR's Genomics Evidence Neoplasia Information  
239 Exchange (GENIE). Mutations in *HRAS* were rare (pan-cancer frequency of non-synonymous  
240 mutations was 1.32%) and about half (0.57%) were oncogenic mutations in codons 12, 13 or 61  
241 that occurred in samples lacking oncogenic *KRAS* (**Figure S8a**). We did, however, identify  
242 multiple rare non-oncogenic *HRAS* mutations in oncogenic KRAS containing lung  
243 adenocarcinomas and tumors of other types (**Figure 5d, Figure S8**). To test whether these mutants  
244 lack the ability to interact with oncogenic KRAS, we used the ReBiL2.0 system. We measured the  
245 ability of four of these *HRAS* mutants, as well as a control Y64A mutant that has been suggested  
246 to reduce HRAS-HRAS dimerization<sup>47</sup>, to inhibit KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interactions. We  
247 identified two HRAS mutants, T50M and R123C, that are unable to reduce KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup>  
248 interactions (**Figure 5e, Figure S10b**). Interestingly, both HRAS<sup>T50</sup> and HRAS<sup>R123</sup> are located  
249 close to the predicted HRAS-KRAS<sup>G12D</sup> interface involving the  $\alpha$ 4 and  $\alpha$ 5 helices (**Figure 5f,**  
250 **Figure S9**). R123 is involved in an intrachain salt bridge with residue E143, which also participates  
251 in the RAS-RAS interface. Mutation to cysteine results in an uncompensated charge on E143,  
252 which may destabilize the RAS-RAS interaction. These findings are consistent with a model in  
253 which wild-type RAS paralogs competitively interacts with oncogenic KRAS and thus suppress  
254 KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interactions.

255 Previous publications have shown that different RAS proteins preferential bind to RAF  
256 proteins and other RAS effectors and thus could function differently in their downstream  
257 signaling<sup>10, 50</sup>. Re-analysis of HRAS and NRAS AP/MS datasets suggests that GTP-bound HRAS  
258 is more similar in its low binding affinity to RAF effectors as GDP-bound rather than GTP-bound  
259 KRAS and NRAS (**Figure 5g**)<sup>10</sup>. To test our hypothesis that the disruption of KRAS<sup>G12D</sup>-  
260 KRAS<sup>G12D</sup> interaction by HRAS suppresses downstream oncogenic signaling, we re-expressed  
261 wild-type HRAS, HRAS<sup>Y64A</sup>, or the two novel patient-derived HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> mutants  
262 in *HRAS*-null lung cancer cells. Re-expression of wild-type HRAS, but not any of the three  
263 mutants, reduced ERK phosphorylation and proliferation (**Figure 5h-i, Figure S10c**). These  
264 results further suggest that RAS paralog imbalance alters oncogenic KRAS signaling via  
265 oncogenic KRAS-wildtype RAS paralog interaction and thus is a driver of lung cancer growth.

266

### 267 **HRAS and NRAS are specific suppressors of oncogenic KRAS-driven lung cancer growth**

268 Our *in vivo* data demonstrate that HRAS and NRAS function as tumor suppressors, and  
269 our cell culture results suggest that these effects may be mediated by interaction of these RAS  
270 paralogs with oncogenic KRAS. If the tumor-suppressive mechanism by which HRAS and NRAS  
271 is mediated through interactions with oncogenic KRAS, then these genes should not be tumor  
272 suppressors in lung adenocarcinoma in which activation of the RAS/RAF/MEK signaling pathway  
273 occurs downstream of KRAS. To test this directly in autochthonous tumors, we initiated tumors  
274 with a sub-pool of barcoded lenti-sgRNA/Cre vectors (Lenti-sgMultiGEMM/Cre) in mouse  
275 models of oncogenic KRAS-driven and oncogenic BRAF-driven lung cancer (**Figure 6a**). In  
276 addition to vectors targeting *Hras* and *Nras*, this pool contained vectors targeting several known  
277 tumor suppressors (*Apc*, *Rbm10*, and *Cdkn2a*) and other KRAS-interacting proteins (*Aldh1a*,

278 *Nme2*), as well as control vectors (**Figure 6a**). We initiated tumors with the Lenti-  
279 sgMultiGEMM/Cre pool in *KT* and *KT;H11<sup>LSL-Cas9</sup>* mice as well as in *BraftT;H11<sup>LSL-Cas9</sup>* mice  
280 which contain a Cre-regulated allele of oncogenic BRAF<sup>V618E</sup> (the mouse equivalent of  
281 BRAF<sup>V600E</sup>)(**Figure 6b**)<sup>51</sup>. 15 weeks after tumor initiation *BraftT;H11<sup>LSL-Cas9</sup>* mice has greater  
282 overall tumor burden than *KT;H11<sup>LSL-Cas9</sup>* mice (**Figure 6c-d**). Analysis of the distribution of  
283 sgInert tumor sizes in the two models using Tuba-seq showed that oncogenic BRAF-driven tumors  
284 were larger than oncogenic KRAS-driven tumors (median sizes of ~3500 cells and ~1000 cells,  
285 respectively). The two distributions had similar maximum tumor sizes, suggesting that the  
286 increased tumor burden is driven by a shift towards larger tumors of relatively uniform size which  
287 is consistent with previous results (**Figure 6e-f**)<sup>51</sup>.

288 Our Tuba-seq data also allowed us to compare the impact of the CRISRP/Cas9 inactivated  
289 genes across oncogenic contexts. Importantly, while inactivation of *Hras* or *Nras* increased the  
290 growth of oncogenic KRAS-driven lung tumors, inactivation of *Hras* or *Nras* had no effect on the  
291 growth of oncogenic BRAF-driven lung cancer (**Figure 6g, Figure S11d-e**). These results were  
292 consistent for both Lenti-sgRNA/Cre vectors targeted each gene. The known tumor suppressor  
293 genes assayed (*Apc*, *Cdkn2a*, and *Rbm10*) generally retained their growth-suppressive effects in  
294 the BRAF-driven model, suggesting that the abrogation of effect observed for *Hras* and *Nras* is  
295 not due to some generic inability of additional alterations to increase BRAF-driven lung tumor  
296 growth (**Figure 6h, Figure S11d-e**). Thus, HRAS and NRAS function as specific suppressors of  
297 oncogenic KRAS-driven tumor growth *in vivo*.

298 Assessing the impact of genomic alterations on the growth of lung cancer driven by distinct  
299 oncogenes was illuminating in two other regards. First, we identify instances of oncogene-tumor  
300 suppressor epistasis (e.g., *Apc* inactivation has a greater effect on BRAF-driven lung cancer

301 whereas *Rbm10* inactivation has a greater effect on KRAS-driven lung cancer) (**Figure 6h, Figure**  
302 **S11d-e**). Thus, the consequences of inactivating tumor suppressor pathways can depend on the  
303 oncogenic context. Second, inactivation of *Nme2*, *Fnta*, and *Aldh1a* reduced initiation and growth  
304 of both oncogenic KRAS-driven and oncogenic BRAF-driven lung cancer, suggesting that they  
305 are generally required for optimal lung cancer growth *in vivo* (**Figure S11**). Thus, our paired  
306 screens not only localized the effect of *Hras* and *Nras* inactivation, but also highlighted the value  
307 of this approach in uncovering alterations that have effects within or across oncogenic contexts.

308

## 309 **DISCUSSION**

310 Oncogenic KRAS-driven lung cancer is a leading cause of cancer-related deaths. However,  
311 despite the identification of oncogenic RAS almost half a century ago, the functions of many RAS-  
312 interacting proteins remain largely unknown. By integrating AP/MS data from human cancer cells  
313 with somatic cell CRISPR/Cas9-editing in autochthonous mouse models, we assess the functional  
314 impact of inactivating a panel of KRAS-interacting proteins on lung cancer *in vivo* in a multiplexed  
315 manner. Our results support a model in which heterotypic interactions between RAS paralogs  
316 suppress oncogenic KRAS-driven lung cancer growth.

317 All RAS family proteins, HRAS, NRAS and KRAS (including both the KRAS4A and  
318 KRAS4B splice isoforms), have been reported to form dimers and nanoclusters<sup>16, 46-48</sup>.  
319 Importantly, both *in vitro* and *in vivo* studies suggest that KRAS-KRAS interactions are required  
320 for effector protein activation, cellular transformation, and optimal tumor growth<sup>45</sup>. Furthermore,  
321 oncogenic KRAS-wild-type KRAS interactions influence lung cancer initiation, progression, and  
322 therapeutic sensitivity<sup>37</sup>. Multiple lines of evidence, including oncogenic *KRAS* copy number gain  
323 and loss of the wild-type *KRAS* allele in human tumors, as well as functional studies in mouse

324 models, suggest that wild-type KRAS is tumor-suppressive (also called “RAS allelic imbalance”),  
325 although the exact role of wild-type KRAS in lung cancer is still debated<sup>3, 38, 41, 52, 53</sup>. Recent data  
326 also suggest that interactions among H-, N- and KRAS occur, thus raising the question of the roles  
327 of wild-type HRAS and NRAS in oncogenic KRAS-driven cancer<sup>10, 11, 16</sup>.

328 In this study, we identified wild-type HRAS and NRAS as potent KRAS-specific tumor  
329 suppressors that interact with oncogenic KRAS, disrupt KRAS-KRAS interactions, and suppress  
330 RAS/MAPK signaling. Inactivation of *HRAS* or *NRAS* in the context of oncogenic KRAS led to  
331 an increase in downstream MAPK signaling (**Figure 4**). The impact of RAS paralog imbalance  
332 extends beyond lung cancer and KRAS codon 12 mutations. Germline *Hras* deletion increases the  
333 development of *Kras*-driven pancreatic cancer, skin papilloma, and carcinogen induced KRAS<sup>Q61</sup>  
334 lung cancer<sup>53-55</sup>. Interestingly, we also identified two rare, patient-derived *HRAS* mutations,  
335 HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup>, which are incapable of disrupting KRAS clustering, and would  
336 therefore likely confer fitness advantages to oncogenic KRAS-driven cancer. These results suggest  
337 that modulating RAS protein interactions, such as by skewing the stoichiometry of oncogenic to  
338 wild-type RAS or forcing inter-paralog competition, could lead to novel therapeutic strategies.  
339 However, the dynamics of intracellular RAS interactions, as well as the importance of these  
340 mutations in oncogenesis requires further study.

341 Given the complexity of RAS signaling, other non-mutually exclusive mechanisms by  
342 which RAS paralogs could reduce oncogenic KRAS-driven cancer growth should be considered.  
343 For example, it has been reported that upstream regulators, such as SOS1, could bridge the  
344 interaction between oncogenic and wild-type RAS<sup>56</sup>. GDP-bound wild-type HRAS and NRAS  
345 could also compete with oncogenic KRAS for upstream guanine nucleotide exchange factors and  
346 thus reduce RAS signaling<sup>57</sup>. In addition, although we provide evidence that inactivation of *Hras*

347 and *Nras* has no impact on oncogenic BRAF-driven lung cancer, it is possible that they could  
348 compete with oncogenic KRAS for other BRAF-independent downstream effectors. Whether  
349 HRAS and NRAS also function through these alternative routes, and how different mechanisms  
350 are synchronized to execute their tumor-suppressive functions, will require additional  
351 investigation.

352         The National Cancer Institute "RAS Pathway V2.0", contains more than 200 proteins  
353 known or suspected to be involved in RAS signaling. Characterizing the role of these proteins in  
354 tractable *in vivo* models of RAS-driven cancer remains a challenge. Our study outlines a  
355 technological avenue to study KRAS-specific signaling components in a multiplexed manner. By  
356 harnessing the power of Tuba-seq, we were able to quantify the tumor suppressive and promoting  
357 effects of more than a dozen putative RAS pathway genes simultaneously, highlighting the  
358 function of HRAS and NRAS as tumor suppressors. Furthermore, by performing paired screens in  
359 oncogenic KRAS-driven and oncogenic BRAF-driven mouse lung cancer models, we localized  
360 the growth suppressive effects of these RAS paralogs to lung cancer driven specifically by  
361 oncogenic KRAS. Our study thus demonstrates the feasibility of performing *in vivo* genetic  
362 interaction screening, and the power of such an approach to provide insight into the mechanisms  
363 of tumor suppression. Future studies of this type should enable a more quantitative understanding  
364 of the role of RAS pathway components in RAS-driven oncogenicity.

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370 **FIGURE LEGEND:**

371 **Figure 1. Multiplexed identification of KRAS-interacting proteins that impact KRAS<sup>G12D</sup>-**  
372 **driven lung cancer growth in vivo.**

373 **a.** Candidate mediators of KRAS-driven lung tumor growth were identified on the basis of their  
374 interactions with GTP- and GDP-locked Kras in multiple AP/MS-based protein-protein interaction  
375 screens and their expression in a mouse model of Kras-driven lung adenocarcinoma.

376 **b.** Selected KRAS-interacting proteins interact with either GTP- or GDP-locked KRAS (shown as  
377 NSAF in A549 cells) and their homolog is expressed in KRAS<sup>G12D</sup>-driven lung cancer (shown as  
378 TPM).

379 **c.** Bubble plot of two AP/MS experiments with GTP- and GDP-locked mutant GTPases as baits  
380 (rows), showing the enrichment of selected candidate KRAS-interacting proteins (columns). Dark  
381 borders indicate FDR < 0.05.

382 **d.** Schematic of tumor initiation with a pool of barcoded Lenti-sgRNA/Cre vectors (Lenti-  
383 sgKrasIP-Pool/Cre). The lentiviral pool includes four Inert sgRNAs that are either non-targeting  
384 (NT) or target a functionally inert locus (Neo1-3, targeting *NeoR* in the *R26<sup>LSL-tdTomato</sup>* allele). Each  
385 barcoded lentiviral vector contains an sgRNA, Cre, and a two-component barcode composed of an  
386 sgRNA identifier (sgID) and a random barcode (BC). This design allows inactivation of multiple  
387 target genes in parallel followed by quantification of the resulting tumor size distributions through  
388 high-throughput sgID-BC sequencing.

389 **e.** Tumors were initiated in cohorts of *KT*, *KT;H11<sup>LSL-Cas9</sup>* and *KT;p53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>* mice  
390 through intratracheal delivery of Lenti-sgKrasIP-Pool/Cre. Tuba-seq was performed on each  
391 tumor-bearing lung 12 weeks after initiation, followed by analyses of sgID-BC sequencing data to  
392 characterize the effects of inactivating each gene.

393 **f.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
394 tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9</sup>* mice. Genes are ordered by 95<sup>th</sup>  
395 percentile tumor size, with sgInerts on the left. sgInerts are in gray, and the line at y=1 indicates  
396 no effect relative to sgInert. Error bars indicate 95% confidence intervals. Percentiles that are  
397 significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence  
398 intervals and P-values were calculated by bootstrap resampling.

399 **g.** Comparison of 95<sup>th</sup> percentile tumor size for each sgRNA relative to the size the 95<sup>th</sup> percentile  
400 tumor size of sgInert-containing tumors in *KT;H11<sup>LSL-Cas9</sup>* mice versus *KT;p53<sup>flox/flox</sup>;H11<sup>LSL-Cas9</sup>*  
401 mice. Error bars indicate 95% confidence intervals calculated by bootstrap resampling.

402

403 **Figure 2. HRAS and NRAS are potent suppressors of KRAS<sup>G12D</sup>-driven lung cancer growth**  
404 **in vivo**

405 **a,b.** A pool of barcoded Lenti-sgRNA/Cre vectors (Lenti-sgValidation/Cre) targeting candidate  
406 mediators of KRAS-driven lung tumor growth identified in the initial KRAS-interacting protein  
407 Tuba-seq screen was used to initiate tumors in validation cohorts of *KT* and *KT;H11<sup>LSL-Cas9</sup>* mice.  
408 This lentiviral pool includes four Inert sgRNAs, as well as sgRNAs targeting *Lkb1*, *Rb1*, and  
409 *Rbm10* as tumor suppressor controls. Each candidate gene from the initial screen is targeted with  
410 three sgRNAs. Tumors were initiated through intratracheal delivery of Lenti-sgValidation/Cre,  
411 and Tuba-seq was performed on each tumor-bearing lung 12 weeks after initiation, followed by  
412 analyses of sgID-BC sequencing data to characterize the effects of inactivating each gene (**b**).

413 **c.** Fluorescence images of representative lung lobes 12 weeks after tumor initiation. Scale bars =  
414 5 mm. Lung lobes are outlined with a white dashed line.

- 415 **d.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
416 tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9</sup>* mice. Genes are ordered by 95<sup>th</sup>  
417 percentile tumor size, with sgInerts on the left. Note that *sgLkb1* is plotted on a separate scale  
418 to facilitate visualization of sgRNAs with lesser magnitudes of effect. Dashed line indicates no  
419 effect relative to sgInert. Error bars indicate 95% confidence intervals. 95% confidence intervals  
420 and P-values were calculated by bootstrap resampling. Percentiles that are significantly different  
421 from sgInert (2-sided FDR-adjusted  $p < 0.05$ ) are in color.
- 422 **e.** Targeting *Hras* and *Nras* significantly increases mean tumor size relative to sgInerts, assuming  
423 a log-normal distribution of tumor sizes (LNmean). Error bars indicate 95% confidence intervals  
424 calculated by bootstrap resampling.
- 425 **f.** Schematic of tumor initiation with individual Lenti-sgRNA/Cre vectors. Mouse number and titer  
426 of the lentiviral vectors are indicated.
- 427 **g.** Representative fluorescence images of lungs from *KT;H11<sup>LSL-Cas9</sup>* mice after tumor initiation  
428 with Lenti-sgRNA/Cre vectors as indicated. Scale bar = 5 mm.
- 429 **h.** Representative H&E images of lungs from *KT;H11<sup>LSL-Cas9</sup>* mice after tumor initiation with Lenti-  
430 sgRNA/Cre vectors as indicated. Tumor area (percentage of total lung area) from each mouse is  
431 shown as Mean  $\pm$  SD. \*:  $p < 0.05$ ; Scale bar = 5 mm.
- 432 **i.** Tumor burden in *KT;H11<sup>LSL-Cas9</sup>* mice with tumors initiated with Lenti-sgRNA/Cre vectors as  
433 indicated. Each dot represents relative tumor area (percentage of total lung area) from one mouse.  
434 \*:  $p < 0.05$
- 435 **j.** Representative BrdU staining images of lungs from *KT;H11<sup>LSL-Cas9</sup>* mice after tumor initiation  
436 with Lenti-sgRNA/Cre vectors as indicated. Number of BrdU<sup>pos</sup> cells per field is shown as Mean  $\pm$   
437 SD. \*\*:  $p < 0.01$ ; Scale bar = 100  $\mu$ m.

438 **k.** Quantification of proliferation cells in *KT;H1<sup>LSL</sup>-Cas9* mice with tumors initiated with Lenti-  
439 sgRNA/Cre vectors as indicated. Each dot represents a tumor. \*\*: p<0.01

440

441 **Figure 3. Wildtype HRAS or NRAS constrain the growth of human KRAS-driven cancer**  
442 **cell lines.**

443 **a.** Inactivation of wild type HRAS or NRAS increases growth of KRAS-mutant H23 (G12C) and  
444 H727 (G12V) cells. Wildtype (sg*SAFE*) or *HRAS*- or *NRAS*-knockout cells were seeded in 96 well  
445 plates and cultured under limited serum (1%). Cell numbers were measured via CCK8 assay.  
446 Points are Mean±SD of 12 wells normalized to Day 0. \*\*: p<0.01

447 **b.** Re-expression of wild type HRAS suppresses proliferation of HRAS-null H23 and H727 cells.  
448 TRE-*HRAS* cells were seeded in 96 well plates and cultured under limited serum (1%) with or  
449 without 50 ng/ml Doxycycline (Dox) and cell numbers were measured via CCK8 assay. Points are  
450 Mean±SD of 12 wells normalized to Day 0. \*\*: p<0.01

451 **c-d.** Inactivation of HRAS or NRAS increases H23 colony formation. Wildtype (sg*SAFE*), *HRAS*-  
452 knockout (sg*HRAS*), or *NRAS*-knockout (sg*NRAS*) H23 cells were seeded at 1000 cells/well in 6-  
453 well plates and grown for two weeks. Cells were stained with crystal violet. **c.**

454 Representative images. Scale bar = 5mm. **d.** Mean±SD of colony number of 12 fields. \*\*: p<0.01

455 **e-f.** Re-expression of wild type HRAS suppresses HRAS-null H23 cell colony formation. TRE-  
456 Ctrl or TRE-*HRAS* H23 cells were seeded at 1000 cells/well in 6-well plates and grown with or  
457 without 50 ng/ml Dox for two weeks. Cells were stained with crystal violet. **e.** Representative  
458 images. Scale bar = 5mm. **f.** Mean±SD of colony number of 12 fields. \*\*: p<0.01

459 **g-k.** Inactivation of wild type HRAS or NRAS increases H23 cell growth after transplantation. **g.**  
460 Schematic of tumor initiation with subcutaneous (SubQ) or intravenous (IV) transplantation of

461 H23 cells with inactivation of HRAS or NRAS in NSG mice. Mouse number, cell number, and  
462 tumor growth time after transplantation are indicated. **h.** Tumor weight from SubQ transplantation  
463 of indicated cells. Each dot represents a mouse. Mean value was shown. **i.** Ki67<sup>pos</sup> cell number in  
464 tumor section from SubQ transplantation of indicated cells was shown as Mean±SD value of 20  
465 view fields. **j.** Tumor area (percentage of h-mitochondriapos area) from IV transplantation of  
466 indicated cells. Each dot represents a tumor. Mean value was shown. **k.** Ki67<sup>pos</sup> cell number in  
467 tumor section from IV transplantation of indicated cells is shown as Mean±SD value of 20 view  
468 fields (200x). \*: p<0.05; \*\*: p<0.01; ns: not significant.

469

#### 470 **Figure 4. Wildtype RAS paralogs suppress RAS signaling**

471 **a.** Representative image of pERK staining in *KT;H11<sup>LSL-Cas9</sup>* mice with tumors initiated with Lenti-  
472 sgRNA/Cre vectors as indicated. Quantification of pERK<sup>pos</sup> cells per tumor was shown as  
473 Mean±SD of 20 tumors. \*: p<0.05; \*\*: p<0.01; Scale bar: 100 μm

474 **b.** Representative image of pERK staining in subcutaneous tumor transplanted with H23 cells as  
475 indicated. Quantification of pERK<sup>pos</sup> cells per field was shown as Mean±SD of 20 fields. \*\*:   
476 p<0.01; Scale bar: 100 μm. HSP90 shows loading.

477 **c.** Western blot analysis of sorted cancer cells from *KT;H11<sup>LSL-Cas9</sup>* mice transduced with Lenti-  
478 sgRNA/Cre vectors as indicated. Multiple tumors were pooled and Tomato<sup>pos</sup> cancer cells were  
479 sorted prior to and protein extraction. HSP90 shows loading.

480 **d.** Western blot analysis of murine lung adenocarcinoma cell line that was transduced with Lenti-  
481 sgRNA vectors as indicated and selected with puromycin to generate stable knockout cell lines.  
482 Wildtype cells (*sgNeo*) or HRAS- or NRAS-knockout cells (*sgHras*, *sgNras*) were cultured under  
483 limited serum (1%) for 2 days before protein extraction. HSP90 shows loading.

484 e. Western blot analysis of cultured human lung adenocarcinoma cell lines transduced with Lenti-  
485 sgRNA vectors as indicated and selected with puromycin to generate stable knockout cell lines.  
486 Wildtype cell (sg*SAFE*) or HRAS- or NRAS-knockout cells (sg*HRAS*, sg*NRAS*) were  
487 cultured under limited serum (1%) for 2 days before protein extraction. HSP90 shows loading.

488 f. Western blot analysis of human lung adenocarcinoma cell lines re-expression HRAS (TRE-  
489 HRAS) under Doxycycline (Dox) treatment. HRAS-null cells were generated as described in  
490 **Figure 3a**. HRAS-null cells were re-transduced with lentiviral vector expressing TRE-HRAS at  
491 high MOI (>5) to generate stable HRAS re-expression cells (sgHRAS-TRE-HRAS). To re-express  
492 HRAS, cells were treated with 0, 1, or 2ng/ml Dox and cultured under limited serum (1%) for 2  
493 days before protein extraction. HSP90 shows loading.

494 g. Comparison of GI50 values to MEK inhibitors trametinib among wildtype and HRAS-null H23  
495 cells under treatment of indicated dose of trametinib for four days. Cell numbers were measured  
496 via CCK8 assay and normalized to cells treated with vehicle. Each data point was  
497 shown as Mean±SD of 12 wells.

498 h. Comparison of GI50 values to MEK inhibitors trametinib among HRAS-null H23 cells (H23-  
499 sgHRAS) re-expressing HRAS in presence (HRAS+Dox) or absence (HRAS) of Doxycycline plus  
500 indicated dose of trametinib for four days. Cell numbers were measured via CCK8 assay and  
501 normalized to cells treated with vehicle. Each data point was shown as Mean±SD of 12 wells.

502

503 **Figure 5. Wildtype RAS paralogs fine-tune RAS signaling through interaction with**  
504 **oncogenic KRAS.**

505 a. Bubble plot of three AP/MS experiments with H-, K-, and N-RAS as baits (rows), showing the  
506 enrichment of their paralogs (columns).

507 **b.** Diagram of the ReBiL2.0 system. KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interactions were quantified by  
508 normalized luminescent signal generated by membrane association facilitated interaction of the  
509 split-luciferase that is fused to the N-terminus of KRAS<sup>G12D</sup> (upper). Split-luciferase that is fused  
510 to the last four amino acids of KRAS (CVIM) is applied as control for background split-luciferase  
511 interaction on the membrane (lower). Adapted from Li et al. 2020.

512 **c.** All three RAS proteins are able to disrupt KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interaction. U2OS-764 (nl-  
513 KRAS<sup>G12D</sup>/cl-KRAS<sup>G12D</sup>) or U2OS-794 (nl-CVIM/cl-CVIM) cells expressing KRAS, HRAS, or  
514 NRAS were cultured in limited serum (1%) under 100 ng/ml Doxycycline (Dox) for 24 hours.  
515 ReBiL2.0 assay were performed as previously described and detailed in Methods. Points are  
516 Mean±SD ReBiL2.0 score of 36 wells normalized to cells transduced with empty lentiviral vector.  
517 \*\*: p<0.01

518 **d.** Pan-cancer frequency of HRAS mutations in patients with wildtype and oncogenic KRAS-  
519 tumors from Project GENIE. Known oncogenic HRAS mutations are highlighted in red. The  
520 dashed line indicates equal mutation frequency in KRAS-wildtype and mutant samples. Four  
521 candidate mutations that were chosen for further validation in this study were highlighted.

522 **e.** HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> are novel RAS-RAS interaction deficient mutations. U2OS-764 (nl-  
523 KRAS<sup>G12D</sup>/cl-KRAS<sup>G12D</sup>) cells expressing wildtype or rare mutant HRAS were cultured in limited  
524 serum (1%) under 100 ng/ml Dox for 24 hours. Points are Mean±SD ReBiL2.0 score of 12 wells  
525 normalized to cells transduced with empty lentiviral vector (upper). \*\*: p<0.01; ns: not significant.  
526 HRAS (wildtype and mutant) protein expression level in corresponding cells were shown by  
527 Western blot analysis (lower).

528 **f.** HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> are located close to the predicted HRAS-KRAS interaction interface.  
529 HRAS is shown in light orange and KRAS<sup>G12D</sup> is shown in blue. Residue R123 (in magenta) makes  
530 an intrachain salt bridge with E143 (in cyan).

531 **g.** Prey RAF proteins enriched in each experiment with the indicated baits in A549 cells (for K-,  
532 H-, or N-RAS) or HEK293 cells (for KRAS). Yellow color indicates higher values of NSAF. Both  
533 GTP- and GDP-bound HRAS behave like GDP-bound KRAS in their RAF interactions.

534 **h.** Western blot analysis of cultured HRAS-null HOP62 cells (HOP62-Cas9-sgHRAS) re-  
535 expressing wildtype or mutants (T50M, Y64A, or R123C) under Dox treatment. Cells were  
536 cultured under limited serum (1%) for 2 days before protein extraction. Re-expression of HRAS  
537 mutations have no effects on ERK phosphorylation.

538 **i.** Cell proliferation of cultured HRAS-null HOP62 cells (HOP62-Cas9-sgHRAS) re-expressing  
539 wildtype or mutants (T50M, Y64A, or R123C) under Dox treatment. Cells were cultured in limited  
540 serum (1%) with or without Dox for 4 days. Cell viability was measured via CCK8 assay and  
541 normalized to cells treated with vehicle. Re-expression of HRAS mutants have no effects on cell  
542 proliferation.

543

544 **Figure 6. Paired screens in KRAS-driven and BRAF-driven lung cancer models validates**

545 **HRAS and NRAS as KRAS-specific tumor suppressors.**

546 **a-b.** Schematic of pairwise screen of tumor suppressive function in KRAS- and BRAF-driven lung  
547 cancer. A pool of barcoded Lenti-sgRNA/Cre vectors targeting top mediators of KRAS-driven  
548 lung tumor growth (Lenti-sgMultiGEMM/Cre) was used to initiate tumors in cohorts of  
549 *KT;H11<sup>LSL-Cas9/+</sup>* and *Braf<sup>CA/+</sup>T;H11<sup>LSL-Cas9/+</sup>* (*BrafT;H11<sup>LSL-Cas9/+</sup>*) mice. Each regulator of KRAS-  
550 driven tumor growth (*Hras*, *Nras*, *Nme2* and *Fnta*) was targeted by two sgRNAs (those with the



551 largest effect size in the validation screen). The pool also included four Inert sgRNAs, as well as  
552 sgRNAs targeting *Apc*, *Cdkn2a*, and *Rbm10* as tumor suppressor controls (**a**). Tumors were  
553 initiated through intratracheal delivery of Lenti-sgMultiGEMM/Cre, and Tuba-seq was performed  
554 on each tumor-bearing lung 15 weeks after initiation, followed by analysis of sgID-BC sequencing  
555 data to characterize the effects of inactivating each gene (**b**).

556 **c**. Fluorescence images of representative lung lobes 15 weeks after tumor initiation. Scale bars =  
557 5 mm. Lung lobes are outlined with a white dashed line.

558 **d**. Total lung weight in *KT;H11<sup>LSL-Cas9/+</sup>* and *Braft;H11<sup>LSL-Cas9/+</sup>* mice 15 weeks after tumor  
559 initiation. Each dot is a mouse and mean value is indicated. \*\*: p<0.01

560 **e-f**. Size distribution of sgInert tumors in *KT;H11<sup>LSL-Cas9/+</sup>* and *Braft;H11<sup>LSL-Cas9/+</sup>* mice. In **e**., each  
561 dot represents a tumor, and the area of each dot is proportional to the number of cancer cells in  
562 that tumor. To prevent overplotting a random sample of 1,000 tumors from each of five  
563 representative *KT;H11<sup>LSL-Cas9/+</sup>* and *Braft;H11<sup>LSL-Cas9/+</sup>* mice are plotted. In **f**., the empirical  
564 cumulative distribution function of tumor sizes across all *KT;H11<sup>LSL-Cas9/+</sup>* and *Braft;H11<sup>LSL-Cas9/+</sup>*  
565 mice are plotted. Tumors >500 cells in size are shown.

566 **g**. Inactivation of *Hras* and *Nras* increases tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* but not *Braft;H11<sup>LSL-</sup>*  
567 *Cas9/+* models. Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-  
568 containing tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (left, white background)  
569 and *Braft;H11<sup>LSL-Cas9/+</sup>* (right, gray background) mice. Line at y=1 indicates no effect relative to  
570 sgInert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different  
571 from sgInert (two-sided FDR-adjusted p < 0.05) are in color. Confidence intervals and P-values  
572 were calculated by bootstrap resampling.

573 **h.** Comparison of the effects of inactivation of known tumor suppressors (*Rbm10*, *Apc*, and  
574 *Cdkn2a*) on tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* models. Tumor sizes at  
575 indicated percentiles for each sgRNA relative to the size of sgInert-containing tumors at the  
576 corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (left, white background) and *BrafT;H11<sup>LSL-Cas9/+</sup>*  
577 (right, gray background) mice. Line at  $y=1$  indicates no effect relative to sgInert. Error bars indicate  
578 95% confidence intervals. Percentiles that are significantly different from sgInert (two-sided FDR-  
579 adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap  
580 resampling.

581 **i.** Wildtype RAS paralogs function as tumor suppressors in oncogenic KRAS-driven lung cancer.  
582 Left panel, in oncogenic KRAS-driven lung cancer cells, wildtype RAS paralogs competitively  
583 interact with oncogenic KRAS and suppress oncogenic KRAS clustering. Right panel, inactivation  
584 of wildtype RAS allele, or “RAS paralog imbalance”, hyper-activate oncogenic KRAS signaling  
585 and promotes lung cancer growth.

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596 **Supplemental Figure 1. Prioritize candidate KRAS-interacting proteins for this study.**

597 **a.** Flow chart for prioritizing candidate KRAS-interacting proteins for this study. Candidate  
598 KRAS-interacting proteins were chosen based on multiple criteria including their interaction with  
599 KRAS, their homolog mRNA expression in *Kras*<sup>G12D</sup>-driven lung cancer in mouse model, and the  
600 consistency for them to bind different RAS-GTPase. RADIL is added at the last step due to its  
601 validated importance in KRAS-mutant human cell lines.

602 **b.** Candidate proteins interact with KRAS from two protein-protein interaction analyses (Kelly,  
603 Kostyrko, Han et al. 2020; Brodye, Simpson, Murray et al. 2020). Shared KRAS-interaction  
604 proteins are shown as their log10NSAF and SigMap Score.

605 **c.** Homolog mRNA expression (TPM) of candidate KRAS-interacting proteins in *Kras*<sup>G12D</sup>-driven  
606 lung cancer in mouse model (Chuang et al. 2017).

607 **d.** Bubble plot of eight AP/MS experiments with GTP- and GDP-locked mutant GTPases as baits  
608 (rows), showing the enrichment of selected candidate KRAS-interacting proteins (columns). Dark  
609 borders indicate FDR < 0.05.

610 **e.** Mutation frequencies of these 13 candidate genes in lung adenocarcinoma (data from TCGA,  
611 Nat. Genet. 2016).

612

613 **Supplemental Figure 2. Tumor barcoding coupled with barcode sequencing (Tuba-seq) can**  
614 **uncover engineered alterations that reduce tumor number and growth.**

615 **a-b.** Schematic of the Tuba-seq approach to measure the effects of essential gene inactivation on  
616 tumor growth. Lentiviral-sgRNA/Cre vectors with inert sgRNAs (gray) or sgRNAs targeting  
617 known essential genes (navy) were diversified with a two component sgID-BC. A vector targeting  
618 known tumor suppressor *Apc* was included as a positive control (**a**). Tumors were initiated with

619 this barcoded Lenti-sgEssential/Cre pool in *KT* and *KT;H1<sup>LSL-Cas9</sup>* mice. Tuba-seq was performed  
620 on each tumor-bearing lung 12 weeks after initiation, followed by analyses of sgID-BC sequencing  
621 data to characterize the effects of inactivating each gene (**b**).

622 **c.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
623 tumors at the corresponding percentiles. Line at  $y=1$  indicates no effect relative to sgInert. Error  
624 bars indicate 95% confidence intervals. Percentiles that are significantly different from sgInert  
625 (two-sided FDR-corrected  $p < 0.05$ ) are in color. Confidence intervals and P-values were  
626 calculated by bootstrap resampling.

627 **d.** The impact of each sgRNA on mean tumor size relative to sgInerts, assuming a log-normal  
628 distribution of tumor sizes (LNmean). sgRNAs with two-sided  $P < 0.05$  after FDR-adjustment are  
629 in bold.

630 **e.** The impact of each sgRNA on tumor burden (number of neoplastic cells aggregated across all  
631 tumors of a genotype) relative to sgInerts and normalized to the same statistic in *KT* mice to  
632 account for representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  $y=1$   
633 indicates no effect. Error bars indicate 95% confidence intervals. Relative burdens significantly  
634 different from sgInert (two-sided FDR-corrected  $p < 0.05$ ) are in color. Confidence intervals and P-  
635 values were calculated by bootstrap resampling.

636 **f.** The impact of each sgRNA on tumor number relative to sgInerts and normalized to the same  
637 statistic in *KT* mice to account for representation of each sgRNA in the viral pool. sgInerts are in  
638 gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative  
639 tumor numbers significantly different from sgInert (two-sided FDR-corrected  $p < 0.05$ ) are in color.  
640 Confidence intervals and P-values were calculated by bootstrap resampling.

641 **g.** The impact of each sgRNA on tumor number plotted against its impact on LNmean tumor size.  
642 The lines at  $y=1$  and  $x=1$  indicate no effect relative to sgInert on tumor number and size,  
643 respectively. *sgRsp19* and *sgPcna* cluster in the lower left quadrant near  $x=1$ , indicating that  
644 targeting essential genes strongly reduces tumor number but only moderately decreases average  
645 tumor size. Error bars indicate 95% confidence intervals calculated by bootstrap resampling.

646

647 **Supplemental Figure 3. Inactivation of KRAS-interacting proteins has similar impacts on**  
648 **tumor growth in p53-proficient and p53-deficient contexts.**

649 **a.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
650 tumors at the corresponding percentiles in *KT* mice. *KT* mice lack Cas9, thus all sgRNAs are  
651 functionally equivalent to sgInerts. Genes are ordered as in **Figure 1f**. Line at  $y=1$  indicates no  
652 effect relative to sgInert. Error bars indicate 95% confidence intervals. Confidence intervals and  
653 P-values were calculated by bootstrap resampling. As expected, no percentiles were significantly  
654 different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ).

655 **b.** The impact of each sgRNA on mean tumor size relative to sgInerts in *KT;H11<sup>LSL-Cas9</sup>*, assuming  
656 a log-normal distribution of tumor sizes (LNmean). sgRNAs with two-sided  $P < 0.05$  after FDR-  
657 adjustment are in bold. P-values were calculated by bootstrap resampling.

658 **c-d.** The impact of each sgRNA on tumor burden (**c**) and number (**d**) relative to sgInerts in  
659 *KT;H11<sup>LSL-Cas9</sup>* mice, normalized to the corresponding statistic in *KT* mice to account for  
660 representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  $y=1$  indicates  
661 no effect. Error bars indicate 95% confidence intervals. Relative tumor burdens and numbers  
662 significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence  
663 intervals and P-values were calculated by bootstrap resampling.

664 e. Tumor sizes at the indicated percentiles for each sgRNA relative to the size of sgInert-containing  
665 tumors in *KT;p53<sup>flox/flox</sup>;H1I<sup>LSL-Cas9</sup>* mice. Genes are ordered as in **Figure 1f**. Dashed line indicates  
666 no effect relative to sgInert. Error bars indicate 95% confidence intervals. Percentiles that are  
667 significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence  
668 intervals and P-values calculated by bootstrap resampling.

669 **f-h.** Comparison of the impact of each sgRNA on relative LNmean tumor size (**f**), tumor burden  
670 (**g**) and tumor number (**h**) in *KT;H1I<sup>LSL-Cas9</sup>* and *KT;p53<sup>flox/flox</sup>;H1I<sup>LSL-Cas9</sup>* mice. Error bars indicate  
671 95% confidence intervals calculated by bootstrap resampling.

672

673 **Supplemental Figure 4. Top candidate KRAS-interacting proteins from initial Tuba-seq**  
674 **screen impact multiple metrics of tumor growth in validation cohort.**

675 **a.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
676 tumors at the corresponding percentiles in *KT* mice. *KT* mice lack Cas9, thus all sgRNAs are  
677 functionally equivalent to sgInerts. Genes are ordered as in **Figure 2d**, but note the change in axis  
678 scaling. Line at  $y=1$  indicates no effect relative to sgInerts. Error bars indicate 95% confidence  
679 intervals. Confidence intervals and P-values were calculated by bootstrap resampling. As expected,  
680 no percentiles were significantly different from sgInert (FDR-adjusted  $p < 0.05$ ).

681 **b.** The impact of each sgRNA on mean tumor size relative to sgInerts, assuming a log-normal  
682 distribution of tumor sizes (LNmean). Two-sided P-values were calculated by bootstrap  
683 resampling. sgRNAs with  $P < 0.05$  after FDR-adjustment are in bold. Note that this data for the  
684 sgInerts, *sgHras#1-3* and *sgNras#1-3* is also plotted in **Figure 2e**.

685 c. The impact of each sgRNA on tumor burden relative to sgInerts in *KT;H11<sup>LSL-Cas9</sup>* mice,  
686 normalized to the corresponding statistic in *KT* mice to account for representation of each sgRNA  
687 in the viral pool. sgInerts are in gray and the line at  $y=1$  indicates no effect. Error bars  
688 indicate 95% confidence intervals. Relative tumor burdens significantly different from sgInert  
689 (two-sided FDR-adjusted  $p<0.05$ ) are in color. Confidence intervals and P-values were calculated  
690 by bootstrap resampling.

691 d. The impact of each sgRNA on tumor number relative to sgInerts in *KT;H11<sup>LSL-Cas9</sup>* mice,  
692 normalized to the corresponding statistic in *KT* mice to account for representation of each sgRNA  
693 in the viral pool. sgInerts are in gray and the line at  $y=1$  indicates no effect. Error bars  
694 indicate 95% confidence intervals. Relative tumor numbers significantly different from sgInert  
695 (two-sided FDR-adjusted  $p<0.05$ ) are in color. Confidence intervals and P-values were calculated  
696 by bootstrap resampling.

697

698 **Supplemental Figure 5. Dependency of human LUAD cell lines on RAS family members.**

699 a. Comparison of RAS family member dependency scores between KRAS mutant and KRAS  
700 wildtype human LUAD cell lines. \*\*\*\* ( $p < 0.0001$ ), ns (not-significant).

701 b. Volcano plot showing the effects of RAS gene knockouts in A549 cells. The T-score represents  
702 the normalized effect of multiple sgRNAs targeting a gene. A positive T-score indicates a tumor  
703 suppressive effect. The effects of each gene relative to SAFE sgRNAs were tested via Mann–  
704 Whitney U (MWU) test, corrected via Benjamini-Hochberg procedure and shown as -  
705  $\log_{10}(\text{MWU-Adjusted P-val})$ . (Data source: Kelly, Kostyrko, Han *et al.* 2020)

706 c. Volcano plot showing effects of RAS gene knockouts in KRAS-mutant human LUAD cells  
707 (left: H2009, mid: H23, right: H1975) in 3D culture. The T-score represents the normalized effect

708 of multiple sgRNAs targeting a gene. A positive T-score indicates a tumor suppressive effect. The  
709 effects of each gene relative to SAFE sgRNAs were tested via two-side t-test, corrected via  
710 Benjamini-Hochberg procedure and shown as  $-\log_{10}(Q\text{-val})$ . (Data source: Han *et al.* 2020)

711

712 **Supplemental Figure 6. Inactivation of wild type HRAS or NRAS increases H23 cell growth**  
713 **after transplantation.**

714 **a.** Representative image of subcutaneous tumor size four weeks after transplantation with H23  
715 cells as indicated. Quantification was shown in **Figure 3h**. Scale bar: 2 mm

716 **b.** Representative image of Ki67 staining from subcutaneous tumor four weeks after  
717 transplantation with H23 cells as indicated. Quantification was shown in **Figure 3i**. Scale bar: 100  
718  $\mu\text{m}$

719 **c.** Representative image of HE (upper) and human mitochondria (lower) staining from lung tumor  
720 four weeks after intravenous transplantation with H23 cells as indicated. Quantification was shown  
721 in **Figure 3j**. Scale bar: 500  $\mu\text{m}$

722 **d.** Representative image of Ki67 staining from lung tumor four weeks after intravenous  
723 transplantation with H23 cells as indicated. Quantification was shown in **Figure 3k**. Scale bar: 200  
724  $\mu\text{m}$

725

726 **Supplemental Figure 7. Wildtype RAS paralogs finetune RAS signaling.**

727 **a.** Quantification of pERK<sup>pos</sup> cells in *KT;H11<sup>LSL-Cas9</sup>* mice with tumors initiated with Lenti-  
728 sgRNA/Cre vectors as indicated in **Figure 4a**. Each dot represents a tumor. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$

729 **b.** Quantification of pERK<sup>pos</sup> cells per field of indicated cells from **Figure 4b**. Each dot represents  
730 a view field. \*\*:  $p < 0.01$



731 **c-f.** Raw images for western blots from **Figure 4c-f**. HRAS expression on **Figure 4f** were detected  
732 using same lysis on a different gel with increased loading.

733

734 **Supplemental Figure 8. Identification of rare HRAS mutations in oncogenic KRAS-mutant**  
735 **tumors.**

736 **a.** Pan-cancer frequency of *HRAS* mutations in patients with *KRAS*-wildtype and oncogenic *KRAS*-  
737 mutant tumors from Project GENIE. Mutations that are intergenic, intronic, silent, or fall in the 3'  
738 or 5' UTR were excluded. Oncogenic *KRAS* mutants were defined as tumors having missense  
739 mutations in codons 12, 13 or 61. Known oncogenic *HRAS* mutations are highlighted in red. The  
740 dashed line indicates equal mutation frequency in *KRAS*-wildtype and mutant samples. Non-  
741 oncogenic mutations occurring at least once in patients with oncogenic *KRAS* mutations are  
742 annotated. *HRAS* mutants selected for analysis of ability to disrupt  $KRAS^{G12D}$ - $KRAS^{G12D}$   
743 interactions are highlighted in bold.

744 **b.** Characteristic of samples with rare *HRAS* mutants selected for analysis of their ability to disrupt  
745  $KRAS^{G12D}$ - $KRAS^{G12D}$  interactions using the ReBiL2.0 system.

746

747 **Supplemental Figure 9. Modeling RAS-RAS dimer.**

748 **a.** Homodimers of RAS present in crystals of *HRAS*, *KRAS*, and *NRAS* in the Protein Data Bank.  
749 Dimers were downloaded from the Protein Common Interface Database (ProtCID)<sup>58</sup>, which  
750 clusters interfaces present in different crystals of homologous proteins. The  $\alpha 4$ - $\alpha 5$  dimer shown  
751 is present in 84 entries of *HRAS*, 13 entries of *KRAS*, and one entry of *NRAS* (PDB 5UHV).

752 **b.** Models of a homodimer of  $KRAS^{G12D}$  and heterodimers of  $KRAS^{G12D}$  with *HRAS*,  $HRAS^{T50M}$ ,  
753 and  $HRAS^{R123C}$ . The  $\alpha 4$ - $\alpha 5$  *HRAS* dimer from PDB entry 3K8Y was used as a template.

754 KRAS<sup>G12D</sup> from PDB entry 5USJ was superposed with the program PyMol on one or both  
755 monomers of 3K8Y to form the heterodimers and the homodimer respectively. Residues T50 and  
756 R123 were mutated with PyMol. All four structures were relaxed with the program Rosetta using  
757 the FastRelax protocol with the Ref2015 scoring function)<sup>59</sup>. Rosetta uses the backbone-dependent  
758 rotamer library of Shapovalov and Dunbrack to repack side chains around the mutated sites<sup>60</sup>. The  
759 resulting energies were: KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup>, -1122.8 kcal/mol; HRAS-KRAS<sup>G12D</sup>, -1144.8  
760 kcal/mol; HRAS<sup>T50M</sup>-KRAS<sup>G12D</sup>, -1135.5 kcal/mol; HRAS<sup>R123C</sup>-KRAS<sup>G12D</sup>, -1130.9 kcal/mol.  
761 Residues T50 (magenta) and R123 (orange) are indicated in sticks.

762

763 **Supplemental Figure 10. Wildtype RAS paralogs finetune RAS signaling through interaction**  
764 **with oncogenic KRAS.**

765 **a.** Raw images for western blots of split-luciferase (HA-tag) expression for ReBiL2.0 from **Figure**  
766 **5c.** HA-tag expression were detected using same lysis on a different gel with increased loading.  
767 **b.** Raw images for western blots of split-luciferase (HA-tag) expression for ReBiL2.0 from **Figure**  
768 **5e.** HA-tag expression were detected using same lysis on a different gel with increased loading.  
769 **c.** Raw images for western blots from **Figure 5h.** HRAS expression were detected using same lysis  
770 on a different gel with increased loading.

771

772 **Supplemental Figure 11. Paired screen in KRAS-driven and BRAF-driven lung cancer**  
773 **models validates HRAS and NRAS as KRAS-specific tumor suppressors.**

774 **a-c.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing  
775 tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (**a**), *BrafT;H11<sup>LSL-Cas9/+</sup>* (**b**) and *KT*  
776 mice (**c**). Genes are ordered by 95<sup>th</sup> percentile tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* mice, with sgInerts

777 on the left. sgInerts are in gray, and line at  $y=1$  indicates no effect relative to sgInert. Error bars  
778 indicate 95% confidence intervals. Percentiles that are significantly different from sgInert (two-  
779 sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by  
780 bootstrap resampling. The negative effects of sgRNAs targeting *Fnta* and *Nme2* in the *KT* mice  
781 (c) are unexpected and indicate a potential bias in the size distributions of tumors with these  
782 genotypes. We note that the same bias may be present in the *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-</sup>*  
783 *Cas9/+* data; however, sgRNAs targeting these genes in previous experiments showed consistent  
784 negative effects on tumor size, suggesting that the observed effects in this *KT;H11<sup>LSL-Cas9/+</sup>* cohort  
785 are not solely the product of this bias.

786 **d.** The impact of each sgRNA on tumor burden relative to sgInerts in *KT;H11<sup>LSL-Cas9/+</sup>* (top) and  
787 *BrafT;H11<sup>LSL-Cas9/+</sup>* (bottom) mice, normalized to the corresponding statistic in *KT* mice to account  
788 for representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  
789  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor burdens  
790 significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence  
791 intervals and P-values were calculated by bootstrap resampling.

792 **e.** The impact of each sgRNA on tumor number relative to sgInerts in *KT;H11<sup>LSL-Cas9/+</sup>* (top) and  
793 *BrafT;H11<sup>LSL-Cas9/+</sup>* (bottom) mice, normalized to the corresponding statistic in *KT* mice to account  
794 for representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  
795  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor numbers  
796 significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence  
797 intervals and P-values were calculated by bootstrap resampling.

798

799

## 800 **METHODS**

### 801 *Cells, Reagents and Plasmids:*

802 H23, H727, and HOP62 cells were originally purchased from ATCC; HC494(KPT) lung  
803 adenocarcinoma cells were generated in the Winslow Lab; U2OS-134-764np (nl-KRAS<sup>G12D</sup> cl-  
804 KRAS<sup>G12D</sup>; KRAS<sup>G12D</sup> was fused to the N-termini of split luciferase proteins) and U2OS-134-794p  
805 (nl-CVIM cl-CVIM; CVIM represents the C-terminal last 20 amino acids of KRAS4B) cells were  
806 generated in the Wahl lab by Dr. Yao-Cheng Li (Salk Institute for Biological Studies). HC494  
807 cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin and 100 µg/mL  
808 streptomycin. A549, H460 and H82 cells were cultured in RPMI1640 media containing 10% FBS,  
809 100 units/mL penicillin and 100 µg/mL streptomycin. U2OS cells were cultured in DMEM/F12  
810 (Thermo Fisher; phenol-red free), 10% (vol/vol) FBS, and 10 µg/mL ciprofloxacin. All cell lines  
811 were confirmed to be mycoplasma negative (MycoAlert Detection Kit, Lonza).

812 Trametinib was purchased from MedChemExpress (HY-10999); 5-Bromo-2'-deoxyuridine  
813 (10280879001) and D-Luciferin (L9504-5MG) was purchased from Sigma-Aldrich. All plasmids  
814 used in this study were listed in supplementary Table 1 and will be donated to Addgene.

815

### 816 *Design, generation, barcoding, and production of lentiviral vectors*

817 The sgRNA sequences targeting the putative tumor suppressor genes were designed using  
818 CRISPick (<https://portals.broadinstitute.org/gppx/crispick/public>). All sgRNA sequence are  
819 shown in Supplementary Table 2. Each desired sgRNA vector was modified from our previously  
820 published pll3-U6-sgRNA-Pgk-Cre vector via site-directed mutagenesis (New England Biolabs,  
821 E0554S). The generation of the barcode fragment containing the 8-nucleotide sgID sequence and

822 20-nucleotide degenerate barcode, and subsequent ligation into the vectors were performed as  
823 previously described.  
824 Lentiviral vectors were produced using polyethylenimine (PEI)-based transfection of 293T cells  
825 with delta8.2 and VSV-G packaging plasmids in 150-mm cell culture plates. Sodium butyrate  
826 (Sigma Aldrich, B5887) was added 8 hours after transfection to achieve a final concentration of  
827 20 mM. Medium was refreshed 24 hours after transfection. 20 mL of virus-containing supernatant  
828 was collected 36, 48, and 60 hours after transfection. The three collections were then pooled and  
829 concentrated by ultracentrifugation (25,000 rpm for 1.5 hours), resuspended overnight in 100  $\mu$ L  
830 PBS, then frozen at  $-80^{\circ}\text{C}$  and were thawed and pooled at equal ratios immediately prior to delivery  
831 to mice.

832

### 833 *Mice and tumor initiation*

834 The use of mice for the current study has been approved by Institutional Animal Care and  
835 Use Committee at Stanford University, protocol number 26696.

836  $\text{Kras}^{\text{LSL-G12D/+}}$  (RRID:IMSR\_JAX:008179),  $\text{R26}^{\text{LSL-tdTomato}}$  (RRID:IMSR\_JAX:007909),  
837 and  $\text{H11}^{\text{LSL-Cas9}}$  (RRID:IMSR\_JAX:027632) mice have been previously described. They were on  
838 a C57BL/6:129 mixed background. The  $\text{B6.129P2(Cg)-Braf}^{\text{tm1Mmcm/J}}$  ( $\text{BRAF}^{\text{F-V600E}}$ ) mice  
839 were initially generated by Dankort et al. and obtained from the Jackson Laboratory  
840 (RRID:IMSR\_JAX: 017837). Tumors were initiated by intratracheal delivery of 60  $\mu$ l of  
841 lentiviral vectors dissolved in PBS.

842 For the initial experiments in Figure 1 and 2, tumors were allowed to develop for 12 weeks after  
843 viral delivery of a lentiviral pool that contained 19 barcoded Lenti-sgRNA/Cre vectors (Lenti-

844 sgKrasIP/Cre). Tumors were initiated in  $Kras^{LSL-G12D}$ ;  $R26^{LSL-tdTomato}$  (*KT*),  $KT;H11^{LSL-Cas9}$ ; or  
845  $KT;p53^{fl/fl};H11^{LSL-Cas9}$  mice with  $1.95 \times 10^5$  infectious units (ifu)/mouse.

846 For the validation experiments in Figure 3, tumors were allowed to develop for 15 weeks after  
847 viral delivery of a lentiviral pool that contained 26 barcoded Lenti-sgRNA/Cre vectors (Lenti-  
848 sgValidation/Cre). Tumors were initiated in  $Kras^{LSL-G12D}$ ;  $R26^{LSL-tdTomato}$  (*KT*) or  $KT;H11^{LSL-Cas9}$ ;  
849 mice with  $3 \times 10^5$  ifu/mouse.

850 For the individual sgRNA tumor initiation experiments in **Figure 3**, tumors were allowed to  
851 develop for 12 weeks after viral delivery of individual sgRNA expressing lentiviral vector that  
852 targeting Neo2, Hras, or Nras. Tumors were initiated in  $KT;H11^{LSL-Cas9}$ ; mice with  $1 \times 10^5$   
853 ifu/mouse.

854 For the paired screen experiments in Figure 6, tumors were allowed to develop for 15 weeks after  
855 viral delivery of a lentiviral pool that contained 15 barcoded Lenti-sgRNA/Cre vectors (Lenti-  
856 sgMultiGEMM/Cre). Tumors were initiated in  $KT;H11^{LSL-Cas9/+}$  or  $Braf^{V600E};R26^{LSL-tdTomato};H11^{LSL-Cas9/+}$   
857 mice with  $3 \times 10^5$  ifu/mouse. Note that  $KT;H11^{LSL-Cas9/+}$  rather than  $KT;H11^{LSL-Cas9/LSL-Cas9}$   
858 mice were used in this experiment to match the Cas9 dosage of the  $BrafT;H11^{LSL-Cas9/+}$   
859 mice, whereas  $KT;H11^{LSL-Cas9/LSL-Cas9}$  mice were used in all other experiments. To evaluate the  
860 effects of Cas9 dosage on the tumor suppressive effects of the Lenti-sgMultiGEMM/Cre pool, we  
861 also initiated tumors in a small cohort of  $KT;H11^{LSL-Cas9/LSL-Cas9}$  mice. Reductions in the magnitude  
862 of the effects of various sgRNAs were observed in the  $KT;H11^{LSL-Cas9/+}$  cohort relative to the  
863  $KT;H11^{LSL-Cas9/LSL-Cas9}$  cohort, underscoring the importance of matching Cas9 dosage and  
864 suggesting that Cas9 can be limiting in  $H11^{LSL-Cas9/+}$  mice.

865

866 *Tuba-seq library generation*

867 Genomic DNA was isolated from bulk tumor-bearing lung tissue from each mouse as previously  
868 described. Briefly, benchmark control cell lines were generated from LSL-YFP MEFs transduced  
869 by a barcoded Lenti-sgNT3/Cre vector (NT3: an inert sgRNA with a distinct sgID) and purified  
870 by sorting YFP<sup>pos</sup> cells. Three benchmark control cell lines (500,000 cells each) were added to  
871 each mouse lung sample prior to lysis to enable the calculation of the absolute number of neoplastic  
872 cells in each tumor from the number of sgID-BC reads. Following homogenization and overnight  
873 protease K digestion, genomic DNA was extracted from the lung lysates using standard phenol-  
874 chloroform and ethanol precipitation methods. Subsequently, Q5 High-Fidelity 2x Master Mix  
875 (New England Biolabs, M0494X) was used to amplify the sgID-BC region from 32 µg of genomic  
876 DNA in a total reaction volume of 800 µl per sample. The unique dual-indexed primers used were  
877 Forward: AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-8 nucleotides for i5 index-ACA  
878 CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT-6 to 9 random nucleotides for increased  
879 diversity-GCG CAC GTC TGC CGC GCT G and Reverse: CAA GCA GAA GAC GGC ATA  
880 CGA GAT-6 nucleotides for i7 index- GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG  
881 ATC T-9 to 6 random nucleotides for increased diversity-CAG GTT CTT GCG AAC CTC AT.  
882 The PCR products were purified with Agencourt AMPure XP beads (Beckman Coulter, A63881)  
883 using a double size selection protocol. The concentration and quality of the purified libraries were  
884 determined using the Agilent High Sensitivity DNA kit (Agilent Technologies, 5067-4626) on the  
885 Agilent 2100 Bioanalyzer (Agilent Technologies, G2939BA). The libraries were pooled based on  
886 lung weight to ensure even reading depth, cleaned up again using AMPure XP beads, and  
887 sequenced (read length 2x150bp) on the Illumina HiSeq 2500 or NextSeq 550 platform (Admera  
888 Health Biopharma Services).

889

890 *Generation of Stable Cell Lines:*

891 Parental cells were seeded at 50% confluency in a 6-well plate the day before transduction (day  
892 0). The cell culture medium was replaced with 2 mL fresh medium containing 8 µg/mL  
893 hexadimethrine bromide (Sigma Aldrich, H9268-5G), 20 µL ViralPlus Transduction Enhancer  
894 (Applied Biological Materials Inc., G698) and 40 µL concentrated lentivirus and cultured  
895 overnight (Day 1). The medium was then replaced with complete medium and cultured for another  
896 24 hours (Day 2). Cells were transferred into a 100 mm cell culture dish with appropriate amounts  
897 of antibiotic (Blasticidin doses: U2OS: 10 µg/mL; HOP62: 50 µg/mL; H727: 10 µg/mL; H23: 15  
898 µg/mL; Puromycin doses: HC494: 5 µg/mL; U2OS: 1 µg/mL; HOP62: 5 µg/mL; H727: 5 µg/mL;  
899 H23: 5 µg/mL) and selected for 48 hours (Day 3).

900

901 *Western Blot*

902 Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, and  
903 0.1% SDS) and incubated at 4 °C with continuous rotation for 30 minutes, followed by  
904 centrifugation at 12,000 × rcf for 10 minutes. The supernatant was collected, and the protein  
905 concentration was determined by BCA assay (Thermo Fisher Scientific, 23250). Protein extracts  
906 (10–50 µg) were dissolved in 10% SDS-PAGE and transferred onto PVDF membranes. The  
907 membranes were blocked with 5% non-fat milk in TBS with 0.1% Tween 20 (TBST) at room  
908 temperature for one hour, cut according to the molecular weight of target protein (with at least two  
909 flanking protein marker), followed by incubation with primary antibodies diluted in TBST  
910 (1:1000) at 4 °C overnight. After three 10-minutes washes with TBST, the membranes were  
911 incubated with the appropriate secondary antibody conjugated to HRP diluted in TBST (1:10000)  
912 at room temperature for 1 hour. After three 10-minutes washes with TBST, Protein expression



913 was quantified with enhanced chemiluminescence reagents (Fisher Scientific, PI80196). For AKT  
914 and ERK, phosphorylated proteins were detected first and the membrane were striped, blocked,  
915 and incubated with 1<sup>st</sup> and 2<sup>nd</sup> antibodies for pan protein detections.

916 Antibodies used in this study: HSP90 (BD Biosciences, 610418), pAKT (Cell Signaling, 4060S),  
917 pERK (Cell Signaling, 4370L), ERK (Cell Signaling, 9102S), AKT (Cell Signaling, 4691S),  
918 HRAS (Thermo Fisher Scientific, 18295-1-AP), NRAS (Santa Cruz Biotechnology, sc-31), HA-  
919 tag (Santa Cruz Biotechnology, sc-7392).

920

#### 921 *Histology and immunohistochemistry (IHC)*

922 Lung lobes were fixed in 4% formalin and paraffin embedded. Hematoxylin and eosin staining  
923 was performed using standard methods. IHC was performed on 4- $\mu$ m sections with IHC was  
924 performed using Avidin/Biotin Blocking Kit (Vector Laboratories, SP-2001), Avidin-Biotin  
925 Complex kit (Vector Laboratories, PK-4001), and DAB Peroxidase Substrate Kit (Vector  
926 Laboratories, SK-4100) following standard protocols.

927 The following primary antibodies were used: Ki-67 (BD Pharmingen, 550609), BrdU (BD  
928 Pharmingen, 555627), human mitochondria (Abcam, ab92824), pERK (Cell Signaling, 4370L).

929 Total tumor burden (tumor area/total area  $\times$  100%), mitochondria<sup>pos</sup> tumor burden (mitochondria<sup>pos</sup>  
930 area/total area  $\times$  100%), BrdU<sup>pos</sup> cell number, Ki67<sup>pos</sup> cell number, and pERK<sup>pos</sup> cell number were  
931 calculated using ImageJ.

932

#### 933 *Cell proliferation assay (CCK8)*

934 For cell proliferation assays, cells were seeded in 96-well plates at a density of 5000 cells per well  
935 and allowed to adhere overnight in regular growth media (Day 0). Cells were then cultured in

936 media as indicated on each figure panel for 7 days. Relative cell number were measured every  
937 other day using Cell Counting Kit-8 (Bimake, B34304) according to the manufacturer's  
938 instructions.

939

#### 940 *Colony formation assay*

941 For clonogenic assays, cells were seeded in 6-well plates at a density of 500 cells per well  
942 and allowed to adhere overnight in regular growth media. Cells were then cultured in media as  
943 indicated on each figure panel for 14 days. Growth media with or without drugs was replaced every  
944 2 days. At the end point, cells were stained with 0.5% crystal violet in 20% methanol. Colony  
945 numbers were calculated using ImageJ

946

#### 947 *Allograft studies in immunocompromised mice*

948 For intravenous transplants into immunocompromised NSG mice,  $5 \times 10^5$  H23 cells were injected  
949 into one of lateral tail veins. Mice were sacrificed 28 days post-injection and lung lobe were fixed  
950 in 4% formalin and paraffin embedded. For subcutaneous transplants into immunocompromised  
951 NSG mice,  $2 \times 10^6$  of each H23 cells (sgSAFE, sgHRAS, and sgNRAS) were re-suspended in  
952 200uL Matrigel<sup>®</sup> Basement Membrane Matrix (Corning, 354234) and injected into three parallel  
953 sites per mouse. Mice were sacrificed 28 days post-injection. Tumors were dissected and the  
954 weight, height, width, and length, of each tumor was measured. Tumor volume was roughly  
955 calculated via the formula:  $V = (4/3) \times \pi \times (L/2) \times (L/2) \times (D/2)$ .

956 Institute of Medicine Animal Care and Use Committee approved all animal studies and procedures.

957

#### 958 *ReBiL2.0 assay*

959 ReBiL2.0 assay was performed as previously described<sup>16</sup>. ReBiL cells (U2OS-134-764np or U2OS-  
960 134-794p with overexpression of KRAS4b, HRAS or NRAS) were seeded in i) 96-well plates at  
961 density of  $2 \times 10^4$ , and ii) 6-well plates at density of  $1 \times 10^6$  and allowed to adhere overnight in regular  
962 growth media (DMEM/F12, 10% FBS, and 10  $\mu\text{g}/\text{mL}$  ciprofloxacin). The next day, cells were  
963 then cultured in serum limited media (DMEM/F12, 1% FBS, and 10  $\mu\text{g}/\text{mL}$  ciprofloxacin)  
964 containing 100 ng/mL doxycycline for 24 hours. Upon termination of the ReBiL assay, i) to  
965 measure raw luciferase activity, 300  $\mu\text{M}$  D-luciferin was added to 96-well plate culture and  
966 incubate in  $37^\circ\text{C}$  for 30mins and raw luminescent data collected by a Tecan microplate reader; ii)  
967 to measure viable cell numbers, CCK-8 assay were performed in the same 96-well plate culture  
968 and raw cell number data collected by a Tecan microplate reader; iii) to quantify the 1/2luc fusion  
969 proteins, ReBiL cells from 6-well plate culture were harvested with RIPA lysis buffer for protein  
970 extraction and western blot was performed for HA-tag and HSP90 expression. Then the ReBiL2.0  
971 score was calculated via the formula:

$$972 \text{ ReBiL2.0 score} = ([\text{Raw Luminescence}]/[\text{Cell number}]) / ([1/2\text{luc Least}]/[\text{HSP90}])$$

973

974 *Analysis of human lung adenocarcinoma cancer genome sequencing data (for HRAS rare*  
975 *mutations)*

976 To assess evidence that *HRAS* functions as a Kras-specific tumor suppressor in human cancer, we  
977 queried publicly available cancer genomic datasets. GENIE Release 9.1-public was accessed  
978 through the Synapse platform and data on somatic mutations (data\_mutations\_extended.txt),  
979 sample- and patient-level clinical data (data\_clinical\_sample.txt and data\_clinical\_patient.txt), and  
980 genotyping panel information (genomic\_information.txt) were downloaded. While it is unclear  
981 how our findings may extrapolate to cancer types beyond lung adenocarcinoma, Hras mutations

982 are exceedingly rare (occurring at a frequency of just  $\sim 0.008$  in GENIE samples) so we performed  
983 a pan-cancer analysis. Each sample was assigned to its patient of origin and annotated for the  
984 presence of both oncogenic Kras mutations (defined as missense mutations in Kras exons 12, 13  
985 or 61) and for the presence of potentially functional Hras mutations (variants that were silent,  
986 intergenic, intronic, or fell in the 3' or 5' UTRs were excluded from this analysis). When multiple  
987 samples were derived from the same patient, the patient in question was annotated as having a  
988 mutation if it occurred in at least one of their associated samples. From this information we  
989 produced a list of the frequency of all Hras variants in patients with and without oncogenic Kras  
990 in both datasets. The genotyping panel information was used to identify GENIE patients that were  
991 not genotyped at Hras and exclude these from the frequency calculation.

992

### 993 *Process paired-end reads to identify the sgID and barcode*

994 Sequencing of Tuba-seq libraries produces reads that are expected to contain an 8-nucleotide sgID  
995 followed by a 30-nucleotide barcode (BC) of the form  
996 GCNNNNNTANNNNNGCNNNNNTANNNNNGC, where each of the 20 Ns represent random  
997 nucleotides. Each sgID has a one-to-one correspondence with an sgRNA in the viral pool; thus,  
998 the sgID sequence identifies the gene targeted in a given tumor. Note that all sgID sequences in  
999 the viral pool differ from each other by at least three nucleotides such that incorrect sgID  
1000 assignment (and thus, inference of tumor genotype) due to PCR or sequencing error is extremely  
1001 unlikely. The random 20-nucleotide portion of the BC is expected to be unique to each lentiviral  
1002 integration event, and thus tags all cells in a single clonal expansion. Note that the length of the  
1003 barcode ensures a high theoretical potential diversity ( $\sim 4^{20} > 10^{12}$  barcodes per vector), so while  
1004 the actual diversity of each Lenti-sgRNA/Cre vector is dictated by the number of colonies

1005 generated during the plasmid barcoding step, it is very unlikely that we will observe the same BC  
1006 in multiple clonal expansions.

1007 FASTQ files were parsed using regular expressions to identify the sgID and BC for each read. To  
1008 minimize the effects of sequencing error on BC identification, we required the forward and reverse  
1009 reads to agree completely within the 30-nucleotide sequence to be further processed. We also  
1010 screened for barcodes that were likely to have arisen due to errors in sequencing the barcodes of  
1011 genuine tumors. Given the low rate of sequencing error, we expect these spurious “tumors” to have  
1012 read counts that are far lower than the read counts of the genuine tumors from which they arise.  
1013 While it is impossible to eliminate these spurious tumors, we sought to minimize their effect by  
1014 identifying small “tumors” with barcodes that are highly similar to the barcodes of larger tumors.  
1015 Specifically, if a pair of “tumors” had barcodes that were within a Hamming distance of two, and  
1016 if one of the tumors had less than 5% as many reads as the other, then the reads associated with  
1017 the smaller tumor were attributed to the larger tumor.

1018 After these filtering steps, the read counts associated with each barcode were converted to absolute  
1019 neoplastic cell numbers by normalizing to the number of reads in the “spike-in” cell lines added  
1020 to each sample prior to lung lysis and DNA extraction. The median sequencing depth across  
1021 experiments was ~1 read per 6.4 cells.

1022 For statistical comparisons of tumor genotypes, we applied a minimum tumor size cutoff of 100  
1023 cells. In selecting a cutoff, we sought to include tumors that are large enough to be consistently  
1024 detected despite differences in sequencing depth among mice, while using as many tumors as  
1025 possible to maximize the statistical power. Importantly, we analyzed each Tuba-seq dataset with  
1026 multiple minimum tumor size cut-offs (50, 100, 200, 500 cells) and found that our findings were  
1027 robust.

1028

1029 *Summary statistics for overall growth rate*

1030 To assess the extent to which a given gene ( $X$ ) affects tumor growth, we compared the distribution  
1031 of tumor sizes produced by vectors targeting that gene ( $sgX$  tumors) to the distribution produced  
1032 by our negative control vectors ( $sgInert$  tumors). We relied on two statistics to characterize these  
1033 distributions: the size of tumors at defined percentiles of the distribution (specifically the 50<sup>th</sup>, 60<sup>th</sup>,  
1034 70<sup>th</sup>, 80<sup>th</sup>, 90<sup>th</sup>, and 95<sup>th</sup> percentile tumor sizes), and the log-normal mean size (LN mean). The  
1035 percentile sizes are nonparametric summary statistics of the tumor size distribution. In considering  
1036 percentiles corresponding to the right tail of the distribution, we focus on the growth of larger  
1037 tumors, thereby avoiding issues stemming from potential variation in cutting efficiency among  
1038 guides. The LN mean is the maximum-likelihood estimate of mean tumor size assuming a log-  
1039 normal distribution. Previous work found that this statistic represents the best parametric summary  
1040 of tumor growth based on the maximum likelihood quality of fit of various common parametric  
1041 distributions.

1042

1043 To quantify the extent to which each gene suppressed or promoted tumor growth, we normalized  
1044 statistics calculated on tumors of each genotype to the corresponding inert statistic. The resulting  
1045 ratios reflect the growth advantage (or disadvantage) associated with each tumor genotype relative  
1046 to the growth of  $sgInert$  tumors.

1047

1048 For example, the relative  $i^{\text{th}}$  percentile size for tumors of genotype X was calculated as:

1049

1050

$$\left| \text{Relative size at } i^{\text{th}} \text{ percentile}_{sgX} = \frac{i^{\text{th}} \text{ percentile of } sgX \text{ distribution}}{i^{\text{th}} \text{ percentile of } sgInert \text{ distribution}} \right|$$

1051

1052 Likewise, the relative LN mean size for tumors of genotype X was calculated as:

1053

1054 
$$\text{Relative LNmean}_{sgX} = \frac{\text{LNmean of sgX distribution}}{\text{LNmean of sgInert distribution}}$$

1055

1056 *Summary statistics for relative tumor number and relative tumor burden*

1057 In addition to the tumor size metrics described above, we characterized the effects of gene  
1058 inactivation on tumorigenesis in terms of the number of tumors and total neoplastic cell number  
1059 (“tumor burden”) associated with each genotype. Unlike the aforementioned metrics of tumor size,  
1060 tumor number and burden are linearly affected by lentiviral titer and are thus sensitive to  
1061 underlying differences in the representation of each Lenti-sgRNA/Cre vector in the viral pool.  
1062 Critically, each Tuba-seq experiment included a cohort of *KT* control mice. *KT* mice lack  
1063 expression of Cas9, thus all Lenti-sgRNA/Cre vectors are functionally equivalent in these mice,  
1064 and the observed tumor number and burden associated with each sgRNA reflects the make-up of  
1065 the viral pool.

1066 To assess the extent to which a given gene (*X*) affects tumor number, we therefore first normalized  
1067 the number of sgX tumors in *KT;H11<sup>LSL-Cas9</sup>* mice (also *KT;p53<sup>lox/lox</sup>;H11<sup>LSL-Cas9</sup>* and *Braf<sup>LSL-V600E/+</sup>T; H11<sup>LSL-Cas9</sup>* mice in the initial Kras-interacting protein screen and the paired screen,  
1068 respectively) to the number of sgX tumors in the *KT* mice:

1070

1071 
$$\text{Tumor number}_{sgX} = \frac{\text{Number of sgX tumors in } KT;H11^{LSL-Cas9} \text{ mice}}{\text{Number of saX tumors in } KT \text{ mice}}$$

1072

1073 As with the tumor size metrics, we then calculated a relative tumor number by normalizing this  
1074 statistic to the corresponding statistic calculated using sgInert tumors:

1075

$$1076 \quad \left| \text{Relative tumor number}_{sgX} = \frac{\text{Tumor number}_{sgX}}{\text{Tumor number}_{sgInert}} \right.$$

1077

1078 Genes that influence relative tumor number modify the probability of tumor initiation and/or the  
1079 very early stages of oncogene-driven epithelial expansion, which prior work suggests are  
1080 imperfectly correlated with tumor growth at later stages. Relative tumor number thus captures an  
1081 additional and potentially important aspect of tumor suppressor gene function.

1082

1083 Analogous to the calculation of relative tumor number, we characterized the effect of each gene  
1084 on tumor burden by first normalizing the sgX tumor burden in Cas9-expressing mice to the burden  
1085 in KT mice:

1086

$$1087 \quad \left| \text{Tumor burden}_{sgX} = \frac{\text{Total neoplastic cell number for sgX in KT; H11}^{LSL-Cas9} \text{ mice}}{\text{Total neoplastic cell number for sgX in KT mice}} \right.$$

1088

1089 We then calculated a relative tumor burden by normalizing this number to the corresponding  
1090 statistic calculated using sgInert tumors:

1091

$$1092 \quad \left| \text{Relative tumor burden}_{sgX} = \frac{\text{Tumor burden}_{sgX}}{\text{Tumor burden}_{sgInert}} \right.$$

1093



1094 Tumor burden is an integration over tumor size and number, and thus reflects the total neoplastic  
1095 load in each mouse. Tumor burden is thus more strongly related to morbidity than are our metrics  
1096 of tumor size and is closely related to traditional measurements of tumor progression such as  
1097 duration of survival and tumor area. While intuitively appealing, tumor burden is notably noisier  
1098 than our metrics of tumor size as it is strongly determined by the size of the largest tumors.

1099

#### 1100 *Calculation of confidence intervals and P-values for tumor growth and number metrics*

1101 Confidence intervals and P-values were calculated using bootstrap resampling to estimate the  
1102 sampling distribution of each statistic. To account for both mouse-to-mouse variability and  
1103 variability in tumor size and number within mice, we adopted a two-step, nested bootstrap  
1104 approach where we first resampled mice, and then resampled tumors within each mouse in the  
1105 pseudo-dataset. 10,000 bootstrap samples were drawn for all reported P-values. 95% confidence  
1106 intervals were calculated using the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentile of the bootstrapped statistics. Because  
1107 we calculate metrics of tumor growth that are normalized to the same metrics in sgInert tumors,  
1108 under the null model where genotype does not affect tumor growth, the test statistic is equal to 1.  
1109 Two-sided p-values were thus calculated as followed:

$$1110 \overline{p} = 2 * \min \{ \Pr(T > 1), \Pr(T < 1) \}$$

1111 Where T is the test statistic and  $\Pr(T > 1)$  and  $\Pr(T < 1)$  were calculated empirically as the proportion  
1112 of bootstrapped statistics that were more extreme than the baseline of 1. To account for multiple  
1113 hypothesis testing, p-values were FDR-adjusted using the Benjamini-Hochberg procedure as  
1114 implemented in the Python package stats models.

1115

#### 1116 *AP-MS data visualization*

1117 AP-MS data was analyzed as described (Ding et al 2016). Briefly, protein spectral  
1118 matches (PSMs; Kelly et al 2020) were normalized by protein length and total spectral matches  
1119 per experiment. These normalized spectral abundance factors (NSAFs) were then normalized to  
1120 NSAFs of matched prey proteins from a large cohort of unrelated AP/MS experiments to produce  
1121 a Z-score. Z-scores are proportional to the areas of circles in bubble plots. In cluster diagrams,  
1122 NSAFs are binarized by statistical significance ( $FDR > 0.5$ ), similarities between interactome  
1123 profiles are determined by cosine distance, and dendrogram topology is determined by UPGMA.

1124

#### 1125 *Modeling RAS-RAS dimer*

1126 Potential templates for modeling the heterodimers were obtained from the ProtCID database.  
1127 ProtCID is built from clustering interfaces of homologous proteins obtained from domain-domain  
1128 contacts within protein crystals in the Protein Data Bank. Hierarchical clustering of interfaces is  
1129 performed with a Jaccard-index similarity metric based on the contacts shared between different  
1130 interfaces. Models for the structure of the HRAS/KRAS heterodimer were built by superposing a  
1131 structure of KRAS-G12D (PDB: 5USJ) onto a monomer of the HRAS homodimer in PDB entry  
1132 3K8Y.

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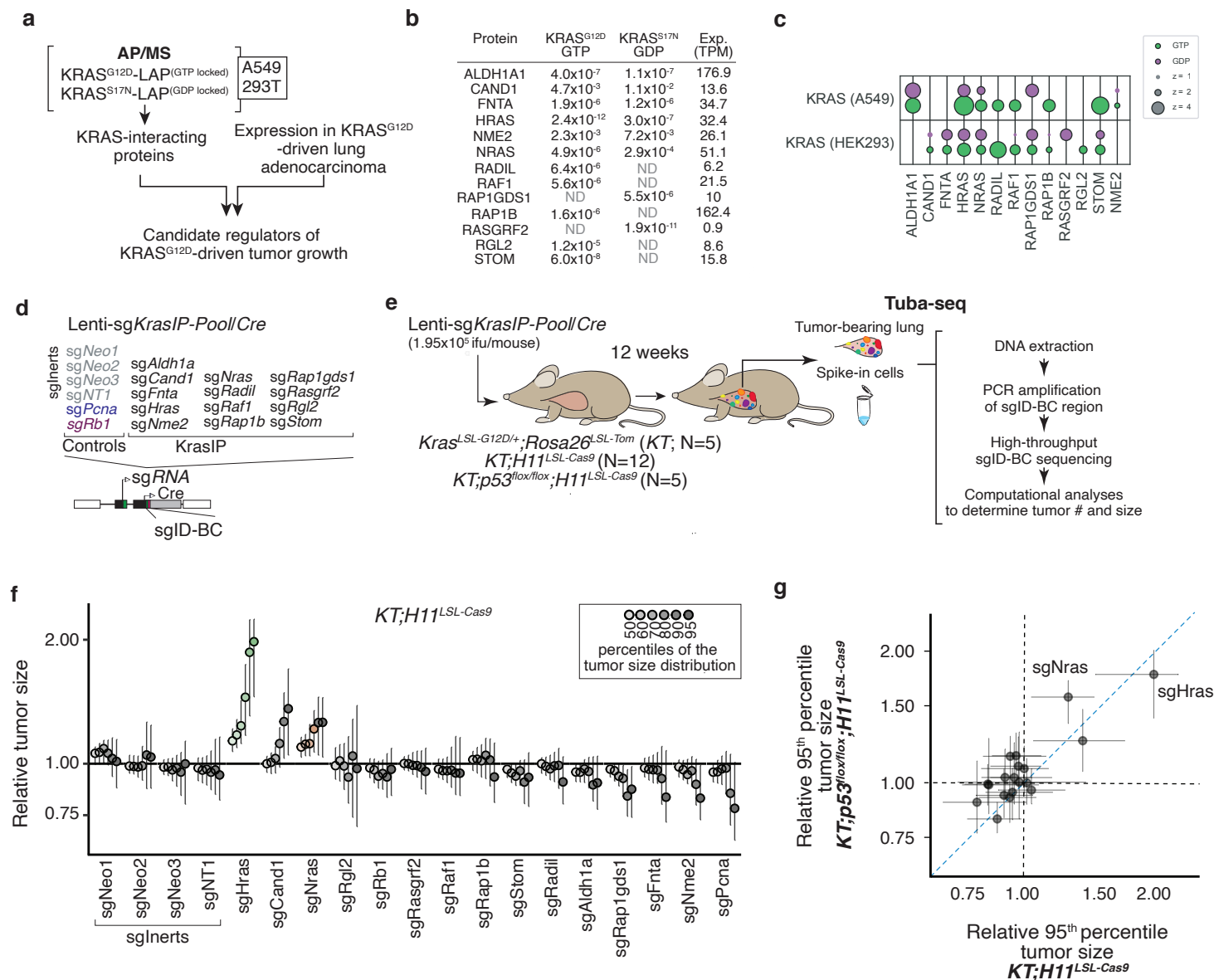
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1293

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**Figure 1. Multiplexed identification of KRAS-interacting proteins that impact KRAS<sup>G12D</sup>-driven lung cancer growth *in vivo*.**

**a.** Candidate mediators of KRAS-driven lung tumor growth were identified on the basis of their interactions with GTP- and GDP-locked Kras in multiple AP/MS-based protein-protein interaction screens and their expression in a mouse model of Kras-driven lung adenocarcinoma.

**b.** Selected KRAS-interacting proteins interact with either GTP- or GDP-locked KRAS (shown as NSAF in A549 cells) and their homolog is expressed in KRAS<sup>G12D</sup>-driven lung cancer (shown as TPM).

**c.** Bubble plot of two AP/MS experiments with GTP- and GDP-locked mutant GTPases as baits (rows), showing the enrichment of selected candidate KRAS-interacting proteins (columns). Dark borders indicate FDR < 0.05.

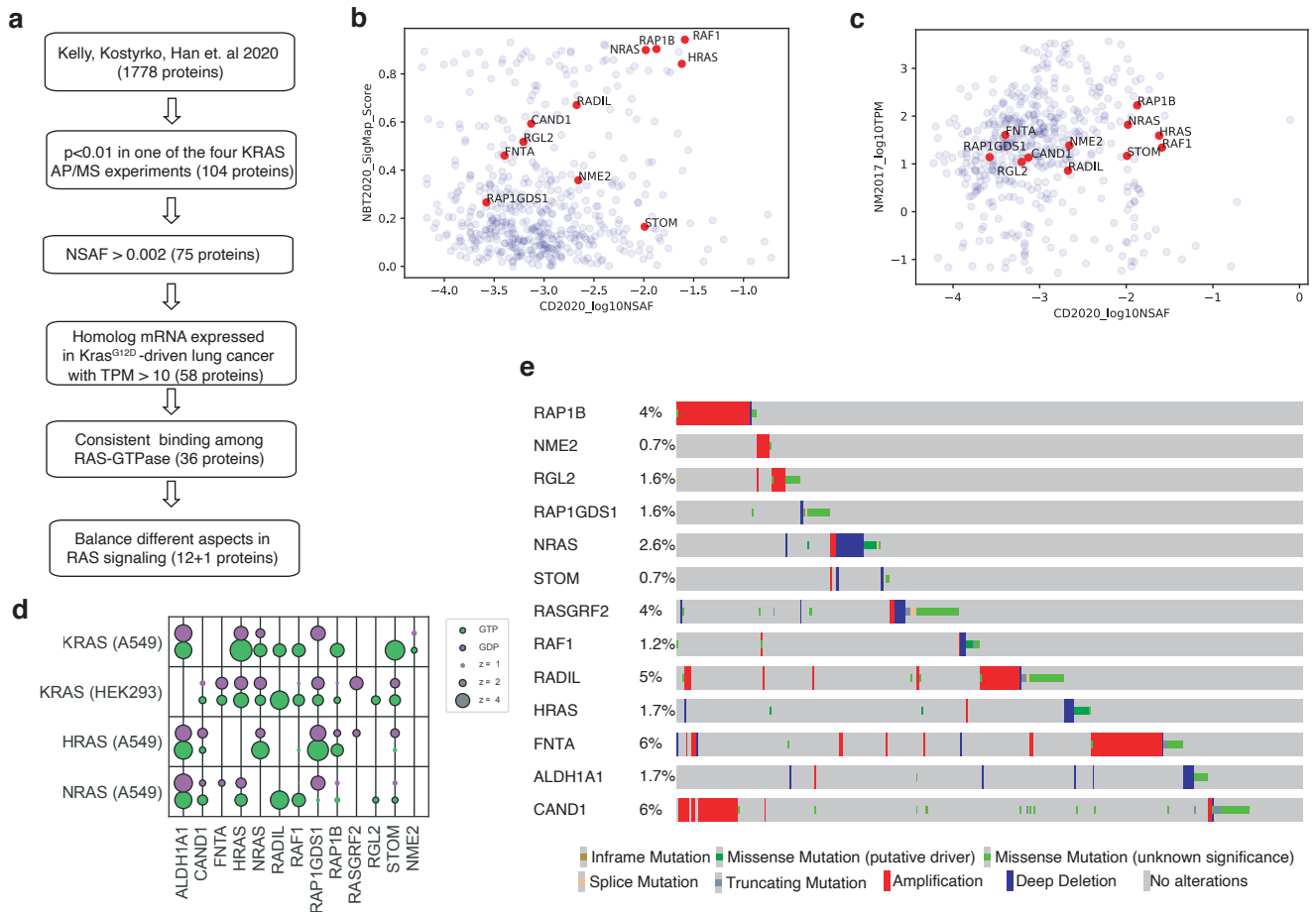
**d.** Schematic of tumor initiation with a pool of barcoded Lenti-sgRNA/Cre vectors (Lenti-sgKrasIP-Pool/Cre). The lentiviral pool includes four Inert sgRNAs that are either non-targeting (NT) or target a functionally inert locus (Neo1-3, targeting NeoR in the *R26<sup>SL-IdTomato</sup>* allele). Each barcoded lentiviral vector contains an sgRNA, Cre, and a two-component barcode composed of an sgRNA identifier (sgID) and a random barcode (BC). This design allows inactivation of multiple target genes in parallel followed by quantification of the resulting tumor size distributions through high-throughput sgID-BC sequencing.

**e.** Tumors were initiated in cohorts of *KT*, *KT;H11<sup>LSL-Cas9</sup>* and *KT;p53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>* mice through intratracheal delivery of Lenti-sgKrasIP-Pool/Cre. Tuba-seq was performed on each tumor-bearing lung 12 weeks after initiation, followed by analyses of sgID-BC sequencing data to characterize the effects of inactivating each gene.

**f.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sglNert-containing tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9</sup>* mice. Genes are ordered by 95<sup>th</sup> percentile tumor size, with sglNerts on the left. sglNerts are in gray, and the line at  $y=1$  indicates no effect relative to sglNert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sglNert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**g.** Comparison of 95<sup>th</sup> percentile tumor size for each sgRNA relative to the size the 95<sup>th</sup> percentile tumor size of sglNert-containing tumors in *KT;H11<sup>LSL-Cas9</sup>* mice versus *KT;p53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>* mice. Error bars indicate 95% confidence intervals calculated by bootstrap resampling.





**Supplemental Figure 1. Prioritize candidate KRAS-interacting proteins for this study.**

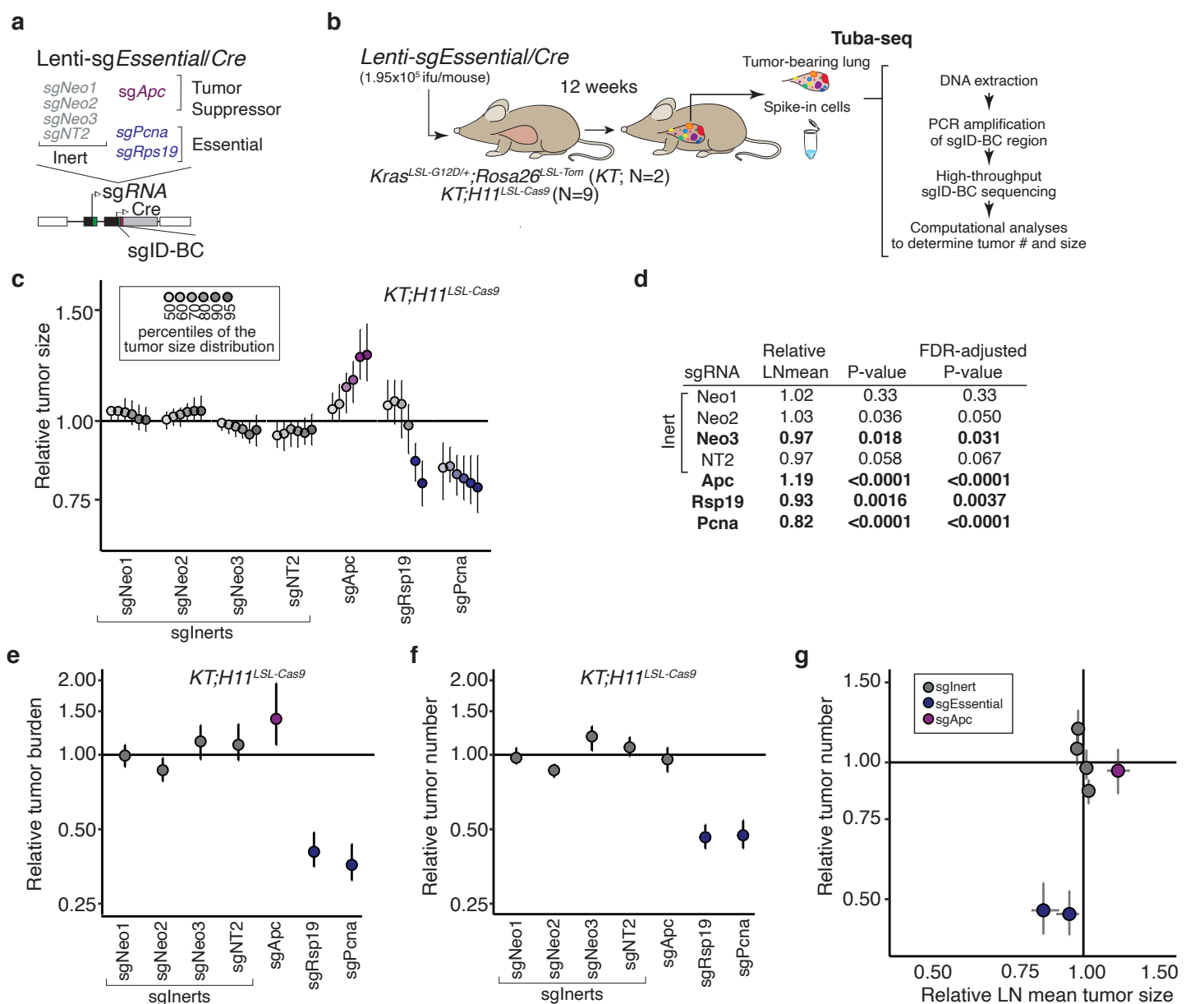
**a.** Flow chart for prioritizing candidate KRAS-interacting proteins for this study. Candidate KRAS-interacting proteins were chosen based on multiple criteria including their interaction with KRAS, their homolog mRNA expression in *Kras*<sup>G12D</sup>-driven lung cancer in mouse model, and the consistency for them to bind different RAS-GTPase. RADIL is added at the last step due to its validated importance in KRAS-mutant human cell lines.

**b.** Candidate proteins interact with KRAS from two protein-protein interaction analyses (Kelly, Kostyrko, Han et al. 2020; Brody, Simpson, Murray et al. 2020). Shared KRAS-interaction proteins are shown as their log<sub>10</sub>NSAF and SigMap Score.

**c.** Homolog mRNA expression (TPM) of candidate KRAS-interacting proteins in *Kras*<sup>G12D</sup>-driven lung cancer in mouse model (Chuang et al. 2017).

**d.** Bubble plot of eight AP/MS experiments with GTP- and GDP-locked mutant GTPases as baits (rows), showing the enrichment of selected candidate KRAS-interacting proteins (columns). Dark borders indicate FDR < 0.05.

**e.** Mutation frequencies of these 13 candidate genes in lung adenocarcinoma (data from TCGA, Nat. Genet. 2016).



**Supplemental Figure 2. Tumor barcoding coupled with barcode sequencing (Tuba-seq) can uncover engineered alterations that reduce tumor number and growth.**

**a-b.** Schematic of the Tuba-seq approach to measure the effects of essential gene inactivation on tumor growth. Lentiviral-sgRNA/Cre vectors with inert sgRNAs (gray) or sgRNAs targeting known essential genes (navy) were diversified with a two component sgID-BC. A vector targeting known tumor suppressor *Apc* was included as a positive control (**a**). Tumors were initiated with this barcoded Lenti-sgEssential/Cre pool in KT and *KT;H11<sup>LSL-Cas9</sup>* mice. Tuba-seq was performed on each tumor-bearing lung 12 weeks after initiation, followed by analyses of sgID-BC sequencing data to characterize the effects of inactivating each gene (**b**).

**c.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sglInert-containing tumors at the corresponding percentiles. Line at  $y=1$  indicates no effect relative to sglInert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sglInert (two-sided FDR-corrected  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

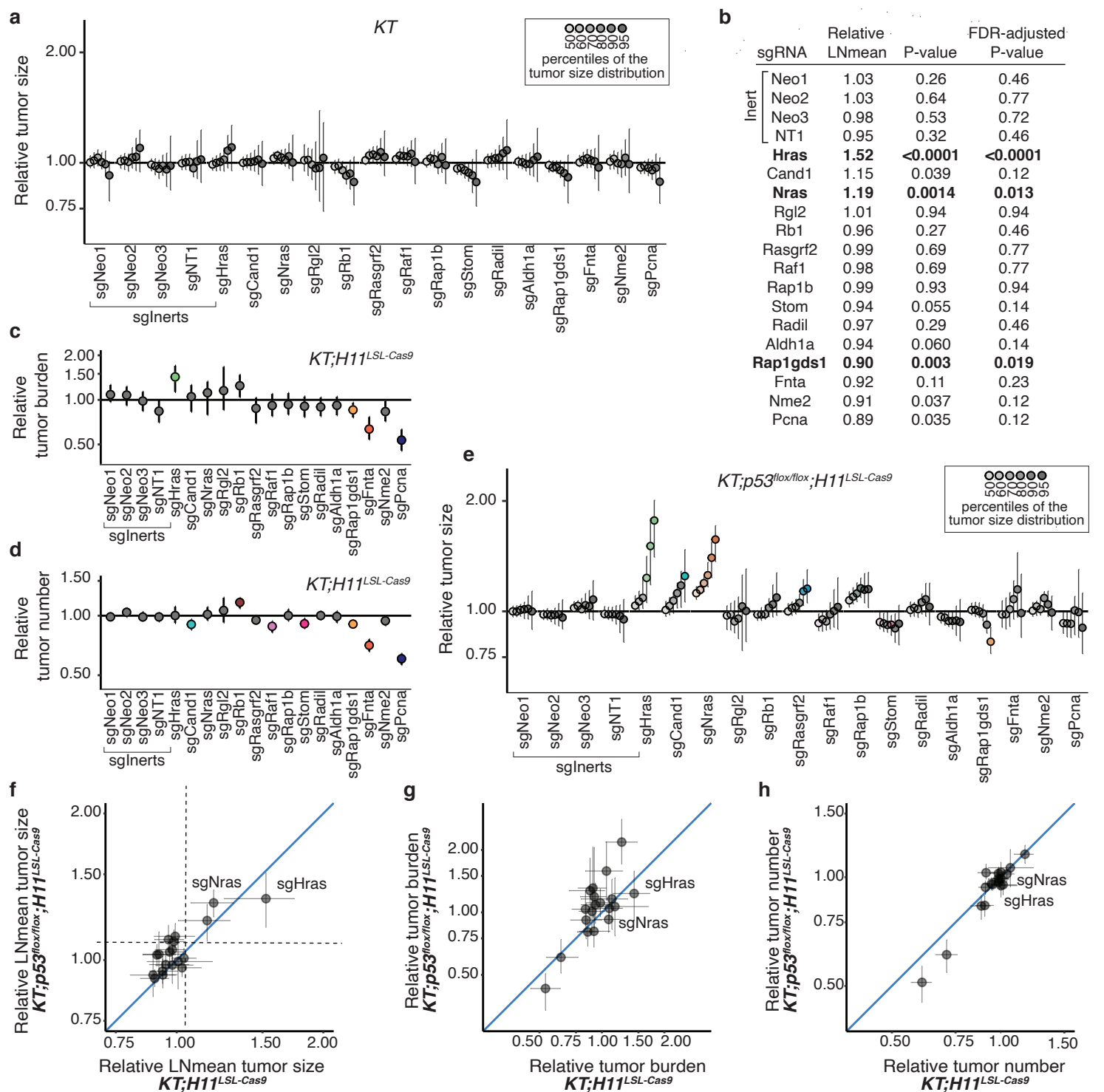
**d.** The impact of each sgRNA on mean tumor size relative to sglInerts, assuming a log-normal distribution of tumor sizes (LNmean). sgRNAs with two-sided  $P < 0.05$  after FDR-adjustment are in bold.

**e.** The impact of each sgRNA on tumor burden (number of neoplastic cells aggregated across all tumors of a genotype) relative to sglInerts and normalized to the same statistic in *KT* mice to account for representation of each sgRNA in the viral pool. *sgInerts* are in gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative burdens significantly different from sglInert (two-sided FDR-corrected  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**f.** The impact of each sgRNA on tumor number relative to sglInerts and normalized to the same statistic in *KT* mice to account for representation of each sgRNA in the viral pool. *sgInerts* are in gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor numbers significantly different from sglInert (two-sided FDR-corrected  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**g.** The impact of each sgRNA on tumor number plotted against its impact on LNmean tumor size. The lines at  $y=1$  and  $x=1$  indicate no effect relative to sglInert on tumor number and size, respectively. *sgRsp19* and *sgPcna* cluster in the lower left quadrant near  $x=1$ , indicating that targeting essential genes strongly reduces tumor number but only moderately decreases average tumor size. Error bars indicate 95% confidence intervals calculated by bootstrap resampling.

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**Supplemental Figure 3. Inactivation of KRAS-interacting proteins has similar impacts on tumor growth in p53-proficient and p53-deficient contexts.**

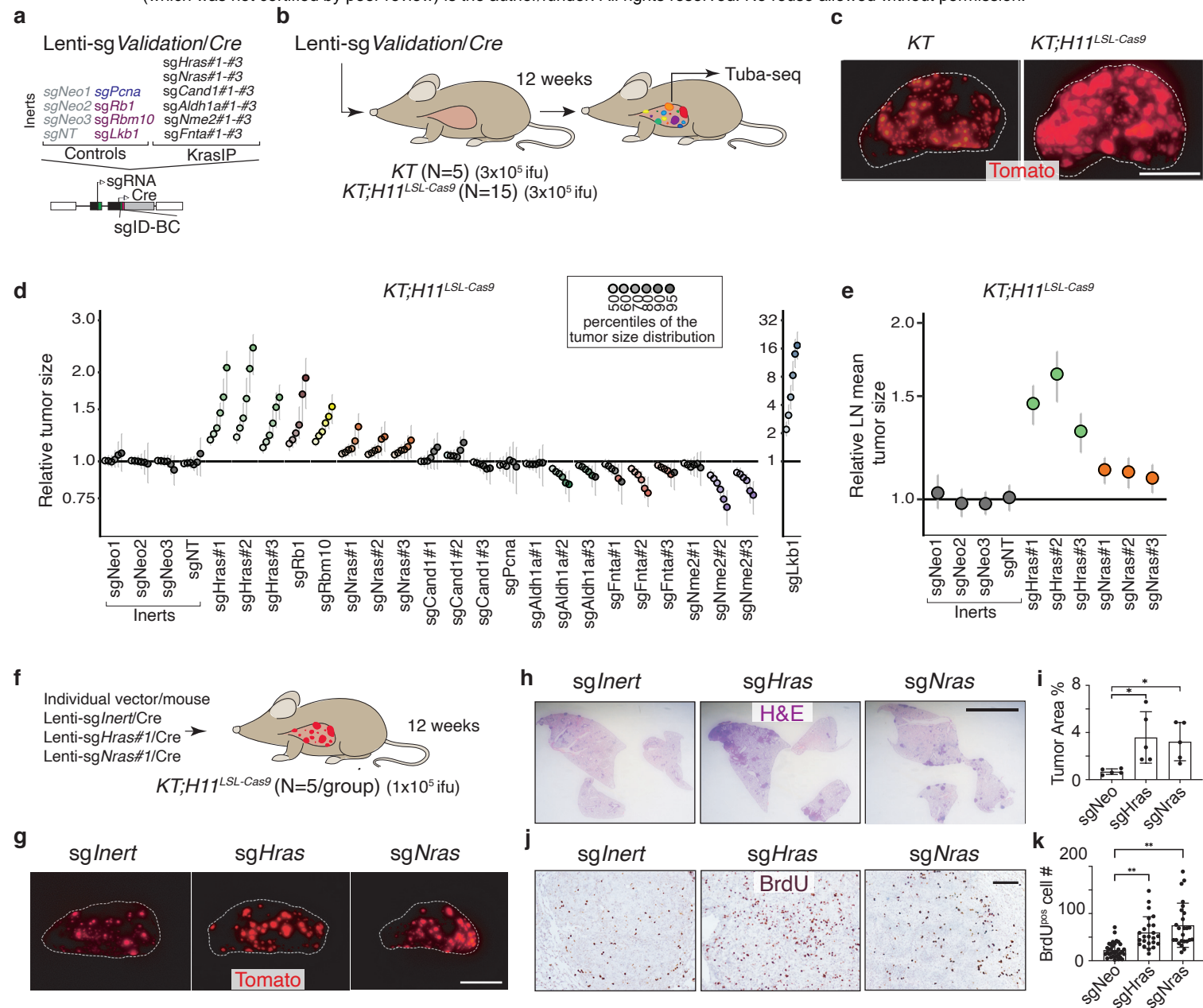
**a.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sglntert-containing tumors at the corresponding percentiles in *KT* mice. *KT* mice lack Cas9, thus all sgRNAs are functionally equivalent to sglnterts. Genes are ordered as in Figure 1f. Line at  $y=1$  indicates no effect relative to sglntert. Error bars indicate 95% confidence intervals. Confidence intervals and P-values were calculated by bootstrap resampling. As expected, no percentiles were significantly different from sglntert (two-sided FDR-adjusted  $p < 0.05$ ).

**b.** The impact of each sgRNA on mean tumor size relative to sglnterts in *KT;H11<sup>LSL-Cas9</sup>*, assuming a log-normal distribution of tumor sizes (LNmean). sgRNAs with two-sided  $P < 0.05$  after FDR-adjustment are in bold. P-values were calculated by bootstrap resampling.

**c-d.** The impact of each sgRNA on tumor burden (**c**) and number (**d**) relative to sglnterts in *KT;H11<sup>LSL-Cas9</sup>* mice, normalized to the corresponding statistic in *KT* mice to account for representation of each sgRNA in the viral pool. sglnterts are in gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor burdens and numbers significantly different from sglntert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**e.** Tumor sizes at the indicated percentiles for each sgRNA relative to the size of sglntert-containing tumors in *KT;p53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>* mice. Genes are ordered as in Figure 1f. Dashed line indicates no effect relative to sglntert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sglntert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values calculated by bootstrap resampling.

**f-h.** Comparison of the impact of each sgRNA on relative LNmean tumor size (**f**), tumor burden (**g**) and tumor number (**h**) in *KT;H11<sup>LSL-Cas9</sup>* and *KT;p53<sup>fllox/fllox</sup>;H11<sup>LSL-Cas9</sup>* mice. Error bars indicate 95% confidence intervals calculated by bootstrap resampling.



**Figure 2. HRAS and NRAS are potent suppressors of KRAS<sup>G12D</sup>-driven lung cancer growth *in vivo***

**a,b.** A pool of barcoded Lenti-sgRNA/Cre vectors (Lenti-sgValidation/Cre) targeting candidate mediators of KRAS-driven lung tumor growth identified in the initial KRAS-interacting protein Tuba-seq screen was used to initiate tumors in validation cohorts of KT and KT;H11<sup>LSL-Cas9</sup> mice. This lentiviral pool includes four Inert sgRNAs, as well as sgRNAs targeting *Lkb1*, *Rb1*, and *Rbm10* as tumor suppressor controls. Each candidate gene from the initial screen is targeted with three sgRNAs. Tumors were initiated through intratracheal delivery of Lenti-sgValidation/Cre, and Tuba-seq was performed on each tumor-bearing lung 12 weeks after initiation, followed by analyses of sgID-BC sequencing data to characterize the effects of inactivating each gene (**b**).

**c.** Fluorescence images of representative lung lobes 12 weeks after tumor initiation. Scale bars = 5 mm. Lung lobes are outlined with a white dashed line.

**d.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing tumors at the corresponding percentiles in KT;H11<sup>LSL-Cas9</sup> mice. Genes are ordered by 95<sup>th</sup> percentile tumor size, with sgInerts on the left. Note that sgLkb1 is plotted on a separate scale to facilitate visualization of sgRNAs with lesser magnitudes of effect. Dashed line indicates no effect relative to sgInert. Error bars indicate 95% confidence intervals. 95% confidence intervals and P-values were calculated by bootstrap resampling. Percentiles that are significantly different from sgInert (2-sided FDR-adjusted  $p < 0.05$ ) are in color.

**e.** Targeting *Hras* and *Nras* significantly increases mean tumor size relative to sgInerts, assuming a log-normal distribution of tumor sizes (LNmean). Error bars indicate 95% confidence intervals calculated by bootstrap resampling.

**f.** Schematic of tumor initiation with individual Lenti-sgRNA/Cre vectors. Mouse number and titer of the lentiviral vectors are indicated.

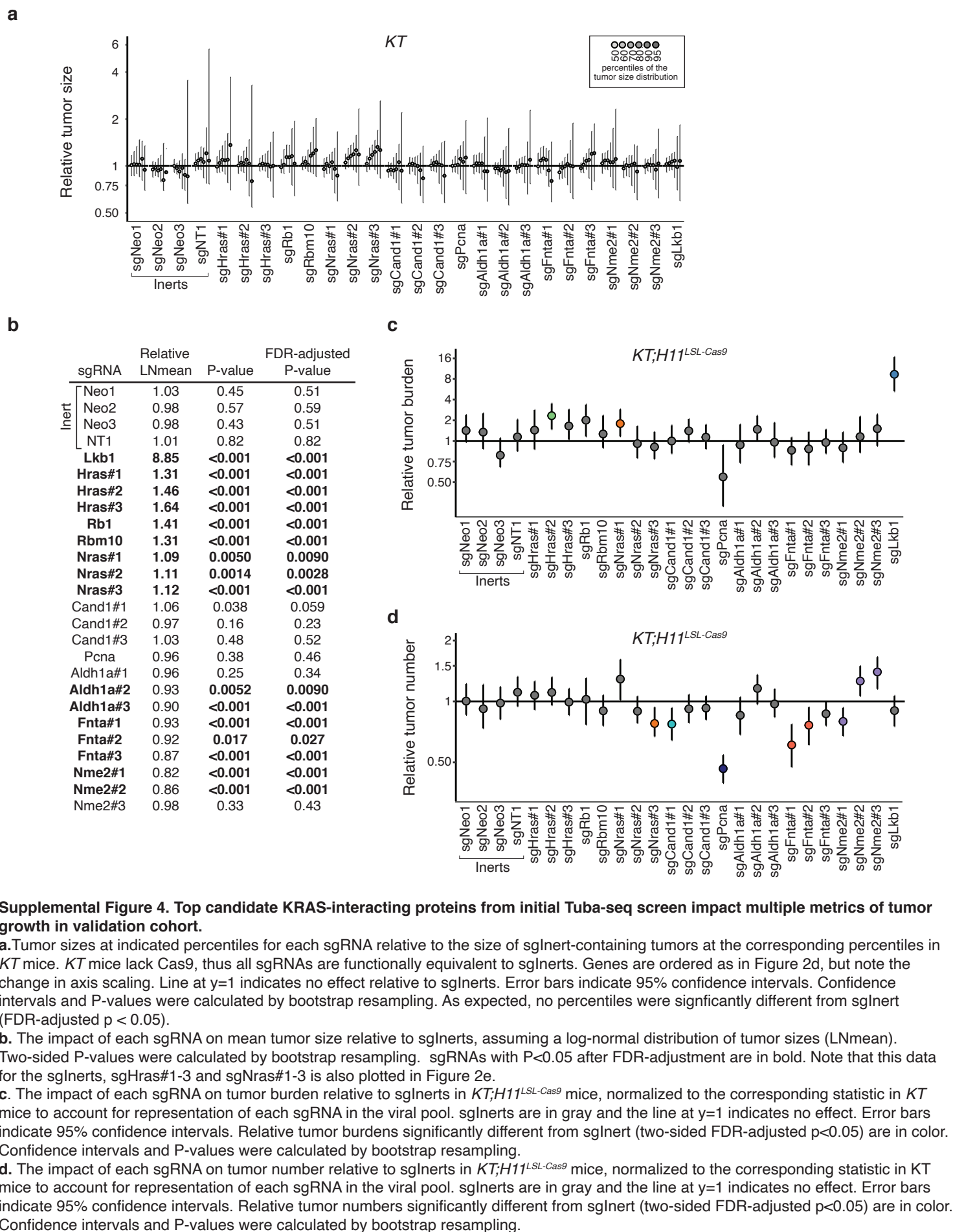
**g.** Representative fluorescence images of lungs from KT;H11<sup>LSL-Cas9</sup> mice after tumor initiation with Lenti-sgRNA/Cre vectors as indicated. Scale bar = 5 mm.

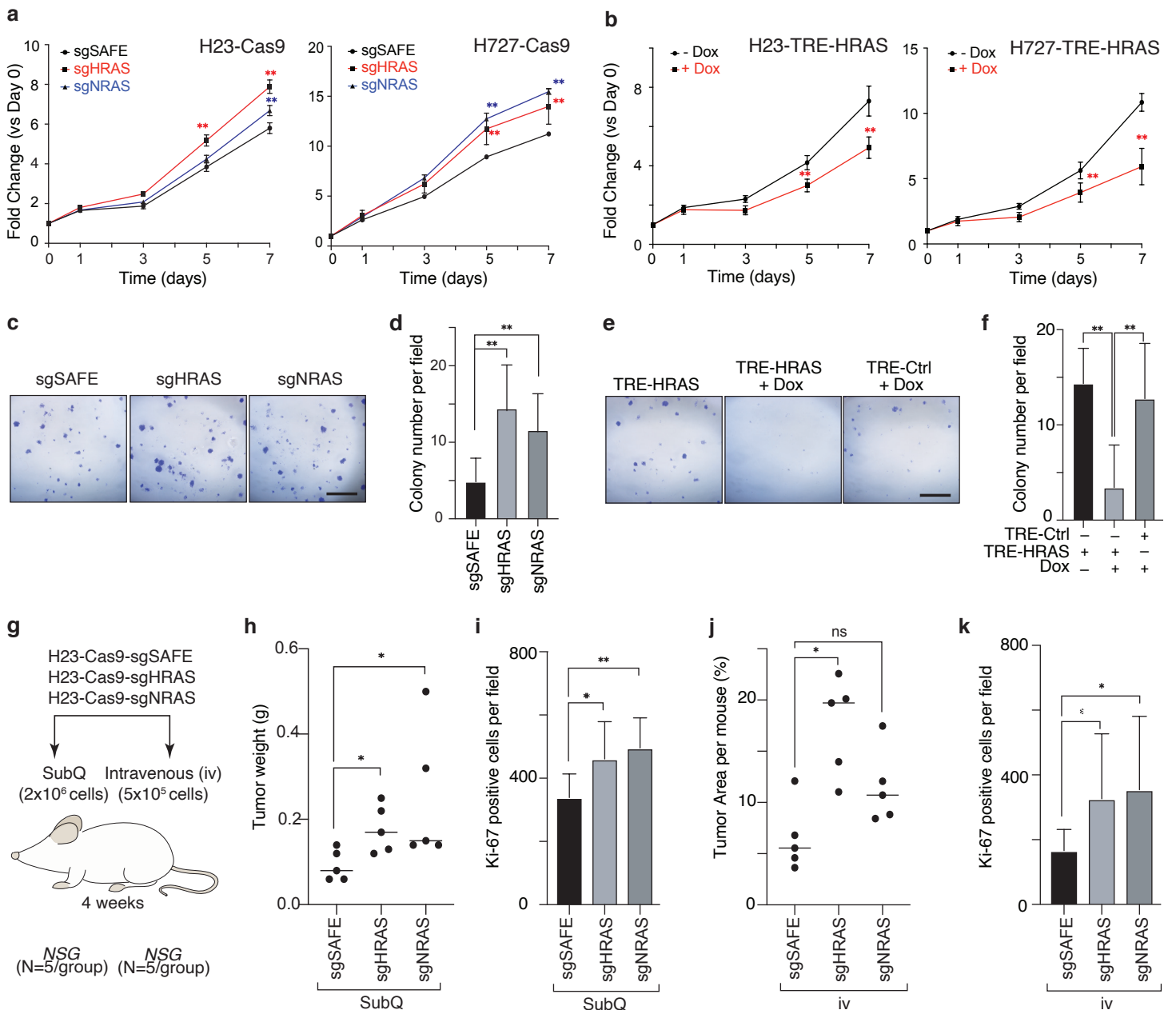
**h.** Representative H&E images of lungs from KT;H11<sup>LSL-Cas9</sup> mice after tumor initiation with Lenti-sgRNA/Cre vectors as indicated. Tumor area (percentage of total lung area) from each mouse is shown as Mean  $\pm$  SD. \*:  $p < 0.05$ ; Scale bar = 5 mm.

**i.** Tumor burden in KTC mice with tumors initiated with Lenti-sgRNA/Cre vectors as indicated. Each dot represents relative tumor area (percentage of total lung area) from one mouse. \*:  $p < 0.05$

**j.** Representative BrdU staining images of lungs from KT;H11<sup>LSL-Cas9</sup> mice after tumor initiation with Lenti-sgRNA/Cre vectors as indicated. Number of BrdU<sup>+</sup> cells per field is shown as Mean  $\pm$  SD. \*\*:  $p < 0.01$ ; Scale bar = 100  $\mu$ m.

**k.** Quantification of proliferation cells in KTC mice with tumors initiated with Lenti-sgRNA/Cre vectors as indicated. Each dot represents a tumor. \*\*:  $p < 0.01$





**Figure 3. Wildtype HRAS or NRAS constrain the growth of human KRAS-driven cancer cell lines.**

**a.** Inactivation of wild type HRAS or NRAS increases growth of KRAS-mutant H23 (G12C) and H727 (G12V) cells. Wildtype (sgSAFE) or HRAS- or NRAS-knockout cells were seeded in 96 well plates and cultured under limited serum (1%). Cell numbers were measured via CCK8 assay. Points are Mean±SD of 12 wells normalized to Day 0. \*\*: p<0.01

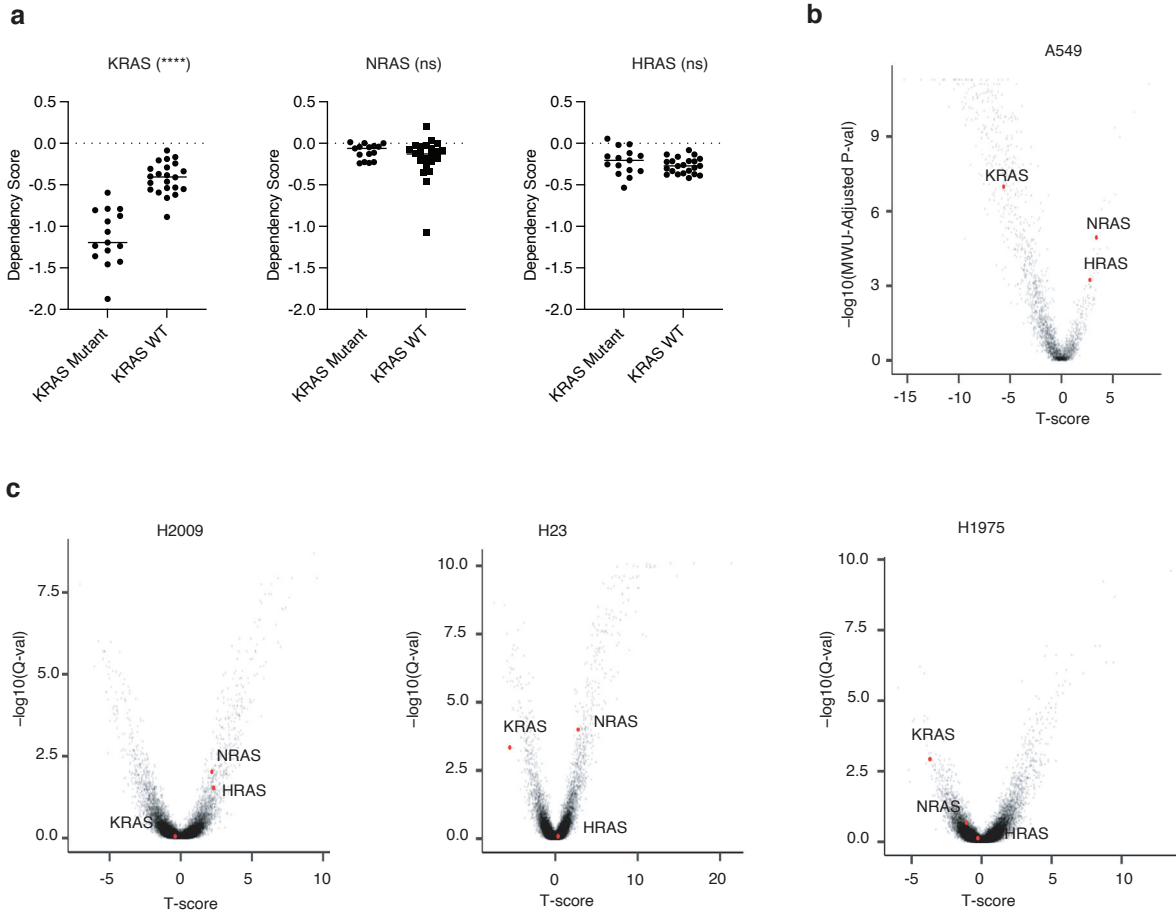
**b.** Re-expression of wild type HRAS suppresses proliferation of HRAS-null H23 and H727 cells. TRE-HRAS cells were seeded in 96 well plates and cultured under limited serum (1%) with or without 50 ng/ml Doxycycline (Dox) and cell numbers were measured via CCK8 assay. Points are Mean±SD of 12 wells normalized to Day 0. \*\*: p<0.01

**c-d.** Inactivation of HRAS or NRAS increases H23 colony formation. Wildtype (sgSAFE), HRAS-knockout (sgHRAS), or NRAS-knockout (sgNRAS) H23 cells were seeded at 1000 cells/well in 6-well plates and grown for two weeks. Cells were stained with crystal violet. **c.** Representative images. Scale bar = 5mm. **d.** Mean±SD of colony number of 12 fields. \*\*: p<0.01

**e-f.** Re-expression of wild type HRAS suppresses HRAS-null H23 cell colony formation. TRE-Ctrl or TRE-HRAS H23 cells were seeded at 1000 cells/well in 6-well plates and grown with or without 50 ng/ml Doxycycline (Dox) for two weeks. Cells were stained with crystal violet. **e.** Representative images. Scale bar = 5mm. **f.** Mean±SD of colony number of 12 fields. \*\*: p<0.01

**g-k.** Inactivation of wild type HRAS or NRAS increases H23 cell growth after transplantation. **g.** Schematic of tumor initiation with subcutaneous (SubQ) or intravenous (IV) transplantation of H23 cells with inactivation of HRAS or NRAS in NSG mice. Mouse number, cell number, and tumor growth time after transplantation are indicated. **h.** Tumor weight from SubQ transplantation of indicated cells. Each dot represents a mouse. Mean value was shown. **i.** Ki67<sup>pos</sup> cell number in tumor section from SubQ transplantation of indicated cells was shown as Mean±SD value of 20 view fields. **j.** Tumor area (percentage of h-mitochondria<sup>pos</sup> area) from IV transplantation of indicated cells. Each dot represents a tumor. Mean value was shown. **k.** Ki67<sup>pos</sup> cell number in tumor section from IV transplantation of indicated cells is shown as Mean±SD value of 20 view fields (200x). \*: p<0.05; \*\*: p<0.01; ns: not significant.

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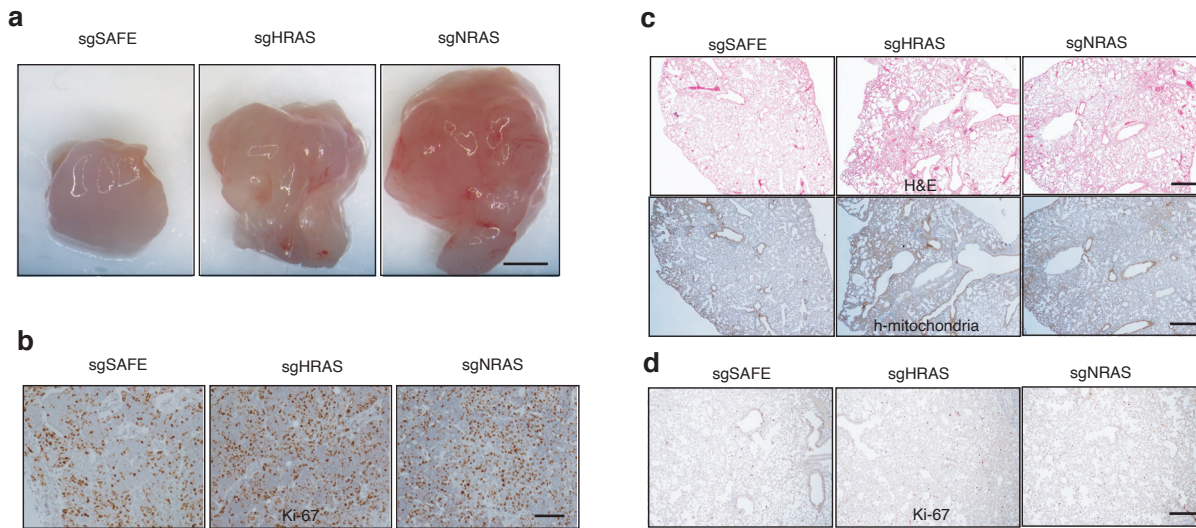
**Supplemental Figure 5. Dependency of human LUAD cell lines on RAS family members.**

**a.** Comparison of RAS family member dependency scores between KRAS mutant and KRAS wildtype human LUAD cell lines. \*\*\*\* ( $p < 0.0001$ ), ns (not-significant). (Data source: DepMap)

**b.** Volcano plot showing the effects of RAS gene knockouts in A549 cells. The T-score represents the normalized effect of multiple sgRNAs targeting a gene. A positive T-score indicates a tumor suppressive effect. The effects of each gene relative to SAFE sgRNAs were tested via Mann–Whitney U (MWU) test, corrected via Benjamini-Hochberg procedure and shown as  $-\log_{10}(\text{MWU-Adjusted P-val})$ . (Data source: Marcus Robert Kelly, Kaja Kostyrko, Kyuho Han, et al. 2020)

**c.** Volcano plot showing effects of RAS gene knockouts in KRAS-mutant human LUAD cells (left: H2009, mid: H23, right: H1975) in 3D culture. The T-score represents the normalized effect of multiple sgRNAs targeting a gene. A positive T-score indicates a tumor suppressive effect. The effects of each gene relative to SAFE sgRNAs were tested via two-side t-test, corrected via Benjamini-Hochberg procedure and shown as  $-\log_{10}(\text{Q-val})$ . (Data source: Kyuho Han, et al. 2020)

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**Supplemental Figure 6. Inactivation of wild type HRAS or NRAS increases H23 cell growth after transplantation.**

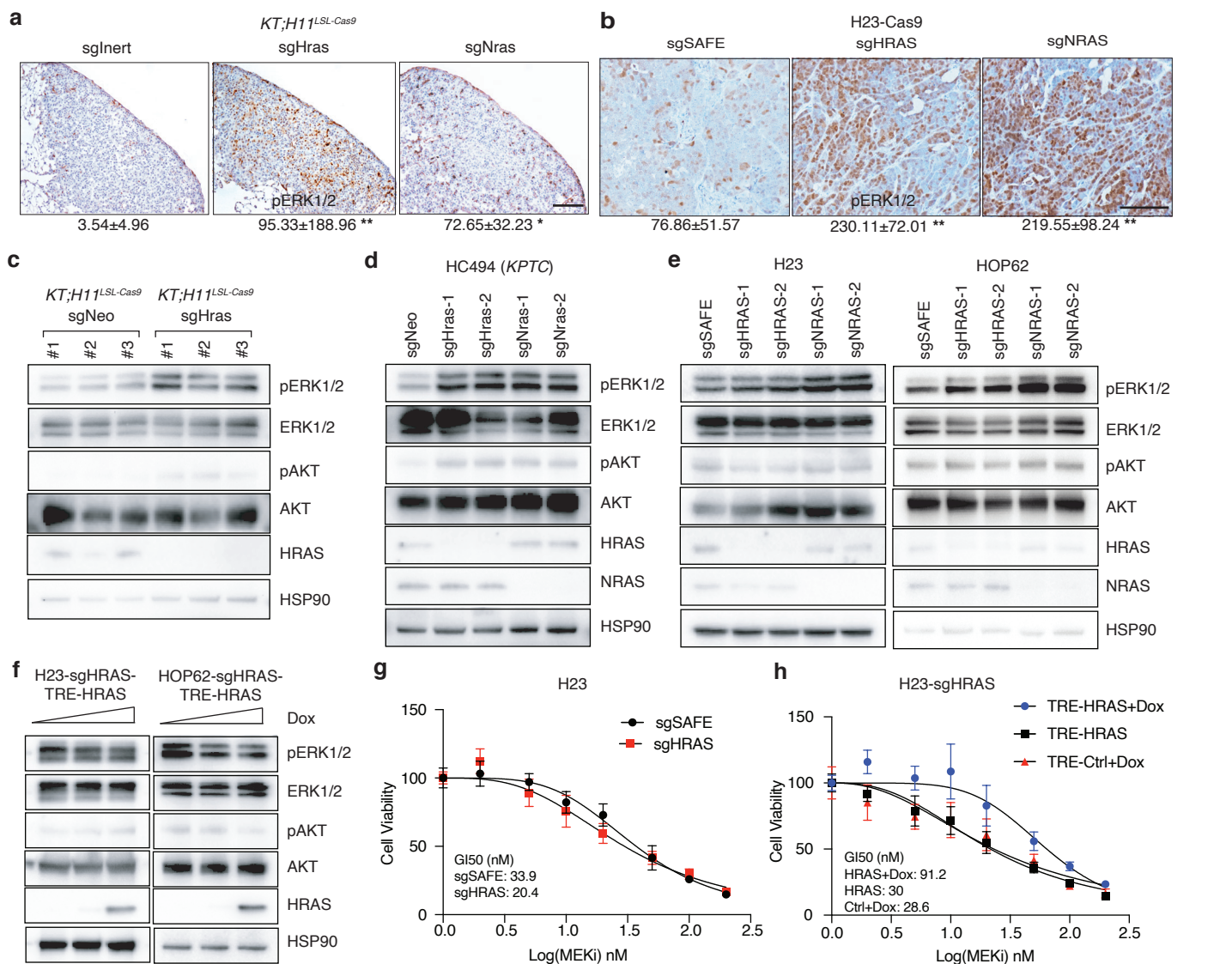
**a.** Representative image of subcutaneous tumor size four weeks after transplantation with H23 cells as indicated. Quantification was shown in **Figure 3h**. Scale bar: 2 mm

**b.** Representative image of Ki67 staining from subcutaneous tumor four weeks after transplantation with H23 cells as indicated. Quantification was shown in **Figure 3i**. Scale bar: 100  $\mu$ m

**c.** Representative image of HE (upper) and human mitochondria (lower) staining from lung tumor four weeks after intravenous transplantation with H23 cells as indicated. Quantification was shown in **Figure 3j**. Scale bar: 500  $\mu$ m

**d.** Representative image of Ki67 staining from lung tumor four weeks after intravenous transplantation with H23 cells as indicated. Quantification was shown in **Figure 3k**. Scale bar: 200  $\mu$ m





#### Figure 4. Wildtype RAS paralogs suppress RAS signaling

**a.** Representative image of pERK staining in KTC mice with tumors initiated with Lenti-sgRNA/Cre vectors as indicated. Quantification of pERK<sup>pos</sup> cells per tumor was shown as Mean±SD of 20 tumors. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; Scale bar: 100  $\mu$ m

**b.** Representative image of pERK staining in subcutaneous tumor transplanted with H23 cells as indicated. Quantification of pERK<sup>pos</sup> cells per field was shown as Mean±SD of 20 fields. \*\*:  $p < 0.01$ ; Scale bar: 100  $\mu$ m. HSP90 shows loading.

**c.** Western blot analysis of sorted cancer cells from *KT;H11<sup>LSL-Cas9</sup>* mice transduced with Lenti-sgRNA/Cre vectors as indicated. Multiple tumors were pooled and Tomato-positive cancer cells were sorted prior to and protein extraction. HSP90 shows loading.

**d.** Western blot analysis of murine lung adenocarcinoma cell line that was transduced with Lenti-sgRNA vectors as indicated and selected with puromycin to generate stable knockout cell lines. Wildtype cells (sgNeo) or HRAS- or NRAS-knockout cells (sgHras, sgNras) were cultured under limited serum (1%) for 2 days before protein extraction. HSP90 shows loading.

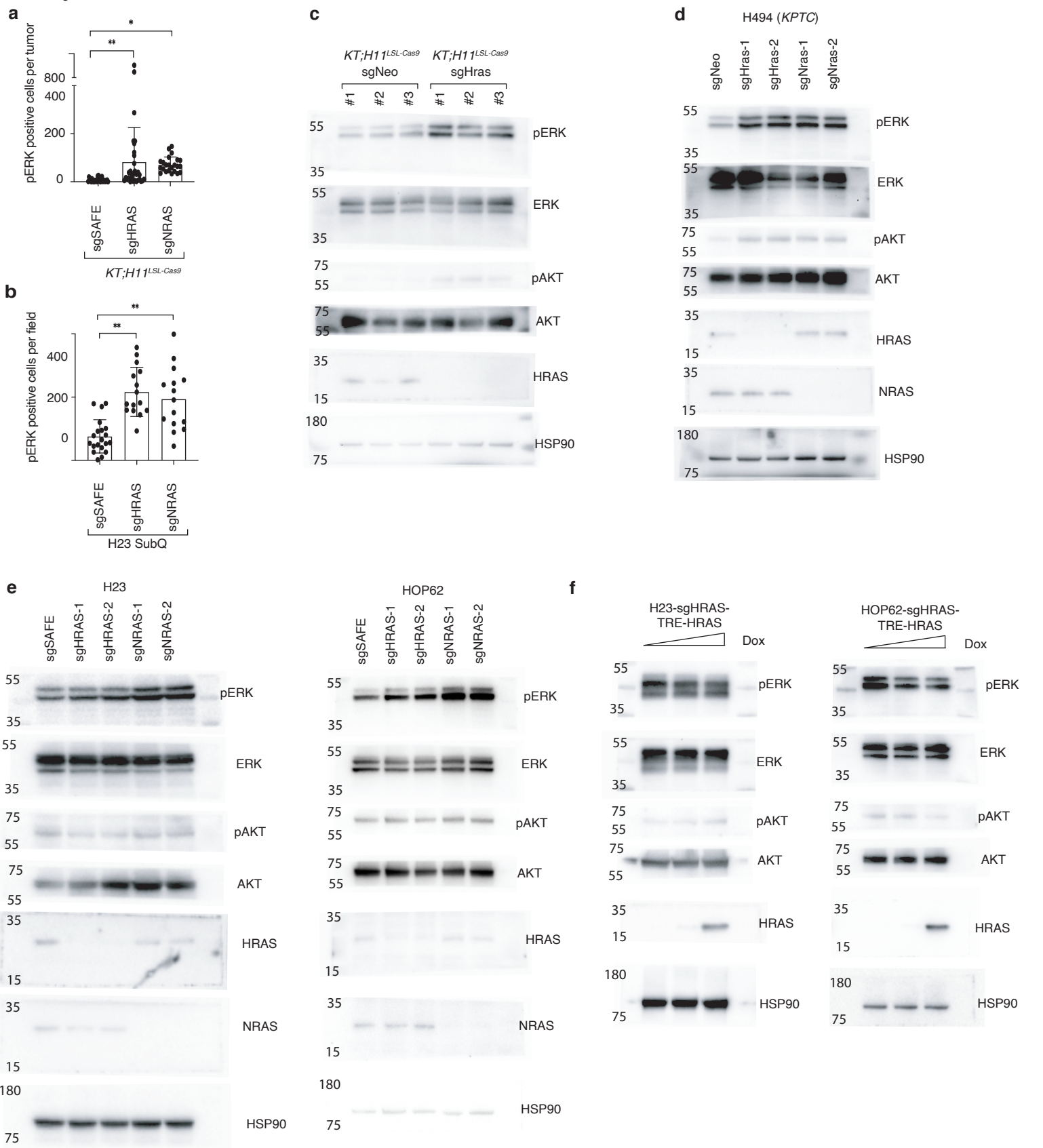
**e.** Western blot analysis of cultured human lung adenocarcinoma cell lines transduced with Lenti-sgRNA vectors as indicated and selected with puromycin to generate stable knockout cell lines. Wildtype cell (sgSAFE) or HRAS- or NRAS-knockout cells (sgHRAS, sgNRAS) were cultured under limited serum (1%) for 2 days before protein extraction. HSP90 shows loading.

**f.** Western blot analysis of human lung adenocarcinoma cell lines re-expression HRAS (TRE-HRAS) under Doxycycline (Dox) treatment. HRAS-null cells were generated as described in Figure 3a. HRAS-null cells were re-transduced with lentiviral vector expressing TRE-HRAS at high MOI (>5) to generate stable HRAS re-expression cells (sgHRAS-TRE-HRAS). To re-express HRAS, cells were treated with 0, 1, or 2ng/ml Dox and cultured under limited serum (1%) for 2 days before protein extraction. HSP90 shows loading.

**g.** Comparison of GI50 values to MEK inhibitors trametinib among wildtype and HRAS-null H23 cells under treatment of indicated dose of trametinib for four days. Cell number were measured via CCK8 assay and normalized to cells treated with vehicle. Each data point was shown as Mean±SD of 12 wells.

**h.** Comparison of GI50 values to MEK inhibitors trametinib among HRAS-null H23 cells (H23-sgHRAS) re-expressing HRAS in presence (HRAS+Dox) or absence (HRAS) of Doxycycline plus indicated dose of trametinib for four days. Cell number were measured via CCK8 assay and normalized to cells treated with vehicle. Each data point was shown as Mean±SD of 12 wells.

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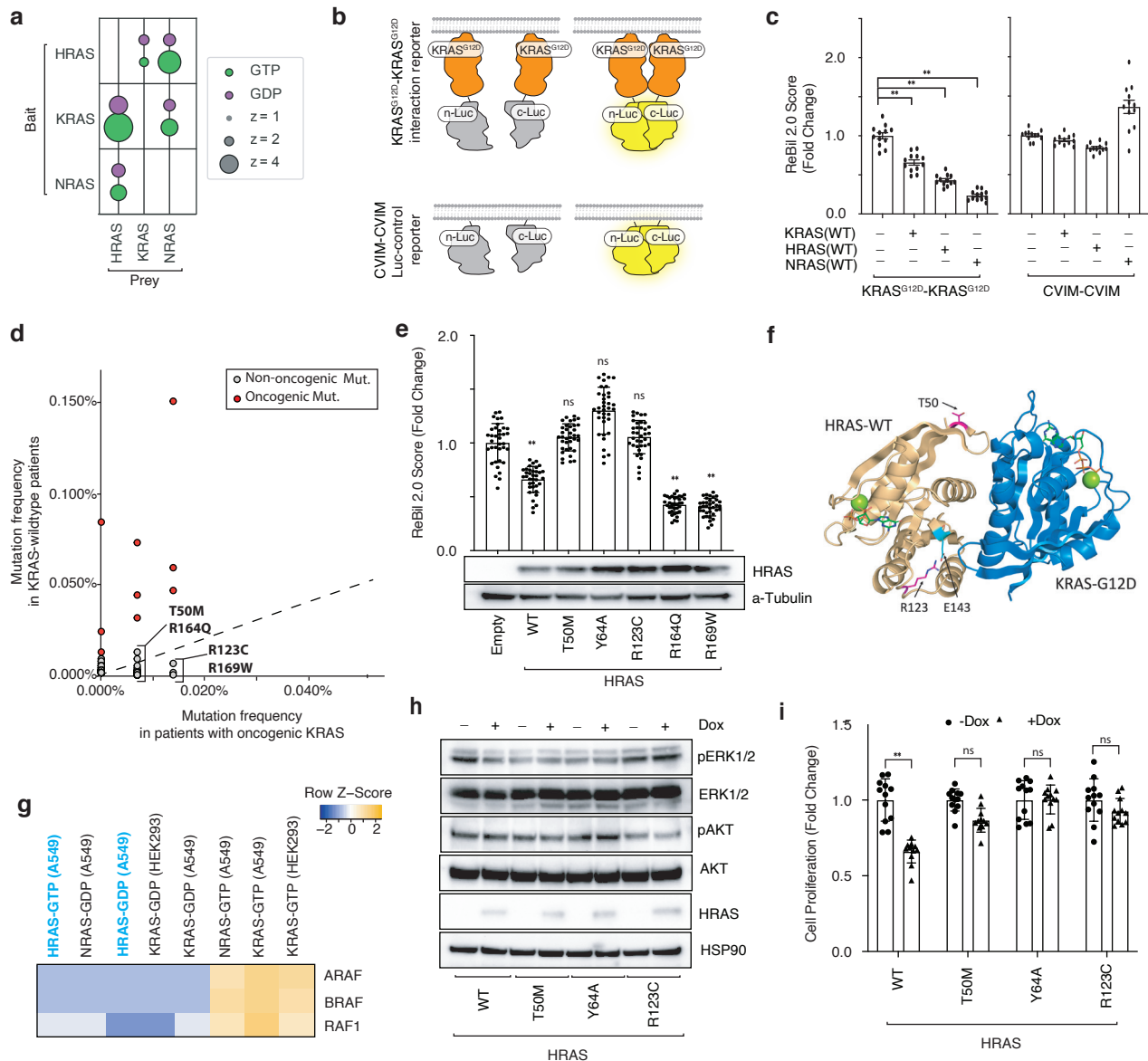
**Supplemental Figure 7. Wildtype RAS paralogs finetune RAS signaling.**

**a.** Quantification of pERK<sup>pos</sup> cells in *KT;H11<sup>LSL-Cas9</sup>* mice with tumors initiated with Lenti-sgRNA/Cre vectors as indicated in **Figure 4a**. Each dot represents a tumor.

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$

**b.** Quantification of pERK<sup>pos</sup> cells per field of indicated cells from **Figure 4b**. Each dot represents a view field. \*\*:  $p < 0.01$

**c-f.** Raw images for western blots from **Figure 4c-f**. HRAS expression on **Figure 4f** were detected using same lysis on a different gel with increased loading.



**Figure 5. Wildtype RAS paralogs finetune RAS signaling through interaction with oncogenic KRAS.**

**a.** Bubble plot of three AP/MS experiments with H-, K-, and N-RAS as baits (rows), showing the enrichment of their paralogs (columns).

**b.** Diagram of the ReBiL2.0 system. KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interactions were quantified by normalized luminescent signal generated by membrane association facilitated interaction of the split-luciferase that is fused to the N-terminus of KRAS<sup>G12D</sup> (upper). Split-luciferase that is fused to the last four amino acids of KRAS (CVIM) is applied as control for background split-luciferase interaction on the membrane (lower). Adapted from Li et al. 2020.

**c.** All three RAS proteins are able to disrupt KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup> interaction. U2OS-764 (nl-KRAS<sup>G12D</sup>/cl-KRAS<sup>G12D</sup>) or U2OS-794 (nl-CVIM/cl-CVIM) cells expressing KRAS, HRAS, or NRAS were cultured in limited serum (1%) under 100 ng/ml Doxycycline (Dox) for 24 hours. ReBiL2.0 assay were performed as previously described and detailed in Methods. Points are Mean±SD ReBiL2.0 score of 36 wells normalized to cells transduced with empty lentiviral vector. \*\*: p<0.01

**d.** Pan-cancer frequency of HRAS mutations in patients with KRAS-wildtype and oncogenic KRAS-mutant tumors from Project GENIE. Known oncogenic HRAS mutations are highlighted in red. The dashed line indicates equal mutation frequency in KRAS-wildtype and mutant samples. Four candidate mutations that were chosen for further validation in this study were highlighted.

**e.** HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> are novel RAS-RAS interaction deficient mutations. U2OS-764 (nl-KRAS<sup>G12D</sup>/cl-KRAS<sup>G12D</sup>) cells expressing wildtype or rare mutant HRAS were cultured in limited serum (1%) under 100 ng/ml Dox for 24 hours. Points are Mean±SD ReBiL2.0 score of 12 wells normalized to cells transduced with empty lentiviral vector (upper). \*\*: p<0.01; ns: not significant. HRAS (wildtype and mutant) protein expression level in corresponding cells were shown by Western blot analysis (lower).

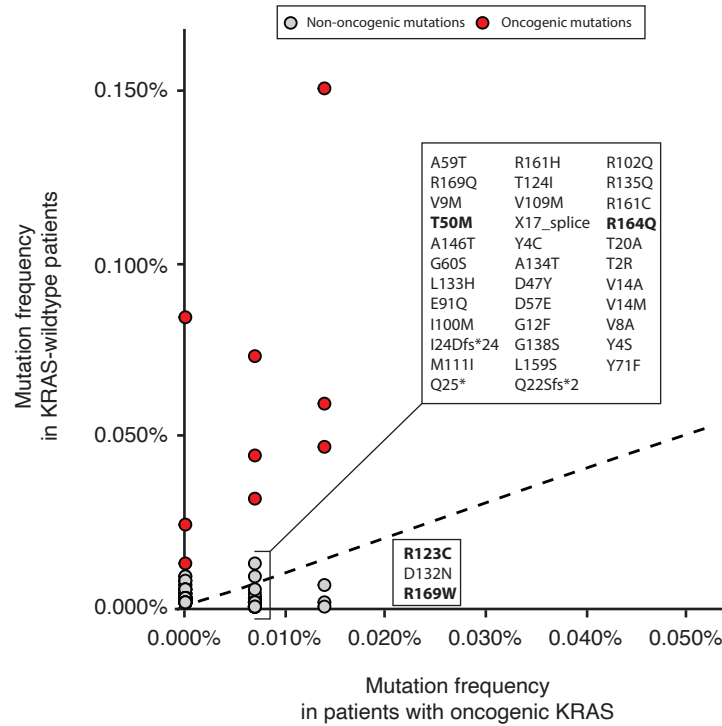
**f.** HRAS<sup>T50M</sup> and HRAS<sup>R123C</sup> are located close to the predicted HRAS-KRAS interaction interface. HRAS is shown in light orange and KRAS<sup>G12D</sup> is shown in blue. Residue R123 (in magenta) makes an intrachain salt bridge with E143 (in cyan).

**g.** Prey RAF proteins enriched in each experiment with the indicated baits in A549 cells (for K-, H-, or N-RAS) or HEK293 cells (for KRAS). Both GTP- and GDP-bound HRAS behave like GDP-bound KRAS in their RAF interactions.

**h.** Western blot analysis of cultured HRAS-null HOP62 cells (HOP62-Cas9-sgHRAS) re-expressing wildtype or mutant HRAS (T50M, Y64A, or R123C) under Dox treatment. Cells were cultured under limited serum (1%) for 2 days before protein extraction. Re-expression of HRAS mutants have no effects on ERK phosphorylation.

**i.** Cell proliferation of cultured HRAS-null HOP62 cells (HOP62-Cas9-sgHRAS) re-expressing wildtype or mutant HRAS (T50M, Y64A, or R123C) under Dox treatment. Cells were cultured in limited serum (1%) with or without Dox for 4 days. Cell viability was measured via CCK8 assay and normalized to cells treated with vehicle. Re-expression of HRAS mutants have no effects on cell proliferation.

a



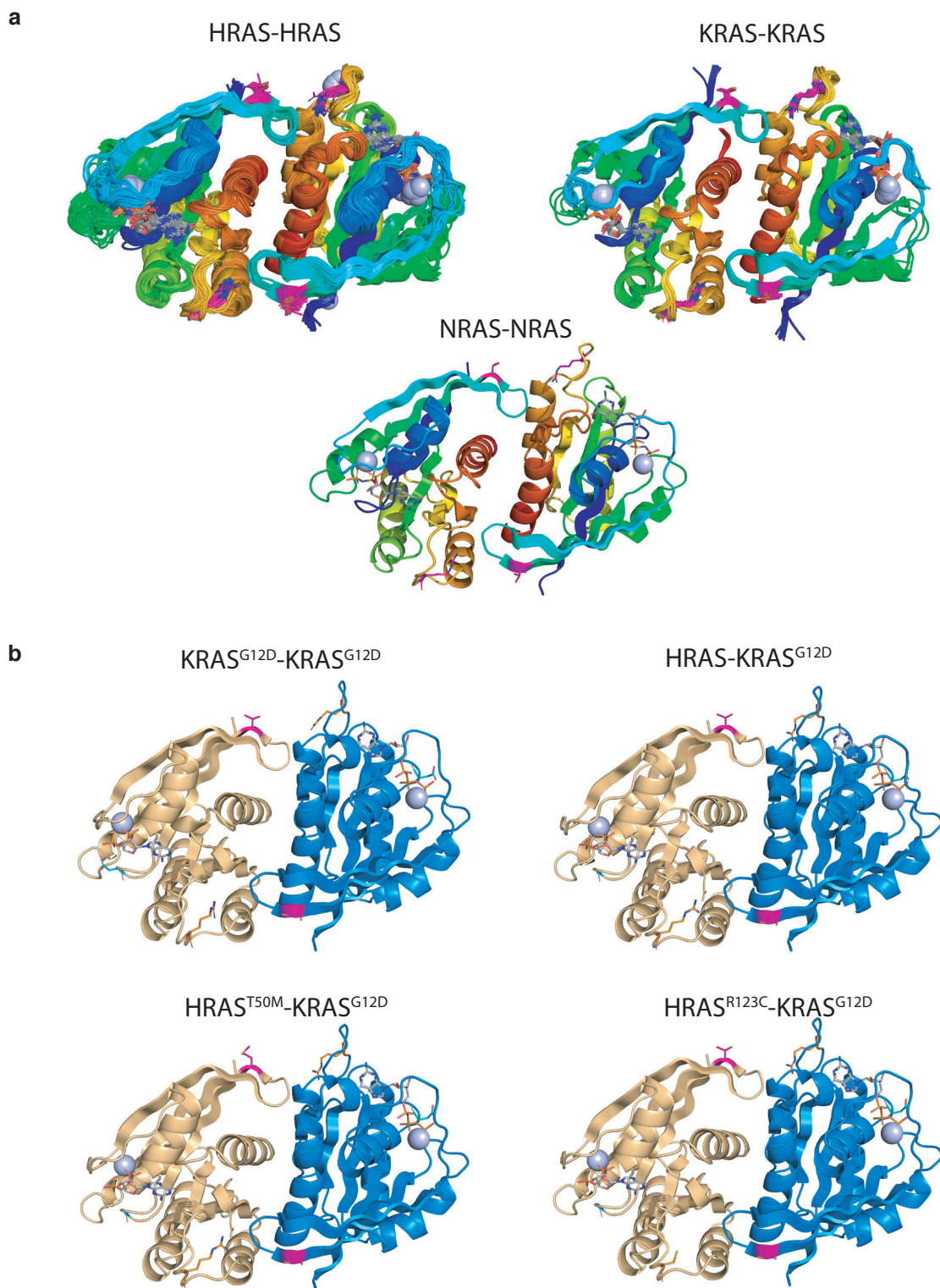
b

Mutation	Cancer type	Sample type(s)	Co-mutated with oncogenic <i>Kras</i> ?
T50M	Lung adenocarcinoma	Primary tumor, metastasis	Yes
	Ampullary Carcinoma	Primary tumor	No
	Leiomyosarcoma	Primary tumor	No
	Malignant Fibrous Histiocytoma	Primary tumor	No
R123C	Rectal Adenocarcinoma	Primary tumor	Yes
	Rectal Adenocarcinoma	Primary tumor	Yes
	Colorectal Adenocarcinoma	Primary tumor	No
	Head and Neck Squamous Cell Carcinoma	Primary tumor	No
	Lung Adenocarcinoma	Primary tumor	No
	Mixed Germ Cell Tumor	Primary tumor	No
	Papillary Thyroid Cancer	Primary tumor	No
R164Q	Colorectal Adenocarcinoma	Metastasis	Yes
R169W	Colon Adenocarcinoma	Primary tumor	Yes
	Colon Adenocarcinoma	Metastasis	Yes

**Supplemental Figure 8. Identification of rare *HRAS* mutations in oncogenic *KRAS*-mutant tumors.**

a. Pan-cancer frequency of *HRAS* mutations in patients with *KRAS*-wildtype and oncogenic *KRAS*-mutant tumors from Project GENIE. Mutations that are intergenic, intronic, silent, or fall in the 3' or 5' UTR were excluded. Oncogenic *KRAS* mutants were defined as tumors having missense mutations in codons 12, 13 or 61. Known oncogenic *HRAS* mutations are highlighted in red. The dashed line indicates equal mutation frequency in *KRAS*-wildtype and mutant samples. Non-oncogenic mutations occurring at least once in patients with oncogenic *KRAS* mutations are annotated. *HRAS* mutants selected for analysis of ability to disrupt *KRAS*<sup>G12D</sup>-*KRAS*<sup>G12D</sup> interactions are highlighted in bold.

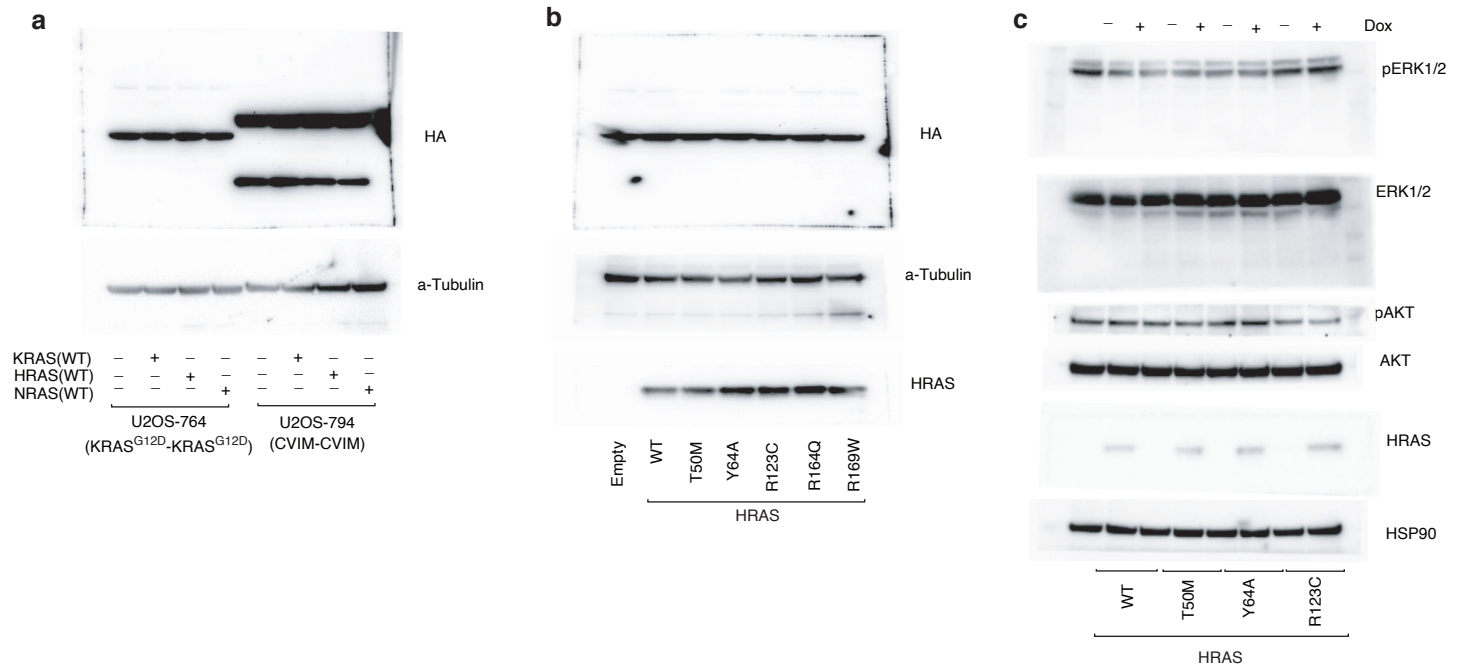
b. Characteristic of samples with rare *HRAS* mutants selected for analysis of ability to disrupt *KRAS*<sup>G12D</sup>-*KRAS*<sup>G12D</sup> interactions using the ReBiL2.0 system.



**Supplemental Figure 9. Modeling RAS-RAS dimer.**

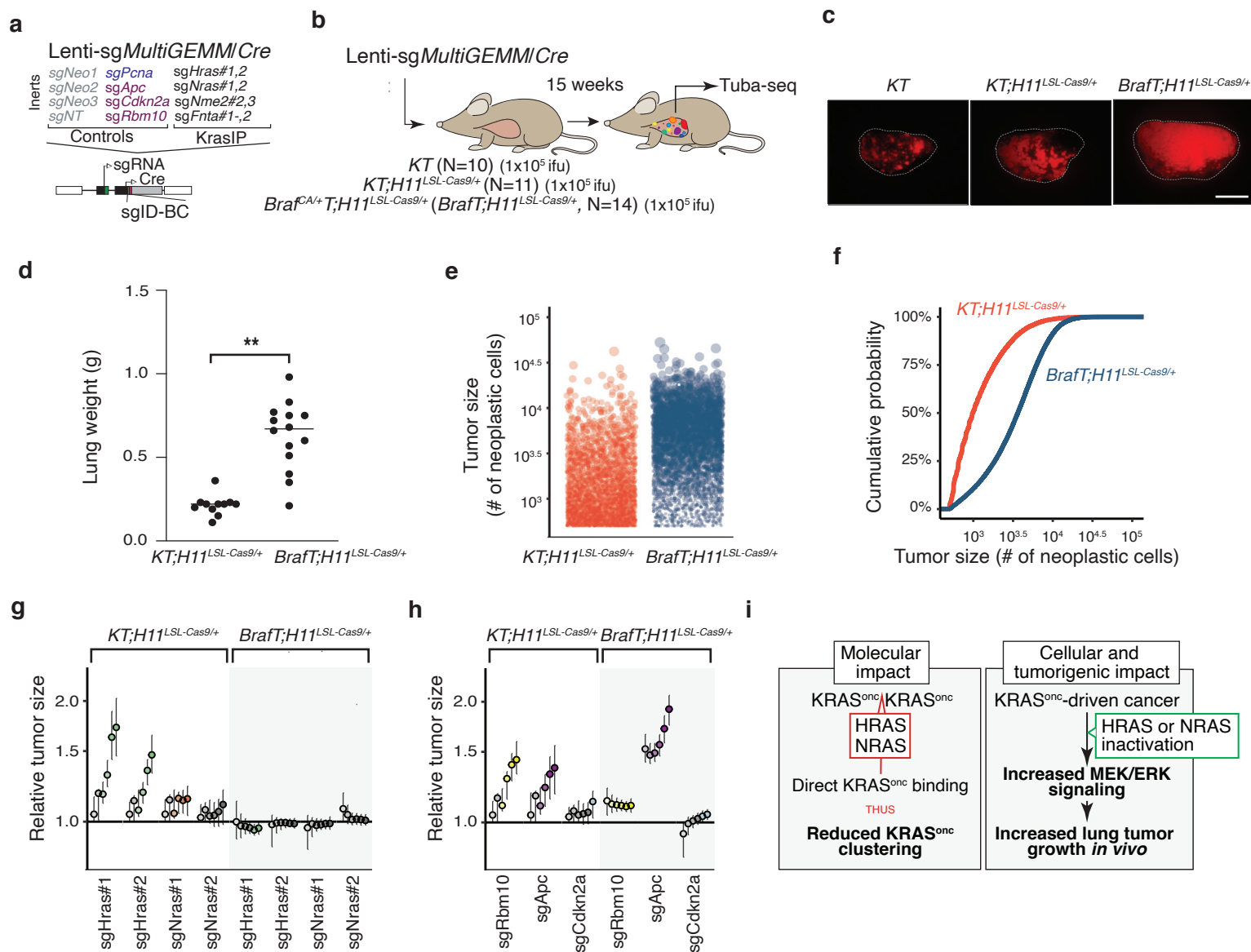
**a.** Homodimers of RAS present in crystals of HRAS, KRAS, and NRAS in the Protein Data Bank. Dimers were downloaded from the Protein Common Interface Database (ProtCID), which clusters interfaces present in different crystals of homologous proteins. The  $\alpha$ 4- $\alpha$ 5 dimer shown is present in 84 entries of HRAS, 13 entries of KRAS, and one entry of NRAS (PDB 5UHV).

**b.** Models of a homodimer of KRAS<sup>G12D</sup> and heterodimers of KRAS<sup>G12D</sup> with HRAS, HRAS<sup>T50M</sup>, and HRAS<sup>R123C</sup>. The  $\alpha$ 4- $\alpha$ 5 HRAS dimer from PDB entry 3K8Y was used as a template. KRAS<sup>G12D</sup> from PDB entry 5USJ was superposed with the program PyMol on one or both monomers of 3K8Y to form the heterodimers and the homodimer respectively. Residues T50 and R123 were mutated with PyMol. All four structures were relaxed with the program Rosetta using the FastRelax protocol with the Ref2015 scoring function). Rosetta uses the backbone-dependent rotamer library of Shapovalov and Dunbrack to repack side chains around the mutated sites. The resulting energies were: KRAS<sup>G12D</sup>-KRAS<sup>G12D</sup>, -1122.8 kcal/mol; HRAS-KRAS<sup>G12D</sup>, -1144.8 kcal/mol; HRAS<sup>T50M</sup>-KRAS<sup>G12D</sup>, -1135.5 kcal/mol; HRAS<sup>R123C</sup>-KRAS<sup>G12D</sup>, -1130.9 kcal/mol. Residues T50 (magenta) and R123 (orange) are indicated in sticks.



**Supplemental Figure 10. Wildtype RAS paralogs finetune RAS signaling through interaction with oncogenic KRAS.**

- a.** Raw images for western blots of split-luciferase (HA-tag) expression for ReBiL2.0 from **Figure 5c**. HA-tag expression were detected using same lysis on a different gel with increased loading.
- b.** Raw images for western blots of split-luciferase (HA-tag) expression for ReBiL2.0 from **Figure 5e**. HA-tag expression were detected using same lysis on a different gel with increased loading.
- c.** Raw images for western blots from **Figure 5h**. HRAS expression were detected using same lysis on a different gel with increased loading.



**Figure 6. Paired screen in KRAS-driven and Braf-driven lung cancer models validates Hras and Nras as Kras-specific tumor suppressors.**

**a-b.** Schematic of pairwise screen of tumor suppressive function in Kras- and Braf-driven lung cancer. A pool of barcoded Lenti-sgRNA/Cre vectors targeting top mediators of Kras-driven lung tumor growth (Lenti-sgMultiGEMM/Cre) was used to initiate tumors in cohorts of *KT;H11<sup>LSL-Cas9/+</sup>* and *Braf<sup>CA/+</sup>;H11<sup>LSL-Cas9/+</sup>* (*BrafT;H11<sup>LSL-Cas9/+</sup>*) mice. Each mediator of KRAS-driven tumor growth (*Hras*, *Nras*, *Nme2* and *Fnta*) was targeted by two sgRNAs (those with the largest effect size in the validation screen). The pool also included four Inert sgRNAs, as well as sgRNAs targeting *Apc*, *Cdkn2a*, and *Rbm10* as tumor suppressor controls (**a**). Tumors were initiated through intratracheal delivery of Lenti-sgMultiGEMM/Cre, and Tuba-seq was performed on each tumor-bearing lung 15 weeks after initiation, followed by analysis of sgID-BC sequencing data to characterize the effects of inactivating each gene (**b**).

**c.** Fluorescence images of representative lung lobes 15 weeks after tumor initiation. Scale bars = 5 mm. Lung lobes are outlined with a white dashed line.

**d.** Total lung weight in *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* mice 15 weeks after tumor initiation. Each dot is a mouse and mean value is indicated. \*\*:  $p < 0.01$

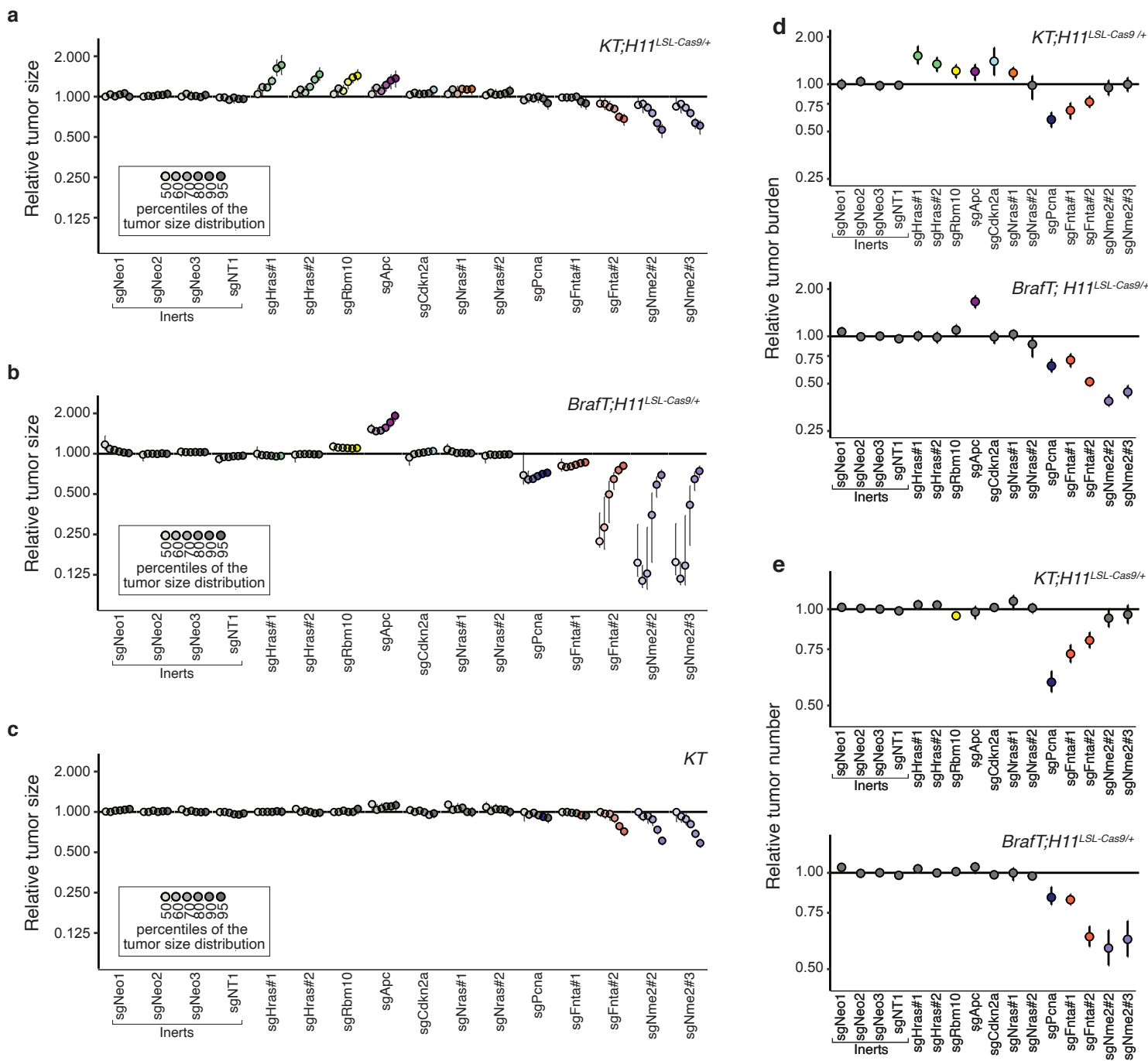
**e-f.** Size distribution of sglInert tumors in *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* mice. In **e**, each dot represents a tumor, and the area of each dot is proportional to the number of cancer cells in that tumor. To prevent overplotting a random sample of 1,000 tumors from each of five representative *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* mice are plotted. In **f**, the empirical cumulative distribution function of tumor sizes across all *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* mice is plotted. Tumors >500 cells in size are shown.

**g.** Inactivation of *Hras* and *Nras* increases tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* but not *BrafT;H11<sup>LSL-Cas9/+</sup>* models. Tumor sizes at indicated percentiles for each sgRNA relative to the size of sglInert-containing tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (left, white background) and *BrafT;H11<sup>LSL-Cas9/+</sup>* (right, gray background) mice. Line at  $y=1$  indicates no effect relative to sglInert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sglInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**h.** Comparison of the effects of inactivation of known tumor suppressors (*Rbm10*, *Apc*, and *Cdkn2a*) on tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* and *BrafT;H11<sup>LSL-Cas9/+</sup>* models. Tumor sizes at indicated percentiles for each sgRNA relative to the size of sglInert-containing tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (left, white background) and *BrafT;H11<sup>LSL-Cas9/+</sup>* (right, gray background) mice. Line at  $y=1$  indicates no effect relative to sglInert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sglInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**i.** Schematic of wildtype RAS paralogs function as tumor suppressors in oncogenic KRAS signaling. Left panel, in oncogenic KRAS-driven lung cancer cells, wildtype RAS paralogs competitively interact with oncogenic KRAS and suppress oncogenic KRAS clustering. Right panel, inactivation of wildtype RAS allele, or "RAS paralog imbalance", hyper-activated oncogenic KRAS signaling and promotes lung cancer growth.

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**Supplemental Figure 11. Paired screen in KRAS-driven and Braff-driven lung cancer models validates Hras and Nras as Kras-specific tumor suppressors.**

**a-c.** Tumor sizes at indicated percentiles for each sgRNA relative to the size of sgInert-containing tumors at the corresponding percentiles in *KT;H11<sup>LSL-Cas9/+</sup>* (**a**), *BraffT;H11<sup>LSL-Cas9/+</sup>* (**b**) and *KT* mice (**c**). Genes are ordered by 95<sup>th</sup> percentile tumor size in *KT;H11<sup>LSL-Cas9/+</sup>* mice, with sgInerts on the left. sgInerts are in gray, and line at  $y=1$  indicates no effect relative to sgInert. Error bars indicate 95% confidence intervals. Percentiles that are significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling. The negative effects of sgRNAs targeting *Fnta* and *Nme2* in the *KT* mice (**c**) are unexpected and indicate a potential bias in the size distributions of tumors with these genotypes. We note that the same bias may be present in the *KTC* and *BraffTC* data; however, sgRNAs targeting these genes in previous experiments showed consistent negative effects on tumor size, suggesting that the observed effects in this *KTC* cohort are not solely the product of this bias.

**d.** The impact of each sgRNA on tumor burden relative to sgInerts in *KT;H11<sup>LSL-Cas9/+</sup>* (top) and *BraffT;H11<sup>LSL-Cas9/+</sup>* (bottom) mice, normalized to the corresponding statistic in *KT* mice to account for representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor burdens significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.

**e.** The impact of each sgRNA on tumor number relative to sgInerts in *KT;H11<sup>LSL-Cas9/+</sup>* (top) and *BraffT;H11<sup>LSL-Cas9/+</sup>* (bottom) mice, normalized to the corresponding statistic in *KT* mice to account for representation of each sgRNA in the viral pool. sgInerts are in gray and the line at  $y=1$  indicates no effect. Error bars indicate 95% confidence intervals. Relative tumor numbers significantly different from sgInert (two-sided FDR-adjusted  $p < 0.05$ ) are in color. Confidence intervals and P-values were calculated by bootstrap resampling.