# Synthetic gene circuits that selectively target RAS-driven cancers

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# Abstract

Therapies targeting mutated RAS, the most frequently mutated oncogene in human cancers, could benefit millions of patients. Recently approved RAS inhibitors represent a breakthrough, but are limited to a specific $KRAS^{G12C}$ mutation and prone to resistance. Synthetic gene circuits offer a promising alternative by sensing and integrating cancer-specific biomolecular inputs, including mutated RAS, to selectively express therapeutic proteins in cancer cells. A key challenge for these circuits is achieving high cancer selectivity to prevent toxicity in healthy cells. To address this challenge, we present a novel approach combining multiple RAS sensors into RAS-targeting gene circuits, which allowed us to express an output protein in cells with mutated RAS with unprecedented selectivity. We implemented a modular design strategy and modelled the impact of individual circuit components on output expression. This enabled cell-line specific adaptation of the circuits to optimize selectivity and fine-tune expression. We further demonstrate the targeting capabilities of the circuits by employing them in different RAS-driven cancer cells. This work highlights the potential of synthetic gene circuits as a novel therapeutic strategy for RAS-driven cancers, advancing the application of synthetic biology in oncology.

# Introduction

RAS (Rat sacroma) mutations are the most common oncogenic alterations in human cancers[1], accounting for 19% of all cases[2]. The RAS gene family consists of HRAS, KRAS and NRAS, with KRAS being the most frequently mutated isoform[3]. Considered "undruggable" for many years, recent approval of several KRASG12C inhibitors[4] has demonstrated the efficacy of targeting RAS in cancer treatment. However, these therapies are limited to cancers with KRASG12C mutations, which narrows the target range and makes the inhibitors susceptible to resistance development due to the emergence of other RAS mutations[5].

Additional RAS-targeting strategies have encompassed protein engineering, RAS silencing, and artificial promoters. Synthetic proteins and endogenous RAS effectors (proteins that interact with active RAS and transduce its signaling) were engineered to bind[6], inhibit[7], or degrade RAS[8,9]. Additionally, miRNAs modulated by oncogenic RAS were identified as potential drug targets[10] and siRNA against KRAS was used to target RAS-driven cancers in mice[11]. Artificial promoters or synthetic transcription factor response elements responsive for transcription factors downstream of RAS were developed to interrogate RAS signaling[12] and target cancer cells with RAS mutations by expressing a toxin-antitoxin system [13,14].

Gene circuits that logically integrate multiple biomolecular inputs are an emergent modality that allows discriminating between malignant and healthy cells and selectively eliciting a therapeutic response only in target cells[15–17]. Logic gene circuit-based therapeutic prototypes already demonstrated efficacy and safety in animal tumor models[18]. Some of the previously-explored approaches for sensing and targeting RAS[10,12–14,19] are potentially

compatible with the logic gene circuit paradigm. Two circuits have already been shown to build upon, or directly sense, RAS signaling. Gao and colleagues demonstrated a split protease approach to sense over-activation of proteins upstream of RAS. By fusing one domain of a protease to HRAS and the other domain to a RAS binding domain (RBD) of rapidly accelerated fibrosarcoma type C (CRAF), they created a sensor for overexpressed mutated epidermal growth factor receptor (EGFR) or son of sevenless 1 (Sos-1) in HEK293 cells[20]. Vlahos and colleagues adapted this approach to directly sense RAS activation by fusing either part of a split protease to an RBD domain, enabling RAS-dependent interleukin-12 secretion in HEK293T cells overexpressing KRASG12V.[21] Further advancing synthetic circuits for therapeutic applications against RAS-driven cancer requires achieving high selectivity for mutated RAS and robust function in heterogeneous cancer cells. High selectivity is required to achieve strong output expression in cancer cells while minimizing off-target effects and toxicity in healthy cells with wild-type RAS. In addition, eventual translatability of these approaches to the clinic requires improved robustness in the face of tumor heterogeneity.

In this study, we address these challenges by developing a set of versatile RAS sensors and RAS-dependent transcription factor response elements to create synthetic gene circuits that use RAS activation as input. By combining these direct and indirect RAS sensors in an AND-gate configuration, we develop RAS-targeting circuits with high dynamic range and high selectivity towards cells with mutant RAS. The modular design allows fine-tuning the circuits to specific target and off-target cells and balancing their activation strength versus leakiness. Finally, we demonstrate that our new RAS-targeting circuits function as cancer cell classifiers, selectively expressing an output protein in a wide range of cancer cell lines with RAS-overactivating mutations while maintaining minimal output in cell lines with wild-type RAS.

# Results

## Design of a synthetic RAS sensor

The endogenous RAS signaling pathway is initiated by the binding of cytoplasmic effector proteins to activated RAS-GTP. The effectors sense RAS-GTP dimerization and propagate the signal downstream through a phosphorylation cascade. Post-activation, GTPase-activating proteins, such as neurofibromin 1 (NF-1), facilitate the hydrolysis of RAS-bound GTP to GDP, thereby terminating the signaling. Cancer-associated mutations in RAS render it insensitive to this hydrolysis, resulting in constitutively active RAS[22] that drives uncontrolled cell proliferation and tumor growth.

Inspired by the natural function of CRAF, a key effector of RAS[23], we designed a sensor that exploits the selective binding to RAS-GTP of CRAF's RBDCRD (**R**AS-**b**inding **d**omain/**c**ysteine **r**ich **d**omain) domain. In our sensor, we fuse the RBDCRD domain to engineered truncated and mutated NarX variants originally derived from the bacterial two-component system, namely $NarX^{379-598}$H399Q and $NarX^{379-598}$N509A (Fig. 1a). We showed previously that these NarX variants are able to transphosphorylate in mammalian cells, but only upon forced dimerization via fused protein domains. Therefore, NarX variants can functionally replace CRAF's own dimerization domain when fused to RBDCRD, while enabling orthogonal signaling in mammalian cells via a humanized NarL response regulator[24]. We call these chimeric constructs "RBDCRD-NarX fusions".

We surmised that the RAS sensor would function as follows: activation of RAS (whether endogenous or mutation-driven) would elevate RAS-GTP levels, which would in turn bind the RBDCRD domain of the RBDCRD-NarX fusion. Resulting dimerization of RBDCRD would lead

to a forced dimerization of the fused NarX, leading to NarX$^{H399Q}$ transphosphorylating NarX$^{N509A}$, in turn phosphorylating NarL. Phosphorylated NarL would bind to its response element on the NarL-responsive promoter and induce the expression of an output protein, here mCerulean (Fig. 1b). In order to test these assumptions, we constructed the necessary components including the two complementary RBDCRD-NarX fusions, the humanized NarL and the NarL-reponsive promoter coupled to mCerulean. Initially, we chose HEK293 cells as a test system. To emulate the presence of mutant RAS we transfected HEK293 cells with a plasmid expressing KRAS$^{G12D}$, and to emulate high levels of wild-type RAS we transfected them with a plasmid-encoded wild type KRAS (KRAS$^{WT}$). (Note that HEK293 cells express low endogenous levels of wild-type KRAS, HRAS and NRAS.) In the first set of tests, delivery of all the sensor components to HEK293 cells using transient transfection showed significantly higher output expression in cells expressing KRAS$^{G12D}$ than in cells transfected with KRAS$^{WT}$ (Fig. 1c). In HEK293 cells transformed with the mutant KRAS, sensor response increased upon increase in the dose of the sensor-encoding plasmids (Fig. 1d). Further, sensor function depended on RAS binding, because mutations in the RAS binding domain (RBD) and the cysteine-rich domain (CRD) strongly decreased output expression. Interestingly, while the RBD$^{R89L}$ mutant still exhibited dose-dependent leakiness, the CRD$^{C168S}$ and the double RBD$^{R89L}$CRD$^{C168S}$ mutant reduced output expression to background levels (Fig. 1d). In agreement with previous reports[25] and with our observation that the sensor employing RBDCRD instead of RBD alone shows increased downstream signaling (Supplementary Fig. 1), this result confirms that both RBD and CRD domains are involved in RAS binding and sensor activation.

We then characterized sensor response to increasing input levels. As expected, increased levels of mutant KRAS drove higher output expression, whereas wild-type KRAS resulted in some sensor activation but at much higher expression levels (Fig. 1e). Finally, we

measured the RAS-GTP protein levels in HEK293 cells using a RAS-pulldown ELISA assay and were able to directly correlate higher RAS-GTP levels with higher output expression (Fig. 1f). Collectively, this dataset provides evidence that the RAS sensor is directly activated by RAS-GTP, and therefore it can selectively sense mutant RAS because the hallmark of this mutant is highly increased RAS-GTP level. Nonetheless, wild-type RAS also binds GTP, which explains sensor response in HEK293 cells that overexpress wild-type RAS and contain relatively high levels of RAS-GTP (Fig. 1f).

## Mechanism of Action

To recapitulate, our design anticipates that the function of the RAS sensor requires the following steps: delivery and expression of the RAS sensor components; RAS-GTP binding of RBDCRD-NarX fusion proteins via their RBDCRD domain, resulting in forced dimerization of the NarX domains and transphosphorylation; NarL phosphorylation; and NarL-mediated output expression. To quantify the effect of RAS activation on these steps, we manipulated the RAS-GTP levels in HEK293 cells in a variety of ways: (i) we overexpressed wild-type KRAS to show the effect of high non-mutant KRAS concentration on downstream sensor response and quantify sensor activation in the presence of RAS-GTP associated with wild-type KRAS; (ii) we overexpressed mutant KRAS in order to measure sensor response to saturating levels of the intended mutant KRAS input; (iii) we overexpressed NF1, an endogenous GTPase-activating protein that deactivates residual endogenous wild-type RAS in HEK293 cells and allows to quantify non-specific sensor response in the absence of RAS-GTP; and (iv) we expressed Sos-1, a guanine nucleotide exchange factor that activates RAS by generating high levels of RAS-GTP, to quantify the upper bound on sensor output.

In order to quantify signal propagation along this cascade, we generated a number of reporter constructs. To quantify the expression of the RBDCRD-NarX fusion, we further fused this component to SYFP2 and measured YFP fluorescence in HEK293 cells with various RAS-GTP levels. We observed that overexpression of $KRAS^{G12D}$ or overactivation of endogenous RAS with Sos-1 resulted in YFP increase and thus elevated expression of the RBDCRD-NarX fusion, compared to cells with baseline RAS-GTP. Conversely, inactivation of endogenous RAS with NF1 resulted in a lower YFP/RBDCRD-NarX expression (Fig. 2a). Second, we used the same construct to visualize RAS binding of the RBDCRD-NarX. Because RAS is a membrane protein[26], we expected the binding of RBDCRD-NarX to KRAS-GTP to result in YFP accumulation at the membrane. Indeed, RAS-GTP increase by overexpressing $KRAS^{G12D}$ or via Sos-1 led to higher membrane-to-total-signal ratio, compared to cells with endogenous RAS-GTP levels or with NF1-deactivated RAS (Fig. 2b). Third, we examined the dimerization of the RAS sensor by fusing the two parts of a split mVenus to RBDCRD-NarX proteins and measuring the signal from reconstituted mVenus. Again, RAS activation increased the signal from dimerized RBDCRD-NarX-mVenus, while RAS deactivation decreased it (Fig. 2c). Last, we investigated the effect of RAS-GTP levels on mCerulean sensor output of the complete RAS sensor. We observed the expected trend whereas the magnitude of the effect was amplified compared to the effects of individual sensing step, suggesting signal amplification in the synthetic sensing pathway (Fig. 2d).

While the dependency of RAS binding, dimerization, and output expression of the sensor on RAS-GTP levels was expected, we did not expect that the expression of the RBDCRD-NarX fusion itself (Fig. 2a) would be RAS-GTP dependent. Possible explanations could include the presence of transcription factor binding sites activated by RAS signaling in the elongation factor 1a (EF1a) promoter driving the expression of RBDCRD-NarX, or RAS-dependent non-

specific activation of protein synthesis. EF1a promoter sequence contains potential binding sites of CREB, c-Myc, SRF, AP1 and Elk-4 (Supplementary Fig. 2)[27]. Moreover, reports that (over-)activated RAS or MAPK signaling can increase protein synthesis[28–31], suggesting that both mechanisms may be involved. Whatever the cause, the fact that functional binding of RBDCRD to RAS is required for sensor activation and output generation (Fig. 1e), and the much stronger increase in sensor output (Fig.2d) compared to the increase in RBDCRD-NarX levels (Fig.2a), suggests that this effect is only a minor contributor to overall sensor response.

## Optimization of activation efficiency and sensor tunability

The RAS sensor is activated by RAS-GTP and therefore by both wild-type and mutant RAS. Cells with mutant RAS have higher RAS-GTP and higher sensor activation than cells with wild-type RAS. Nevertheless, residual sensor activation in healthy cells with wild-type RAS may cause toxicity in a clinical implementation of the sensor. Therefore, we aimed to further improve the RAS sensor's ability to discriminate between wild-type and mutant RAS and optimized the transfer function between RAS-GTP input and mCerulean output. Structural predictions with AlphaFold2[32] revealed that longer linkers between the sensor's RBDCRD and NarX-derived domains could bring the NarX domains into closer proximity, potentially improving dimerization and transphosphorylation efficiency (Fig. 3a). Our measurements showed that up to a certain size, longer and/or more rigid linkers indeed increased output expression, but not the dynamic range between $KRAS^{G12D}$ and $KRAS^{WT}$ (Fig. 3b).

We also explored alternative natural and synthetic RAS binding domains. Among the tested binding domains, the Ras association domain (RA) of the natural RAS effector Rassf5, the RAS association domain 2 (RA2) of the phospholipase C epsilon (PLCe)[33], and the synthetic

RAS binder K55[34] showed a slightly higher or similar dynamic range. To better understand how different binding domains affect RAS-specific activation, we deactivated endogenous $RAS^{WT}$ in HEK293 using NF-1. RBDCRD was the only binding domain that showed a pronounced decrease in output expression in cells with NF1 compared to cells with $KRAS^{WT}$, indicating low RAS-unspecific activation for RBDCRD but also its activation in cells with $KRAS^{WT}$. The other binding domains showed one of two responses: (1) already very low activation in cells with $KRAS^{WT}$ which was not further decreased in cells with NF1, for example in RA(Rassf5) and K55; (2) background activation in cells with NF-1 indicating RAS-unspecific activation, for example in RA2(PLCe) (Fig. 3c).

Fusing stronger transactivation domains to NarL markedly increased output expression of the RAS sensor without changing its dynamic range. Specifically, we replaced the initial VP48 with either F.L.T., a fusion of three partial transactivation domains, FoxoTAD (Forkhead-Box-Protein-O3$^{604-664}$), LMSTEN ($MYB^{251-330}$), and TA1 ($RelA^{521-331}$) or with $RelA^{342-551}$ with all its transactivation domains (Schweingruber et al. in preparation). In cells co-transfected with NF1, the sensor with NarL-F.L.T. exhibited low output expression similar to the initial sensor variant with NarL-VP48. In contrast, the sensor with NarL-RelA had high background in cells with NF1 suggesting RAS-unspecific activation when using RelA as a transactivation domain (Fig. 3d). Overall, the testing of alternative sensor parts did not increase the dynamic range between $KRAS^{G12D}$ and $KRAS^{WT}$ but it showed that the RAS sensor was modular. Different linkers, binding domains, and transactivation domains could be used to construct the sensor and tune the absolute strength of output expression without affecting the dynamic range.

## Multi-input RAS-targeting circuits with improved selectivity for $KRAS^{G12D}$

Mutations in signaling proteins, such as RAS, often lead to increased activation of downstream transcription factors[35]. We hypothesized that using transcription factors from the mitogen-activated protein kinase pathway (MAPK) downstream of RAS as inputs could add an additional layer of selectivity to RAS-targeting circuits. Regulating the expression of RAS sensor components via MAPK-dependent response elements allowed to create a coherent type-1 feed-forward motif with AND-gate logic[36] (Fig. 4a). This motif has been shown to act as a noise repressor[37] and as a persistence detector with a delayed onset that is only activated by persisting input stimulation[36]. Both are desired properties in RAS-targeting circuits to detect constitutively active mutated RAS while minimizing output expression from the more transient activation of wild-type RAS[38,39].

As an initial step, we designed synthetic response elements with binding sites for transcription factors shown to be activated by mutated RAS, including Elk-1[10], c-Fos & c-Jun[40], c-Myc[41], and SRF[42]. For each response element, multiple binding sites of MAPK transcription factors[12,43,44], minimal promoters from genes downstream of RAS[12], or sequences from existing RAS-responsive promoters[13,19,45] were encoded upstream of a low leakage minimal promoter[46] (Fig. 4b & Supplementary Fig. 3).

To assess the functionality of the response elements, we cloned the response elements directly upstream of a fluorescent mScarlet protein and transfected them into HEK293 cells. The minimal promoter of c-fos (pFos)[12], the polyomavirus enhancer domain (PY2)[19], and the serum response element (SRE)[12] were the response elements exhibiting the highest dynamic range between cells with $KRAS^{G12D}$ and cells with $KRAS^{WT}$ (Fig. 4c). The response elements demonstrated a similar dependency on $KRAS^{G12D}$ and $KRAS^{WT}$ to the one observed with the

binding-based RAS sensor in Fig. 1e. All three response elements contain binding sites for Ets-like 1 protein (elk-1). However, the Elk response element consisting of only elk binding sites showed low mScarlet expression and low dynamic range. To better understand this apparent contradiction, we further investigated the Elk response element. Overexpressing elk-1 with the Elk response reporter increased the mScarlet expression, indicating that endogenous elk-1 levels are not high enough for efficient activation of the Elk response element. With overexpressed elk-1, we observed high selectivity for cells with $KRAS^{G12D}$ and low RAS-independent background activation in cells overexpressing NF-1 (Supplementary Fig. 4). A RAS titration provided more evidence for the RAS dependence of the Elk response element (Fig. 4e).

Superior performance of pFos, SRE, and PY2 response elements demonstrate that inclusion of additional transcription factor binding sites can amplify transcriptional sensor response, potentially due to synergy[47]. Consistent with this and previous reports that the combination of AP-1 and elk-1 binding sites is critical for maximal RAS responsiveness in the polyomavirus enhancer[13,48], we found that combining elk-1 with additional transcription factors allows the design of efficient RAS-dependent response elements.

Finally, we combined these MAPK response elements with the RAS sensor using AND-gate logic, by using the response elements to regulate the expression of binding-triggered RBDCRD-NarX sensor. The resulting RAS-sensing circuits showed improved selectivity for $KRAS^{G12D}$ with the best performer exhibiting an 81-fold dynamic range between $KRAS^{G12D}$ and $KRAS^{WT}$ compared to the 8-fold dynamic range of the binding-based RAS sensor alone (Fig. 4f).

## Modularity allows to create RAS sensor circuits with high dynamic range

As shown above, RAS-sensing circuits can utilize a variety of MAPK response elements, RAS binding domains, linkers, and transactivator domains. To further optimize the transfer function

between the input and the output and improve the discrimination between cells with mutant and wild-type RAS, we performed a screening campaign to examine the effect of different combinations of circuit components on the dynamic range. In this screen we tested combinations of various building blocks: RBDCRD, K55, and RA(Rassf5) as binding domains fused to the NarX; VP48 or F.L.T as transactivation domain of NarL; and pFos, PY2 and SRE as MAPK response elements. The response elements regulated the expression of either the NarX fusion proteins only, or of the NarL protein only, while the other part was constitutively driven by EF1a. For the EF1a-driven RBDCRD-NarX proteins we tested two versions with either a 6x flexible or a 3x rigid linker. This set resulted in a total of 222 unique conditions, over two experimental batches. Almost thirty conditions resulted in high dynamic ranges of >100-fold, with the highest circuit variants using the combination of PY2, RBDCRD, and F.L.T. (Fig. 5a).

We found that PY2 was the most prevalent response element among the high-performing hits, followed by pFos, while SRE only occurred in three hits. The prevalence of the transactivation domain varied depending on whether the NarX or the NarL proteins were regulated by a MAPK response element. While F.L.T. was more abundant among the hits where NarL was regulated by the response elements, VP48 dominated in the circuits where the response elements regulated the NarX fusion proteins. The K55 and RBDCRD binding domains were found in an equal number of hits (Fig. 5b). However, we observed that circuits with F.L.T. as the transactivation domain performed best with RBDCRD as the binding domain, while there was no significant difference between RBDCRD and K55 for circuits with VP48 (Supplementary Fig. 5).

To further improve our understanding of the effect of different building blocks, we fitted a linear regression model (Fig. 5c) and compared it with the experimental data (Supplementary Fig. 6-7). This revealed that the effect of the MAPK response elements depended on which

sensor components they regulated. Expression of NarL-F.L.T. under the control of MAPK response elements strongly increased the output expression in cells with $KRAS^{G12D}$, while, except for the SRE_NarL-F.L.T. circuit, the activation in cells with $KRAS^{WT}$ remained low. In contrast, MAPK control of NarL-VP48 decreased the output in $KRAS^{G12D}$ and even more so in $KRAS^{WT}$ cells. Expression of the NarX fusion proteins increased the circuit activation in cells with $KRAS^{G12D}$, but also in cells with $KRAS^{WT}$. (Fig. 5c, & Supplementary Fig. 6a-f).

In this campaign, we focused on circuits where only NarL or only the NarX proteins were regulated by the response elements. Next, we also implemented the regulation of all circuit components by PY2. The PY2_NarX & NarL-F.L.T. circuit led to the highest output in cells with $KRAS^{G12D}$, with only marginally higher activation with $KRAS^{WT}$ than PY2_NarX_F.L.T. or PY2_NarL-F.L.T. and still lower than the RAS_Sensor_F.L.T. (Fig. 5d&e).

In summary, the tests revealed that the RAS-targeting circuits are composable, with various combinations of parts leading to circuits with high dynamic ranges that diverge in their transfer functions and their activation thresholds. The availability of different circuit parts thus allowed to adapt the input-output behavior of the circuits and increased the distinction between cells with mutated and cells with wild-type RAS.

## RAS sensor circuits detect endogenous RAS mutations in cancer cells

To evaluate the response of the RAS sensor circuits to endogenous RAS activation in cancer cell line, we transfected the EF1a-driven, binding-triggered RAS sensor into wild-type HCT-116 cells, a colon cancer cell line harboring a $KRAS^{G13D}$ mutation ($HCT\text{-}116^{WT}$)[49]. To have a

comparable off-target cell line without mutated RAS, we also transfected a commercially available KRAS knock-out HCT-116 cell line (HCT-116$^{k.o.}$).

The RAS sensor was functional in HCT-116 cells and responded to endogenous levels of RAS activation with higher activation in HCT-116 cells with KRAS$^{G13D}$ than in the knock-out cells. Further, loss-of-function mutations in RBDCRD decreased activation (Fig. 6a). However, the dynamic range was only 3-fold. Therefore, we leveraged the modularity of the circuit design to improve selectivity for target HCT-116 cells. We found that the amplified AND-gate circuits with PY2 response element, F.L.T transactivation domain, and RBDCRD as a RAS-binding domain, were most effective in distinguishing HCT-116$^{WT}$ from HCT-116$^{k.o.}$ (Fig. 6b-d). In contrast to what we saw in HEK293 overexpressing RAS (Fig. 5d), the "AND-gate" RAS-targeting circuits do not generate higher output than the EF1a-driven, binding-triggered RAS sensor in HCT-116. Instead, the improved dynamic range results from decreased leakiness in HCT-116$^{k.o.}$. Only with a 3x rigid linker in RBDCRD-NarX fusion did the PY2_NarL-F.L.T circuit show higher output expression compared to the EF1a-driven, binding-triggered RAS sensor. However, while the circuit with the 3x rigid linker still showed a dynamic range of 18-fold it had a decreased dynamic range compared to the same circuit with the 6x flexible linker (57-fold) (Fig. 6e). Taken together, this dataset demonstrates that the RAS-targeting circuits are functional in cancer cells and are able to respond to endogenous levels of mutated RAS. While there are differences between the model systems, we can leverage the availability of multiple circuit parts to adapt the circuits to specific target and off-target cells to improve selectivity.

## RAS circuits can generate selective output in RAS-driven cancer cells

To specifically target RAS-driven cancer, the RAS-targeting circuits need to show selective output expression in cancer cells with mutations over-activating RAS ($RAS^{MUT}$), while maintaining minimal expression in cells without such mutations ($RAS^{WT}$). Testing the most promising RAS-targeting circuits in twelve cancer cell lines showed that all circuits exhibited significantly higher output expression in $RAS^{MUT}$ cells. The PY2_NarX&NarL-F.L.T. circuit had the highest response rate among the $RAS^{MUT}$ cells but displayed slightly increased background activation in $RAS^{WT}$ cells. This was particularly notable in HT-29, a cell line harboring a BRAF mutation[49] and thus representing a $RAS^{WT}$ cell line but with an over-activated MAPK pathway. HT-29 did not show an elevated background in any of the other circuits, indicating a clean AND-gate behavior between the RAS Sensor and the MAPK response element for the circuits that express only NarL-F.L.T. with the response elements (Fig. 6f).

Interestingly, regulating NarL-F.L.T. through different response elements altered the specific $RAS^{MUT}$ cells in which the RAS circuits showed output expression. This allowed us to identify for each $RAS^{MUT}$ cell line, a RAS circuit with higher activation in these cells than in all $RAS^{WT}$ cell lines, demonstrating that the response elements allow to adapt the RAS circuits to the targeted cancer cell type (Fig. 6g & Supplementary Fig. 8). The output expression of the RAS circuits correlated with the direct expression of a fluorescent protein via the MAPK response elements, although not perfectly. This indicates that the response elements are important for the differential activation between the cell lines but are not the only factor (Supplementary Fig. 9). In summary, these results demonstrate that the RAS-targeting circuits are functional $RAS^{MUT}$ cell classifiers with a broad target range that can be adapted using different MAPK response elements.

# Discussion

In this study, we report the design of synthetic gene circuits to target RAS-driven cancers. We developed a set of RAS sensors with interchangeable parts that can be combined to flexibly design RAS-sensing circuits. Using our modular design, we created and characterized gene circuits advancing RAS-targeting circuits on two key performance criteria: selectivity for mutated RAS, and adaptability to different target and off-target cell lines.

To date the only existing synthetic gene circuit that directly targets RAS had a 2-fold dynamic range between HEK cells overexpressing $KRAS^{G12V}$ and $KRAS^{WT}$ and a 4-fold dynamic range when compared to HEK cells with endogenous RAS levels[21]. Our design of RAS-targeting circuit greatly improves specificity for mutant RAS. This is critical given that RAS circuits sense activated RAS-GTP, which is highly overactivated in cancers with RAS mutations[22] but also present in healthy cells with wild-type RAS. Since this could lead to on-target, off-tumor effects, RAS-targeting circuits are designed to sense the different activation dynamics and activation levels[38,39] resulting from the constitutive overactivation of RAS in cancer[22,50]. To achieve high dynamic range between cells with mutated and cell with wild-type RAS, we optimized the transfer function governing the relationship between the RAS-GTP input and sensor output. To this end, we combined binding-triggered RAS sensors and RAS-dependent MAPK transcription factor sensors into a coherent type 1 feed-forward AND-gate. In this design, MAPK sensors enhance the RAS-dependent increase in expression of the binding-triggered RAS sensor components. Thus, cells with wild-type RAS have lower levels of the RAS sensor components than cells with mutant RAS, leading to suppression of RAS-dependent leakage of output production by the binding-triggered RAS sensor. This network motif was shown to delay the onset of output expression but not the output shutdown[36]. This dynamic behavior, termed

sign-sensitive delay [36], acts as a noise repressor[37] and persistence detector [36], which could further explain why this design improves the sensing of constitutively active mutant RAS while minimizing output expression from the more transient activation of wild-type RAS[38,39]. Integrating multiple RAS dependent sensors into the circuit design resulted in a strongly enhanced distinction between cells overexpressing mutant or wildtype RAS and a more than 100-fold higher dynamic range than previous circuits[21].

Validation in cancer cell lines demonstrated that the RAS-targeting can selectively express an output in cancer cells with diverse RAS mutations. The RAS circuits were activated by a variety of mutations, such as $KRAS^{G12D}$, $KRAS^{G12S}$, $KRAS^{G12V}$ and $KRAS^{G13D}$, but also in K562 cells with a BCR-ABL fusion gene that constitutively activates RAS through Sos-1[56]. A broad targeting range is advantageous because tumors exhibit high intra- and intertumoral heterogeneity of RAS mutations[57]. RAS heterogeneity is a limiting factor in current KRAS inhibitors because of its limited target range to only specific tumors with G12C mutations and resistance development due to escape variants with different RAS mutations[5]. The broad target range of the RAS-targeting circuits enabled us to identify a circuit for each mutant RAS cell line that was more active in those cells than in all wild-type RAS cell lines. However, the heterogeneous cancer cell lines showed variable dynamic ranges, showing the need for adaptation of the circuits to the target cells.

Additional input sensors may allow to further enhance the RAS-targeting circuits. Beyond the direct RAS sensors and the MAPK response elements, adding other RAS-dependent sensors, such as ERK sensors[58,59] or RAS-dysregulated miRNAs[10,60] in an AND-gate configuration could further improve specificity. Alternatively, an OR-gate configuration could reduce the risk of resistance development. Off-target effects could be minimized by including

NOT-gates with inputs associated with healthy cells, such as existing p53 sensors[14,61] or new sensors specific for healthy cells with high RAS activation[62,63].

In the context of cancer therapies, synthetic gene circuits can express a variety of therapeutic proteins as output, such as proapoptotic proteins[15,20], enzymes that convert a prodrug into a cytotoxic drug[18], immunotherapeutic proteins[45], or even combination therapies[45]. Exchanging the output protein can be achieved by changing the coding sequence on the output plasmid. However, it may also require adaptation of the expression strength to optimize the killing of cancer cells while minimizing toxicity. Selection of the components used in our RAS circuits tunes the expression strength which may allow for dosing of the therapeutic output proteins. Potent molecules, such as interleukin-12, will require stringent expression with low leakiness to prevent systemic toxicity[4], while less toxic molecules could benefit from stronger expression. We envision that the modularity of our system will allow to tailor the expression profile to the therapeutic output.

In conclusion, this study provides the foundation for the design of RAS-targeting circuits. Our results confirm the feasibility of developing synthetic gene circuits that selectively target RAS-mutated cancer cells and encourage the use of their versatility to adapt the circuits to future challenges during clinical translation. Together, this highlights the potential of RAS-targeting circuits as a new therapeutic strategy that could reshape therapies against RAS-driven cancer.

# Methods

**Plasmid Construction.** Plasmids were cloned using standard cloning techniques, such as Gibson, GoldenGate assembly, or restriction enzyme cloning. DNA fragments were ordered

from TWIST Biosciences or Genewiz (Azenta Life Sciences). Enzymes were purchased from New England Biolabs and Thermo Fischer Scientific. The sequences of all used plasmids are listed in Supplementary Table 1 and the sequences of the individual RAS circuit parts in Supplementary Table 2.

**Cell Culture.** All cell lines used were cultured at 37 °C, 5% CO2 in the medium suggested by the provider with 10% FBS and 1% penicillin/streptomycin solution. Cells were passaged before reaching confluency 1-3 times per week, depending on experimental plans. Details on the individual cell lines, such as provider, medium, and splitting ratios are listed in Supplementary Table 3.

**Transfections.** The transfections for the RAS-GTP pulldown experiment in Fig. 1f and the confocal microscopy experiment in Fig. 2b were performed in 6-well plates, the transfection for the microscopy experiment in Fig. 6a, and the cell line screening in Fig. 6f in 24-well plates, and all other experiments were performed in 96-well plates. The difference in plate size was accounted for by scaling the amount of transfected DNA to the number of seeded cells. The cells were seeded 24h before transfection in 100 µL of medium (500 µL for 24-well; 2.5 mL for 6 well plates). Endotoxin-free (ZR DNA Prep Kit, Zymo Research, cat.no. D4201 & D4212) plasmids were mixed according to the experimental layout (Supplementary Tables 4 & 5), Opti-MEM™ (Thermo Fischer Scientific) was added to reach a volume of 30 µL (50 µL in 24-well plates; 500 µL in 6-well plates). Transfection reagents were mixed with Opti-MEM™ to reach a volume of 20 µL (50 µL in 24-well plates; 500 µL in 6-well plates). After incubation for at least 5 minutes, the mixture was added to the DNA samples, gently vortexed, spun down, and then incubated at room temperature for at least 20 minutes before addition to the cells. The seeding

and transfection conditions for each cell line are shown in Supplementary Table 6 for the cell line screening in Fig. 6f and in Supplementary Table 7 for all other experiments. To minimize the effect of differential evaporation in 96-well plates, only the inner 60 wells were used for samples, while the outer wells were filled with PBS.

For the experiments comparing different cell lines (Fig. 6 & Supplementary Fig. 8, 9 & 13), the DNA amount was optimized to achieve more similar transfection efficiencies, as listed in Supplementary Tables 6-7. In the cancer cell line screening, we adjusted the DNA amount to 1.5x for cell lines with low transfection efficiencies (<20%), 1x DNA for cell lines with moderate efficiency (20-50%), and 0.5x DNA for cell lines with high efficiency (>50%).

**Structure Prediction.** The protein structure of the RBDCRD-linker-NarX fusion proteins was predicted with AlphaFold2 from the amino acid sequence using the Latch Console platform (LatchBio). Using PyMOL (Version 2.5.4, Schrödinger, LLC.), we aligned the NMR-derived structure of two KRAS-RBDCRD dimers tethered to a nanodisc (worldwide Protein Data Bank, PDB accession code: 6PTS[64]) with the NMR structure of a KRAS4B-GTP homodimer on a lipid bilayer nanodisc (PDB accession code: 6W4E[65]). The predicted structure of two RBDCRD-linker-NarX proteins was then aligned to each of the RBDCRD structures. The flexible parts of the RBDCRD and the linkers were rotated, to bring the two NarX domains into proximity.

**Fluorescence Microscopy.** Microscopy pictures were taken 36-48 h after transfection using a Nikon Eclipse Ti inverted microscope equipped with a Nikon Intensilight C-HGFI fiber illuminator, Semrock filter cubes (IDEX Health & Science), a 10x objective, and a Hamamatsu C10600 ORCA-R2 digital camera. Excitation filters, dichroic mirrors, emission filters, and

exposure times are summarized in Supplementary Table 8. The Look-up table (LUT) values were adjusted for ideal contrast and kept constant within an experiment.

**Flow Cytometry.** Cells were prepared for flow cytometry analysis by removing the medium and adding 70-100 µL of Accuatase (Gibco, Thermo Fischer Scientific, cat.no. #A1110501). The cells were incubated for 15-30 min at room temperature and then kept on ice before measurement using a BD LSRFortessa™ with a high-throughput screening device. To avoid potential cell damage and minimize time on ice, the plates were prepared consecutively, right before analysis. Excitation wavelengths, longpass filters, and emission bandpass filters were optimized to reduce crosstalk between different fluorophores and are summarized in Supplementary Table 9. When working with 24-well plates, after removing the medium, 150-200 µL of Accutase was used to detach the cells. After 15-30 minutes of incubation, the cells were detached by gentle pipetting, and the complete cell suspension was transferred to a 96-well plate for analysis using the HTS device.

**RAS-GTP Pulldown ELISA.** RAS-GTP levels were measured using a Ras Activation ELISA assay kit (Merck Millipore cat.no. #17-497) according to the manual. HEK293 cells were seeded in 6-well plates and transfected as described in the 'Transfection' section. Cells were lysed using 250 µL of the provided lysis buffer with added Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, cat.no. #89900). Samples were snap frozen in liquid nitrogen and stored at -80°C. An aliquot was used to quantify the protein amount in the cell lysates using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat.no #23227). The next day, 100 µg of each sample was used for the ELISA. The anti-RAS antibody (Merck Millipore cat.no. #17-497; part no.2006992) was provided in the ELISA kit. Chemiluminescence was measured using a Tecan

Spark Multimode Microplate Reader and measured 20 min after the addition of the substrate with an integration time of 1 s.

**Confocal Microscopy for Quantification of Membrane Binding.** Cells were seeded in a 24-well plate and transfected as described in the 'Transfections' section. After 36 h we detached the cells using 0.25% trypsin and re-seeded 30`000 cells in 200 µL medium into an 8-well glass-bottom plate to have sparsely distributed cells suitable for membrane detection and image analysis. The cells were incubated at 37°C, 5% CO2 to reattach. After 4 h, we stained the membrane of the cells with the CellBrite Steady 685 Membrane Staining Kit (20 µL of 1:100 dilution; Biotium cat.no. #30109-T) and the nuclei with 5 µL NucBlue Live Cell Stain (Thermo Fischer Scientific, cat.no. R37605). The wells were imaged using a Falcon SP8 confocal microscope (Leica Microsystems) with a 20x objective. SYFP2 was measured using an excitation laser at 524 nm and 540-600 nm bandpass filter, NucBlue was measured using the 405 nm laser and 415-460 nm bandpass filter, CellBrite was measured using a 670 nm excitation laser and a 750-800 nm bandpass filter.

**Image analysis for Quantification of Membrane Binding.** Confocal images were analyzed with Python v3.10 using the Scikit-Image v0.19.2, SciPy v1.8.1, NumPy v1.22.4, Pandas v1.4.2, OpenCV v4.5.0 and custom packages built upon these libraries. Cells were segmented based on the membrane (CellBrite Steady 685) and nuclear (NucBlue) signals. The nuclear signals were used to assign the membrane signal to the individual cell. First, each nucleus was identified as a single cell. Second, the membrane signal was assigned to the nearest nucleus and used to create a mask for the membrane of each cell. Third, the membrane masks were post-processed to exclude non-closed objects, such as cell debris or cells with incomplete

membrane staining as well as cells with more than one nucleus per cell. Fourth, the membrane masks were filled to additionally obtain the full cell masks. Fifth, for signal analysis, the total fluorescence in the SYFP2 channel was calculated for both the membrane and the full cell mask of each cell. Finally, the ratio between the membrane and the total cell signal was calculated for each cell. We used untransfected cells stained with NucBlue and CellBright steady 685 to assess background fluorescence. For the analysis, we included all transfected cells with total cell fluorescence above this background.

**Regression Model.** The effect of different circuit parts on the output expression in $HEK^{G12D}$, $HEK^{WT}$ as well as on the dynamic range ($HEK^{G12D}/HEK^{WT}$) was modeled using a Gaussian generalized linear model in R Studio v2023.06.0+421. Because the datasets were skewed toward low values, resulting in a loss of model accuracy at low values (Supplementary Fig. 10), a log transformation of all values was performed before running the models. Linearity and homoscedasticity assumptions were assessed using residual plots (Supplementary Fig. 11a-f). The goodness of fit was evaluated by comparing the fitted and measured values and calculating Pearson ($R^2$) and Spearman correlations using the cor() function in R Studio (Supplementary Fig. 11g-i). For the selection of the independent variables, we tested three models assuming different interactions between the response elements and the parts they express. (Supplementary Table 10). The model with the best correlation (Model 3) was selected to analyze the effect of the different circuit parts in Figure 5c. In addition to the primary variables of interest, we considered potential covariates such as experimental batch, transfection efficiency, and amount of NarX and NarL plasmids. While the experimental batch did not affect the correlation of the model, transfection efficiency and plasmid amounts were included in the regression to ensure the robustness of the model.

**Analysis of Flow cytometry data.**

Flow cytometry data analysis was performed using FlowJo software v10.8.0 (BD Life Sciences). The gating strategy is shown in (Supplementary Fig. 12a-d). When multiple cell lines with different transfection efficiencies were used, the cells were binned using the expression of the mCherry transfection control to include only cells with high transfection levels > $10^3$ (Supplementary Fig. 12d-e). We further validated that there was no direct correlation between transfection efficiency and normalized output in our experimental data (Supplementary Fig. 10). Absolute units (AU) of fluorescence were calculated by multiplying the number of positive cells (frequency of parent) with the mean expression of the fluorophore:

$$Frequency\ of\ Parent_{Fluorophor} * Mean_{Fluorophor}$$

Relative units (RU) of fluorescence were calculated by dividing the AU of the fluorophore of interest by the AU of the transfection control:

$$Fluorescence\ [RU] = \frac{Frequency\ of\ Parent_{Fluorophor\ of\ Interest} * Mean_{Fluorophor\ of\ Interest}}{Frequency\ of\ Parent_{Transfection\ Control} * Mean_{Transfection\ Control}}$$

For the dynamic range, the fold change between the target cell line (HEK$^{G12D}$ or HCT-116$^{WT}$; ON-state) and off-target cell line (HEK$^{G12D}$ or HCT-116$^{WT}$; OFF-state) was calculated. The error bars were calculated using error propagation rules:

$$Propagated\ Uncertainty = Fold\ Change * \sqrt{\left(\frac{SD_{ON-state}}{Mean_{ON-state}}\right)^2 + \left(\frac{SD_{OFF-state}}{Mean_{OFF-state}}\right)^2}$$

The flow cytometry histograms in Fig. 5d and Fig. 6e show all mCerulean-positive cells concatenated from the three biological replicates. The numerical values of the cell number, mean and frequency of parent can be found in Supplementary Tables 11 & 12.

**Analysis and Visualization of Screening Data.**

Data analysis and plotting of the data from the screening of different circuit parts (Fig. 5) and the screening of cancer cell lines (Fig. 6) were performed using R studio v2023.06.0+421. The data was imported from FlowJo analysis and labeled with the corresponding sample descriptors before calculating the mean and SD for the biological replicates. Plots were generated using ggplot2 v3.4.3 and cowplot v1.1.1. Large language models (ChatGPT3.5, ChatGPT4.0; OpenAI) were used to facilitate code writing.

**Statistical Analysis.**

In experiments comparing two groups, unpaired two-tailed Student's t-tests were used to assess significance (Fig. 1c & 6f and Supplementary Fig. 7d-l & 13a). When comparing three or more groups, an ordinary one-way ANOVA was used followed by a Dunnett's multiple comparison test when all groups were compared with a control column (Fig. 2 and Supplementary Fig. 5 & 7a-c) or a Tukey's test when all columns were compared (Supplementary Fig. 6). Significance in the regression model (Fig. 5c) was tested using a Wald test. Data was considered statistically significant at a p-value below 0.05. The number of replicates is provided in the figure captions. Each replicate was taken from a distinct sample. Apart from the large screening experiments (Fig. 5 & 6), data is representative of at least two experiments. The p-values, F-values, t-values, and degrees of freedom (df) from all statistical

comparisons are provided in Supplementary Table 13. Statistical analyses were performed using Prism 10 (GraphPad) or R Studio (v2023.06.0+421).

## Data and Code availability

All data supporting the findings and the code used to calculate membrane localization, analyze the screenings, and for the regression model are available from the corresponding author upon request.

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## Author Contributions

G.S. conceived the study, performed the majority of experiments, analyzed the data, and wrote the manuscript. L.N. planned and performed some experiments and analyzed data. Y.B. conceived the study, analyzed data, wrote the manuscript, and supervised the project.

# Competing Interests

YB is a shareholder and an employee of Pattern Biosciences

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# Figures

## Figure 1

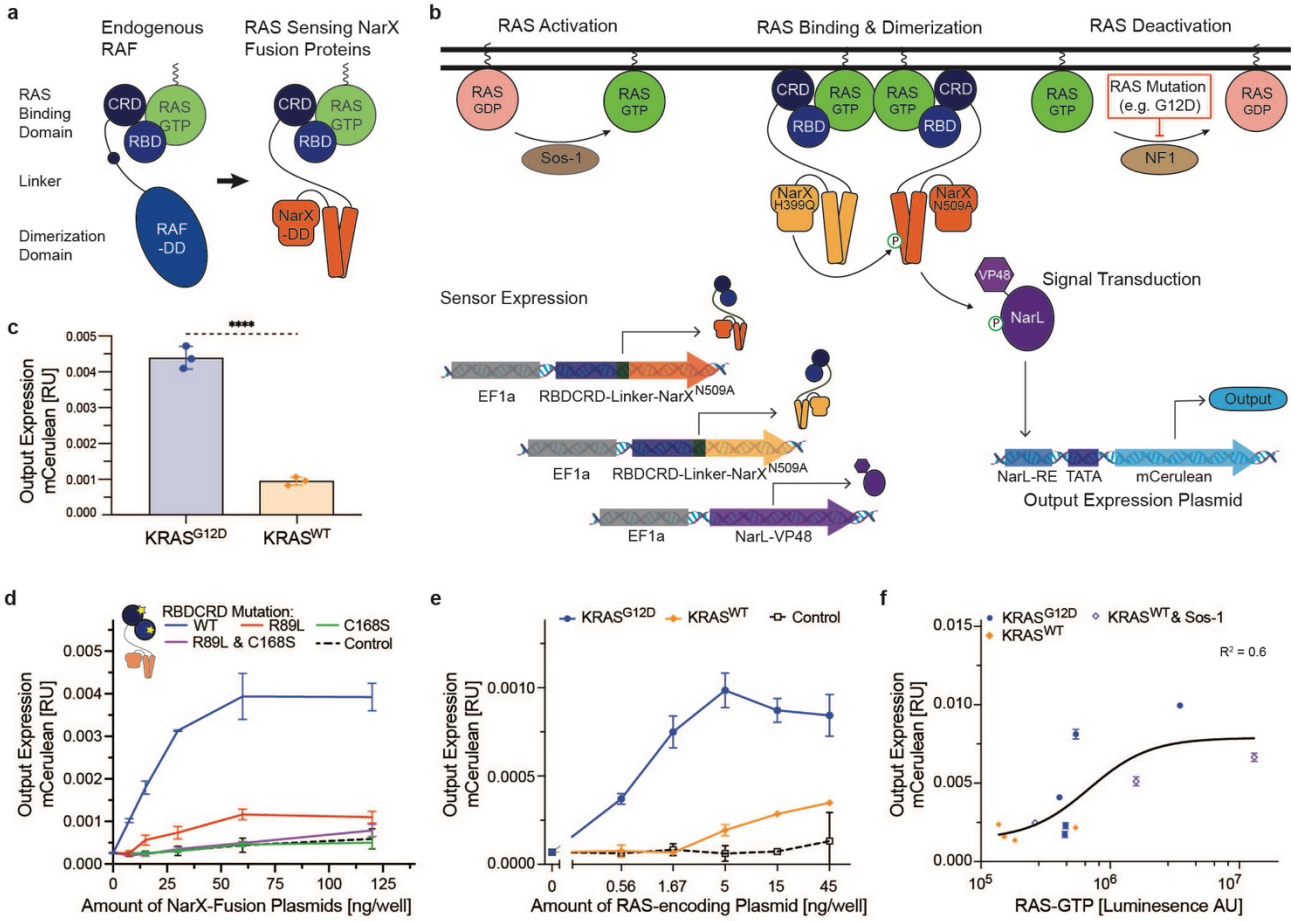


**Figure 1 | Design and Characterization of the RAS Sensor.** (**a**) Design of the binding component of the RAS sensor. Inspired by natural RAF (left), the RAS-binding component of the sensor (right) comprises RAS binding domain (RBDCRD), a linker, and a NarX-derived transphosphorylation domain. (**b**) Schematic of the RAS sensor composition and mechanism of action. The sensor's genetic payload is encoded on four plasmids. Two plasmids express the RAS-binding components: RBDCRD fused, respectively, to NarX$^{N509A}$ or NarX$^{H399Q}$. The third plasmid expresses NarL, and the fourth plasmid encodes the output protein (mCerulean) under the control of a NarL response element (NarL-RE) in front of a minimal promoter (TATA). Upon RAS activation, the RBDCRD domain of the RAS-binding components bind to RAS-GTP. This binding leads to a forced dimerization of the NarX domains and a transphosphorylation of NarX$^{N509A}$, in turn phorsphorylating NarL. Phosphorylated NarL binds its response element on the output plasmid, inducing the expression of the output protein. (**c**) Sensor activation by mutated RAS. The bar chart shows output expression in HEK293 cells co-transfected with the RAS Sensor and either KRAS$^{G12D}$ or KRAS$^{WT}$. (**d**) Dose-response curve and dependence of the RAS sensor on functional RAS binding. Output expression of RAS sensors with either RBDCRD wild-type (blue) or RBDCRD with R89L (red), C168S (green), or both (purple) mutations. The

dashed line represents conditions where the NarX-fusion plasmids were replaced with a non-coding plasmid (control). (**e**) Dependence of sensor output on RAS levels. Output expression of the RAS sensors measured with increasing amounts of $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (orange), or negative control (black) plasmid. (**f**) Input-output curve. Correlation of the output expression with the RAS-GTP levels in HEK293 cells, measured by a luminescence RAS-pulldown ELISA assay. To alter RAS-GTP levels, the cells were transfected with different amounts of either $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (orange), or $KRAS^{WT}$ + Sos-1 (purple) plasmids. Pearson`s correlation is shown as $R^2$. mCerulean output expression was measured by flow cytometry and normalized to a constitutively expressed mCherry transfection control. Mean values were calculated from biological triplicates. Error bars represent +/- SD. Significance was tested using an unpaired two-tailed Student's t-test. ****$p < 0.0001$.

## Figure 2

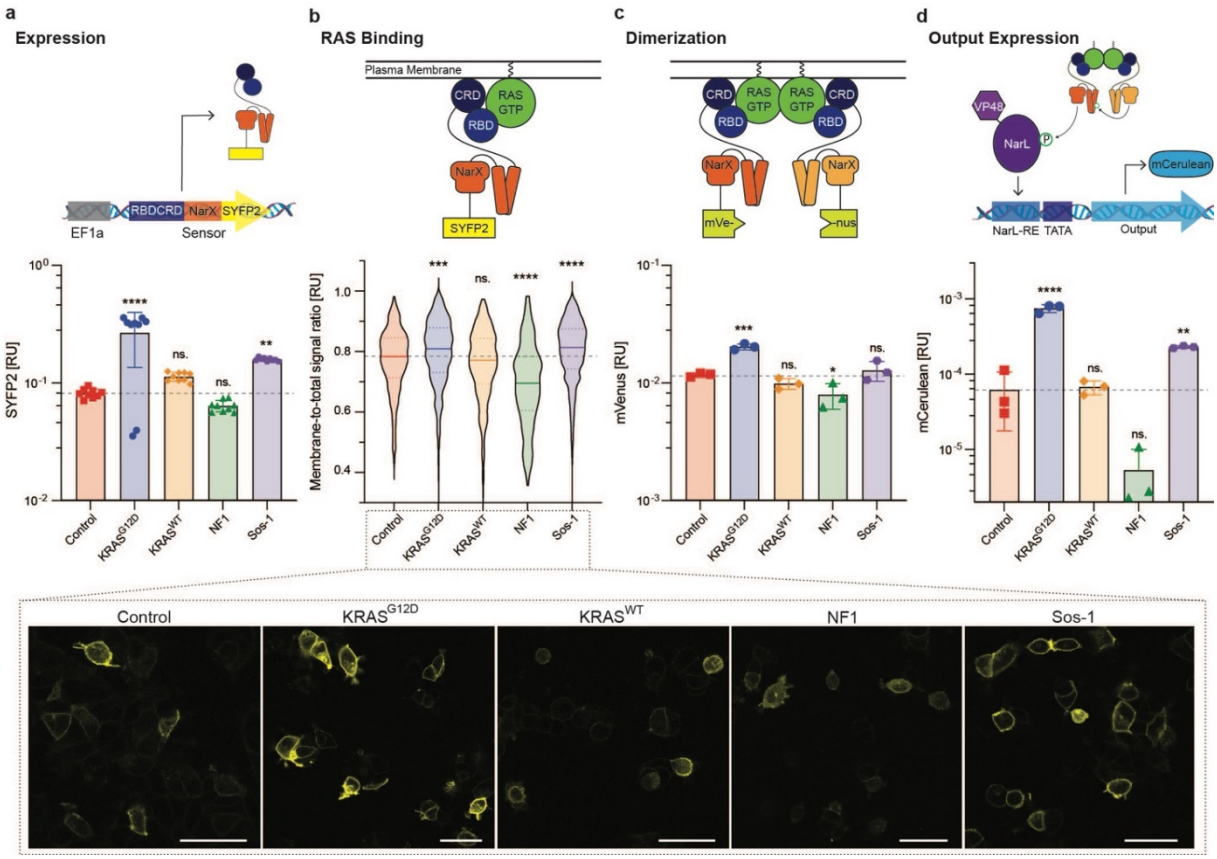


**Figure 2 | Mechanism of Action.** Effect of differential RAS activation on the steps considered necessary for RAS Sensor activation. RAS activation in HEK293 cells was manipulated by co-expressing $KRAS^{G12D}$, $KRAS^{WT}$, Sos-1 (a guanine nucleotide exchange factor that activates endogenous RAS), or NF1 (a GTPase-activating protein that deactivates endogenous RAS). In the control condition the cells are transfected with a non-coding plasmid, here it represents the endogenous RAS activation. Schematics on top of the graphs illustrate how and what part of the Mechanism was investigated. (**a**) Expression levels of the RBDCRD-NarX-SYFP2 fusion protein measured by flow cytometry in the presence of various KRAS modulators (x axis labels). (**b**) RAS binding of the RBDCRD-NarX-SYFP2 fusion protein approximated by calculating the ratio of membrane to total SYFP2 signal for each cell. Intracellular localization of SYFP2 was measured using confocal

microscopy. The micrographs below show representative images for each condition. Scale bars = 50 µm. (**c**) Dimerization of the NarX fusion proteins assessed by transfecting two complementary RBDCRD-NarX-split.mVenus fusions and measuring the mVenus fluorescence by flow cytometry. **(d**) Output expression after transfection with the full RAS Sensor measured by flow cytometry. In **a, c & d** the fluorescent signals were normalized to a constitutively expressed transfection control. Each symbol represents one biological replicate (**a**: n=9, **c-d**: n=3). The error bars represent +/- SD. In **b** the fluorescence at the membrane was normalized to the total fluorescence for each cell. Violin plots in **b** represent 560 ($KRAS^{G12D}$), 322 ($KRAS^{WT}$), 482 (Control), 226 (NF1), or 1194 (Sos-1) cells from three biological replicates. Significance was tested using an ordinary one-way ANOVA with Dunnett`s multiple comparisons to compare each condition with the control condition (endogenous RAS activation). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

## Figure 3

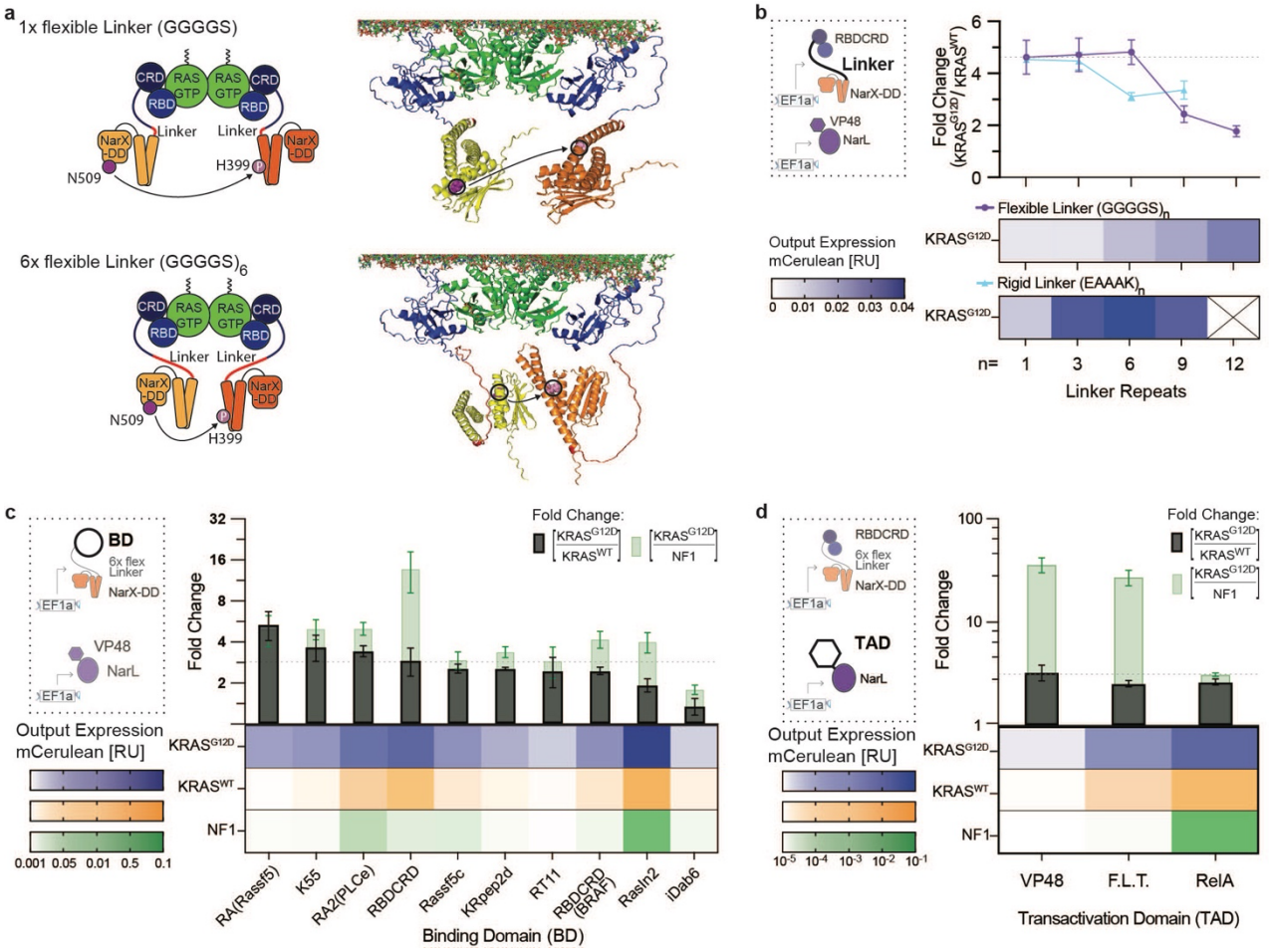


**Figure 3 | Tunability of the RAS sensor.** (**a**) 3D structure of the RAS Sensor dimerizing at the membrane. The structure of the RBDCRD-NarX fusion proteins (orange and yellow) was predicted using AlphaFold and aligned with existing NMR structures of RBDCRD (blue) bound to a KRAS-dimer (green) at the membrane. The ATP-binding site N509 (purple) and the phosphorylation site H399 (pink) are highlighted as spheres. On

the top RBDCRD and NarX are fused with a 1x GGGGS linker and on the bottom with a 6x GGGGS linker. (**b**) Effect of different linkers in the RBDCRD-NarX fusion protein of the RAS Sensor. The heatmaps show the output expression in HEK293 co-transfected with $KRAS^{G12D}$ when using different numbers of repeats of a flexible (GGGGS) or a rigid (EAAAK) linker in the RBDCRD-NarX fusion proteins. The graph above shows the corresponding fold change between output expression in cells with $KRAS^{G12D}$ and $KRAS^{WT}$. (**c**) Effect of different binding domains (BD) fused to NarX in the RAS Sensor. The heatmap shows the output expression in HEK293 co-transfected with 15 ng/well of $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (orange), or NF1 (green), a GTPase-activating protein that deactivates endogenous RAS. The bars above show the corresponding fold changes between cells with $KRAS^{G12D}$ and $KRAS^{WT}$ (black) or $KRAS^{G12D}$ and NF1 (green). (**d**) Effect of different transactivation domains (TAD) fused to NarL in the RAS Sensor. The heatmap shows the output expression in HEK293 co-transfected with $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (yellow) or NF1 (green). The bars above show the corresponding fold changes between cells with $KRAS^{G12D}$ and $KRAS^{WT}$ (black) or $KRAS^{G12D}$ and NF1 (green). mCerulean output expression was measured using flow cytometry and normalized to a constitutively expressed mCherry transfection control. Mean values were calculated from three (**b**) or two (**c-d**) biological replicates. Error bars were calculated using error propagation rules.

# Figure 4

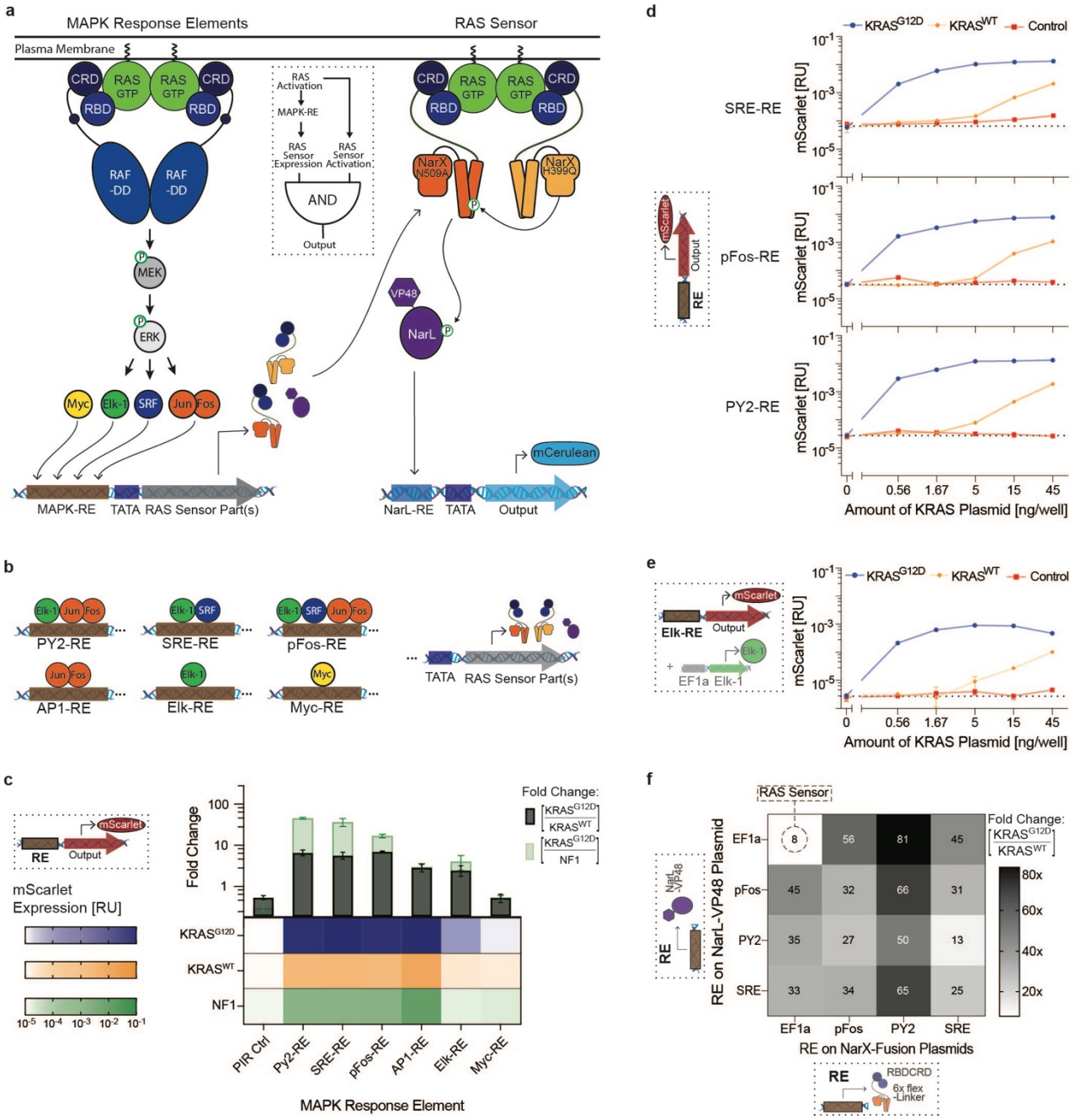


**Figure 4 | Design of Multi-Input RAS-targeting Circuits.** (**a**) Schematic of the RAS-targeting circuit with an AND-gate between mitogen-activated protein kinase (MAPK) sensors and the direct RAS Sensor. Dimerization of RAS activates the MAPK pathway and its downstream transcription factors. These transcription factors bind the synthetic response elements (RE), expressing the parts of the RAS Sensor. The RBDCRD-NarX proteins then bind activated RAS, dimerize, and propagate the signal to NarL, leading to output expression. The logic diagram of the resulting coherent feed-forward loop with AND-gate logic is shown in the dotted box. (**b**) Schematic of the transcription factor binding sites present in the response elements. Multiple repeats of the binding sites were placed upstream of a minimal promoter (TATA) driving expression of the RAS Sensor parts. (**c**) Expression levels with the MAPK response elements. The heatmap shows the direct

expression of mScarlet of the different REs in HEK293 cells co-transfected with 15 ng/well of either $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (orange), or NF1, a protein that deactivates endogenous RAS (green). The bars above show the corresponding fold changes between cells with $KRAS^{G12D}$ and $KRAS^{WT}$ (black) or $KRAS^{G12D}$ and NF1 (green). (**d**) RAS-dependency of the SRE-, pFos-, and PY2- response elements. Direct mScarlet expression of the response elements in HEK293 cells co-transfected with different amounts of $KRAS^{G12D}$ (blue), $KRAS^{WT}$ (orange), or non-coding control plasmids (red). (**e**) RAS-dependency of the Elk-RE when additionally overexpressing Elk-1. RAS titration as described in **d**. (**f**) Dynamic range of the RAS-targeting circuits. Fold change in mCerulean output expression between HEK293 co-transfected with 1.67 ng/well of $KRAS^{G12D}$ and $KRAS^{WT}$. In the RAS targeting circuits, NarL-VP48 and/or the RBDCRD-6xfL-NarX fusion proteins were expressed using different MAPK-REs or a constitutive promoter (EF1a). Fluorescent protein expression was measured by flow cytometry and normalized to a constitutively expressed transfection control. Mean values were calculated from two (**c-e**) or three (**f**) biological replicates. PY2: polyoma virus enhancer domain; SRE: Serum response element; pFos: minimal promoter of c-fos; AP1: activator protein 1; Elk: Ets-like protein; Myc: myelocytomatosis protein. Detailed response element design is shown in Supplementary Fig. 4.

# Figure 5

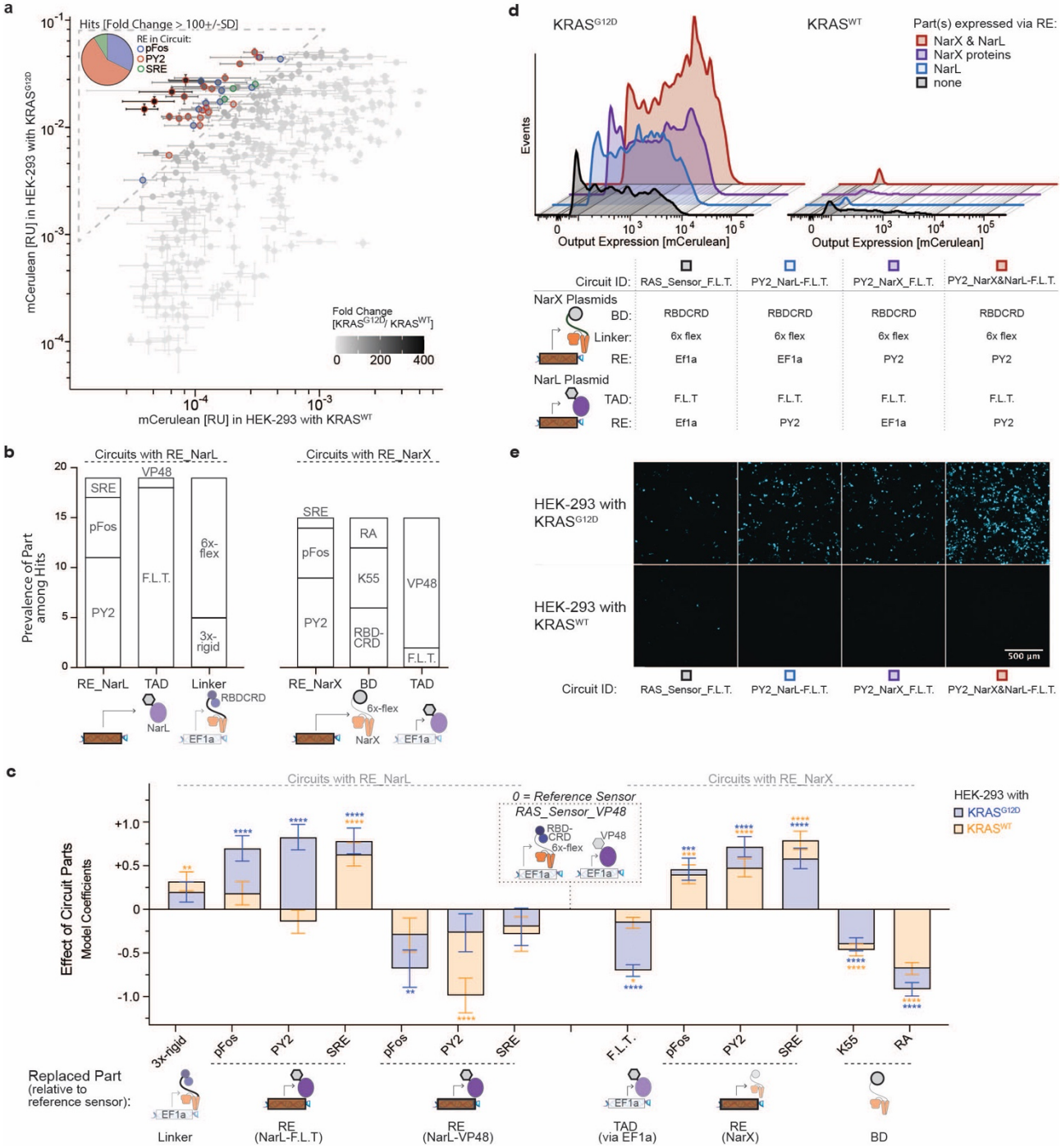


**Figure 5 | Modularity of the RAS-targeting Circuits.** (**a**) Screening of RAS circuit variants with different parts. mCerulean output expression in HEK293 co-transfected with 1.67 ng/well of KRAS$^{G12D}$ versus KRAS$^{WT}$ representing the ON- versus OFF-state of the screened circuits. The gray shading of the symbols represents the dynamic range (fold change between ON and OFF). Circuits with a high dynamic range (> 100+/-SD) are highlighted. The pie chart shows the prevalence of the response elements (RE) among the hits. (**b**) Prevalence of the circuit parts among the hits. On the left for all circuits where the RE expressed NarL (RE_NarL) and on the right where the RE expressed the NarX proteins (RE_NarX). TAD: transactivation domain, BD: binding domain. RA: Ras association domain of Rassf5 (**c**) Effect of different circuit parts on the output expression in

HEK293 with $KRAS^{G12D}$ (blue) and $KRAS^{WT}$ (orange) fitted using a generalized linear regression. The EF1a expressed RAS Sensor with RBDCRD, a 6x flexible linker in the NarX fusion proteins, and VP48 as TAD fused to NarL was set as the reference sensor. The graph shows the model coefficients, which can be interpreted as the effect on output expression when a part in the reference sensor is replaced by the part indicated on the x-axis. (**d**) Expression of all circuit parts via response elements. Fluorescence histograms of mCerulean-positive cells comparing ON- ($KRAS^{G12D}$) and OFF-state ($KRAS^{WT}$) of RAS circuits when either NarL (blue), the NarX proteins (violet), or all parts (red) are expressed via REs. The tested circuits contain RBDCRD as BD, a 6x flexible linker and F.L.T. as TAD, the parts that led to the hits with the highest dynamic range. The table below shows the parts used in each of the tested circuit. (**e**) Microscopy images showing the mCerulean expression of the conditions from **d**. mCerulean output expression was measured by flow cytometry and normalized to a constitutively expressed mCherry transfection control. Each circuit was measured in three biological replicates. Error bars represent +/- SD. Significance in **c** was tested using the Wald test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

# Figure 6

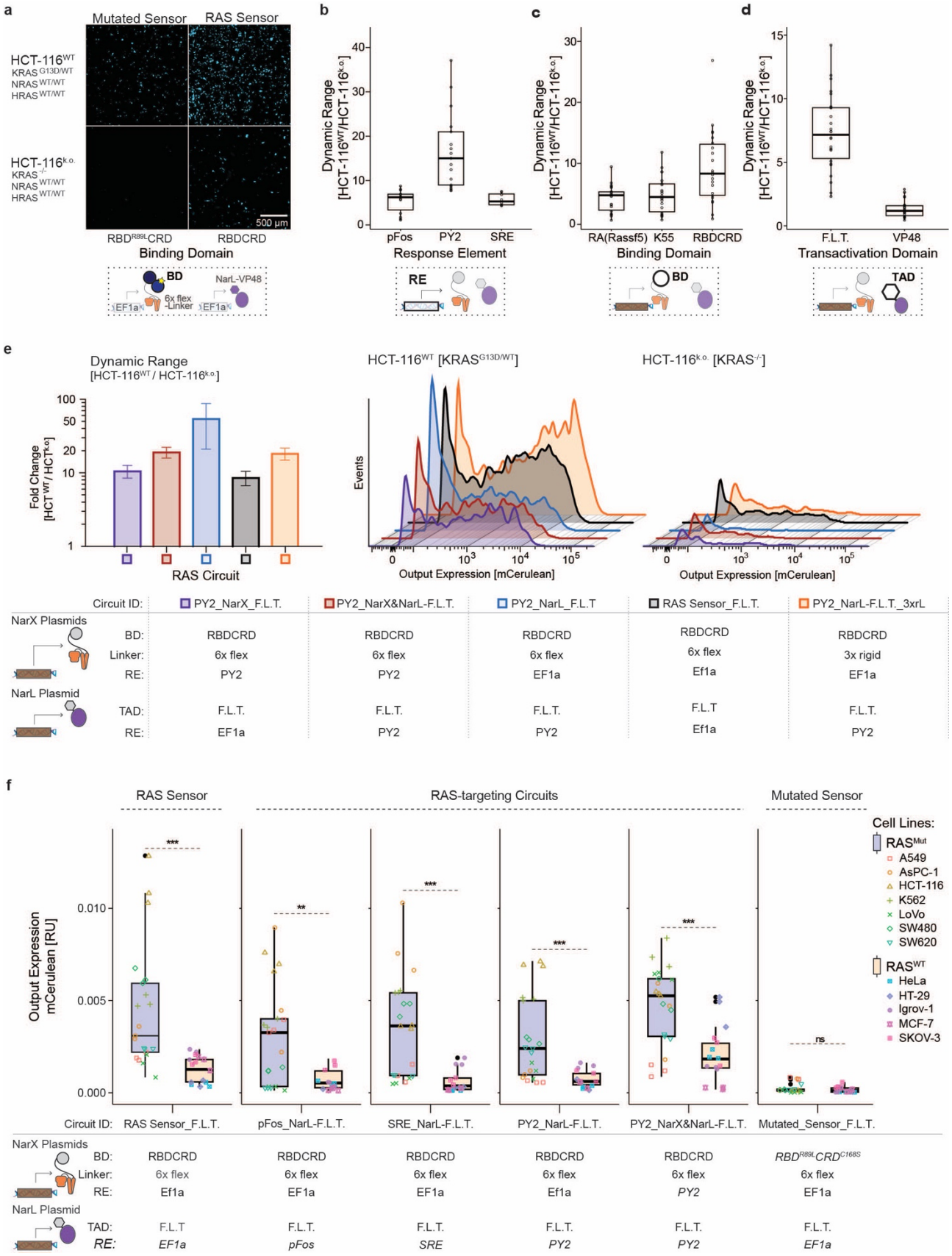
a
Mutated Sensor
RAS Sensor
HCT-116WT
KRAS G13D/WT
NRAS WT/WT
HRAS WT/WT
HCT-116k.o.
KRAS -/-
NRAS WT/WT
HRAS WT/WT
500 µm
RBDR89LCRD
RBDCRD
Binding Domain
BD
NarL-VP48
EF1a
6x flex -Linker
b
Dynamic Range [HCT-116WT/HCT-116k.o.]
pFos
PY2
SRE
Response Element
RE
c
RA(Rassf5)
K55
RBDCRD
Binding Domain
BD
d
F.L.T.
VP48
Transactivation Domain
TAD
e
Dynamic Range [HCT-116WT / HCT-116k.o.]
Fold Change [HCTWT / HCTk.o.]
RAS Circuit
HCT-116WT [KRASG13D/WT]
HCT-116k.o. [KRAS-/-]
Events
Output Expression [mCerulean]
Circuit ID:
PY2_NarX_F.L.T.
PY2_NarX&NarL-F.L.T.
PY2_NarL_F.L.T
RAS Sensor_F.L.T.
PY2_NarL-F.L.T._3xrL
NarX Plasmids
BD:
Linker:
RE:
NarL Plasmid
TAD:
RE:
RBDCRD
6x flex
PY2
EF1a
F.L.T.
3x rigid
Ef1a
f
RAS Sensor
RAS-targeting Circuits
Mutated Sensor
Output Expression mCerulean [RU]
Cell Lines:
RASMut
A549
AsPC-1
HCT-116
K562
LoVo
SW480
SW620
RASWT
HeLa
HT-29
Igrov-1
MCF-7
SKOV-3
Circuit ID:
RAS Sensor_F.L.T.
pFos_NarL-F.L.T.
SRE_NarL-F.L.T.
PY2_NarL-F.L.T.
PY2_NarX&NarL-F.L.T.
Mutated_Sensor_F.L.T.
RBDR89LCRDC168S
ns

**Figure 6 | Translation into Cancer Cells.** (**a**) RAS Sensor activation in colon cancer cells. Microscopy images of the mCerulean output expression in HCT-116 wild-type cells harboring a homozygous $KRAS^{G13D}$ mutation ($HCT\text{-}116^{WT}$; top row) and HCT-116 KRAS knock-out cells ($HCT\text{-}116^{k.o.}$; bottom row) transfected with the initial RAS Sensor (right) or a RAS Sensor with an R89L mutation in the Ras binding domain (left). (**b-d**) Effect of different circuit parts in colon cancer cells. Boxplots of dynamic range of different RAS-targeting circuits grouped by to the circuit parts of interest they contain. The circuit parts investigated were: the response elements in **b**, the binding domain fused to the NarX proteins in **c**, and the transactivation domain fused to NarL in **d**. Each black circle represents a different RAS circuit. (**e**) Best performing RAS-targeting circuits in colon cancer cells. The parts used in each RAS circuit are listed in the table below. The bar graph shows the dynamic range, while the fluorescence histograms show mCerulean-positive cells obtained in the On- ($HCT\text{-}116^{WT}$) and Off-state ($HCT\text{-}116^{k.o.}$) of the circuits. (**f**) RAS-targeting circuits are classifiers for cells with mutated RAS. Output expression of RAS-targeting circuits in different cancer cell lines with ($RAS^{MUT}$ = blue) or without ($RAS^{WT}$ = orange) mutation leading to increased RAS activation. The colored symbols represent biological replicates of the different cell lines. The parts used in each RAS circuit are indicated in the table below. mCerulean output expression was measured by flow cytometry and normalized to a constitutively expressed mCherry transfection control. Dynamic range was calculated as fold change between normalized output expression in $HCT\text{-}116^{WT}$ and $HCT\text{-}116^{k.o.}$. Each circuit was measured in three biological replicates. Error bars were calculated using error propagation rules. Significance was tested using an unpaired two-tailed Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.