SMALL GTPASES
2021, VOL. 12, NO. 1, 67–78
https://doi.org/10.1080/21541248.2019.1611168

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RESEARCH PAPER

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Anchorage-independent growth conditions reveal a differential SOS2 dependence for transformation and survival in *RAS*-mutant cancer cells

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ABSTRACT

The RAS family of genes (*HRAS, NRAS*, and *KRAS*) is mutated in around 30% of human tumours. Wild-type RAS isoforms play an important role in mutant RAS-driven oncogenesis, indicating that RasGEFs may play a significant role in mutant RAS-driven transformation. We recently reported a hierarchical requirement for SOS2 in mutant RAS-driven transformation in mouse embryonic fibroblasts, with KRAS>NRAS>HRAS (Sheffels et al., 2018). However, whether *SOS2* deletion differentially affects mutant RAS isoform-dependent transformation in human tumour cell lines has not been tested. After validating sgRNAs that efficiently deleted *HRAS* and *NRAS*, we showed that the differential requirement for SOS2 to support anchorage-independent (3D) growth, which we previously demonstrated in MEFs, held true in cancer cells. *KRAS*-mutant cells showed a high dependence on SOS2 for 3D growth, as previously shown, whereas *HRAS*-mutant cells did not require SOS2 for 3D growth. This differential requirement was not due to differences in RTK-stimulated WT RAS activation, as SOS2 deletion reduced RTK-stimulated WT RAS/PI3K/AKT signalling in both *HRAS* and *KRAS* mutated cell lines. Instead, this differential requirement of SOS2 to promote transformation was due to the differential sensitivity of RAS-mutated cancer cells to reductions in WT RAS/PI3K/AKT signalling. *KRAS* mutated cancer cells required SOS2/PI3K signaling to protect them from anoikis, whereas survival of both *HRAS* and *NRAS* mutated cancer cells was not altered by SOS2 deletion. Finally, we present an integrated working model of SOS signaling in the context of mutant KRAS based on our findings and those of others.

ARTICLE HISTORY
Received 12 December 2018
Revised 15 April 2019
Accepted 20 April 2019

KEYWORDS
RAS; SOS2; cancer; son of sevenless; MAPK; PI3K; anoikis

RAS in cancer

The RTK–RAS pathway is among the most commonly mutated pathways in cancer[1]. The three RAS genes, *HRAS*, *NRAS*, and *KRAS*, are the most commonly mutated gene family, with mutations found in around 30% of human tumours [2]. Within the RAS gene family, *KRAS* is the most frequently mutated, accounting for 85% of RAS-driven cancers [2], including 95% of pancreatic cancers [3], 42% of colon cancers [4], and 20–30% of lung cancers [5], which are the top three causes of cancer-related death in the United States [2,6]. *H*- and *NRAS* mutations are common in other cancer types, including bladder cancers and skin cancers, respectively [7]. Due to their prevalence in tumours, many efforts have been made to develop therapies to directly target RAS. Thus far, no effective KRAS inhibitors have been developed, with the exception of cross-linking compounds targeting the *KRAS* (G12C) mutant [5,8–10]. Targeting other RTK pathway members has also had limited success in RAS-driven cancers, due to high toxicities and resistance arising from the disruption of feedback mechanisms in the RTK–RAS–RAF pathway [11]. Together, these difficulties in developing treatments for RAS-driven tumours indicate a need for novel therapeutic strategies.

Several studies have found that non-mutated wild-type RAS proteins play an important role in modulating downstream effector signalling during mutant RAS-driven tumorigenesis, but this role differs for the wild-type allele of the mutated RAS gene versus the other two non-mutated RAS family members. Cancers driven by mutant KRAS often show loss of the wild-type *KRAS* allele [12–14]; in *KRAS*-mutant tumours loss of heterozygosity (LOH) at the KRAS locus correlates with increased tumour growth and shortened overall survival [15]. These data suggest that in *KRAS*-mutant tumors, wild-type KRAS may have a tumour suppressor role [16–18], a hypothesis which has been supported by observations *in vitro* [19,20] and in vivo with mouse models [20–22].

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In contrast, the two non-mutated RAS family members may play a tumour-promoting role in *RAS*-mutant tumours. In *RAS*-mutated cancer cell lines, wild-type RAS isoforms promote mutant RAS-driven proliferation and transformation [23–26]. Wild-type RAS isoforms may contribute to oncogenic signalling through their ability to activate effector pathways that the mutant isoform does not strongly activate, making the cellular outcome a product of combined signalling by wild-type and mutant RAS [27]. Activation of wild-type RAS requires interaction with a RasGEF (guanine nucleotide exchange factor), such as Son of Sevenless 1 and 2 (SOS1 and SOS2), which are required for normal RTK-dependent RAS activation [28]. SOS1 can also be activated by allosteric binding of RAS-GTP [29], providing a potential link between constitutively active mutant RAS and wild-type RAS activation. The importance of wild-type RAS in mutant RAS-driven cancer indicates that SOS1 and SOS2 may be novel therapeutic targets for RAS-driven tumours. Defining their independent and combined roles in mutant RAS-dependent oncogenesis is key to determining the value of SOS1 and SOS2 as therapeutic targets in RAS-mutated tumours.

SOS2 is hierarchically required for transformation in RAS-mutant cancer cells

We recently showed that in immortalized mouse embryo fibroblasts (MEFs) there was a hierarchal requirement for SOS2 in RAS-driven transformation (KRAS > NRAS > HRAS), with KRAS being the most SOS2-dependent RAS isoform [30]. We also demonstrated that *KRAS*-mutant lung and pancreatic cancer cell lines required SOS2 to fully maintain their transformed phenotype. To complement our recent findings in *KRAS*-mutant cancer cells, we investigated whether *HRAS*- and *NRAS*-mutant cancer cell lines were dependent on SOS2 to maintain their transformed phenotype. We began our investigation using the *HRAS* (G12V) mutant T24 bladder cancer cell line and the *NRAS* (Q61H) mutant RD rhabdomyosarcoma line, as they were previously used to establish the contribution of wild-type RAS signalling to anchorage-dependent proliferation of RAS-mutant cancer lines by Young et al. [24] .

Before testing *HRAS* and *NRAS* mutated cancer cell lines for SOS2 dependence, we first established the dependence of these cell lines on oncogenic RAS expression for both proliferation and transformation. To identify sgRNAs that would allow us to efficiently delete oncogenic *HRAS* or *NRAS* using CRISPR/Cas9, we tested four putative targeting sgRNAs for each RAS isoform from a previously published genome-wide CRISPR screen, which used 18–20 sgRNAs per gene [31]. To target *HRAS*, we selected four sgRNAs that showed specific growth inhibition in a MET-amplified cancer cell line that showed a strong HRAS dependence, and for *NRAS* sgRNAs we tested four sgRNAs that showed specific growth inhibition in an *NRAS*-mutant cancer cell line.

Out of the four *HRAS*-targeting sgRNAs we tested, three (#1, #2, and #3) successfully deleted *HRAS* in >90% of cells, as indicated by the decrease in protein abundance compared to a non-targeting (NT) sgRNA construct (Figure 1). We next examined whether *HRAS* deletion by each sgRNA would inhibit anchorage-dependent (2D) proliferation and anchorage-independent (3D) transformation in *HRAS*-mutant T24 cells. For each sgRNA that successfully deleted *HRAS*, we observed a significant decrease in anchorage-dependent (2D) proliferation, as expected, though proliferation was not completely blocked. *HRAS* deletion also led to the reversion of the transformed phenotype, as measured by a cancer stem cell (CSC) frequency assay [32]. Here, serially diluted T24 cells were seeded in ultra-low attachment 96-well flat-bottomed plates (1–1000 cells/well), cultured for 7–10 days, and scored for the formation of cancer spheres. Wells containing cancer spheres that had grown to a diameter greater than 100 μm were scored as positive, and the frequency of cancer stem cells in the population of T24 cells was then calculated by extreme limiting dilution analysis [33]. When *HRAS* was deleted, the frequency of cancer stem cells decreased, indicating a dependence on HRAS for transformation. As sgRNA #3 had the most consistent effect on HRAS abundance and HRAS-dependent proliferation and transformation, we chose this sgRNA for further studies.

In *NRAS*-mutant RD cells, three out of four of the NRAS sgRNAs tested (#1, #2, and #4) successfully deleted *NRAS*, again as assessed by protein abundance compared to a non-targeting construct. Similar to what we observed in T24 cells following *HRAS* deletion, *NRAS* deletion reduced anchorage-dependent proliferation in RD cells. Furthermore, *NRAS* deletion also reduced, but did not completely block, anchorage-independent (3D) growth of cancer spheroids, indicating that in RD cells, transformation is not fully dependent on mutant NRAS expression. For *NRAS*, sgRNA #4 showed the most consistent decrease in protein abundance and so was chosen for further studies. These results indicate that *HRAS*-mutant T24 cells and *NRAS*-mutant RD cells are both dependent on mutant RAS expression for full 2D and 3D growth.

Having established sgRNAs that allow us to efficiently delete *HRAS* and *NRAS* to use as positive controls, we then investigated the effect of deleting *SOS2* using one of two

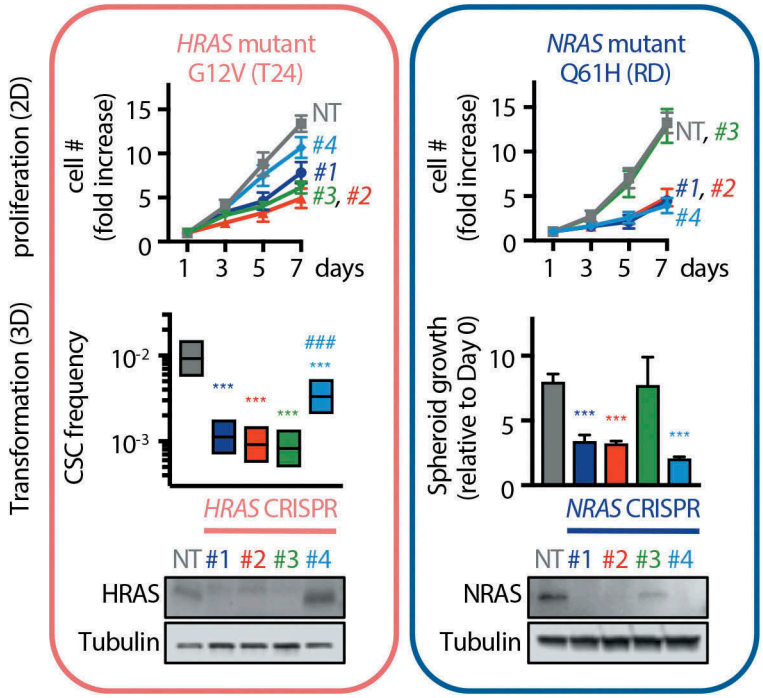

Figure 1. Mutant RAS is required for 2D and 3D growth in *HRAS* and *NRAS*-mutant cancer cells. (a) *HRAS* (G12V) mutant T24 bladder cancer cells were transduced with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT) or one of four sgRNAs designed to target *HRAS*. Infected cells were assessed for 2D proliferation (top) and cancer stem cell frequency as a measure of transformation (middle). Whole cell lysates (WCLs) were analysed by Western blotting with antibodies specific for HRAS and β-actin (bottom). (b) *NRAS* (Q61H) mutant RD rhabdomyosarcoma cells were transduced with lentiviruses with Cas9 and either a non-targeting sgRNA (NT) or one of four sgRNAs designed to target NRAS. Infected cells were assessed for 2D proliferation (top) and 3D spheroid growth (middle). Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for NRAS and β-actin (bottom). Data are mean ± SD from three independent experiments. Blots and images are representative of 3 independent experiments. Statistical significance was determined by ANOVA using the Tukey's method to correct for multiple comparisons. * $P < 0.05$, *** $P < 0.001$ versus NT; ### $P < 0.001$ versus HRAS #1, #2, #3.

different sgRNAs on proliferation and transformation in T24 cells, RD cells, and H358 cells, a *KRAS*-mutated cancer cell line. We first confirmed efficient SOS2 and mutant RAS deletion by the appropriate sgRNA constructs by Western blot (Figure 2, bottom), which did not alter the expression of SOS1 or β-actin. In each cell line, deletion of the mutated *RAS* gene significantly reduced both anchorage-dependent (2D) proliferation and anchorage-independent (3D) transformation, confirming the *RAS* dependence of each cell line for both 2D and 3D growth. Consistent with our previous observations using MEFs, *SOS2* deletion had no effect on 2D proliferation in cells expressing mutated *HRAS, NRAS*, or *KRAS* (Figure 2, top), indicating that mutant RAS-driven anchorage-dependent growth is independent of SOS2.

When we assessed anchorage-independent transformation, we observed a differential effect of *SOS2* deletion on cellular transformation that was again similar to our previous observations in MEFs. *SOS2* deletion had no effect on 3D growth in *HRAS*-mutant T24 cells, consistent with our previous observations in MEFs (Figure 2, middle). In *NRAS*-mutant RD cells, *SOS2* deletion led to a non-significant decrease in 3D growth compared to the non-targeting sgRNA construct, indicating *NRAS*-mutant cancer cells do not require SOS2 for 3D growth, but may have an intermediate dependence between *HRAS*- and *KRAS*-mutant cells similar to the intermediate dependence established in MEFs. In *KRAS*-mutated H358 cells, *SOS2* deletion significantly reduced transformation, as we had previously observed in *KRAS* mutated YAPC pancreatic cancer cells [30].

To confirm that the differential requirement for SOS2 in promoting RAS isoform-dependent transformation in human cancer cell lines was generalizable across multiple *RAS* mutated cancer cell lines, we tested the effects of *SOS2* deletion on anchorage-independent growth in six more cancer cell lines, two each expressing mutated *HRAS, NRAS*, or *KRAS* (Figure 3, see Table 1 for cell line information). As expected, we found that *SOS2* was necessary for full anchorage-independent growth for in each of the additional *KRAS*-mutated cell lines we tested (H23 and SW620) but that *SOS2* deletion had no effect on anchorage-independent growth in either of the *HRAS*-mutant

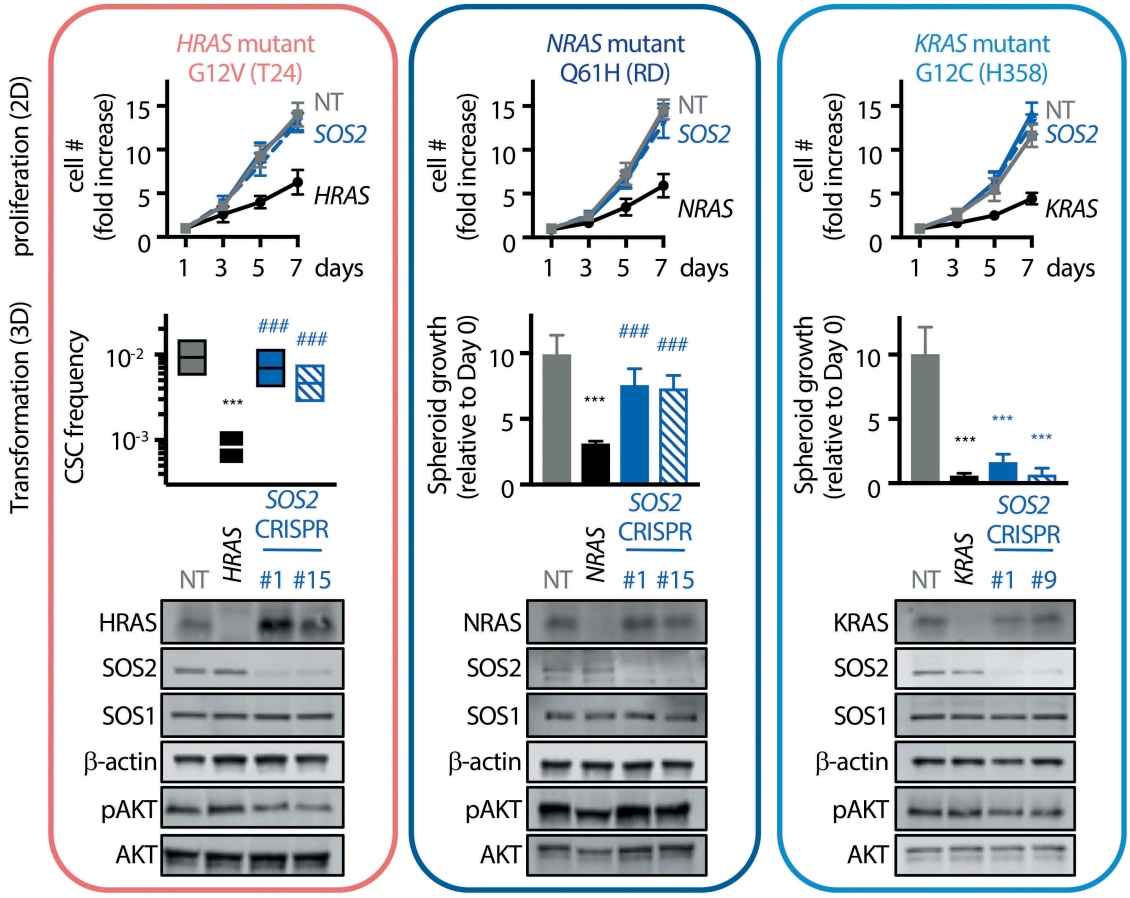

Figure 2. *KRAS*-mutant cancer cells depend on SOS2 for anchorage-independent, but not anchorage-dependent, growth. *HRAS* (G12V) mutant T24 bladder cancer cells (salmon), *NRAS* (Q61H) mutant RD rhabdomyosarcoma cells (dark blue), or *KRAS* (G12C) mutant H358 non-small cell lung cancer (NSCLC) cells (light blue) were transduced with lentiviruses expressing Cas9 and either an NT sgRNA, an sgRNA targeting mutant *RAS*, or one of two sgRNAs targeting *SOS2*. Post-selection, cells were assessed for 2D proliferation (top) and 3D transformation (middle). WCLs were analysed by Western blotting with antibodies specific for SOS1, SOS2, HRAS, NRAS, KRAS, β-actin, pAKT, and AKT (bottom). Data are mean ± SD from three independent experiments. Blots and images are representative of three independent experiments. Statistical significance was determined by ANOVA using the Tukey's method to correct for multiple comparisons. * $P < 0.05$, *** $P < 0.001$ versus NT; ### $P < 0.001$ versus mutant *RAS* deletion.

cell lines (RL95-2 and NCI-H1915). We again saw an intermediate SOS2 dependence in *NRAS*-mutant cell lines. While *SOS2* deletion showed no effect on oncogenic transformation in SK-Mel-2 cells, we observed a significant decrease in anchorage-independent growth in NCI-H1299 NSCLC cells. These data are consistent with the intermediate SOS2 dependence on *NRAS*-driven transformation we had originally observed in MEFs.

We had previously described an RTK/SOS2/WT RAS/PI3K signalling pathway that was important for transformation in MEFs expressing mutated *KRAS*, and demonstrated that in *KRAS* mutated YAPC pancreatic cancer cells SOS2 is necessary for full RTK-dependent PI3K/AKT pathway activation. To determine whether this held generally true in *RAS* mutated cancer cells, we assessed AKT phosphorylation in cycling T24, RD, and H358 cells. We found a significant decrease in AKT phosphorylation in both *HRAS* mutated T24 cells and in *KRAS* mutated H358 cells (Figure 2). In contrast, AKT phosphorylation was not reduced by *SOS2* deletion in RD cells, perhaps due to their already high levels of AKT phosphorylation (Figure 2, middle) and consistent with previous reports where AKT phosphorylation was shown to be independent of WT RAS in RD cells [24]. To determine whether the decreased AKT phosphorylation we observed was due to reduced RTK signalling, we assessed EGF-stimulated PI3K/AKT signalling in T24 and H358 cells after overnight serum deprivation. We found that in both T24 and H358 cells, EGF-stimulated AKT phosphorylation was significantly reduced after *SOS2* deletion (Figure 4).

We further assessed whether *SOS2* was required for RTK-stimulated WT RAS activation in *HRAS* mutated (T24) and in *KRAS* (H23) mutated cancer cells (Figure 5) using RAS-binding domain (RBD) pull-downs after

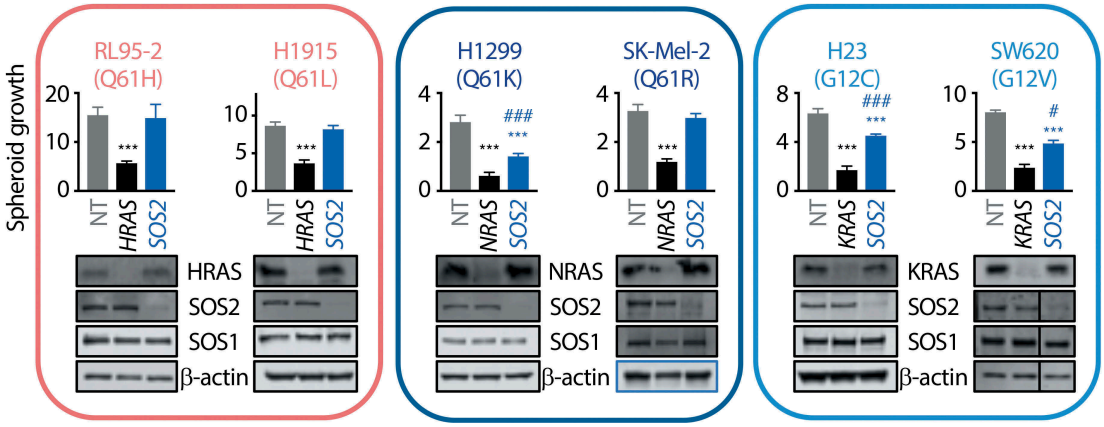

Figure 3. There a differential requirement for SOS2 to promote anchorage-independent growth in *RAS*-mutant cancer cell lines. *HRAS* (Q61H) mutant RL95-2 endometrial carcinoma cells and *HRAS* (Q61L) mutant NCI-H1915 NSCLC cells (salmon), *NRAS* (Q61K) mutant NCI-H1299 NSCLC cells and *NRAS* (Q61R) mutant SK-Mel-2 melanoma cells (dark blue), or *KRAS* (G12C) mutant H358 NSCLC cells and *KRAS* (G12V) mutant SW620 colorectal adenocarcinoma cells (lite blue) were transduced with lentiviruses expressing Cas9 and either an NT sgRNA, an sgRNA targeting mutant *RAS*, or an sgRNA targeting *SOS2*. Post-selection, cells were assessed for and 3D transformation. WCLs were analysed by Western blotting with antibodies specific for SOS1, SOS2, HRAS, NRAS, KRAS, and β-actin. Data are mean ± SD from three independent experiments. Blots and images are representative of three independent experiments. Images of SOS1, SOS2, and β-actin Western blots for SW620 cells were assembled from separate parts of the same image, as these samples were separated on the original Western blots (black line). Statistical significance was determined by ANOVA using the Tukey's method to correct for multiple comparisons. *** $P < 0.001$ versus NT; ### $P < 0.05$, ### $P < 0.001$ versus mutant *RAS* deletion.

Table 1. RAS mutation status and tissue of origin of the cancer cell lines studied.

Cell line	RAS mutation	Origin
T24	HRAS G12V	Transitional cell carcinoma
RL95-2	HRAS Q61H	Endometrial carcinoma
NCI-H1915	HRAS Q61L	NSCLC
RD	NRAS Q61H	Muscle rhabdomyosarcoma
NCI-H1299	NRAS Q61K	NSCLC
SK-MEL-2	NRAS Q61R	Melanoma
NCI-H358	KRAS G12C	NSCLC
NCI-H23	KRAS G12C	NSCLC
SW620	KRAS G12V	Colorectal adinocarcinoma

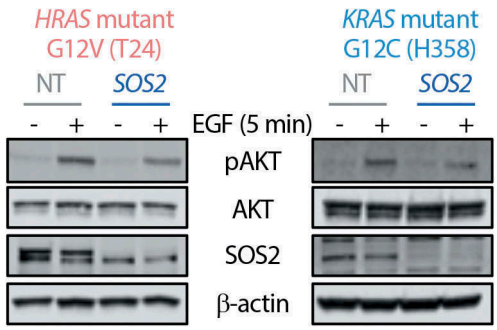

Figure 4. SOS2 is required for full RTK-stimulated AKT phosphorylation in *RAS* mutated cancer cells. *HRAS* (G12V) mutant T24 bladder cancer cells or *KRAS* (G12C) mutant H358 NSCLC cells were transduced with lentiviruses expressing Cas9 and either an NT sgRNA, an sgRNA targeting mutant *RAS*, an sgRNA targeting *SOS2*. Post-selection, cells were starved overnight and then stimulated with EGF 100 ng/mL for 5 min prior to lysis. Multiplex western blotting for pAKT (Ser[472]), AKT, SOS2, and β-actin was performed on a LI-COR Odyssey machine.

overnight serum deprivation. In *KRAS* mutated cancer cells, we found that *SOS2* deletion inhibited RTK-stimulated HRAS activation and reduced RTK-stimulated NRAS activation, showing the requirement for *SOS2* in WT RAS activation in the setting of mutated KRAS. Furthermore, in *HRAS* mutated cancer cells, we observed a reduction in RTK-stimulated KRAS activation following *SOS2* deletion. In contrast, *SOS2* deletion had no effect on basal WT RAS activation in cells expressing either mutated *KRAS* or *HRAS*. These data confirm the importance of SOS2 to RTK-stimulated WT RAS activation in *RAS* mutated cancer cells.

During oncogenic transformation, one of the major roles of the PI3K/AKT pathway is to promote survival by protecting cancer cells from anoikis, a form of apoptosis associated with loss of extracellular matrix (ECM) contact which must be overcome by cancer cells in order for them to grow in an anchorage-independent environment and to invade and metastasize [34,35]. To determine whether SOS2 was required for anchorage-independent survival, *HRAS*, *NRAS*, or *KRAS* mutated cancer cells expressing either a NT sgRNA or sgRNAs that deleted either SOS2 or oncogenic RAS were seeded as spheroids in 96-well ultra-low attachment round-bottomed plates and counted using trypan blue to assess cell viability 4 h after plating. As expected, oncogenic *RAS* deletion reduced cell

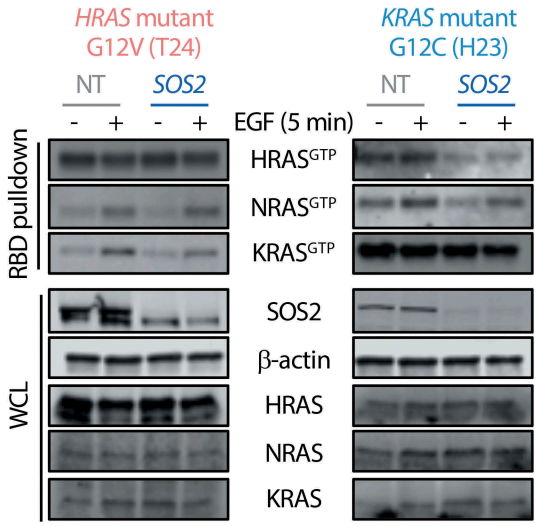

Figure 5. SOS2 is required for full RTK-stimulated WT RAS activation in *RAS* mutated cancer cells. *HRAS* (G12V) mutant T24 bladder cancer cells or *KRAS* (G12C) mutant H23 NSCLC cells were transduced with lentiviruses expressing Cas9 and either an NT sgRNA, an sgRNA targeting mutant *RAS*, or an sgRNA targeting *SOS2*. Post-selection, cells were starved overnight and then stimulated with EGF 100 ng/mL for 5 min prior to lysis. Lysates were subjected to GST-RAS binding domain pull-downs and were analysed by Western blotting with an antibody specific for HRAS, NRAS, or KRAS to assess activation (GTP loading) of endogenous RAS. WCLs were subjected to Multiplex Western blotting for HRAS, NRAS, KRAS, SOS2, and β-actin on a LI-COR Odyssey machine.

viability for all RAS mutated cancer cells. In contrast, *SOS2* deletion reduced cell viability in *KRAS* mutated cancer cells, but not *HRAS* or *NRAS* mutated cancer cells (Figure 6(a)). These data are consistent with the differential requirement for *SOS2* to support the transformation of *KRAS* mutated cancer cells (see Figures 2 and 3). We further assessed the effect of *SOS2* deletion over time on anchorage-independent cell survival in *HRAS* versus *KRAS* mutated cancer cells, and found not only that SOS2 was preferentially required for anchorage-independent survival in KRAS mutated cancer cells, but that *SOS2* deletion reduced cell survival under anchorage-independent conditions similar to *KRAS* deletion (Figure 6(b)).

Taken together, these results demonstrate the differential dependence on SOS2 for RAS-dependent transformation originally established in MEFs [30] is also generally applicable in *RAS*-mutated cancer cell lines. The ability of *HRAS* and *NRAS*-mutant cells to grow in 3D conditions in the absence of SOS2 indicates that SOS2 is likely not an effective therapeutic target in HRAS- and NRAS-mutant tumours. It also indicates that promising drug targets like SOS2 [33], RTKs [36–39], or Shp2 [40–42], which synergize with MEK inhibition in *KRAS*-mutant cancer cells, may not have the same effect in HRAS and NRAS-driven cancers, suggesting a continued need for new therapeutic strategies for these tumors.

In addition to demonstrating the differential requirement for SOS2 in RAS-mutant cancer cell

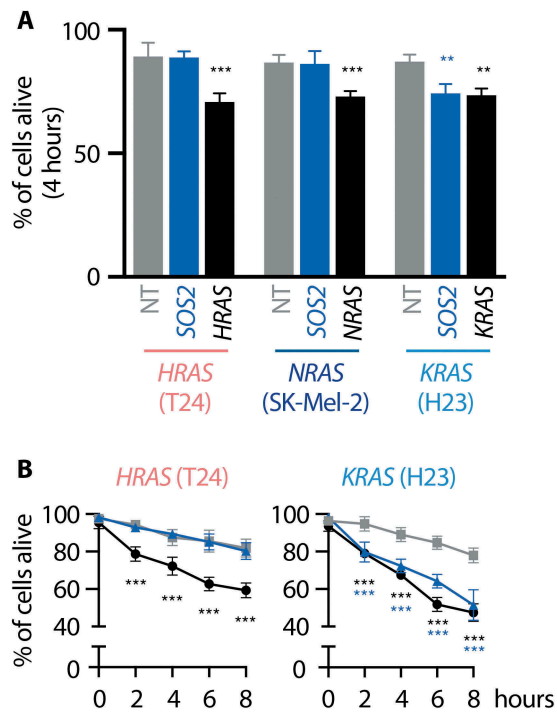

Figure 6. *HRAS* (G12V) mutant T24 bladder cancer cells, and *NRAS* (Q61R) mutant SK-Mel-2 melanoma cells, or *KRAS* (G12C) mutant H23 NSCLC cells were transduced with lentiviruses expressing Cas9 and either an NT sgRNA, an sgRNA targeting mutant *RAS*, or an sgRNA targeting *SOS2*. Post-selection, cells were counted using trypan blue to assess cell viability at time 0, and then, plated in 96-well ultra-low attachment plates for 4 h (a) or for 0,2, 4, 6, or 8 h (b). Forming spheroids were dislodged into a single cell suspension by pipetting, and cells were counted using trypan blue to assess cell viability. ** $P < 0.01$, *** $P < 0.001$ versus NT.

lines, our findings also emphasize the importance of using 3D growth assays to screen for vulnerabilities in cancers. Though all three RAS-mutant cell lines used here were dependent on their oncogene for both 2D and 3D growth, the dependence of *KRAS*-mutant cells on SOS2 was only revealed in 3D growth conditions. Here, our data indicate that RTK/SOS2/WT RAS/PI3K signalling is a critical survival signal in *KRAS* mutated cancer cells. New approaches for treating RAS-driven cancers, such as synergistic inhibition of MEK and upstream RTK signaling, are still needed to improve patient survival, and screens using assays that more closely mimic the tumor environment are more likely to detect effective therapeutic targets.

Toward an integrated working model of SOS signalling in mutant RAS-driven cancers

Previous studies have shown that RAS isoforms have differing abilities to activate the two major RAS effectors, RAF and PI3K. KRAS has a higher ability to activate RAF than HRAS, and conversely, HRAS has a higher ability to activate PI3K than KRAS; in each case, NRAS is intermediate between the other two isoforms (Figure 7(a)) [23,43–45]. *BRAF* and *KRAS* mutations are generally mutually exclusive in cancer (cBioPortal) [46,47], suggesting that mutant KRAS optimally activates the RAF pathway for proliferation and transformation, and higher levels of RAF–MEK–ERK pathway activation that would come from additional *BRAF* mutations may lead to senescence and apoptosis. In contrast, *HRAS* and *BRAF* mutations significantly co-occur, as do *NRAS* and *BRAF* mutations, indicating that additional activation of the RAF pathway may improve the survival or transformation in HRAS or NRAS-driven cancers.

Unlike *BRAF* mutations, PI3K activating mutations are not mutually exclusive with *KRAS* or *HRAS* mutations in tumours, suggesting that neither activated RAS isoform activates PI3K optimally (cBioPortal) [46,47]. In fact, *PIK3CA* mutations and *HRAS* or *KRAS* mutations co-occur in cancers more frequently than would be expected by random mutation (cBioportal) [46,47], indicating that RAS-independent PI3K activation may play an important role in driving RAS-mutant cancers. However, HRAS does have a higher ability to activate PI3K than KRAS, with NRAS again intermediate [43] (Figure 7(a)), suggesting that HRAS may be less reliant on other sources of PI3K pathway activation. Indeed, we previously established that MEFs expressing mutated HRAS are less sensitive to PI3K inhibition than those expressing mutated KRAS in anchorage-independent growth conditions. On the other hand,

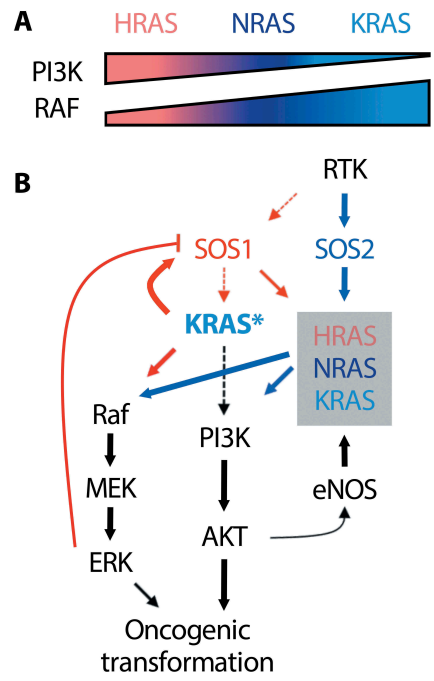

Figure 7. An integrated working model of SOS signalling in RAS-mutated cancer cells. (a) The three RAS isoforms have different relative abilities to activate key downstream effectors RAF and PI3K. KRAS activates RAF more strongly than HRAS, whereas HRAS activates PI3K more strongly than KRAS. NRAS has intermediate activation ability for both effectors. (b) Schematic of SOS-dependent signalling in mutant KRAS-driven cancers.

mutant HRAS-expressing cells were more sensitive to MEK inhibition than mutant KRAS-expressing cells [30]. These patterns in downstream effector pathway activation and sensitivity to effector inhibition suggest that mutant RAS-driven cancers may be vulnerable to therapeutic strategies targeting effector pathways that are not fully engaged by mutant RAS.

These differences in mutant RAS-dependent effector signalling help explain why *SOS2* deletion specifically affects transformation by KRAS, but not HRAS. We previously showed that at the level of effector signalling, *Sos2* deletion reduced RTK-dependent AKT phosphorylation in MEFs expressing all mutant RAS isoforms [30], and here we show that this holds true in *HRAS* and *KRAS* mutated cancer cell lines. However, when we examined the effect of PI3K inhibition on MEFs expressing mutant RAS isoforms or in *RAS* mutated cancer cell lines, we found that there was a hierarchical requirement for PI3K signalling in promoting RAS-driven transformation (KRAS ≥ NRAS > HRAS) that mirrored the hierarchical requirement for SOS2. We hypothesize this is due to the relative inability of KRAS to activate the PI3K pathway, making cells expressing mutated KRAS more sensitive to alterations in PI3K signalling than cells expressing mutated NRAS or HRAS. Furthermore, KRAS-driven transformation could be

rescued in *Sos2*$^{-/-}$ MEFs by the introduction of an activated PI3K catalytic subunit, suggesting that *Sos2* deletion specifically reduced PI3K–AKT signalling to block oncogenic transformation.

Based on our previous results [30] and those of others, we propose a working model of SOS signalling in mutant KRAS-driven cancers (Figure 7(b)). In cells expressing mutated KRAS, SOS proteins can be activated by two distinct mechanisms: (i) RKT-dependent recruitment of SOS to the plasma membrane by Grb2 and (ii) allosteric binding of SOS by mutant KRAS setting up a mutant KRAS^{GTP}–SOS1–wild-type RAS positive feedback loop. For SOS1, KRAS-dependent RAF–MEK–ERK signalling sets up a signalling environment where competing signals titrate the activity of SOS1. Mutant KRAS binds an allosteric pocket on SOS1 [29], relieving SOS1 autoinhibition [48] and thereby activating a mutant KRAS^{GTP}–SOS1–wild-type RAS positive feedback loop that helps drive cell proliferation [49]. In parallel, constitutive RAF–MEK–ERK signalling causes multiple ERK- and RSK-dependent negative feedback phosphorylation events [50–60] including phosphorylation of SOS1 that result in both ERK-dependent dissociation of the Grb2/SOS1 complex [53–56] and RSK-dependent cytoplasmic sequestration of SOS1 by 14–3–3 [50,51]. This ERK- and RSK-dependent feedback phosphorylation may remove a portion of SOS1 from the pool of Grb2-associated SOS, making SOS1 less available for RTK-dependent signalling to wild-type RAS.

In contrast to SOS1, these feedback mechanisms do not seem to regulate the activity of SOS2. SOS2 cannot be phosphorylated by ERK [54] or RSK [50], so the activity of SOS2 is not curtailed by RAF–MEK–ERK feedback inactivation. Furthermore, we have shown that the allosteric (RAS^{GTP}-dependent) SOS2 signalling does not contribute to wild-type HRAS activation or mutant KRAS-dependent transformation [30]. Because of this, the entire pool of SOS2 is available for RTK signalling, where SOS2 plays a critical role in RTK-dependent PI3K–AKT pathway activation [30]. Interestingly, while *SOS2* deletion has a marked effect on RTK-stimulated AKT phosphorylation, it does not alter RTK-stimulated RAF–MEK–ERK pathway activation. We hypothesize that this is due to the markedly differing thresholds of RTK stimulation required to fully activate the PI3K–AKT versus RAF–MEK–ERK cascades, where a much stronger stimulus is required to activate PI3K–AKT than ERK [61]. Because of this difference, we posit that after *SOS2* deletion the 'active' pool of SOS1, those SOS1 molecules not phosphorylated and inhibited by ERK and RSK, remains sufficient to support full RAF–MEK–ERK pathway activation but is insufficient to activate PI3K–AKT signalling. Alternatively, the specificity of signalling from SOS2 to PI3K through RAS may also be influenced either (i) by colocalization of specific signalling components at the membrane [62–66] which has been proposed as a mechanism of regulation for RAS signalling or (ii) by activation of Rac/p110β signalling downstream of SOS2. Previous studies have shown that in addition to its RasGEF activity, the combined DH/PH domain of SOS1 can act as a RacGEF in some settings [28,67,68]. Functionally, association of the proline-rich (PR) regions of SOS1 with different signalling complexes may regulate Ras/ERK (SOS1/Grb2) versus Rac/JNK (SOS1/E3b1/Abi-1) signalling [69]. In primary MEFs, combined deletion of SOS1 and SOS2 is required to reduce RTK-stimulated RAC activation [70]. Furthermore, activating mutations in the DH domain of SOS2 are associated with Noonan Syndrome, indicating the functional importance of this domain in SOS2 [71,72]. However, whether either of these mechanisms can fully explain the differential effect of SOS2 deletion on PI3K–AKT versus RAF–MEK–ERK pathway activation requires further study.

Combined inhibition of PI3K and MEK effectively blocks *KRAS*-mutant cancer growth, but inhibiting these two key pathways has high toxicity in patients, necessitating alternative approaches. *SOS2* deletion reduces RTK-dependent PI3K pathway signalling, indicating that SOS2 is a potential alternative therapeutic target to direct PI3K inhibition. We previously demonstrated that deletion of *SOS2* synergized with the MEK inhibitor trametinib to block the transformed phenotype in *KRAS*-mutant tumor cell lines [30]. These results indicate that SOS2-dependent PI3K signalling plays an important role in mutant KRAS-driven transformation, and that SOS2 may be a therapeutic target in KRAS-driven cancers. While SOS2 is a potential therapeutic target in KRAS-drive cancer, the lack of effect of *SOS2* deletion on anchorage-independent growth in *HRAS* or *NRAS*-mutant cancer cell lines indicates that SOS2 is unlikely to be a viable therapeutic in these cancers. Additionally, SOS2 is likely only a therapeutic target in KRAS mutated tumours that have wild-type PI3K/PTEN signalling, since the expression of an activated p110α catalytic subunit restored KRAS-dependent transformation in *Sos2*$^{-/-}$ MEFs. Therefore, further study on *RAS*-mutated cancer cell lines with different origin sites and co-mutations will need to be done to determine the range of applicability of SOS2 inhibition as a therapeutic strategy.

In addition to further defining the potential for SOS2 as a therapeutic target, our data presented here also

indicates the importance of using anchorage-independent growth assays to investigate potential therapeutic vulnerabilities in RAS-mutated cancer cells. The differential dependence on SOS2 for promoting RAS-dependent transformation is only revealed in anchorage-independent conditions (Figures 2 and 3), which more closely model the in vivo tumour environment, and the synergistic effects of MEK inhibition and SOS2 deletion are only revealed under anchorage-independent conditions [30]. These data suggest a new paradigm where future large-scale screens that search for therapeutic vulnerabilities/synthetic lethal interactions to inhibit cancer growth should be performed under anchorage-independent conditions in order to reveal novel, tractable therapeutic vulnerabilities that would not be identified in anchorage-dependent screens.

Materials and methods

Cell culture

RD and RL95-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM); NCI-H358, NCI-H1915, NCI-H1299, and NCI-H23 cells were maintained in Roswell Park Memorial Institute medium (RPMI); SK-MEL-2 cells were maintained in Eagle's Minimal Essential medium (EMEM); and T24 cells were maintained in McCoy's 5A (Modified) medium. Each medium was supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 0.1 mM minimum essential medium with non-essential amino acids, and 1% penicillin-streptomycin.

Production of recombinant lentiviruses

Lentiviruses were produced by co-transfecting MISSION lentiviral packaging mix (Sigma) into 293 T cells using calcium phosphate. At 48 to 72 h post-transfection, viral supernatants were collected and filtered. Viral supernatants were then either stored at −80°C or used immediately to infect cells in combination with polybrene at 8 μg/mL. All cell lines were selected with 4 μg/mL Puromycin (Invitrogen).

sgRNA studies

A non-targeting (NT) single guide RNA (sgRNA), a KRAS-targeted sgRNA, the three confirmed SOS2-targeted sgRNAs, the four potential HRAS-targeted sgRNAs, and the four potential NRAS-targeted sgRNAs were each cloned into pLentiCRISPRv2 as previously described. sgRNA sequences are given in supplemental table S1. Lentiviruses were produced as described above. Forty-eight hours post-infection, cells were selected in 4 μg/mL Puromycin. Ten days after selection, cells were analysed for KRAS, HRAS, NRAS, and/or SOS2 expression and plated for proliferation and transformation assays.

Cell lysis and western blot analysis

Cells were lysed in RIPA buffer (1% NP-40, 0.1% SDS, 0.1% Na-deoxycholate, 10% glycerol, 0.137 M NaCl, 20 mM Tris pH [8.0], protease and phosphatase inhibitor cocktails (Biotool)) for 20 min at 4°C and spun at 10,000 RPM for 10 min. Clarified lysates were boiled in SDS sample buffer containing 100 mM DTT for 10 min prior to Western blotting. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose or PVDF membranes. Western blots were developed by multiplex Western blotting using anti-SOS1 (Santa Cruz sc-256; 1:500), anti-SOS2 (Santa Cruz sc-258; 1:500), anti-b-actin (Sigma AC-15; 1:5,000), anti-KRAS (Sigma WH0003845M1; 1:100), anti-HRAS (Santa Cruz sc-250; 1:100), anti-NRAS (Santa Cruz sc-31; 1:100), anti-pERK1/2 (Cell Signaling 4370; 1:1,000), anti-ERK1/2 (Cell Signaling 4696; 1:1000), anti-pAKT Ser^{472} (Cell Signaling 4060; 1:1000), or anti-AKT (Cell Signaling 4691; 1:1000) primary antibodies. Anti-mouse and anti-rabbit secondary antibodies conjugated to IRDye680 or IRDye800 (LI-COR; 1:10,000) were used to probe primary antibodies. Protein bands were detected and quantified by Western blotting with the Odyssey system (LI-COR).

RAS pull-downs

For RAS pull-downs, cells were lysed on ice for 20 min in RAS-PD lysis buffer (1% NP-40, 50 mM Tris pH 7.5, 200 mM NaCl, 2.5 mM MgCl2, protease and phosphatase inhibitor cocktails (Biotool)), and spun at 10,000 RPM for 10 min. GST-RBD bound to glutathione-sepharose beads (Millipore) was used to isolate RAS-GTP from lysates by rotating incubation for 1 h at 4°C. Samples were washed four times in RAS-PD lysis buffer. All samples were boiled in 2× SDS sample buffer containing 40 mM DTT for 10 min prior to Western blotting.

Proliferation studies

For growth assays, 2 × 10^3 cells were seeded on cell culture-coated 96-well plates (CellTreat). Cells were lysed with CellTiter-Glo 2.0 reagent (Promega), and luminescence was read using a Synergy H1 Hybrid Reader (BioTek). Cell number was assessed 24 h after plating to account for any discrepancies in plating, and then every 48 h for 7 days. Data were analysed as an increase in luminescence over Day 0.

Transformation studies

For spheroid growth in ultra-low attachment 96-well round-bottomed plates (Corning Costar #7007), cells were seeded at 500–1000 cells per well. Cell number was assessed 18 h after plating to allow spheroids to form (day 0), and then at day 7 or 14 using CellTiter-Glo 2.0 reagent (Promega), which measures ATP content as a surrogate of overall cell number. Spheroid growth for each cell line was normalized to the CellTitre Glo signal at day 0 and is expressed as a fold-increase over day 0.

To determine the cancer stem cell (CSC) frequency, serially diluted T24 cells were seeded in ultra-low attachment 96 well flat-bot[3]tomed plates (1–1000 cells/well), cultured for 7–10 days, and scored for the formation of cancer spheres [32]. Wells containing cancer spheres that had grown to a diameter greater than 100 µm were scored as positive, and the frequency of cancer stem cells in the population of T24 cells was then calculated by extreme limiting dilution analysis [33].

Anoikis studies

Cells were plated in ultra-low attachment 96-well round-bottomed plates (Corning Costar #7007) at 10,000 cells per well. At the times indicated, cells were counted using trypan blue to indicate cell death. Percentage of live cells was calculated by dividing the live cell count by the combined living and dead cell counts.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Congressionally Directed Medical Research Programs [LC160222].

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