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The RasGEFs SOS1 and SOS2 are Potential Therapeutic Targets in RAS-Driven Cancers

by

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ABSTRACT

The RasGEFs SOS1 and SOS2 are Potential Therapeutic Targets in RAS-Driven Cancers:

Erin Sheffels, Doctor of Philosophy, 2020

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The RAS family of genes—*HRAS*, *NRAS*, and *KRAS*—are mutated in about a third of human cancers, with limited treatment options available. Wild-type RAS isoforms cooperate with mutant RAS to promote oncogenic signaling to downstream effectors, proliferation, and transformation. Activation of wild-type RAS involves the RAS guanine nucleotide exchange factors (RasGEFs) SOS1 and SOS2, creating a dependence on upstream signaling despite constitutive activation of mutant RAS. Two models have been established for activation of wild-type RAS in RAS-mutated cancer. In the first model, constitutively active mutant RAS increases the activity of SOS through an allosteric RAS binding site. The increased activity of SOS leads to activation of wild-type RAS. In the second model, wild-type RAS is activated by receptor tyrosine kinase (RTK) signaling through SOS. RTK-mediated wild-type RAS signaling supplements mutant RAS signaling to downstream effects. These models are not mutually exclusive, and both have been demonstrated in RAS-driven cancer cells. However, the relative contribution

of each model to wild-type RAS activation and the roles of SOS1 and SOS2 in each model are unknown. We confirmed the previously established requirement for SOS1 in KRAS-driven proliferation and demonstrated its role in proliferation driven by HRAS and NRAS. We also showed that SOS1 is necessary for RAS-driven transformation for all RAS isoforms. Proliferation required both SOS1 GEF activity and its allosteric RAS binding site, indicating that feedback from RAS to SOS1 is important for mutant-RAS driven proliferation (model 1).

We established a distinct role for SOS2 in mutant RAS-driven signaling as well, despite the perception in the field that SOS2 is redundant to SOS1. Using anchorage-independent growth assays, we showed that SOS2 is required for KRAS-driven transformation, but not HRAS-driven transformation, and intermediately required for NRAS-driven transformation. We found that SOS2 was critical for RTK-stimulated wild-type RAS and PI3K activation in mouse embryonic fibroblasts expressing mutant RAS and in RAS-mutated cancer cells, regardless of isoform (model 2). We showed that *KRAS*-mutated cells were more sensitive to PI3K inhibition than *HRAS*- and *NRAS*-mutated cells, paralleling their dependence on SOS2. The hierarchical dependence on PI3K activity also paralleled the abilities of the RAS isoforms to activate PI3K, with KRAS activating PI3K poorly and HRAS activating PI3K well. We propose that *KRAS*-mutated cells require wild-type RAS to activate PI3K due to the relative inability of KRAS to activate PI3K. Thus, SOS2 contributes to KRAS-driven transformation by activating PI3K through wild-type RAS, protecting cells from anoikis.

Combined inhibition of PI3K and MEK limits *KRAS*-mutated tumor growth and promotes apoptosis. Unfortunately, high toxicities from direct inhibition of both

pathways prevent the use of this therapeutic strategy in patients. Indirect blocking of PI3K signaling through inhibition of specific RTKs is effective but resistance mechanisms arise through activation of other RTKs. SOS2 is a common intermediate of RTKs and SOS2 inhibition is unlikely to cause toxicity in adults, since SOS2 is not necessary for normal cell function. We found that *SOS2* deletion synergized with MEK inhibition to limit KRAS-driven anchorage-independent growth, serving as an indirect PI3K inhibition. Overall, we demonstrated that both SOS1 and SOS2 are potential therapeutic targets in RAS-driven cancers. Due to the stronger requirement for SOS2 over SOS1 in KRAS-driven cancers, we propose that developing inhibitors for SOS2 in addition to current SOS1 inhibitors will improve treatment efficacy. Our results also demonstrate the importance of using three-dimensional anchorage-independent growth assays to evaluate potential targets.

TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER 1: Introduction	1
The RAS pathway	2
RAS	5
RAS regulation	6
Post-translational modifications and localization	6
Inactivation by RasGAPs	8
Activation by RasGEFs	8
RAS in disease	9
RAS in cancer	9
SOS	11
SOS regulation	13
SOS in disease	16
Wild-type RAS in RAS-mutated cancer	17
RAS effector pathways	19
Raf/MEK/ERK pathway	20
PI3K/AKT pathway	21
Treating RAS-driven cancers	22
Directly targeting RAS	22
Indirectly targeting RAS	22
Summary	24
CHAPTER 2: Oncogenic RAS isoforms show a hierarchical requirement for the guanine nucleotide exchange factor SOS2 to mediate cell transformation	25
Abstract	26
Introduction	27
Results	30
Mutant RAS isoforms show a hierarchical requirement for Sos2 to drive transformation	30
SOS2 RASGEF activity is required for KRAS-driven transformation	33
SOS2 promotes EGF-stimulated AKT phosphorylation in cells expressing mutant RAS	36
Mutant RAS isoforms show a differential requirement for PI3K for transformation	37
Activated PI3K rescues KRAS-driven transformation in <i>Sos2</i> ^{-/-} MEFs	40
SOS2 promotes transformation of KRAS-mutant tumor cells	41
Discussion	45
Materials and Methods	49
Figures	55
Supplementary materials	69

CHAPTER 3: Anchorage-independent growth conditions reveal a differential SOS2 dependence for transformation and survival in RAS-mutant cancer cells	79
Abstract	80
RAS in Cancer	81
SOS2 is hierarchically required for transformation in <i>RAS</i> -mutant cancer cells	82
Toward an integrated working model of SOS signalling in mutant RAS-driven cancers	89
Materials and Methods	94
Figures	98
Tables	104
CHAPTER 4: Preliminary findings on the role of SOS1 in oncogenic RAS-driven anchorage-dependent and anchorage-independent growth.....	105
Introduction	106
Materials and Methods	108
Results	112
SOS1 is required for RAS-driven proliferation regardless of isoform	113
SOS1 is partially required for RAS-driven transformation regardless of isoform ..	113
SOS1 GEF activity and allosteric activation by RAS are both required for full proliferation	115
<i>Sos1</i> deletion did not alter RTK-stimulated AKT or ERK phosphorylation	118
Discussion.....	119
Figures	125
CHAPTER 5: Conclusion.....	133
SOS2 contributions to RAS-driven cancer	134
SOS2 is differentially required for RAS-driven transformation	134
SOS2 is required for RTK-stimulated wild-type RAS activation and PI3K signaling for all RAS isoforms	135
The requirement for SOS2 in KRAS-driven transformation is linked to its role in PI3K signaling	136
SOS2 deletion synergizes with MEK inhibition to block anchorage-independent growth in <i>KRAS</i> -mutated cancer cells	137
SOS1 contributions to RAS-driven cancer	138
SOS1 is required for proliferation and partially required for transformation in RAS-mutated cells	138
SOS1 GEF activity and allosteric activation by RAS are required for full proliferation	139
<i>SOS1</i> deletion does not affect RTK-stimulated ERK or AKT phosphorylation	141
REFERENCES	144

LIST OF TABLES

Table 2-S1. sgRNA sequences used in this study.	77
Table 3-1. RAS mutation status and tissue of origin of the cancer cell lines studied	104

LIST OF FIGURES

Fig. 1-1. The RTK/RAS pathway	4
Fig. 1-2. SOS1 and SOS2 have highly similar N-terminal and central domains, but diverge significantly in their C-terminal proline-rich region.	12
Fig. 1-3. SOS is auto-inhibited by internal interactions between its domains.	15
Fig. 2-1. Oncogenic mutant RAS isoforms show a hierarchical requirement for SOS2 to drive transformation in MEFs.	56
Fig. 2-2. SOS2 critically mediates mutant KRAS-driven transformation in MEFs.	57
Fig. 2-3. SOS2 RASGEF activity contributes to KRAS-driven transformation.	59
Fig. 2-4: SOS2 mediates RTK–dependent AKT phosphorylation in cells expressing mutant RAS.	60
Fig. 2-5: Mutant RAS isoforms show a hierarchical requirement for PI3K signaling to drive transformation in MEFs.	62
Fig. 2-6: Activated PI3K (p110 α) cooperates with mutant KRAS to transform <i>Sos2</i> ^{-/-} MEFs.	64
Fig. 2-7: SOS2 critically mediates transformation of <i>KRAS</i> mutant tumor cells.	66
Fig. 2-8: <i>SOS2</i> deletion synergizes with MEK inhibition to revert the transformed phenotype of <i>KRAS</i> mutant tumor cells.....	68
Fig. 2-S1. <i>Sos2</i> deletion does not alter cellular proliferation in MEFs expressing oncogenic RAS.	69
Fig. 2-S2. <i>Sos2</i> deletion does not alter GTP loading of mutant KRAS in MEFs.....	70
Fig. 2-S3. Introduction of SOS2 into <i>Sos2</i> ^{-/-} MEFs using different promoters to drive <i>Sos2</i> expression.	72
Fig. 2-S4: Inhibitor treatment of MEFs expressing mutant H-, N-, and KRAS blocks their corresponding downstream signaling pathways.	73
Fig. 2-S5. There is a hierarchical requirement for SOS2 in promoting mutant RAS-driven cancer spheroid growth.....	74
Fig. 2-S6. Activated PI3K (p110 α) cooperates with KRAS ^{G12V} to transform <i>Sos2</i> ^{-/-} MEFs.	75
Fig. 2-S7. Deletion of SOS2 using CRISPR/Cas9.	76
Fig. 2-S8. Inhibitor treatment of YAPC cells blocks their corresponding downstream signaling pathways.	77
Fig. 3-1. Mutant RAS is required for 2D and 3D growth in <i>HRAS</i> and <i>NRAS</i> -mutant cancer cells.	98
Fig. 3-2. <i>KRAS</i> -mutant cancer cells depend on SOS2 for anchorage-independent, but not anchorage-dependent, growth.....	99
Fig. 3-3. There a differential requirement for SOS2 to promote anchorage-independent growth in <i>RAS</i> -mutant cancer cell lines.....	100
Fig. 3-4. SOS2 is required for full RTK-stimulated AKT phosphorylation in <i>RAS</i> mutated cancer cells.	101
Fig. 3-5. SOS2 is required for full RTK-stimulated WT RAS activation in <i>RAS</i> mutated cancer cells.	102
Fig. 3-6. SOS2 is required for resisting anoikis in <i>KRAS</i> -mutated cancer cells.....	103
Fig. 3-7. An integrated working model of SOS signaling in RAS mutated cancer cells.	104

Fig. 4-1. SOS1 is required for mutant-RAS-driven anchorage-dependent (2D) proliferation and anchorage-independent (3D) transformation in cells expressing oncogenic RAS family isoforms.	126
Fig. 4-2. SOS1 GEF-activity and its allosteric RAS-GTP binding site are both required for proliferation.	127
Fig. 4-3. A competitive growth assay confirms the requirement for SOS1 GEF-activity and its allosteric RAS-GTP binding site.	128
Fig. 4-4. Silent mutation of the NGG PAM motif in SOS1 restores SOS1 expression and KRAS-driven transformation following SOS1 deletion by CRISPR/Cas9.....	129
Fig. 4-5. <i>Sos2</i> deletion reduces EGF-stimulated AKT phosphorylation, whereas combined <i>Sos1/2</i> deletion is required to reduce ERK phosphorylation in MEFs expressing oncogenic KRAS ^{G12V}	130
Fig. 4-6. <i>Sos2</i> deletion reduces EGF-stimulated AKT phosphorylation, whereas combined <i>Sos1/2</i> deletion is required to reduce ERK phosphorylation in MEFs expressing oncogenic HRAS ^{G12V} or NRAS ^{G12V}	131

CHAPTER 1: Introduction

The RAS family of genes are the most commonly mutated gene family in cancer (168; 218), including high incidence of mutations in lung (235), colon (91), and pancreatic cancers (130), three of the top four causes of cancer-related death (135). While advances in targeting specific mutations have been made (132), the majority of RAS-mutant tumors remain resistant to currently available treatments (58; 191; 235; 273). Novel strategies for targeting the pathway are necessary to provide effective therapeutics to the majority of patients with RAS mutations. Here we evaluate the potential of targeting upstream RAS activators SOS1 and SOS2 as a therapeutic strategy, including determining the contributions of SOS1 and SOS2 to RAS-driven proliferation, transformation, and signaling.

THE RAS PATHWAY

The RAS pathway contributes to diverse cellular outcomes including proliferation, differentiation, senescence, apoptosis, and transformation (2; 18; 57). The pathway can be activated by many inputs, including G-protein coupled receptors (GPCRs) and calcium signaling (73), but the canonical pathway involves signaling to RAS from growth factor-stimulated receptor tyrosine kinases (RTKs), such as the epidermal growth factor (EGF) receptor (EGFR). RTK activation by growth factor leads to trans-autophosphorylation on tyrosine residues in the cytoplasmic domain of the receptor. These phosphotyrosines are recognized by SH2 domain-containing proteins, including the adaptor protein Grb2, which consists of one SH2 domain and two SH3 domains. Its SH3 domains bind the proline-rich regions of the RAS guanine nucleotide exchange factors (RasGEFs) SOS1 and SOS2. Recruitment of Grb2 to activated RTKs brings SOS to the membrane, opening it from its autoinhibited folded state (98; 310). The

dual SH3 domains of Grb2 may allow it to facilitate the formation of larger RTK/SOS/RAS complexes at the membrane (123; 125), though the physiological relevance of this potential scaffolding remains unclear. Once at the membrane and opened, SOS can catalyze the exchange of GDP for GTP on RAS, activating RAS. Active RAS then interacts with a variety of RAS binding domain (RBD)-containing proteins, inducing conformational changes that allow the effectors to be further modified to remain active and interact with their downstream targets (Fig. 1-1).

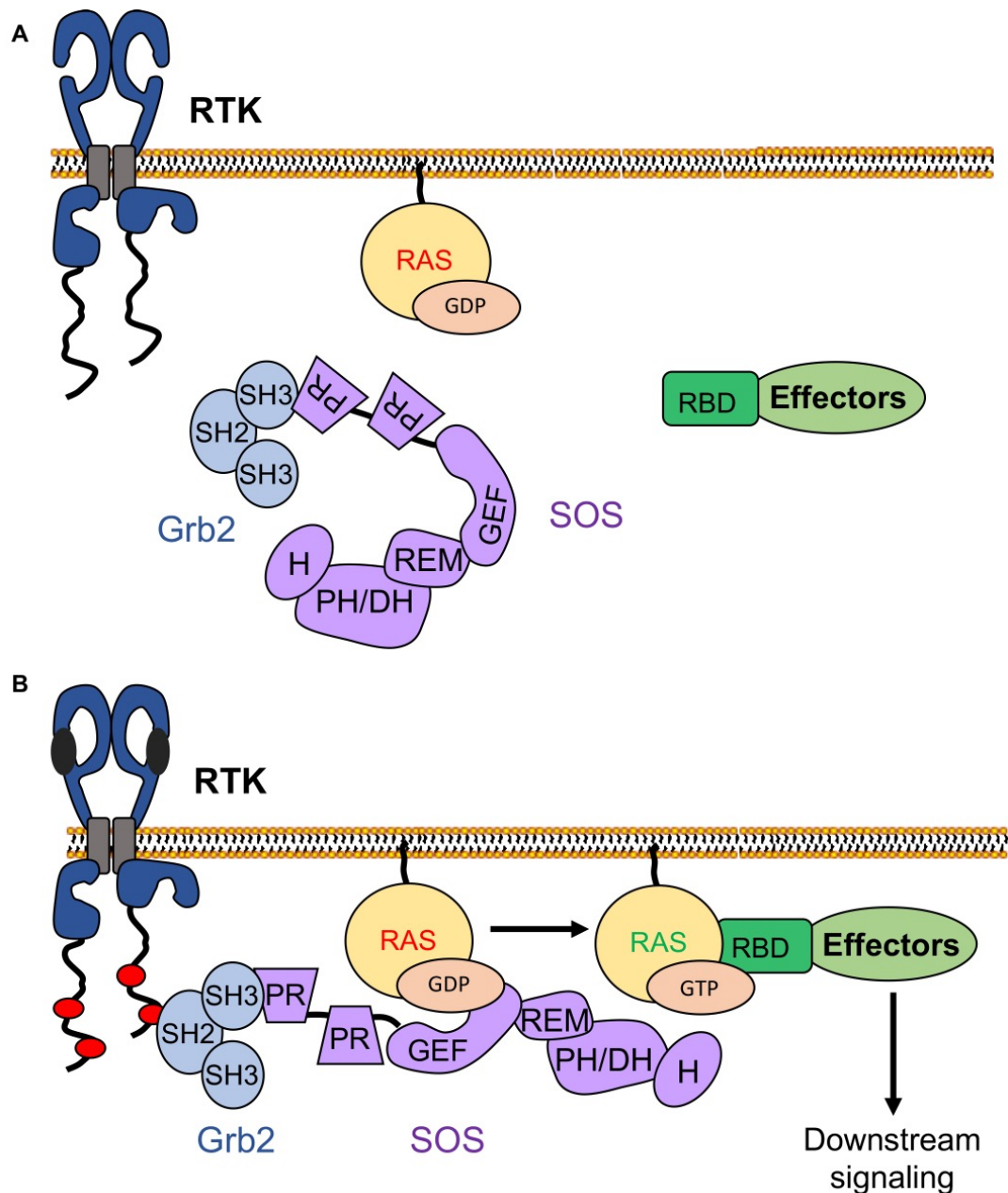


Fig. 1-1. The RTK/RAS pathway

(A) Inactive SOS is constitutively bound to Grb2 in the cytoplasm in the absence of activated RTKs. (B) RTK is activated by ligand binding to RTK dimers, leading to phosphorylation of tyrosines on RTK (red circles). The adaptor protein Grb2 binds the phosphorylated tyrosines and brings SOS to the membrane, where it opens. SOS catalyzes the exchange of GDP for GTP on RAS. RAS-GTP interacts with the RAS binding domain (RBD) on RAS effectors, leading to downstream signaling.

RAS

The RAS family of genes includes three genes, *HRAS*, *NRAS*, and *KRAS*. *HRAS* and *KRAS* were identified independently as retroviral genes transduced from the rodent genome that lead to formation of sarcomas in rats (113; 147), while *NRAS* was identified later from human neuroblastoma (102; 263). *KRAS* encodes two splice variants, *KRAS4A* and *KRAS4B*, the latter of which is predominantly expressed (220; 286; 298). The RAS proteins have highly conserved sequences, both between isoforms (134) and across eukaryotes (13). The majority of the sequence encodes the G domain, which is 90% similar between isoforms and includes the effector lobe and the allosteric lobe. The effector lobe includes the active site for the intrinsic GTPase activity and two switch regions that hold the nucleotide in the binding pocket, which are key for interactions with effectors (104; 271). The allosteric lobe links the effector lobe to the C-terminal highly variable region (HVR), which mediates interactions with the membrane. The HVR consists of 20-25 residues that diverge significantly between isoforms (12; 134), although the HVR for each isoform is conserved across some eukaryotic species (76). The HVR for each isoform encodes two membrane targeting signals and also plays a role in orienting the protein to the membrane for interaction with other signaling proteins (1; 133).

RAS plays an important role in normal cellular signaling and function. Despite their similarity in sequence, only *KRAS4B* is necessary for mouse development (80; 139; 149; 222). Replacing *KRAS* with *HRAS* at the *KRAS* loci allows for normal development, suggesting that the requirement for *KRAS* is due to locus expression during development, not necessarily specific biochemical properties of the protein (208; 224). In adult cells,

the RAS genes are highly abundant and expressed ubiquitously, although levels vary between tissues (169; 208; 277). Conditional knockout of all three RAS genes leads to multiorgan failure in adult mice (70). The different RAS isoforms do have differences in function, including different intrinsic GTPase activity (138) and different abilities to signal to downstream effectors (7; 122; 293; 312).

RAS regulation

Post-translational modifications and localization

RAS proteins are only active at the membrane (165; 307), where they are anchored with lipid tethers. Post-translational modifications of RAS proteins influence their ability to interact with the membrane and their localization within different membrane compartments, including the plasma membrane and endomembranes (17; 45; 48). By directing the localization of RAS, these modifications regulate the ability of RAS to be activated and its access to both upstream and downstream signaling components (4; 237; 326; 327).

All four RAS proteins share a CAAX domain at their C-terminus, which is farnesylated and carboxymethylated as part of protein maturation and contributes to membrane targeting (51; 60; 99). KRAS and NRAS, but not HRAS, can also be geranylgeranylated, allowing them to maintain the ability to be activated in the presence of farnesyl transferase inhibitors (FTIs) (306). In addition to farnesylation of their CAAX domain, NRAS and KRAS4A both have one palmitoylation site in their HVRs (8; 51), and HRAS has two (109; 110). The palmitoylated proteins are trafficked through the endoplasmic reticulum (ER) and Golgi pathways, where they are regulated by cycling between the Golgi and the inner leaflet of the plasma membrane based on their

palmitoylation state (89; 212; 237). KRAS4B, on the other hand, lacks a palmitoylation site, and instead has a lysine-rich region whose positive charge contributes to its interaction with the plasma membrane. After translation, it is brought to the membrane from the ER through interactions with PDE δ (253), potentially involving microtubule transport (43; 281). PDE δ can remove KRAS4B and NRAS from the membrane as part of their regulation, but not KRAS4A or HRAS (201). In addition to modification with lipid moieties, RAS can undergo many other post-translational modifications (reviewed in (3)), including phosphorylation (14; 18), SUMOylation (49), acetylation (313), nitrosylation (166), and ubiquitination (141), all of which have been implicated in regulating the localization or activation of RAS.

The differences in membrane localization and regulation may contribute to differences in signaling between the isoforms (18; 48; 108; 188), and therefore differences in cellular outcome when each isoform is mutated (45). The isoforms show generally consistent patterns in their ability to activate their different downstream effectors. KRAS activates Raf well and PI3K poorly, while HRAS activates PI3K well and Raf poorly, and NRAS has intermediate ability to activate both effectors (171; 293; 312). These differences are not correlated with differences in binding affinity for the effectors (118) or isoform stability (180). How different localization and trafficking of RAS leads to different signaling and outcomes is not completely understood, but many studies have indicated that it involves co-localization and clustering of the RAS isoforms with different activators and effectors in different contexts (30; 75; 116; 215; 216).

Inactivation by RasGAPs

RAS is activated by exchange of bound GDP for GTP, and inactivated by hydrolysis of GTP to GDP. As a GTPase, RAS catalyzes the hydrolysis of GTP to GDP, but its enzymatic activity is low, and the hydrolysis reaction must be further catalyzed to achieve physiologic levels of RAS inactivation. This catalysis is provided by RAS GTPase activating proteins (RasGAPs), which work through diverse mechanisms to stabilize the configuration of the RAS amino acid residues and water required for hydrolysis of the nucleotide (21; 92). There are multiple RasGAPs in mammalian cells, including neurofibromin-1 (NF-1), a key negative regulator of RAS (251), which is inactivated in neurofibromatosis type 1, a rare disease involving tumor formation in the nervous system due to RAS overactivation. Disruption of RAS interactions with RasGAPs is a major mechanism of RAS activation in cancers.

Activation by RasGEFs

In the absence of growth factor stimulation, RAS is generally GDP-bound. Its activation is catalyzed by RAS guanine nucleotide exchange factors (RasGEFs), which stabilize the open configuration of the switch regions of RAS that hold the nucleotide in the binding pocket, allowing the nucleotide to freely diffuse out (20; 104; 195). The loading of GTP also takes place by simple diffusion, relying on the much higher concentration of GTP in the cytoplasm compared to the concentration of GDP. The binding of GTP leads to a conformational change in RAS, allowing it to interact with the RAS binding domains of its targets (179). Without catalysis by a RasGEF, the release of GDP from RAS is slow, locking the protein in its inactive state (290). The primary RasGEFs in mammalian cells are SOS1 and SOS2. Other RasGEFs include Ras guanine

nucleotide releasing factor 1 (RasGRF1) and RasGRF2, which are primarily expressed in the central nervous system (328), and members of the Ras guanyl releasing protein (RasGRP) family, which are important for lymphocyte development and function (reviewed (275)). RasGRPs may also contribute to initial RAS activation (241), but SOS1 or SOS2 is required for normal RTK-dependent RAS activation (25; 252).

RAS in disease

In addition to being a frequently mutated oncogene, RAS and RAS pathway signaling are dysregulated in several congenital syndromes, referred to as RASopathies (234). RAS mutations commonly found in tumors are generally embryonic lethal (288), likely because overactivation of the RAS pathway leads to senescence in normal cells (16). Viable germline mutations in RAS and other pathway members tend to induce more moderate activation of the pathway (256), and are generally associated with facial abnormalities, heart defects, and impaired growth (279). These symptoms are seen in Noonan's syndrome, where 2-4% of patients have mutations in *KRAS* (29; 322), as well as Costello syndrome, which is caused by germline mutations in *HRAS* (93) and associated with a predisposition to rhabdomyosarcoma and neuroblastoma.

RAS in cancer

Oncogenic somatic RAS mutations are found in around 30% of human tumors (218; 299) and promote tumor initiation and maintenance (47). The wide array of pathways activated by RAS signaling means that RAS activation alone can lead cells to attain many of the hallmarks of cancer, including survival and unregulated growth (227; 228). However, some studies indicate that activating mutations or overexpression of RAS are not sufficient to overcome p53-mediated cell cycle arrest (228; 258; 288), and p53 is

commonly co-mutated with KRAS in tumors (178). The three RAS genes are mutated at different frequencies and each RAS isoform induces a different tumor spectrum when mutated (71). *KRAS* is mutated in 85% of RAS-mutant tumors, with mutations commonly found in lung, colorectal, and pancreatic tumors. *NRAS* mutations are common in melanomas and *HRAS* mutations are more frequent in head and neck cancer (120). The preference for different isoforms in different tissues is not fully understood, but may be due to their different signaling abilities (101; 225; 232) or exposure to different types of mutagens (233).

Most RAS mutations occur at codons 12, 13, or 61, with frequencies of specific substitutions varying between isoforms (200; 300) and between tissues (58; 100; 196). The mutant RAS proteins have structures nearly identical to wild-type RAS (156; 285), but the substitutions impair GTPase activity, leading to constitutive activation of RAS. Mutations at these codons may also affect nucleotide binding (137; 267) and interactions of RAS with the membrane and downstream effectors (189). The different properties of each mutation can lead to differences in downstream signaling and tumor growth (reviewed (200; 203; 225)).

Codons 12 and 13 are structural residues normally encoding glycines. G13 mutants make up a small fraction of RAS mutations overall. G12 mutations are common in *KRAS* and *HRAS* (226), where the glycine is frequently replaced with aspartate, valine, and cysteine, depending on the tissue (127; 308). G12 and G13 substitutions sterically impede interactions between RasGAPs and the catalytic residues on RAS (250; 289), leaving RAS constitutively bound to GTP. Codon 61 encodes a glutamine that coordinates water for nucleophilic attack on the γ -phosphate of GTP (26; 211). Mutations

at Q61 are less prevalent overall than G12 mutations, but are common in HRAS and NRAS (226). Q61 mutants have lower intrinsic GTPase activity and are less dependent on RasGEFs for maintaining an active state than G12 and G13 mutants are (126). Common substitutions at codon 61 include arginine, histidine, and leucine. Even at the same codon, the different substitutions impair GTPase activity to different extents, which some studies have correlated with their capacity to transform cells, with greater deficiencies in GTPase activity leading to more robust tumor initiation and growth; however, the exact reason behind different frequencies of substitutions remains unclear (228).

SOS

The primary RasGEFs in mammalian cells are SOS1 and SOS2 (25; 231; 252). They were originally discovered by homology to the *Drosophila* gene Son of sevenless (Sos) (19; 40). SOS1 and SOS2 share the same overall structure and their sequences are 65% similar. They both consist of a central catalytic domain that is sufficient for their activity (20), which contains a RAS exchange motif (REM) domain (159) and a domain homologous to the yeast GEF Cdc25. Their N-terminus includes a histone motif, a Dbl-homology (DH) domain, and a pleckstrin homology (PH) domain. Their C-terminus consists of a proline-rich region (PR), which includes two SH3 recognition sites that interact with the SH3 domain of Grb2 (123). The sequences of their PR domains diverge significantly, with only 40% similarity, which may account for some of their differing roles in RAS signaling (Fig. 1-2, (79)).

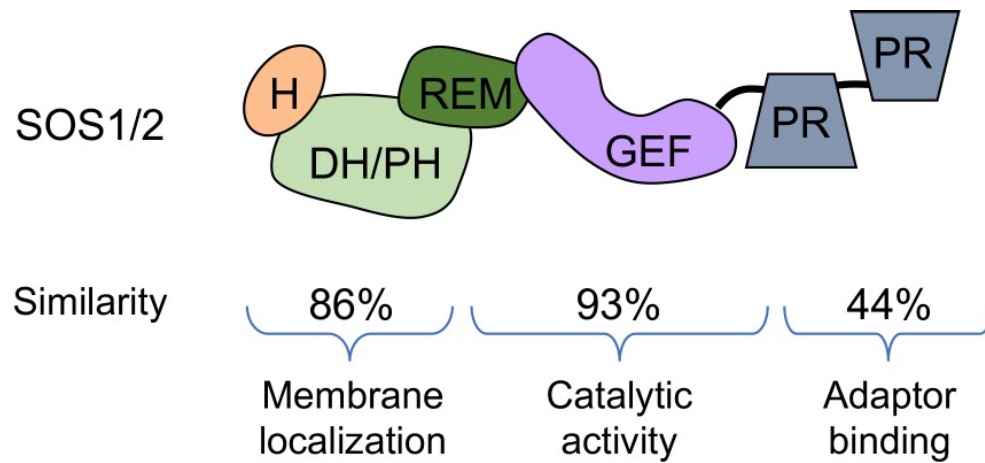


Fig. 1-2. SOS1 and SOS2 have highly similar N-terminal and central domains, but diverge significantly in their C-terminal proline-rich region.

The N-terminal domains contribute to membrane localization, while the central REM and GEF domains are necessary for catalytic activity. The proline rich region interacts with the SH3 domains of Grb2.

SOS1 and SOS2 play a key role in RAS signaling in normal cells. *Sos1* deletion in mice is embryonic lethal due to the specific requirement for SOS1 in placental development (229). While adult mice with conditional *Sos1* deletions are phenotypically normal (11), some cell types have diminished proliferation (174). *Sos2* deletion has no apparent effect on development or adult mice, but double *Sos* knockout is lethal in adults (11; 79). The discrepancy between requirements for *Sos1* and *Sos2* in development have led to the general perception that SOS2 is a redundant protein, capable of compensating for a loss of SOS1 in adult tissues, but without any unique functions of its own. Thus, most previous research on SOS, including SOS in RAS-driven cancer, has been restricted to SOS1.

SOS regulation

SOS1 and SOS2 are both ubiquitously expressed in mammalian tissues, with no indication of tissue specific variation in expression in adults (94). Both proteins are present at low levels, with around 10-fold fewer copies than most other RTK/RAS/MAPK pathway members, including Grb2 and RAS (262). SOS is constitutively bound to Grb2 in the cytoplasm (319), where the complex can be further sequestered by interactions with Sprouty (107). SOS2 has a higher affinity to Grb2 than SOS1 does due to differences in their proline-rich region, leading to potentially tighter interactions with activated RTKs (314), but the physiological relevance of this difference remains unclear.

In its inactive state, SOS1 is autoinhibited by its N-terminal and PR domains, folding the protein in on itself and blocking interactions with RAS (53; 98; 103; 140;

167) (Fig. 1-3A). Once SOS1 is recruited to an active receptor with Grb2, however, the PH domain interacts with PIP₂ in the membrane (41; 158) and basic residues in the histone motif interact with the membrane's negative charge (268) to partially release the autoinhibited state and orient SOS1 for optimal interactions with RAS (98; 310). To further enhance its activity, SOS1 contains an allosteric RAS-GTP binding site between its REM and GEF domains. Crystal structures of SOS1 and RAS together revealed an unexpected binding site separate from the active site in the GEF domain. When RAS-GTP binds this site, SOS1 is further stabilized in its open conformation and the catalytic activity of SOS1 increases by 80 to 500-fold (186) (Fig. 1-3B). RAS-GDP can also bind the allosteric site, but RAS-GTP binds with higher affinity (186) and activates SOS1 ten times more potently (269). SOS interactions with the membrane may further contribute to SOS activation by orienting SOS for optimal allosteric RAS binding (83). SOS1 activation is dependent on the membrane density of RAS-GTP and PIP₂ (98), supporting the model that these interactions play a key role in SOS activation. SOS1 is inactivated by removal from the membrane by endocytosis (52). While the amino acid residues known to be involved in the allosteric site and other membrane interaction domains are conserved in SOS2, the ability of SOS2 to participate in these regulatory mechanisms has not been tested.

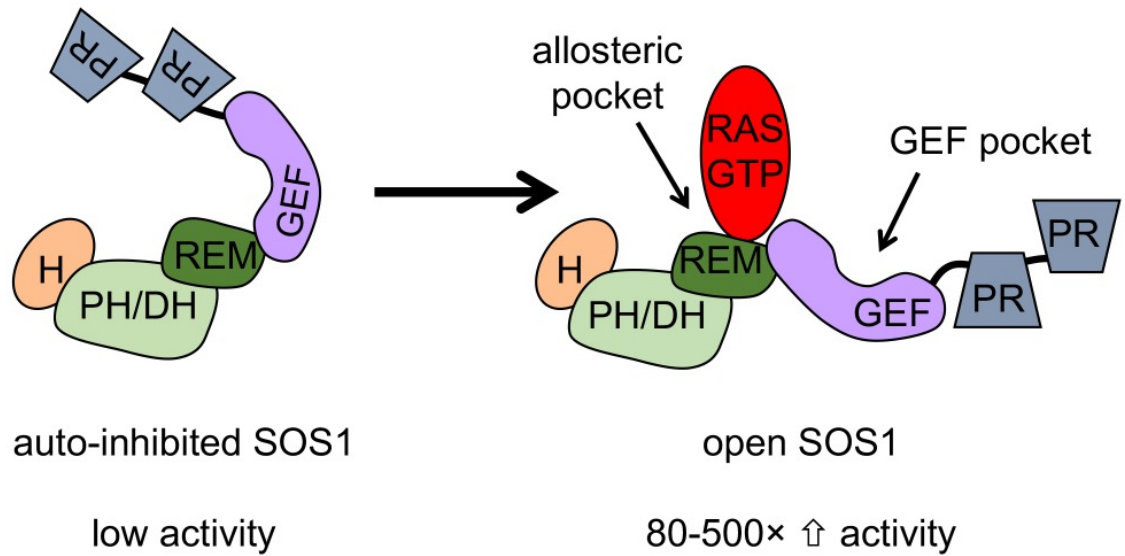


Fig. 1-3. SOS is auto-inhibited by internal interactions between its domains.

Domain interactions block access to its RAS interaction sites. SOS activation is increased by stabilization of the open configuration by RAS-GTP binding to its allosteric site (186).

SOS1 may be uniquely capable of receiving positive feedback from RAS, but it is also subject to negative feedback from ERK and the ERK target RSK. SOS1 contains five ERK phosphorylation sites (46; 223), which cause SOS1 to dissociate from Grb2 when phosphorylated (44; 142; 164). SOS2 only contains one of these sites and its phosphorylation status does not affect the association of SOS2 with Grb2 (54). SOS1 also has two RSK phosphorylation sites in its C-terminal regions (10; 69), which provide inhibitory 14-3-3 docking sites when phosphorylated (69). Neither of these sites is conserved in SOS2 (247). These differences in feedback on the two isoforms may lead to differences in effective activity within the cell and contribute to their different roles in proliferation and RAS signaling.

SOS in disease

SOS1 mutations have been identified in several RASopathies. Gain-of-function mutations in *SOS1* are found in 13-20% of patients with Noonan syndrome (321). Mutations in *SOS2* have also been identified, but are present in less than 3% of cases (311). Mutations isolated from Noonan syndrome patients increased SOS1 activity by increasing affinity for the plasma membrane (205) and relieving SOS1 and SOS2 autoinhibition (55; 98). A different *SOS1* mutation isolated from a patient with Costello syndrome may also increase activity by disrupting autoinhibition (287). A gain-of-function *SOS1* mutation was also identified in hereditary gingival fibromatosis type 1, a rare genetic condition involving benign gingival overgrowth (112). More recently, a somatic *SOS1* mutation was identified in lung cancer (28), indicating that *SOS1* may function as an independent oncogene, in addition to its role in RAS-driven cancer. A

variety of other *SOS1* and *SOS2* mutations are found in both RAS-mutated and wild-type RAS tumors (TCGA, (37; 87)), but they have not been linked to changes in protein function or contributions to the oncogenic phenotype. Likewise, copy number amplifications in *SOS1* and *SOS2* have been identified in tumors, but not at a statistically significant rate compared to other genes, indicating that the copy number variations are likely random products of cancer genome instability, not significant contributors to cancer growth or survival (262). Overall, the expression and function of *SOS1* and *SOS2* do not seem to be altered in most RAS-mutated cancers. No *SOS2*-specific drugs have been developed, but several drugs have been developed to target *SOS1* signaling to RAS, including two recently discovered drugs that block interactions between *SOS1* and RAS (119; 121).

Wild-type RAS in RAS-mutated cancer

Wild-type RAS of all isoforms plays an important role in mutant RAS-driven cancers. Wild-type RAS of the same isoform acts as a tumor suppressor in most contexts (66; 95; 170; 282; 283). Many RAS-mutated tumors have a loss of heterozygosity (LOH) at the mutated RAS gene, leaving them without a wild-type copy of the mutated gene (27; 198; 325). Knockout of a single allele of *KRAS* prior to mutagen exposure lead to increased rate of tumor formation in an *in vivo* model of *KRAS*-driven oncogenesis (324), indicating that the presence of wild-type RAS of the same isoform inhibits tumor formation. LOH is found in about a third of *KRAS*-mutated NSCLC and pancreatic adenocarcinomas, and correlates with increased tumor growth and decreased patient survival in *KRAS*-mutated tumors (230; 318). The tumor suppressor role of the wild-type allele may be due to dimerization of mutant and wild-type RAS, limiting the active

signaling from mutant RAS (6). Similar rates of LOH have been reported for *NRAS* (65; 95; 115; 325) and *d* (81; 204; 282).

On the other hand, wild-type RAS of the non-mutated isoforms contributes positively to oncogenic signaling (22; 136; 317) and is necessary for proliferation and transformation in some RAS-mutated cancers (85; 325). The contributions of wild-type RAS to proliferation and transformation mean that despite the constitutive activation of mutant RAS, cancer cells may still rely on upstream signals. For example, *KRAS*^{G13D} cancers respond to the EGFR inhibitor cetuximab because they rely on strong growth signals from wild-type RAS due to strong binding of *KRAS*^{G13D} with NF-1, preventing wild-type RAS deactivation. EGFR inhibition decreases wild-type RAS activation and limits tumor growth in *KRAS*^{G13D} tumors cells (192).

Two models for wild-type RAS activation in the context of mutant RAS have been established in the literature, both involving SOS. The first model involves the allosteric feedback from active RAS to SOS (186). Allosteric activation of SOS by mutant RAS leads to increased activation of wild-type RAS, increasing signaling to effectors not activated by mutant RAS. This model has been established in *KRAS*-mutated pancreatic cancer cells, where allosteric feedback from mutant RAS to SOS1 is necessary for proliferation (136). The second model involves RTK-mediated activation of wild-type RAS in parallel with constitutive mutant RAS signaling (317). The parallel wild-type RAS signaling supplements mutant RAS signaling to activate effector pathways and promote proliferation (15; 105; 317). The two models are not mutually exclusive and may cooperate in some contexts. For example, positive feedback from RAS to SOS1 potentiates EGF signaling to downstream effectors through SOS *in vitro* (22)

and supports prolonged RAS and ERK activation downstream of T cell and B cell receptors (61; 241). The importance of wild-type RAS in RAS-driven cancer suggests a role for SOS despite being upstream of the active protein.

Due to the different effector activation abilities of the different isoforms, the wild-type RAS isoforms may contribute to oncogenic signaling by activating the effectors that the mutant RAS activates poorly (33). Studies with RAS effector loop mutants that can only interact with one effector pathway have shown that RAS isoforms need to interact with different effectors to induce transformation when mutated (214), indicating that mutant RAS isoforms are contributing to oncogenic signaling through different signaling pathways. Analysis of co-mutation frequencies shows that *KRAS* and *BRAF* mutations are generally mutually exclusive, while *KRAS* and *PIK3CA* mutations co-occur frequently, consistent with the idea that *KRAS* already highly activates the Raf pathway, but requires supplemental signaling in the PI3K/AKT pathway (37; 87). Additionally, in some *KRAS*-mutated colorectal cancer cells, ERK activation decreased when *KRAS* was knocked down, but AKT activation only decreased with RTK inhibition (72), suggesting that PI3K/AKT activity was regulated through RTK-stimulation of wild-type RAS. Thus, wild-type RAS may contribute this supplemental signaling through its ability to activate the pathway that the mutant RAS does not activate well.

RAS EFFECTOR PATHWAYS

RAS interacts with many different effectors to produce its wide-ranging effects on the cell. Pathways and effectors downstream of RAS include Raf (294), PI3K (238), Ral (146), Rac (162; 295), TGF-beta (309), and Wnt (297), among many others (reviewed (145)). However, the Raf/MEK/ERK MAPK pathway and the PI3K/AKT pathway are

generally thought to be the predominant RAS downstream pathways for proliferation and transformation in the context of cancer (157; 245; 248), and both are often required for RAS-driven tumor growth (176).

Raf/MEK/ERK pathway

The Raf/MEK/ERK pathway is one of several mitogen-activated protein kinase (MAPK) pathways in mammalian cells. ERK activation generally leads to either proliferation or differentiation, depending on the intensity and duration of the signal (reviewed (187)), which is modulated by the scaffold KSR-1 (151-153). Raf, MEK, and ERK are all families of serine/threonine kinases that act on the next member in the pathway. There are three Raf family kinases, A-Raf, B-Raf, and C-Raf, with B-Raf and C-Raf primarily contributing to MEK activation downstream of RAS (5). C-Raf interacts with all mutant RAS isoforms with high affinity and is critical for mutant HRAS-driven tumorigenesis, while B-Raf preferentially binds KRAS (280). RAS induces dimerization of B-Raf and C-Raf as part of activation (88; 244; 301). Interactions between RAS-GTP and the RBD of Raf induce a conformational change in Raf, leading to phosphorylation of its kinase domain and release of inhibitory binding by the chaperone protein 14-3-3, which binds Raf to block its activation in the cytoplasm, but also stabilizes active dimers of B-Raf and C-Raf (88) (Raf activation reviewed (64; 303)). Active Raf phosphorylates MEK1 and MEK2 on their activation loops (5), and active MEK1/2 phosphorylates ERK1 and ERK2, which are 87% identical in humans (219).

Active ERK is distributed between the cytoplasm and the nucleus (56) and has a variety of targets involved in increasing proliferation (reviewed (74)), including Elk-1 (305), RSK (114), and cell cycle progression genes (202). MEK, ERK, and RSK also act

on upstream members of the RTK/RAS pathway, creating feedback loops (reviewed (160)). Targets of negative regulation by ERK include RTK (173), SOS1 (46; 54; 223), KSR-1 (193), C-Raf (68), B-Raf (23; 236), and MEK (24; 184). Due to the negative feedback of the pathway, inhibition of MEK or ERK alone is ineffective, and leads to feedback reactivation on the pathway (194; 315). MEK and ERK inhibitors combined increase the degree and duration of pathway suppression and synergize in RAS-mutated cancers (194).

PI3K/AKT pathway

The PI3K/AKT pathway shares downstream targets with the Raf/MEK/ERK pathway, including mTOR signaling, but is generally thought to primarily regulate survival rather than proliferation in the context of mutant RAS signaling (32; 59; 78; 171). RAS activates PI3K through direct binding and recruitment of the p110 catalytic subunit to the membrane (148; 238; 240). RTKs can also directly activate PI3K through direct and adaptor-mediated interactions with the SH2 and SH3 domains of the p85 regulatory subunit, which may be required for PI3K activation by RAS (38; 238). RAS can also activate PI3K indirectly through iNOS signaling (166). Active PI3K goes on to phosphorylate inositol phospholipids, which recruit downstream effectors to the membrane, including PDK1 and AKT. Recruitment to the membrane activates PDK1, which phosphorylates AKT on threonine 308, activating it. AKT is also activated by phosphorylation on serine 473 by mTORC2. AKT activates downstream targets involved in cell survival, growth, and cell cycle regulation (78). RAS interaction with PI3K is required for tumor initiation and maintenance in RAS-mutated lung cancer models (34;

97; 176), and PI3K activation is sufficient for tumor maintenance in some RAS-mutated tumors (176).

TREATING RAS-DRIVEN CANCERS

Directly targeting RAS

Significant efforts have been made to therapeutically target RAS since its identification as an oncogene, with little success. The small, globular nature of RAS and its high affinity for GTP have made developing drugs for direct inhibition difficult (58; 264). The first attempts to target RAS used farnesyl transferase inhibitors (FTIs) to block RAS prenylation, which is necessary for RAS to drive transformation (144). However, only *HRAS*-mutated cells responded to FTI treatment due to the alternative prenylation of *NRAS* and *KRAS* by geranylgeranylation (306). This alternative lipid modification allows *NRAS* and *KRAS* to continue signaling in the absence of farnesyl transferase activity. Currently, two drugs that specifically target mutant *KRAS*^{G12C} through modification of the cysteine have progressed to early clinical trials, but no other mutations or isoforms have been successfully directly targeted (reviewed (209; 261)). Strategies for directly targeting RAS have included intracellular antibodies (143) and antibody mimics (96), siRNA delivery (276), and multivalent binding small molecules (304). Developing methods to specifically and effectively target mutant RAS is an ongoing effort (191).

Indirectly targeting RAS

In addition to biochemical difficulties with directly blocking RAS activity, the networks of feedback in the RAS pathway have presented a challenge for indirect inhibition of RAS activity. Cancer cells either do not respond or quickly develop

resistance to individual inhibition of RAS or its downstream pathways due to relief of negative feedback on upstream pathway members, necessitating multitarget combinations to effectively limit growth (42; 50; 246). Two of the main RAS effector pathways in cancer, the Raf/MEK/ERK pathway and the PI3K/AKT pathway, cooperate to drive proliferation and transformation in *RAS*-mutated cancers (239; 296). Single inhibition of either pathway is ineffective due to relief of negative feedback, but combined inhibition is effective for tumor regression (77; 270). However, direct inhibition of both pathways leads to high toxicities in patients (39). In *KRAS*-mutated tumors, PI3K is downstream of RTK signaling (72; 197). In these tumors, RTK inhibition acts as an indirect inhibitor of PI3K, synergizing with MEK inhibition to limit tumor growth and promote apoptosis without the toxicity associated with PI3K inhibition (72; 183; 302). However, resistance to combined RTK and MEK inhibition develops through activation of alternative RTKs (221; 278; 292) and PIK3CA mutation (203). New strategies for indirect targeting that avoid common resistance mechanisms have high potential to improve patient treatment.

SUMMARY

Successfully targeting oncogenic RAS signaling will likely require both direct and indirect strategies (320), and avoiding resistance mechanisms requires understanding of the complex interactions within the pathway. SOS1 and SOS2 are common intermediates downstream of RTKs and may allow for indirect targeting of the major pathway not activated by mutant RAS through their proposed role in wild-type RAS signaling. Here, we investigate the roles of SOS1 and SOS2 in mutant RAS-driven proliferation and transformation in order to evaluate their potential as therapeutic targets in RAS-driven cancer. Our studies have three main goals:

- 1) To identify the separate and overlapping contributions of SOS1 and SOS2 to mutant RAS-driven signaling and cellular outcomes.
- 2) To determine the mechanisms of contribution of SOS1 and SOS2 to mutant RAS-driven signaling.
- 3) To assess the viability of SOS1 and SOS2 as potential therapeutic targets in RAS-driven cancers.

CHAPTER 2: Oncogenic RAS isoforms show a hierarchical requirement for the guanine nucleotide exchange factor SOS2 to mediate cell transformation

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ABSTRACT

About a third of tumors have activating mutations in *HRAS*, *NRAS*, or *KRAS*, genes encoding guanine triphosphatases (GTPases) of the RAS family. In these tumors, wild-type RAS signaling cooperates with mutant RAS to promote downstream effector activation and cell proliferation and transformation, suggesting that upstream activators of wild-type RAS are important modulators of mutant RAS-driven oncogenesis. The guanine nucleotide exchange factor (GEF) SOS1 mediates KRAS-driven proliferation, but little is understood about the role of SOS2. Here, we found that RAS family members have a hierarchical requirement for the expression and activity of SOS2 to drive cellular transformation. In mouse embryonic fibroblasts (MEFs), SOS2 critically mediated mutant KRAS-driven but not HRAS-driven transformation. *Sos2* deletion reduced epidermal growth factor (EGF)-dependent activation of wild-type HRAS and phosphorylation of the kinase AKT in cells expressing mutant RAS isoforms, and assays using pharmacological inhibitors revealed a hierarchical requirement for signaling by phosphoinositide 3-kinase (PI3K) in promoting RAS-driven cellular transformation that mirrored the requirement for SOS2. KRAS-driven transformation required the GEF activity of SOS2 and was restored in *Sos2*^{-/-} MEFs by expression of constitutively activated PI3K. Finally, CRISPR/Cas9-mediated deletion of *SOS2* reduced EGF-stimulated AKT phosphorylation and synergized with MEK inhibition to revert the transformed phenotype of human *KRAS*-mutant pancreatic and lung tumor cells. These results indicate that SOS2-dependent PI3K signaling mediates mutant KRAS-dependent transformation, revealing therapeutic targets in KRAS-driven cancers. Additionally, our

data reveal the importance of 3-dimensional culture systems in investigating the mediators of mutant KRAS.

INTRODUCTION

The RAS family of small guanine triphosphatases (GTPases) includes three genes, *HRAS*, *NRAS*, and *KRAS*, whose protein products (HRAS, NRAS, KRAS4A, and KRAS4B) are activated by multiple physiological inputs to regulate different cellular outcomes depending on the specific context, including proliferation, differentiation, growth, apoptosis, and cell survival (18; 272). RAS proteins are molecular switches that are active when they are guanine triphosphate (GTP)-bound and inactive when they are guanine diphosphate (GDP)-bound. They are activated by RAS guanine nucleotide exchange factors (RASGEFs) that exchange GDP for GTP on RAS, and are inactivated by their own intrinsic GTPase activity, which is facilitated by RAS GTPase-activating proteins (RASGAPs). Receptor tyrosine kinase (RTK) engagement recruits the RASGEFs Son of Sevenless 1 and 2 (SOS1 and SOS2) to the plasma membrane, where they induce nucleotide exchange and activate RAS. Active RAS then signals through multiple effectors to initiate downstream signaling cascades important for proliferation and survival, including the Raf/MEK/ERK kinase cascade and the PI3K/AKT pathway.

In addition to the role of RAS in RTK-dependent signaling, somatic mutations in *HRAS*, *NRAS*, or *KRAS* drive oncogenesis in approximately 30% of human tumors. These oncogenic *RAS* mutations, which most commonly cause amino acid substitutions at codons 12, 13, or 61, impair RASGAP-mediated GTP hydrolysis leading to constitutive GTP binding and activation. Although this constitutive RAS activation was originally thought to make *RAS* mutant tumors independent of upstream signaling, we now know

that activation of non-mutated wild-type RAS plays an important role in modulating downstream effector signaling during mutant RAS-driven tumorigenesis. The wild-type allele of the corresponding mutated *RAS* isoform is frequently deleted in RAS-driven tumors, suggesting that it may have a tumor suppressor role (65; 95; 170). This hypothesis is supported by observations in vitro (66) and in vivo with mouse models (283; 324). In contrast, the other two non-mutated wild-type RAS family members are necessary for mutant RAS-driven proliferation and transformation in some contexts (85; 177; 317; 325). The wild-type RAS isoforms potentially contribute through their ability to activate effector pathways that the mutant isoform does not strongly activate, making the cellular outcome a product of signaling by both wild-type and mutant RAS (33).

Two models have been proposed to explain how wild-type RAS signaling cooperates with mutant RAS to promote downstream effector activation and RAS-driven oncogenesis. In the first model, RTK-dependent activation of wild-type RAS supplements the basal oncogenic signaling from mutant RAS to fully activate downstream effector pathways and promote proliferation in *RAS* mutant tumor cell lines (15; 105; 317). In the second model, GTP-bound RAS (RAS^{GTP}) binds an allosteric pocket on the RASGEF SOS1 that relieves SOS1 autoinhibition, increasing its catalytic activity up to 80-fold (185). Relief of SOS1 autoinhibition then sets up a positive feedback loop from mutant RAS^{GTP} through SOS1 to wild-type RAS that enhances activation of downstream effectors and is important for proliferation of *KRAS* mutant pancreatic cancer cells (136).

Whereas a role for SOS1 in *KRAS* mutant pancreatic cancer proliferation has been established, a role for SOS2 in mutant *RAS*-driven oncogenesis is not yet understood.

Here, we used immortalized *Sos2*^{-/-} mouse embryo fibroblasts (MEFs) to determine the role of SOS2 in H-, N-, and KRAS-driven transformation. We found that there was a hierarchical requirement for SOS2 in RAS-driven transformation (KRAS > NRAS > HRAS), with KRAS being the most SOS2-dependent RAS isoform. Using mutant SOS2 constructs, we found that KRAS-driven transformation was dependent on SOS2 RASGEF activity, but not on putative SOS2 allosteric activation. SOS2 was required for EGF-stimulated, but not basal, wild-type HRAS activation in cells expressing mutant KRAS. At the level of effector signaling, *Sos2* deletion reduced RTK-dependent AKT phosphorylation in cells expressing all mutant RAS isoforms. However, we also found that there was a hierarchical requirement for PI3K signaling in promoting RAS-driven transformation (KRAS ≥ NRAS > HRAS) that mirrored the hierarchical requirement for SOS2. Furthermore, KRAS-driven transformation could be rescued in *Sos2*^{-/-} MEFs by introduction of an activated PI3K catalytic subunit. Finally, deletion of *SOS2* reduced RTK-dependent AKT phosphorylation and synergized with the MEK inhibitor trametinib to block transformation of *KRAS*-mutant tumor cell lines. These results indicate that SOS2-dependent PI3K signaling plays an important role in mutant KRAS-driven transformation, and that SOS2 may be a therapeutic target in KRAS-driven cancers. Additionally, the specific requirement for SOS2 to promote mutant KRAS-driven proliferation in 3-dimensional (3D), but not 2D, culture suggests that anchorage-independent screens must be used to supplement current 2D screening efforts when investigating therapeutic interventions to treat *KRAS* mutant tumors.

RESULTS

Mutant RAS isoforms show a hierarchical requirement for *Sos2* to drive transformation

Previous work has shown that activation of wild-type RAS promotes mutant RAS-dependent oncogenesis by at least two mechanisms. First, RTK-dependent wild-type RAS activation, presumably via the RASGEFs SOS1 and/or SOS2, cooperates with mutant H-, N-, and KRAS to promote RAS effector activation and cancer cell proliferation (15; 317). Second, mutant KRAS allosterically activates SOS1 (185), generating a feedback loop from GTP-bound KRAS (KRAS^{GTP}) through SOS1 to wild-type RAS that critically mediated proliferation of pancreatic cancer cells (136). While this contribution of SOS1 in mutant *KRAS*-driven cancer cell proliferation has been established, the specific role of SOS2 in *RAS* mutant tumors is not yet clear.

Previous work has shown a role for SOS1, but not SOS2, in anchorage-dependent [2-dimensional (2D)] proliferation in primary MEFs (174); however, we wanted to specifically assess the role of SOS2 in mutant RAS-dependent transformation. Because primary MEFs require cooperating oncogenes in addition to mutant RAS to promote transformation (163), we first generated immortalized MEFs which can be transformed by mutant RAS alone (207). To establish a model system to allow us to examine the specific role of SOS2 in oncogenic transformation, we immortalized *Sos1^{flf}* and *Sos1^{flf}Sos2^{-/-}* MEFs by a 3T6 protocol (152; 284) to generate stable cell lines (hereafter referred to as *Sos2^{+/+}* and *Sos2^{-/-}* MEFs). To assess the role of SOS2 in mutant RAS-driven proliferation and transformation, we then stably expressed HA-tagged HRAS^{G12V}, NRAS^{G12V}, or KRAS4B^{G12V} (hereafter referred to as KRAS^{G12V}) in the *Sos2^{+/+}* and *Sos2^{-/-}* MEFs at 0.2-3 times total endogenous RAS protein abundance (Fig. 2-1A). Whereas

neither *Sos2* deletion nor mutant RAS expression altered SOS1 protein abundance, expression of mutant RAS family members did decrease the expression of EGFR to variable extents, potentially due to feedback regulation on the signaling pathway (Fig. 2-1A).

As expected, expression of oncogenic H-, N-, or KRAS^{G12V} enhanced cell proliferation over vector controls in immortalized MEFs (Fig. 2-S1), and *Sos2* deletion did not affect GTP loading of the mutant RAS protein (Fig. 2-1A), did not alter proliferation driven by mutant NRAS or KRAS, and only modestly reduced proliferation stimulated by mutant HRAS on the last day of a 5-day growth curve (Fig. 2-1B and Fig. 2-S1). These data suggest that SOS2 is not a critical mediator of mutant KRAS or NRAS-driven proliferation and has a minimal effect on proliferation driven by HRAS, in agreement with a previous study in primary MEFs showing that SOS1, but not SOS2, is a critical mediator of proliferation (174). To assess RAS-driven transformation we examined two common features of transformed cells: anchorage-independent growth (soft agar assay, Fig. 2-1C) and loss of contact inhibition (focus-forming assay, Fig. 2-1D). The effect of *Sos2* deletion on RAS-driven transformation differed depending on which mutant RAS isoform was expressed. *Sos2* deletion did not affect HRAS^{G12V}-induced anchorage-independent growth (Fig. 2-1C), loss of contact inhibition (Fig. 2-1D), or morphologic transformation (Fig. 2-1E), indicating that SOS2 was dispensable for HRAS^{G12V}-induced transformation. In contrast, *Sos2* deletion reduced the transforming capacity of NRAS^{G12V} and critically mediated transformation driven by KRAS^{G12V} (Fig. 2-1C-E). *Sos2*^{-/-} MEFs expressing NRAS^{G12V} showed a 50% reduction in colony formation in soft agar (Fig. 2-1C) and qualitatively reduced the proportion of cells

showing loss of contact inhibition (Fig. 2-1D), indicating that SOS2 promoted, but was not required for, transformation by NRAS^{G12V}. *Sos2*^{-/-} MEFs expressing KRAS^{G12V} exhibited minimal anchorage-independent growth (Fig. 2-1C), remained contact inhibited (Fig. 2-1D) and did not show any signs of morphologic transformation (Fig. 2-1E). These data suggest that there is a hierarchical requirement for SOS2 in mutant RAS-driven transformation.

Within the pool of *RAS*-mutant tumors, *KRAS* is the most frequently mutated *RAS* family member (85%). Most *KRAS* mutations occur at codons G12, G13, or Q61, with the frequency of specific mutations varying depending on the tumor type (58; 100). Since our data indicated that *Sos2* deletion has a larger effect on transformation driven by mutant *KRAS* compared to mutant *H-* or *NRAS*, we further investigated the requirement of SOS2 in mutant *KRAS*-driven transformation. To determine whether this requirement is mutation-specific, we expressed either wild-type *KRAS* or one of six common *KRAS* oncogenic mutants (G12C, G12D, G12V, G13D, Q61L, or Q61R) in *Sos2*^{+/+} and *Sos2*^{-/-} MEFs (Fig. 2-2A). For all *KRAS* G12 and G13 oncogenic mutants examined, SOS2 critically mediated mutant *KRAS*-induced anchorage-independent growth (Fig. 2-2B) and loss of contact inhibition (Fig. 2-2C). In contrast, for the *KRAS* Q61 mutants, low amounts of *KRAS*-induced loss of contact inhibition were detectable in the absence of SOS2, indicating that although Q61 mutants require SOS2 for full transformation, they can induce some transformation without SOS2. These data suggest that SOS2 critically mediates full *KRAS*-driven transformation in MEFs, regardless of the specific *KRAS* oncogenic mutation, although it is only partially required for Q61 mutants.

SOS2 RASGEF activity is required for KRAS-driven transformation

KRAS Q61 mutants have lower amounts of GTPase activity than the already reduced activity in G12 and G13 mutants, potentially making them less dependent on GEF activity to restore GTP binding (126). Because of this, we first assessed whether KRAS GTP loading was dependent on SOS2 for KRAS^{G12C}, KRAS^{G12V}, and KRAS^{Q61R} mutant proteins. We did not observe any significant alterations in KRAS^{GTP} abundance upon *Sos2* deletion for any of the mutant KRAS proteins (Fig. 2-S2), indicating that the activation of mutant KRAS is not dependent on SOS2.

Next, we examined the mechanism by which SOS2 contributes to KRAS-driven transformation by restoring SOS2 expression in *Sos2*^{-/-} MEFs, using either wild-type or mutant SOS2 constructs to distinguish the relative contributions of RTK–SOS2–wild-type RAS and KRAS^{GTP}–SOS2–wild-type RAS signaling. However, due to the hypothesized dynamics of SOS signaling, we first created a construct that would allow us to restore SOS2 in *Sos2*^{-/-} MEFs at near endogenous (*Sos2*^{+/+}) protein abundance. In a quantitative proteomic analysis of the core components in the RTK/RAS/MAPK signaling pathway, Shi et al. (262) showed that the absolute abundance of SOS1 and SOS2 are extremely low relative to the other core proteins in the RTK/RAS signaling pathway, leading them to hypothesize that SOS1 and SOS2 may be ‘stoichiometric bottlenecks’ for signal transduction through this pathway. Their findings suggest that exogenous introduction of SOS2 at super-physiologic protein abundance could result in aberrant RAS-dependent signaling, making rescue experiments difficult to interpret. To circumvent this, *SOS2* was cloned into lentiviral vectors containing one of five different promoters, each with different predicted expression levels, and then stably expressed in

Sos2^{-/-} MEFs (Fig. 2-S3A). The high activity EF1 α and UbC promoters, and the moderate activity PGK promoter, lead to SOS2 protein abundance >30-fold and 18-fold higher than endogenous SOS2 found in *Sos2*^{+/+} MEFs, respectively, indicating that these promoters are not optimal for expressing SOS2 at physiologic protein abundances. In contrast, using either an SV40 or a minimal CMV (mCMV) promoter to drive *SOS2* expression lead to SOS2 abundance at only 2-5-fold above that of endogenous SOS2. This near-endogenous SOS2 abundance restored transformation in *Sos2*^{-/-} MEFs expressing KRAS^{G12V} (Fig. 2-S3B). Since the SV40 promoter gave more consistent near-endogenous SOS2 abundance across multiple experiments, we used this promoter for subsequent SOS2 rescue experiments.

Bentley et al (15) previously showed that wild-type N- and HRAS critically mediate transformation of KRAS mutant cancer cells. We hypothesized that SOS2 potentially promotes KRAS-driven transformation by one of two mechanisms: either by RTK-dependent activation of downstream effectors, or as a part of an allosteric KRAS^{GTP}–SOS2–wild-type RAS feedback loop similar to the one previously described for SOS1 (136; 185). To differentiate between these two possibilities, we made constructs with point mutations in *SOS2* that are homologous to mutations previously identified in *SOS1*: SOS2^{F927A}, which is homologous to the SOS1^{F929A} mutant that ablates RASGEF activity (104); and SOS2^{W727E}, which is homologous to the SOS1^{W729E} mutant that renders SOS1 unable to be allosterically activated by RAS^{GTP} (185; 241). If the major contribution of SOS2 is to promote RTK–SOS2–wild-type RAS signaling, then only the SOS2^{F927A} mutant will fail to restore transformation in *Sos2*^{-/-} MEFs expressing mutant KRAS. In contrast, if KRAS^{GTP}–SOS2–wild-type RAS signaling is important for mutant

KRAS-driven transformation, then neither the SOS2^{F927A} nor the SOS2^{W727E} mutants will restore transformation in *Sos2*^{-/-} MEFs expressing mutant KRAS (Fig. 2-3A).

We stably introduced wild-type SOS2, SOS2^{F927A}, or SOS2^{W727E} into in *Sos2*^{-/-} MEFs expressing KRAS^{G12C}, KRAS^{G12V}, or KRAS^{Q61R} (Fig. 2-3B). Wild-type SOS2 restored KRAS-driven anchorage independent growth (Fig. 2-3C), loss of contact inhibition (Fig. 2-3D), and morphologic transformation (Fig. 2-3E) in *Sos2*^{-/-} MEFs expressing each of the mutant RAS constructs. In contrast, RASGEF-dead SOS2 (SOS2^{F927A}) could not restore KRAS-driven transformation in cells expressing either KRAS^{G12C} or KRAS^{G12V} (Fig. 2-3C-E). In cells expressing KRAS^{Q61R}, however, we observed that SOS2^{F927A} enhanced the small incidence of transformation we had observed in *Sos2*^{-/-} MEFs expressing KRAS^{Q61R}, suggesting that although SOS2 did not alter the global GTP loading of KRAS^{Q61R}, the GTPase activity of different oncogenic KRAS mutants may modulate their dependence on SOS2. However, while KRAS^{Q61R} exhibited lower dependence on SOS2 for transformation than either KRAS^{G12C} or KRAS^{G12V} did, SOS2 GEF activity still critically mediated full KRAS^{Q61R}-driven transformation. On the other hand, a SOS2 mutant construct resistant to RAS^{GTP}-dependent feedback activation (SOS2^{W727E}) restored KRAS-driven transformation similarly to wild-type SOS2. Because SOS2 RASGEF activity, but not allosteric RAS^{GTP}-dependent SOS2 activation, was required for mutant KRAS to fully transform MEFs, these data suggest that RTK-dependent SOS2 signaling to wild-type RAS cooperates with basal signaling from mutant KRAS to promote the transformed phenotype.

To directly examine the role of SOS2 in activation of wild-type RAS, we expressed V5-tagged wild-type HRAS in *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing HA-

KRAS^{G12C} and performed GST-RBD pulldowns to directly assess HRAS^{GTP} in both actively cycling cells and upon EGF stimulation after an overnight starve (Fig. 2-3F). Activation of wild type HRAS was significantly decreased in actively cycling *Sos2*^{-/-} MEFs compared to the *Sos2*^{+/+} MEFs, indicating that SOS2 is important for full wild-type HRAS activation (Fig. 2-3F, left). In serum starved cells, the abundance of HRAS^{GTP} was unchanged upon *Sos2* deletion, indicating that basal KRAS^{G12C}-dependent activation of wild-type HRAS was independent of SOS2. EGF-stimulation after an overnight starve showed increased wild-type HRAS activation in *Sos2*^{+/+} MEFs, but not *Sos2*^{-/-} MEFs, suggesting that SOS2-dependent activation of wild-type HRAS is downstream of RTK signaling in cells with mutant KRAS (Fig. 2-3F, right). Furthermore, assessment of V5-HRAS activation in KRAS^{G12C} *Sos2*^{-/-} MEFs expressing wild-type SOS2, SOS2^{F927A}, or SOS2^{W727E} showed that activation of wild-type HRAS was increased in *Sos2*^{-/-} MEFs expressing wild-type SOS2 and SOS2^{W727E} compared to either vector controls or *Sos2*^{-/-} MEFs expressing SOS2^{F927A} (Fig. 2-3G). These data indicate that SOS2 GEF activity, but not allosteric feedback activation of SOS2 by oncogenic KRAS, is key to SOS2-dependent activation of wild-type HRAS downstream of RTKs.

SOS2 promotes EGF-stimulated AKT phosphorylation in cells expressing mutant RAS

The data presented in Fig. 2-3 suggest that RTK-SOS2 signaling through wild-type RAS to downstream Raf/MEK/ERK and PI3K/AKT effector pathways augments basal KRAS^{G12V} signaling to promote oncogenic transformation. To test this possibility, we assessed EGF-stimulated phosphorylation of ERK and AKT in *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing each RAS^{G12V} isoform (Fig. 2-4). Although mutant RAS expression reduced EGFR expression (Fig. 2-1A), *Sos2* deletion did not have any further effects on

EGFR protein abundance, so comparisons of EGF-stimulated signaling in *Sos2*^{+/+} or *Sos2*^{-/-} MEFs expressing any individual mutant RAS isoform were not confounded by alterations in EGFR abundance (Fig. 2-4A). *Sos2* deletion did not significantly alter EGF-stimulated ERK phosphorylation in cells expressing mutant H-, N-, or KRAS, as both peak ERK phosphorylation and prolonged Raf/MEK/ERK signaling were not significantly reduced by *Sos2* deletion (Fig. 2-4A, B). In contrast, *Sos2* deletion significantly reduced EGF-stimulated AKT phosphorylation in cells expressing mutant H-, N-, or KRAS (Fig. 2-4A, B). Loss of SOS2 reduced peak AKT phosphorylation and blocked prolonged PI3K/AKT signaling. These data suggest that SOS2 is important for RTK-stimulated PI3K/AKT, but not Raf/MEK/ERK, signaling. However, these data do not sufficiently explain the differential requirement for SOS2 in RAS isoform-driven transformation, since the reduction in AKT phosphorylation was similar in cells expressing each of the mutant RAS isoforms.

Mutant RAS isoforms show a differential requirement for PI3K for transformation

To reconcile the hierarchical effect of *Sos2* deletion on mutant RAS-dependent transformation with the equivalent decrease in RTK-stimulated AKT phosphorylation, we examined whether MEFs expressing each mutant RAS isoform showed differential sensitivity to PI3K/AKT or Raf/MEK/ERK effector pathway inhibition. Since our initial observation showed that *Sos2* deletion altered transformation but not proliferation, we assessed effector pathway inhibition under both anchorage-dependent and anchorage-independent (transforming) conditions. *Sos2*^{+/+} MEFs expressing H-, N-, or KRAS^{G12V} were seeded on cell-culture treated (anchorage-dependent) and ultra-low attachment (anchorage-independent) 96-well plates and treated with increasing doses of the PI3K

inhibitor LY294002 (Fig. 2-5A), the AKT inhibitor AZD5363 (Fig. 2-5B), or the MEK inhibitor trametinib (Fig. 2-5C). We confirmed that these inhibitors blocked signaling downstream of their intended target by comparing the phosphorylation of downstream targets in treated and untreated MEFs (Fig. 2-S4). We then assessed two measurements of drug potency: the IC_{50} , which measures the drug concentration at half maximal inhibition, and the area under the viability curve (AUC), which takes into account both the potency and total amount of growth inhibition by a given compound. Cells expressing the different mutant RAS isoforms did not show any differences in their responses to PI3K, AKT, or MEK inhibition under anchorage-dependent growth conditions (Fig. 2-5A-C, left). These data suggest that mutant H-, N-, and KRAS-driven anchorage-dependent proliferation depends on these RAS effector pathways to a similar extent.

In contrast to the equivalent effects of effector pathway inhibition on mutant RAS-driven anchorage-dependent growth, RAS-expressing cells showed a hierarchical requirement for PI3K (Fig. 2-5A, right) to promote anchorage-independent growth, mirroring the requirement for SOS2, with $KRAS \geq NRAS > HRAS$. When mutant RAS-expressing MEFs were treated with increasing concentrations of LY294002, anchorage-independent proliferation was inhibited at lower drug concentrations in $KRAS^{G12V}$ expressing MEFs compared to $HRAS^{G12V}$ expressing MEFs, with a significant shift in the dose response curve to lower drug concentrations, resulting in significant decrease in the AUC and a 5-fold decrease in the IC_{50} for LY294002 (Fig. 2-5A). Furthermore, $NRAS^{G12V}$ expressing MEFs showing an intermediate phenotype between MEFs expressing the other two RAS family members. $NRAS^{G12V}$ expressing MEFs showed a 2-

fold decrease in the IC₅₀ for LY294002 but no difference in the AUC compared to HRAS^{G12V} expressing MEFs.

To confirm that these differences were due to inhibition of the PI3K/AKT pathway and not off-target effects of LY294002, RAS^{G12V}-expressing MEFs were treated with the AKT inhibitor AZD5363 (Fig. 2-5B). Similar to what was observed for PI3K inhibition, RAS-isoform expressing MEFs showed a differential requirement for AKT to promote anchorage-independent growth, with KRAS = NRAS > HRAS. Here again, KRAS^{G12V} expressing MEFs showed a decrease in the AUC and a 5-fold decrease in the IC₅₀ for the AKT inhibitor AZD5363 compared to HRAS^{G12V} expressing MEFs. However, unlike the intermediate phenotype the NRAS^{G12V} expressing cells showed for PI3K inhibition, NRAS^{G12V} expressing MEFs were as sensitive to AKT inhibition as KRAS^{G12V} expressing cells.

In contrast to the increased sensitivity of KRAS^{G12V}-expressing cells to PI3K/AKT inhibition, treatment of mutant RAS-expressing MEFs with the MEK inhibitor trametinib revealed that HRAS^{G12V}-expressing MEFs were more sensitive to MEK inhibition compared to either N- or KRAS^{G12V}-expressing MEFs. HRAS^{G12V}-expressing MEFs showed a 2-fold decrease in the IC₅₀ for trametinib compared to N- and KRAS^{G12V}-expressing MEFs. These data suggest that mutant HRAS may have a slightly increased reliance on Raf/MEK/ERK signaling to drive transformation compared to mutant N- or KRAS expressing cells. (Fig. 2-5C).

To further examine the role of PI3K in mutant RAS isoform-driven transformation, we assessed cancer spheroid formation in *Sos2*^{+/+} MEFs expressing H-, N- or KRAS^{G12V} treated with increasing doses of LY294002 (Fig. 2-5D). Cells were

seeded in 96-well ultra-low attachment round-bottomed plates, and imaged 16 hours after plating (Day 0) and again seven days later. In untreated cells, spheroid size increased in all RAS^{G12V}-expressing MEFs over the 7-day period but not in vector controls (Fig. 2-5D), indicating the RAS^{G12V} can induce cancer spheroid growth in MEFs. Mutant RAS-dependent spheroid growth showed the same hierarchical dependence on SOS2 that had been observed in other transformation assays (Fig. 2-5D, right and Fig. 2-S5). Furthermore, when we assessed the effects of LY294002 treatment on cancer spheroid growth in *Sos2*^{+/+} MEFs, we observed a similar hierarchical requirement for PI3K signaling to the one observed in the anchorage-independent proliferation assay, with KRAS > NRAS > HRAS. The LY294002 concentration that inhibited spheroid growth for each mutant RAS isoform corresponded to the IC₅₀ value we observed for inhibiting anchorage-independent proliferation. These data further support the existence of a hierarchical requirement for PI3K signaling to promote RAS-driven transformation that correlates with the requirement for SOS2 expression.

Activated PI3K rescues KRAS-driven transformation in *Sos2*^{-/-} MEFs

The data presented up to this point suggest that RTK–SOS2-dependent PI3K signaling critically mediates mutant KRAS-induced transformation in MEFs. To determine if restoring PI3K/AKT signaling is sufficient to restore mutant KRAS-dependent transformation in *Sos2*^{-/-} MEFs, we expressed an activated form of the p110α catalytic subunit (p110α^{H1047R}) at near-endogenous levels, alone or in combination with KRAS^{G12V}, in *Sos2*^{+/+} and *Sos2*^{-/-} MEFs (Fig. 2-6A). We then assessed transformation using focus forming assays (Fig. 2-6B, C) and by assessing morphologic transformation (Fig. 2-S6). Expression of activated p110α^{H1047R} alone modestly transformed *Sos2*^{+/+}

MEFs as previously reported (63). This p110 α^{H1047R} -driven transformation was unaffected by *Sos2* deletion (Fig. 2-6B), suggesting that PI3K is downstream of, or parallel to, SOS2 in these cells. KRAS^{G12V} robustly transformed immortalized *Sos2*^{+/+} MEFs (Fig. 2-6B), and this transformation was further enhanced by combining p110 α^{H1047R} with KRAS^{G12V} (Fig. 2-6B,C), confirming previous reports of synergic transformation between KRAS^{G12V} and either p110 α (213) or AKT (172). As shown previously, KRAS^{G12V} was unable to transform *Sos2*^{-/-} MEFs alone (Fig. 2-1 and 6B, C). In contrast, combined p110 α^{H1047R} and KRAS^{G12V} expression robustly transformed *Sos2*^{-/-} MEFs similar to the transformation observed in *Sos2*^{+/+} MEFs (Fig. 2-6B). These data suggest that constitutively activated PI3K can substitute for SOS2 to promote KRAS-driven transformation.

SOS2 promotes transformation of KRAS-mutant tumor cells

We next tested whether the requirement of *Sos2* for mutant KRAS-driven transformation in MEFs could be replicated in *KRAS*-mutant human tumor cells. Because transformation assays typically assess growth for 2-4 weeks, we used CRISPR/Cas9 to delete *SOS2* rather than using a transient siRNA approach. We cloned several candidate sgRNAs that were previously used to target *SOS2* in a genome-wide CRISPR/Cas9 screen (199) into the lentiCRISPRv2 vector, which allows for simultaneous expression of the sgRNA and Cas9 (249). Lentiviruses were then produced from these constructs and used to evaluate the efficiency of *SOS2* deletion by each sgRNA in 293T cells. We found seven different sgRNAs that deleted *SOS2* in >80% of 293T cells (Fig. 2-S7). Three of these sgRNAs that target non-overlapping regions of *SOS2* (#1, #9, and #16) were selected and used to examine the role of SOS2 in transformation *KRAS*^{G12V}-mutant YAPC pancreatic cancer cells.

YAPC cells were infected with either a non-targeting sgRNA (NT), an sgRNA targeting *KRAS*, or one of three sgRNAs targeting *SOS2* (#1, #9, or #16). The resulting cells then underwent selection for 10 days after infection to allow for CRISPR/Cas9-mediated gene deletion. The effect of *SOS2* deletion was examined in pooled cultures to avoid any effects of clonal selection. *KRAS* sgRNA expression reduced *KRAS* abundance by >95%, and each *SOS2* sgRNA reduced *SOS2* protein abundance by >80% compared to NT controls, indicating that *SOS2* had been deleted in at least 80% of cells (Fig. 2-7A). *KRAS* deletion completely blocked anchorage-independent growth of YAPC pancreatic cancer cells, confirming the *KRAS* dependence of this cell line. Each of the three *SOS2* sgRNAs reduced the number of anchorage-independent colonies by >70%, and the colonies that did form were generally smaller than the colonies in NT controls, indicating a reversion from the transformed phenotype. These data suggest that *SOS2* critically mediated full mutant *KRAS*-driven oncogenic transformation in *KRAS* mutant pancreatic cancer cells.

To test whether the requirement for *SOS2* to promote mutant *KRAS*-driven transformation might hold true in a *KRAS* mutant tumor cell line from another anatomical site, we assessed whether *SOS2* mediated for transformation in *KRAS*^{G12C}-mutant H358 lung cancer cells. These cells have been used to show the specificity of covalent *KRAS*^{G12C}-specific inhibitors, and are highly dependent on *KRAS* signaling for proliferation and transformation (132). Similar to what we observed in YAPC cells, *KRAS* deletion inhibited cancer spheroid growth in H358 lung cancer cells (Fig. 2-7B). Furthermore, deletion of *SOS2* using two different sgRNAs blocked spheroid growth to a similar extent as deleting *KRAS* itself. These data suggest that *SOS2* is an important

modulator of mutant KRAS-driven transformation in human tumor cells from multiple anatomic sites.

To determine whether the mechanism of SOS2-dependence in *KRAS* mutant cancer cells may be similar to the mechanism in mutant KRAS-expressing MEFs, we tested the activation of the Raf/MEK/ERK and the PI3K/AKT pathways in YAPC pancreatic cancer cells after deletion of *SOS2*. Deletion of *SOS2* with any of the three sgRNAs (#1, #9, or #16) had no effect on ERK phosphorylation in actively cycling cells (Fig. 2-7C), or after EGF stimulation (Fig. 2-7D). These data suggest that, similar to our assessment of the role of *Sos2* in MEFs, deletion of *SOS2* alone does not alter EGF-dependent ERK phosphorylation in *KRAS* mutant cancer cells. In contrast, deletion of *SOS2* significantly decreased AKT phosphorylation in actively cycling cells (Fig. 2-7C) and after EGF stimulation (Fig. 2-7D). These data indicate that, similar to the mechanism we had observed in *Sos2*^{-/-} MEFs, SOS2 mediates RTK-stimulated AKT phosphorylation in a *KRAS* mutant cancer cell line.

With the notable exception of covalent KRAS^{G12C}-specific inhibitors (132), single agent approaches have been broadly unsuccessful in limiting growth of *KRAS* mutant tumors. In contrast, combined inhibition of KRAS effector pathways, such as the combination of MEK and PI3K inhibitors, has shown marked benefit over single agents alone (7; 72; 77). Given that *SOS2* deletion reduced AKT phosphorylation in *KRAS* mutant pancreatic cancer cells (Fig. 2-7C-D), we hypothesized that *SOS2* deletion might act similarly to a PI3K inhibitor and synergize with MEK inhibition to block proliferation and transformation in *KRAS* mutant tumor cells. To understand whether *SOS2* deletion would alter the response of *KRAS* mutant tumor cells to effector pathway

inhibition, we assessed the combination of either *SOS2* or *KRAS* deletion with either the PI3K inhibitor buparlisib (Fig. 2-8A,B) or the MEK inhibitor trametinib (Fig. 2-8C,D) under both anchorage-dependent and anchorage-independent (transforming) conditions. We confirmed that these inhibitors blocked signaling downstream of their intended target by comparing the phosphorylation of downstream targets in treated and untreated YAPC cells (Fig. 2-S8). Deletion of *SOS2* had no significant effect on the IC₅₀ of buparlisib in either YAPC cells (Fig. 2-8A) or H358 cells (Fig. 2-8B), consistent with the idea that *SOS2* deletion and buparlisib treatment both act on the PI3K pathway. Although *SOS2* deletion did significantly decrease the area under the curve (AUC), especially under anchorage-independent conditions (Fig. 2-8A-B, right), this was due to a decrease in the overall cell number after 5 days in culture in *SOS2*-deleted cells (see untreated cells). In contrast, we observed a synergistic effect between *SOS2* deletion and trametinib treatment in inhibiting both anchorage-dependent and anchorage-independent growth. *SOS2* deletion reduced the IC₅₀ for trametinib by 2-3-fold under anchorage-dependent conditions (Fig. 2-8C-D, left) and 5-fold under anchorage-independent growth conditions (Fig. 2-8C-D, right).

To determine whether *SOS2* deletion had a similar effect to PI3K inhibition in synergizing with trametinib to block *KRAS* mutant tumor cell growth, YAPC or H358 cells expressing a non-targeting sgRNA were treated with 100 nM buparlisib, a dose just below the threshold for inhibiting cell growth with buparlisib alone (Fig. 2-8A-B, red arrows), in combination with trametinib. The effect of combining trametinib with low dose buparlisib were similar to the effects of combining trametinib with *SOS2* deletion (Fig. 2-8C-D, compare red and blue curves). These data indicate that, similar to PI3K

inhibition, *SOS2* deletion can synergize with MEK inhibition to block proliferation and transformation of *KRAS* mutant tumor cell lines. Furthermore, these data suggest that *SOS2* is an unappreciated therapeutic target for the treatment of *KRAS* mutant tumors.

DISCUSSION

Driver mutations in the RAS family of GTPases occur in ~30% of human tumors (226; 273). Although these tumors were originally thought to proliferate independently of upstream signaling inputs, we now know that signaling through wild-type RAS cooperates with mutant RAS to activate downstream effector pathways and drive oncogenic proliferation (325). Here, we demonstrated that there is a hierarchical requirement for the RASGEF *SOS2* in RAS isoform-driven transformation, with *KRAS* > *NRAS* > *HRAS*. This requirement for *SOS2* parallels a differential requirement for PI3K signaling to maintain the RAS-transformed phenotype, and in doing so, reveals a previously unappreciated RTK-*SOS2*-RAS-PI3K signaling pathway that supplements mutant *KRAS*-driven PI3K activation to drive oncogenic transformation.

Wild-type RAS isoforms cooperate with oncogenic RAS mutants to promote downstream effector activation and cell proliferation, but the mechanistic underpinnings of this cooperation are unclear. Previous reports have shown two interconnected mechanisms of wild-type RAS activation in cells expressing mutant RAS, and both mechanisms involve RASGEFs as the direct activators of wild-type RAS, which raises the question: Which mechanism underlies RASGEF-dependent wild-type RAS activation in the context of mutant RAS? In the first model, RTK-dependent activation of wild-type RAS acts in an interconnected network with basal mutant RAS^{GTP} signaling to promote G2 checkpoint integrity (90) and proliferation of *RAS* mutant cancer cells (317). Our data

support this model, and suggest that in cells expressing mutant RAS, robust RTK-dependent PI3K/AKT signaling is dependent on SOS2, and this RTK-SOS2-AKT signaling is important for transformation driven by KRAS but not by HRAS. Alternatively, for the second model, crystallographic studies of the RAS:SOS1 complexes show that SOS1 contains an allosteric RAS^{GTP} binding site, distinct from its catalytic RASGEF domain, that relieves SOS1 autoinhibition when occupied (185). This allosteric binding of RAS^{GTP} to SOS1 sets up a potential RAS^{GTP}-SOS1-RAS positive feedback loop that can potentiate EGF signaling to downstream effectors (22), support prolonged T-cell and B-cell receptor-dependent RAS/ERK activation (61; 241), and promote proliferation of *KRAS* mutant cancer cells (136). Our data show that wild-type RAS is activated in cells expressing mutant KRAS independently of RTK signaling and SOS2 expression, suggesting that a SOS1-dependent positive feedback loop plays a role in basal mutant RAS signaling to wild-type RAS.

Mutant H-, N-, and KRAS can all interact with the major RAS effectors PI3K and Raf, thereby constitutively activating the PI3K/AKT and Raf/MEK/ERK pathways. Why, then, would wild-type RAS signaling be required to cooperate with mutant RAS to promote proliferation and oncogenic transformation? Though RAS isoforms interact with the same signaling effectors, they activate these effectors to different extents (293; 312), which are not correlated with a difference in binding affinity (118) or isoform stability (180). A potential role, then, for wild-type RAS is to activate the effector pathways that mutant RAS does not strongly activate, making the cellular outcome a product of signaling by wild-type and mutant RAS (33).

Our study revealed that cells expressing mutant RAS isoforms exhibit differential sensitivity to effector inhibition in maintaining their transformed phenotype. In cells expressing mutant N- or KRAS, isoforms that are relatively poor activators of PI3K (172; 293; 312), anchorage-independent growth was inhibited at lower doses of either the PI3K or AKT inhibitor than the dose required to inhibit growth in cells expressing mutant HRAS (Fig. 2-5). The converse was also true: in cells expressing mutant HRAS, an isoform that poorly activates Raf (172; 293; 312), anchorage-independent growth was inhibited at lower doses of the MEK inhibitor than the dose required in cells expressing mutant NRAS or KRAS. These data suggest that cells expressing mutant RAS isoforms are particularly sensitive to inhibition of RAS effectors that the oncogene poorly activates. Notably, this differential sensitivity to effector pathway inhibition was only observed in anchorage-independent growth assays, not in assays assessing anchorage-dependent (2D) growth, demonstrating that the appropriate culture system must be used to tease apart these drug sensitivities. Multiple studies have shown that *KRAS*-mutant cancer cell lines show a range of *KRAS* dependency for survival in 2D culture (9; 161; 255; 265; 266). However, many of these “*KRAS*-independent” cell lines still require *KRAS* for anchorage-independent growth (86; 190; 242; 323). This finding suggests that we must take care in choosing the appropriate culture system to identify and test novel therapeutic targets to treat *RAS* mutant tumors, and that anchorage-independent 3D growth screens should be used to supplement current 2D screening efforts (242).

The differential sensitivity of 3D-cultured cells expressing mutant RAS isoforms to PI3K inhibition may help explain why *Sos2* deletion showed a differential effect on *RAS* isoform-driven transformation. We found that cells ectopically expressing any of

the three mutant RAS isoforms were reliant on *Sos2* expression for maximal RTK-stimulated AKT phosphorylation (Fig. 2-4). However, because KRAS-expressing MEFs were more reliant on PI3K signaling to promote transformation (Fig. 2-5), the reduced PI3K signaling we observed upon *Sos2* deletion may have been sufficient to block transformation by ectopically expressed mutant KRAS, but not HRAS (Fig. 2-1); alternatively, the relatively high amount of ectopic HRAS in these experiments may mask any role SOS2/PI3K signaling plays in HRAS-driven transformation. Extending these findings into *KRAS* mutant cancer cell lines, we found that KRAS mutant cancer cells were reliant on RTK-SOS2-AKT signaling pathway to supplement basal PI3K activation and promote oncogenic transformation, consistent with our MEF studies. However, whether *SOS2* deletion differentially affects the transformed phenotype in *HRAS* versus *KRAS* mutated cancer cell lines remains to be tested.

The importance of PI3K signaling in KRAS tumors is widely established (157), as is the efficacy of combined MEK and PI3K inhibition in blocking KRAS-driven transformation, since inhibiting either pathway alone leads to activation of the other through relief of negative feedback (77; 270). Unfortunately, this treatment strategy has a high risk of toxicity, since the Raf/MEK/ERK and PI3K/AKT pathways are both key players in normal cell function. To avoid this toxicity, many studies have investigated the efficacy of blocking the PI3K pathway indirectly, or finding other pathways that synergize with MEK or PI3K inhibition (7; 72). For example, Ebi et al (72) show that PI3K signaling is downstream of IGFR in *KRAS*-mutant colon cancer cells, and that IGFR inhibition can indirectly block PI3K signaling. They also show that this indirect block coordinates with MEK inhibition to limit mutant tumor growth and promote

apoptosis to the same extent as direct PI3K inhibition. When we compared the effects of *SOS2* deletion in combination with either PI3K inhibition (buparlisib) or MEK inhibition (trametinib) (Fig. 2-8), we found that *SOS2* deletion had the same effect as an intermediate dose of buparlisib when combined with trametinib. Coupled with the decrease in AKT phosphorylation we observed in the absence of *SOS2*, our findings point to *SOS2* as an alternate target to indirectly block RTK-mediated PI3K signaling in KRAS-mutant cancer cells. *SOS2* inhibition may be more broadly applicable than inhibition of individual RTKs, since different RTKs are predominant in different types of KRAS-mutant cancers. Additionally, *SOS2* is not necessary for development and normal adult cell function in the presence of *SOS1* (11; 79; 155), so inhibition of *SOS2* may have lower toxicity in non-tumor cells than inhibition of PI3K or RTKs. Overall, our findings point to a more complex role of RASGEF signaling in discriminating the effects of RAS isoform-driven transformation than has previously been appreciated and underline the importance of comprehensive examinations of the role of each RASGEF in RAS-driven transformation across RAS isoforms. In addition, our findings suggest that *SOS2* inhibition should be pursued as a potential therapeutic option in KRAS-driven cancers.

MATERIALS AND METHODS

Cell culture. MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) and YAPC and H358 cells were maintained in Roswell Park Memorial Institute medium (RPMI), each supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 0.1 mM minimum essential medium with nonessential amino acids, and 1% penicillin-streptomycin.

RAS construct cloning. HA-HRAS^{G12V} was PCR amplified from pBabePuro and cloned into EcoRI/BamHI digested pCDH-CMV-MCS-EF1-Puro (System Biosciences) using the GeneArt Seamless Cloning and Assembly Kit (Invitrogen). To clone in wild-type KRAS and wild-type NRAS, pCDH-HA-HRAS^{G12V} was digested with BamHI/NotI to remove HRAS but maintain the HA tag, and wild-type KRAS or NRAS were PCR amplified and cloned in using GeneArt. Oncogenic point mutations (NRAS^{G12V} and KRAS (G12C, G12D, G12V, G13D, Q61L, and Q61R)) were then introduced by site-directed mutagenesis. To produce V5-tagged wild-type HRAS, Hs.HRAS (attL1-attL2 clone R999-E10) was cloned into pDest-658 (Blasticidin-resistant, attR4-attR2 lentiviral vector), along with the CMV51p promoter (C453-04, attL4-attL5 sites) and the V5 tag (C514-E24, attR5-attR1 sites) by Gateway Cloning (Invitrogen). All gateway clones were gifts from Dominic Esposito (RAS Project, Frederick National Laboratory).

SOS2 construct cloning. Hs.SOS2 (attL1-attL2 clone 777-E319) was cloned into pDest-658 (Blasticidin-resistant, attR4-attR2 lentiviral vector) along with one of 5 different promoters (attL4-attR1 clones C413-E15 EF1p, C413-E33 UbCp, C413-E21 PGKp, C413-E34 mCMVp, C413-E26 SV40p) by Gateway Cloning (Invitrogen). All gateway clones were gifts from Dominic Esposito (RAS Project, Frederick National Laboratory). SOS2 W727E and F927A point mutations were introduced by site-directed mutagenesis of the wild-type SOS2 entry clone.

Production of recombinant lentiviruses. Lentiviruses were produced by co-transfecting MISSION lentiviral packaging mix (Sigma) into 293T cells using calcium phosphate. Ecotropic p110 α retrovirus was produced by calcium phosphate transfection of pBabePuro (p110 α ^{H1047}) into Phoenix-Eco cells. 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 $8\text{ }\mu\text{g/mL}$. MEFs were selected with $4\text{ }\mu\text{g/mL}$ Puromycin (Invitrogen) or $2.5\text{ }\mu\text{g/mL}$ Blasticidin (Invitrogen). YAPC and H358 cells were selected with $6\text{ }\mu\text{g/mL}$ Puromycin.

Generation of cell lines. Nonimmortalized MEFs were generated from 13.5-day *SosI^{ff}* and *SosI^{ff}Sos2^{-/-}* embryos using a previously described protocol. Cells were maintained in culture according to a 3T6 protocol until immortalized populations of cells emerged (152; 284).

Cell lysis and Western blot analysis. For whole cell lysate (WCL) analysis, cells were either lysed while cycling or starved overnight prior to stimulation with 100 ng/mL EGF for the indicated times. Cells were then 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 minutes at 4°C and spun at $10,000\text{ RPM}$ for 10 minutes. Clarified lysates were boiled in SDS sample buffer containing 100 mM DTT for 10 minutes 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- β -actin (Sigma AC-15; 1:5,000), anti-HA (Santa Cruz sc-805; 1:1000), anti-RAS (Cell Signaling 3965; 1:500), anti-V5 (Cell Signaling 13202; 1:1000), anti-pERK1/2 (Cell Signaling 4370; 1:2,000), anti-ERK1/2 (Cell Signaling 4696; 1:1000), anti-pAKT (Thr³⁰⁸ [Cell Signaling 2965; 1:1000] or Ser⁴⁷³ [Cell Signaling 4060; 1:1000]), anti-AKT (Cell Signaling 2920; 1:1000), anti-p110 α PI3K (Cell Signaling 4249; 1:1000),

anti-KRAS (Sigma WH0003845M1; 1:200), or anti-Tubulin (Cell Signaling; 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). For quantification of SOS2 abundance, samples were normalized to either β -actin or tubulin. For quantification of pERK and pAKT, samples were normalized to a weighted average of β -actin, total ERK1/2, and total AKT (131).

RAS pull-downs. For RAS pull-downs, cells were lysed on ice for 20 minutes in RAS-PD lysis buffer (1% NP-40, 50 mM Tris pH 7.5, 200 mM NaCl, 2.5 mM MgCl_2 , protease and phosphatase inhibitor cocktails (Biotool)), and spun at 10,000 RPM for 10 minutes. GST-RBD bound to glutathione-sepharose beads was either prepared as previously described (35) or purchased (Millipore) and used to isolate RAS-GTP from lysates by rotating incubation for one hour at 4°C. Samples were washed four times in RAS-PD lysis buffer. All samples were boiled in 2× SDS sample buffer containing 100 mM DTT for 10 minutes 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 CellTitre-Glo 2.0 Reagent (Promega), and luminescence was read using a GloMax Discover Plate Reader (Promega). Cell number was assessed 2 hours after plating to account for any discrepancies in plating, and then every 24 hours for 5 days. Data were analyzed as an increase in luminescence over Day 0. For inhibitor studies, 4×10^3 cells were seeded on cell-culture treated 96-well plates (CellTreat) to assess anchorage-dependent growth, and on ultra-low attachment 96-well plates (Corning Costar #3474) to assess anchorage-

independent growth (86). On Day 4, cells were assessed using CellTitre-Glo 2.0 Reagent as described. Inhibitor data are plotted as a scaled dose-response curve for each cell line $(E_{drug} - E_{max}) / (E_0 - E_{max})$, where E_{drug} is the effect of the drug at a given concentration, E_{max} is the maximal effect of the drug, and E_0 is the effect seen in the DMSO control (111; 274). Data were analyzed and IC_{50} and AUC values were calculated using Prism 7.

Transformation Studies: Cells were seeded in 0.32% Nobel agar at 2×10^4 cells per 35-mm dish to assess anchorage-independent growth (soft agar assays) or at 10^5 cells per 6-cm dish to assess the loss of contact inhibition (focus forming assays). Soft agar colonies were counted 21-28 days after seeding; 10 $\times$ images of focus forming assays were taken 14-21 days after seeding, and then the dishes were stained with 1% bromophenol blue (153). Stained focus forming assay dishes were scanned on a Biorad Chemidoc Imaging System and cell density was quantified using Image Lab software. For spheroid growth in ultra-low attachment 96-well round bottomed plates (Corning Costar #7007), cells were seeded at 500 cells per well with the indicated concentration of inhibitor or DMSO. Images were taken 16 hours after plating to assess initial spheroid size, and then 7 or 14 days later to assess the effects of drug treatment on spheroid growth (291). Spheroid size was quantified using ImageJ. In parallel plates, cell number was assessed at day 0, 7, and 14 using CellTitre-Glo 2.0 reagent.

sgRNA studies: A non-targeting (NT) single guide RNA (sgRNA), a *KRAS*-targeted sgRNA, and the 16 potential *SOS2*-targeted sgRNAs were each cloned into pLentiCRISPRv2 as previously described (249). sgRNA sequences are given in supplemental table S1. Lentiviruses were produced as described above. 48 hours post-infection, cells were selected for 4 days in 6 μ g/mL Puromycin, and then shifted into 3

µg/mL Puromycin for an additional 6 days. Ten days after selection, cells were analyzed for KRAS and SOS2 expression and plated for signaling and transformation assays.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism 7 software. Data were analyzed by ANOVA, using the Tukey's method to correct for multiple comparisons.

IC₅₀ and AUC values from dose-response studies were obtained from non-linear regression analysis [log(inhibitor) vs. response (three parameters)]. Data are represented as mean ± SD from a minimum of three independent experiments. Data were considered significant at P<0.05, and are indicated by * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

FIGURES

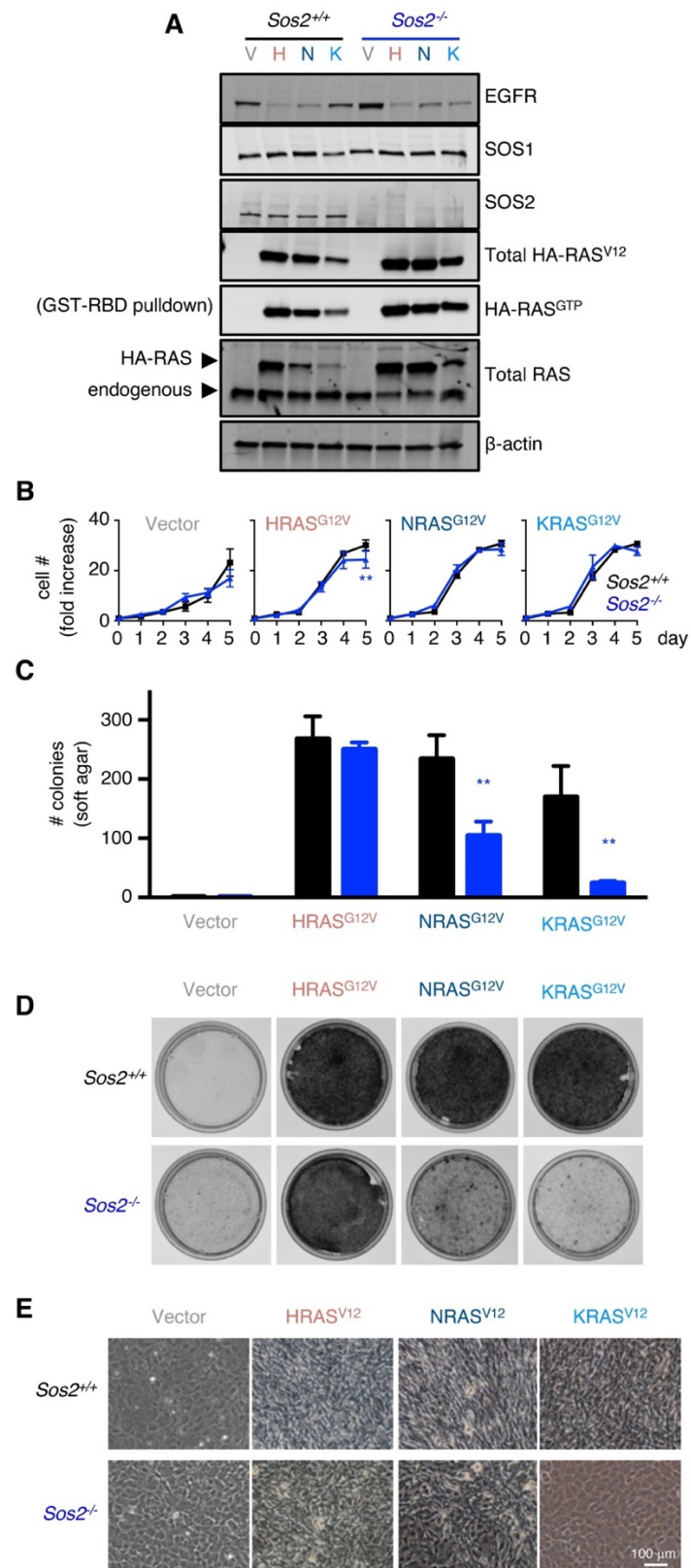


Fig. 2-1. Oncogenic mutant RAS isoforms show a hierarchical requirement for SOS2 to drive transformation in MEFs.

(A) *Sos2*^{+/+} and *Sos2*^{-/-} MEFs were transduced with lentiviruses expressing either empty vector (V) or the indicated HA-tagged mutant RAS isoform (H, N, and K: HRAS^{G12V}, NRAS^{G12V}, and KRAS^{G12V}, respectively). Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for EGFR, SOS1, SOS2, HA (for RAS^{G12V}), total RAS, or β -actin to assess total protein. GST-RAS binding domain pulldowns were analyzed by Western blotting with an antibody specific for the HA epitope to assess activation of mutant HA-RAS^{G12V}. Blots are representative of three independent experiments. (B to D) *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing the indicated mutant RAS isoform were assessed for (B) proliferation in 2D culture plates, (C) colony growth in soft agar to assess anchorage-independent growth, and (D) loss of contact inhibition as assessed by a focus forming assay. Data are mean $\pm$ SD from three independent experiments. ** $P < 0.01$ by ANOVA using the Tukey's method to correct for multiple comparisons. (E) Representative 10 $\times$ images of post-confluent MEFs from (D). Scale bar, 100 μ m. See also Fig. 2-S1 for an overlay of the proliferation curves in (B).

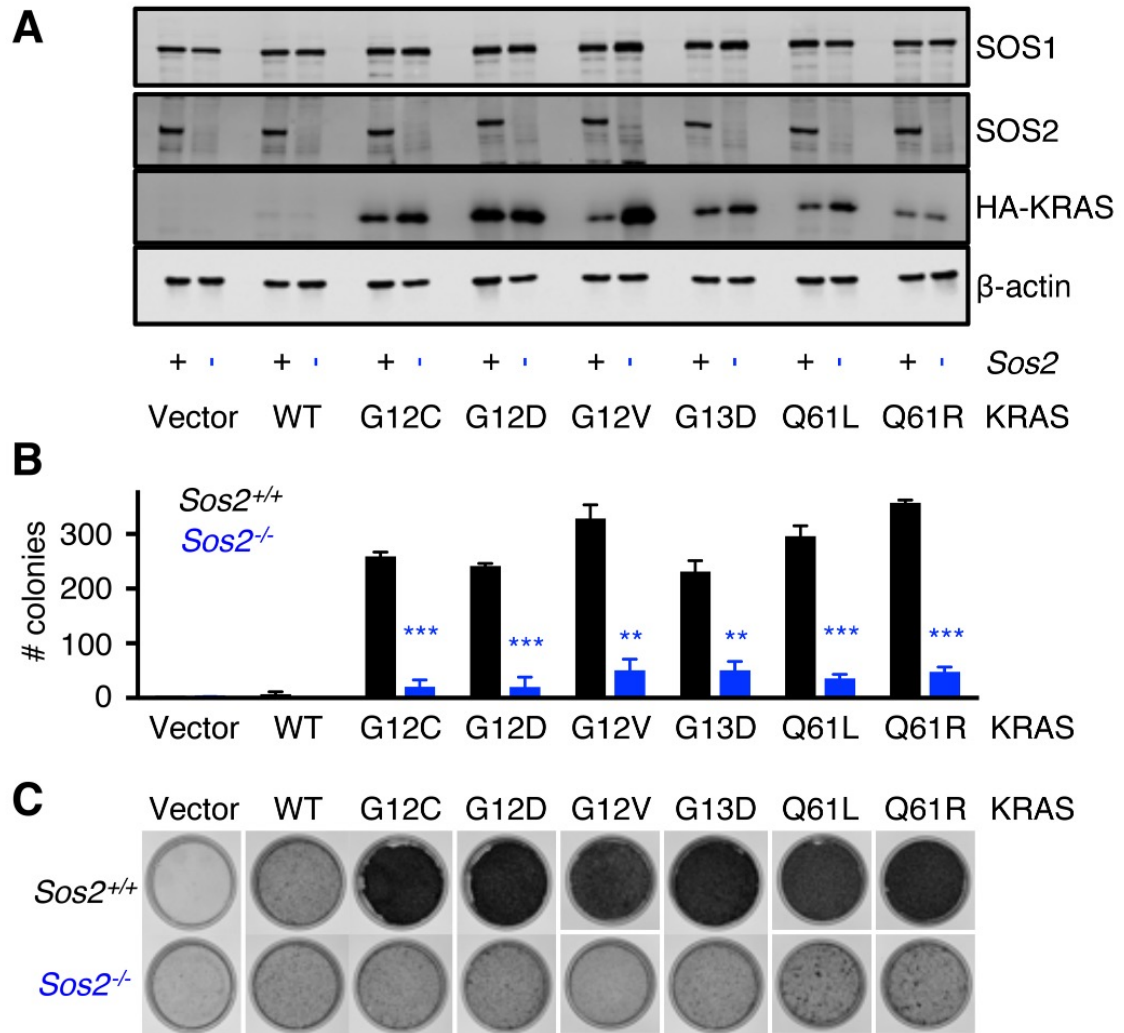


Fig. 2-2. SOS2 critically mediates mutant KRAS-driven transformation in MEFs.

(A) *Sos2*^{+/+} (+) and *Sos2*^{-/-} (-) MEFs were transduced with lentiviruses expressing either empty vector (V), wild-type KRAS, or the indicated HA-tagged mutant KRAS constructs. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for SOS2, HA (KRAS), or β-actin. Blots are representative of three independent experiments. (B and C) *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing the indicated KRAS constructs were assessed for (B) colony growth in soft agar to assess anchorage independent growth, and (C) loss of contact inhibition as assessed by focus forming assay. Data are mean ± SD from three independent experiments. ** P<0.01, *** P<0.001 by ANOVA using the Tukey's method to correct for multiple comparisons.

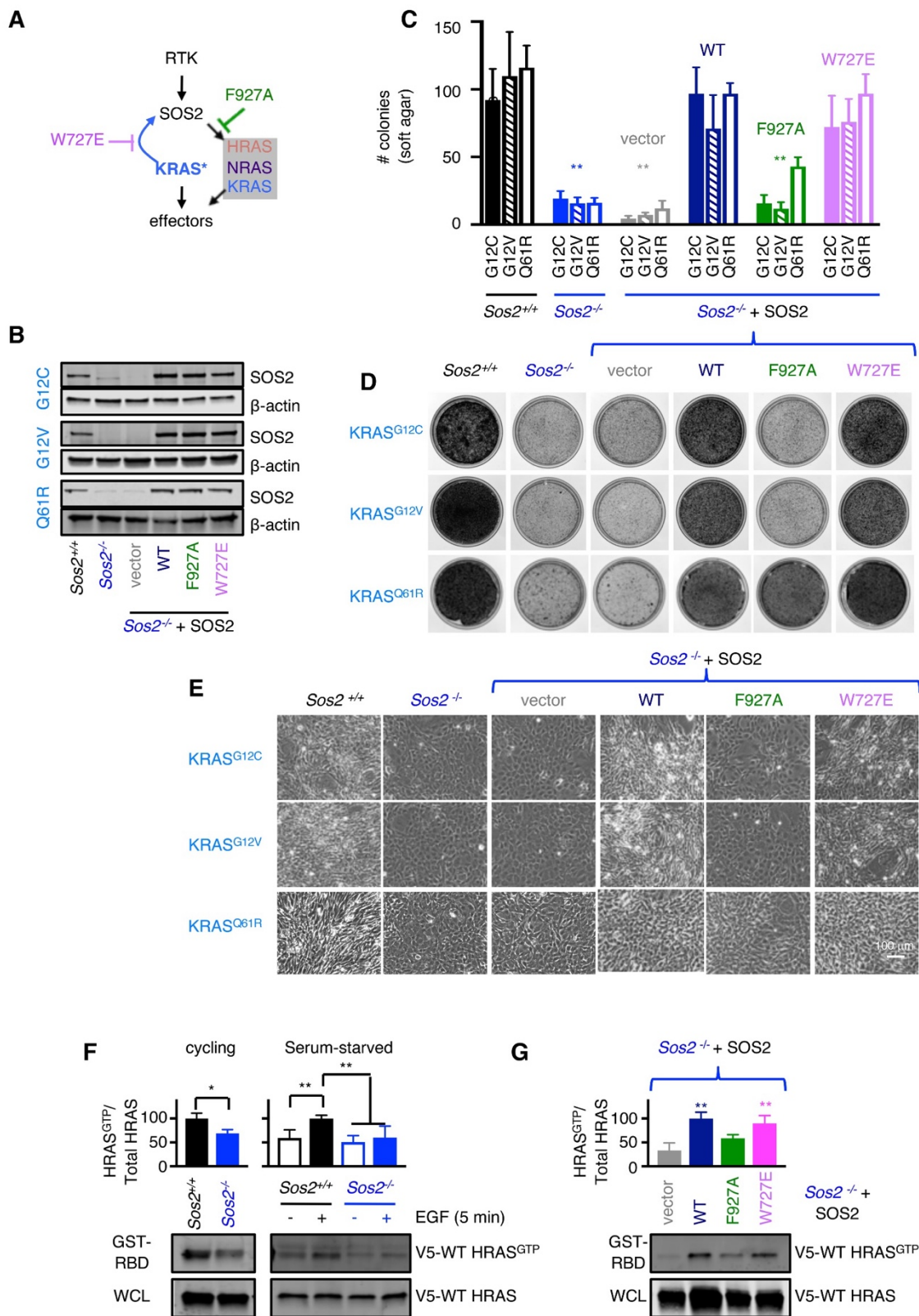


Fig. 2-3. SOS2 RASGEF activity contributes to KRAS-driven transformation.

(A) Schematic showing potential routes of SOS2-dependent wild-type RAS activation in the presence of mutant KRAS. SOS2 point mutants block either RASGEF activity (F927A) or putative allosteric SOS2 activation by KRAS (W727E). **(B)** *Sos2*^{-/-} MEFs expressing KRAS^{G12C}, KRAS^{G12V}, or KRAS^{Q61R} were transduced with lentiviruses expressing either empty vector (V), wild-type SOS2, RASGEF-deficient (F927A) SOS2, or feedback-defective (W727E) SOS2. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for SOS2 or β -actin. **(C and D)** *Sos2*^{+/+} MEFs, *Sos2*^{-/-} MEFs, or *Sos2*^{-/-} MEFs expressing the indicated SOS2 constructs along with either KRAS^{G12C} (closed), KRAS^{G12V} (hashed), or KRAS^{Q61R} (open) were assessed for (C) colony growth in soft agar to assess anchorage-independent growth, and (D) loss of contact inhibition by a focus forming assay. **(E)** Representative 10 × images of post-confluent MEFs from (D). Scale bar, 100 μ m. **(F and G)** Western blotting for activated V5-HRAS from GST-RBD pulldowns (middle, quantified above) or for total V5-HRAS from WCLs (below) from (F) *Sos2*^{+/+} or *Sos2*^{-/-} MEFs expressing HA-KRAS^{G12C} and V5-wild-type HRAS in either actively cycling cells (left) or cells serum starved overnight and then lysed or stimulated with 100 μ g/mL EGF for 5 minutes (right) or (G) *Sos2*^{-/-} MEFs expressing HA-KRAS^{G12C}, V5-wild-type HRAS, and the indicated SOS2 construct. All data are mean $\pm$ SD from three independent experiments; all blots and images are representative of three independent experiments. * P<0.05, ** P<0.01 [for (C), versus *Sos2*^{+/+} and WT; for (G), versus vector and SOS2^{F927A}] by ANOVA using the Tukey's method to correct for multiple comparisons.

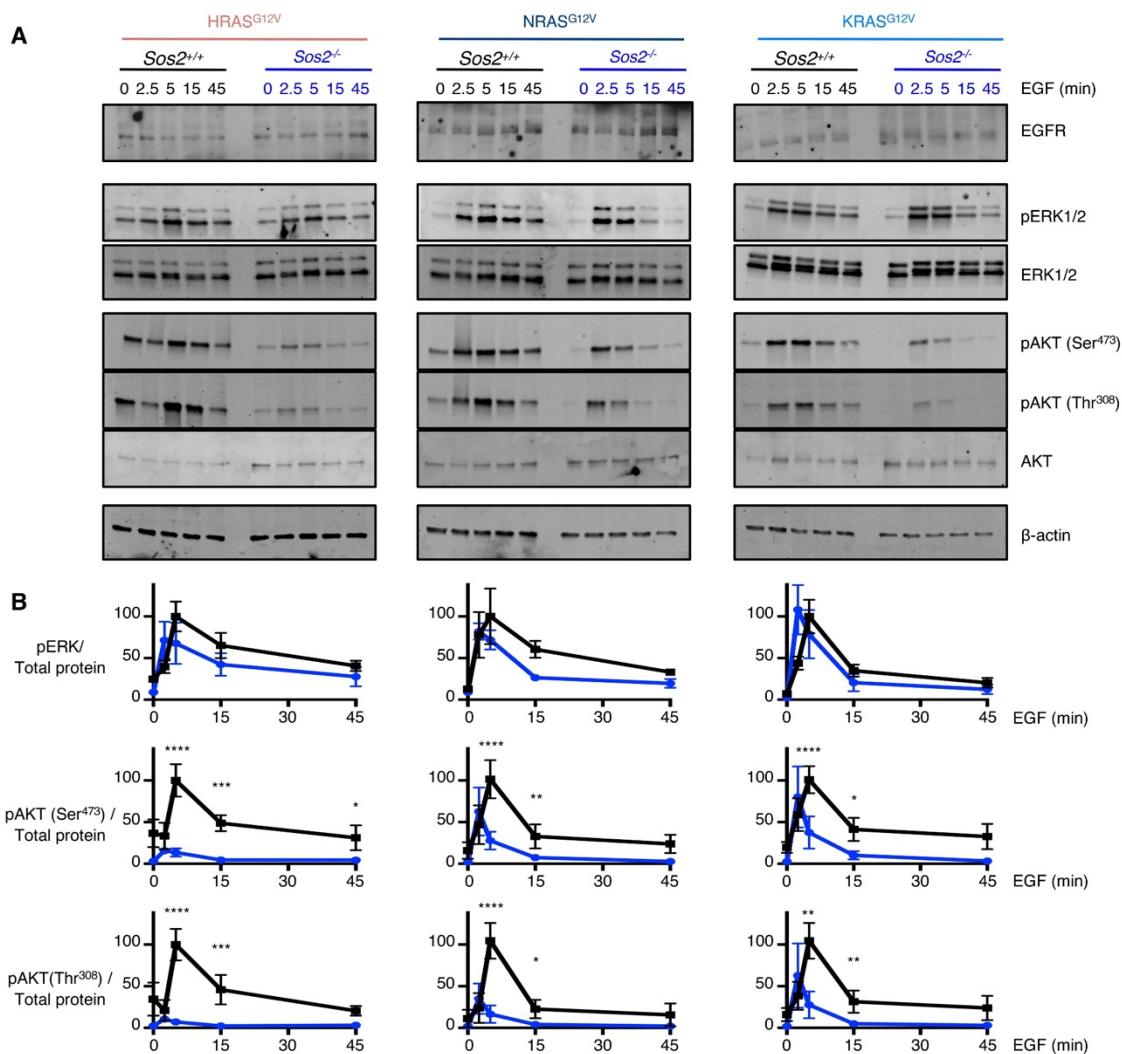


Fig. 2-4: SOS2 mediates RTK-dependent AKT phosphorylation in cells expressing mutant RAS.

Sos2^{+/+} and *Sos2*^{-/-} MEFs expressing the indicated mutant RAS isoforms were placed in serum-free media overnight, and then stimulated with 100 µg/mL EGF for the indicated times. (A) WCLs were analyzed by multiplex Western blotting for pERK1/2, ERK1/2, pAKT (Ser⁴⁷³), pAKT (Thr³⁰⁸), AKT, and β-actin on a LI-COR Odyssey machine. Blots are representative of three independent experiments. (B) Quantification of pERK1/2, pAKT (Ser⁴⁷³), and pAKT (Thr³⁰⁸) abundance versus a weighted average of total proteins (ERK1/2, AKT, and β-actin). Data are mean ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 by ANOVA using the Tukey's method to correct for multiple comparisons.

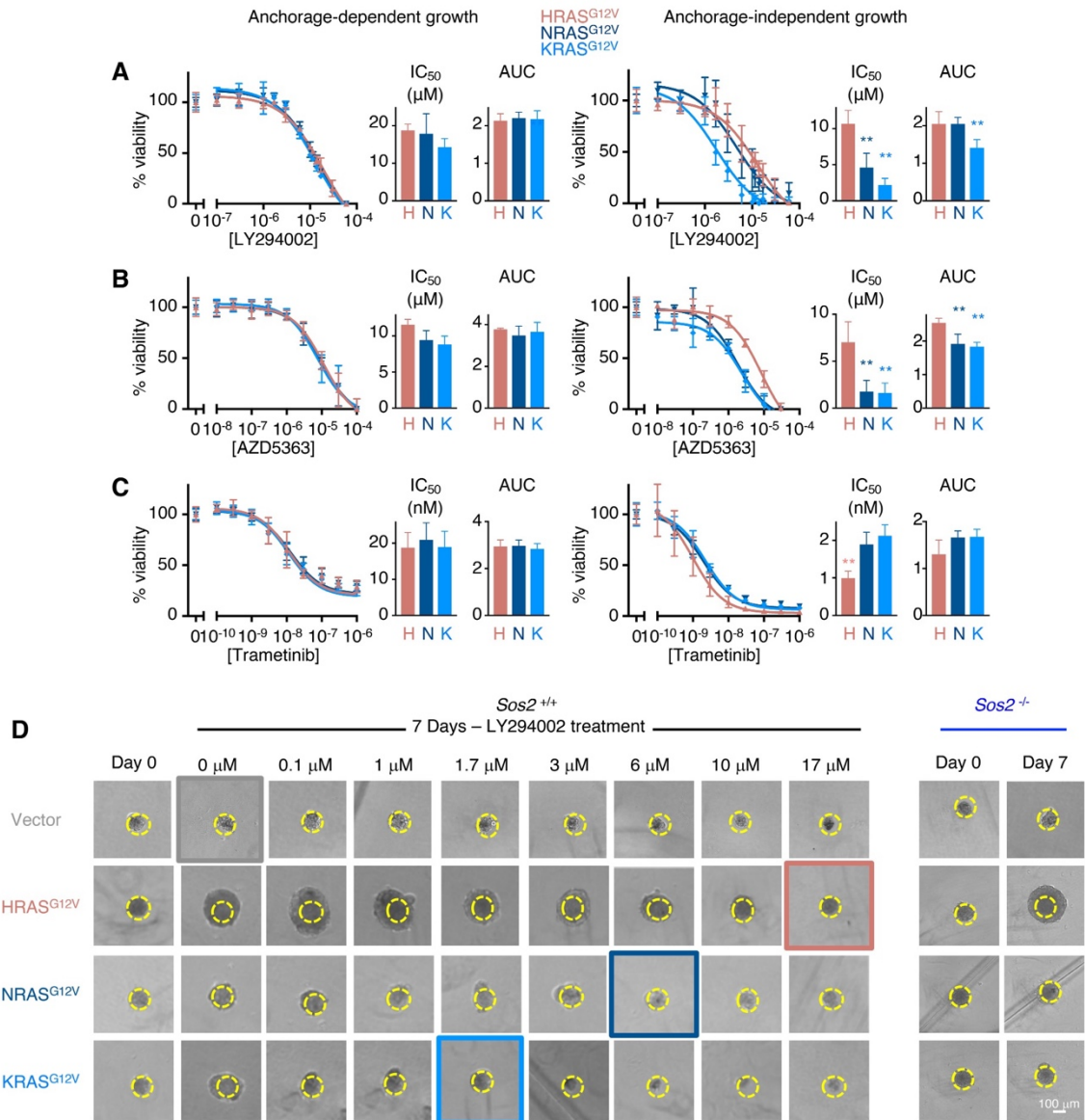


Fig. 2-5: Mutant RAS isoforms show a hierarchical requirement for PI3K signaling to drive transformation in MEFs.

(A to C) *Sos2*^{+/+} MEFs expressing the indicated mutant RAS isoforms were seeded onto either tissue-culture treated 96-well plates to assess anchorage-dependent growth or low-attachment 96-well plates to assess anchorage-independent growth. Cells were treated with the indicated concentrations of (A) the PI3K inhibitor LY294002, (B) the AKT inhibitor AZD5363, or (C) the MEK1/2 inhibitor trametinib for four days, and cell number was assessed. IC₅₀ values and AUC measurements are shown. Data are mean ± SD four independent experiments, presented relative to vehicle-treated controls. ** P<0.01 versus HRAS^{G12V}. HRAS^{G12V} (salmon triangles), NRAS^{G12V} (purple inverted triangles), KRAS^{G12V} (blue diamonds), by ANOVA using the Tukey's method to correct for multiple comparisons. (D) *Sos2*^{+/+} MEFs expressing the indicated mutant RAS isoform were seeded in low-attachment 96-well round-bottomed plates and treated with the indicated concentrations of the PI3K inhibitor LY294002 to assess the effects of PI3K inhibition on RAS-induced cancer spheroid formation (left). *Sos2*^{-/-} MEFs expressing the indicated mutant RAS isoforms were seeded in parallel and left untreated for comparison (right). Images of spheroids were taken 16 hours after plating (Day 0) and again seven days later, and are representative of three independent experiments. Scale bar, 100 μm. The outlined image for each cell line represents the LY294002 concentration where cancer spheroid size did not increase relative to day 0. Images are representative of three independent experiments. See Fig. 2-S4 for inhibition of downstream protein phosphorylation by specific inhibitors, and Fig. 2-S5 for quantification of spheroid growth between *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing mutant RAS.

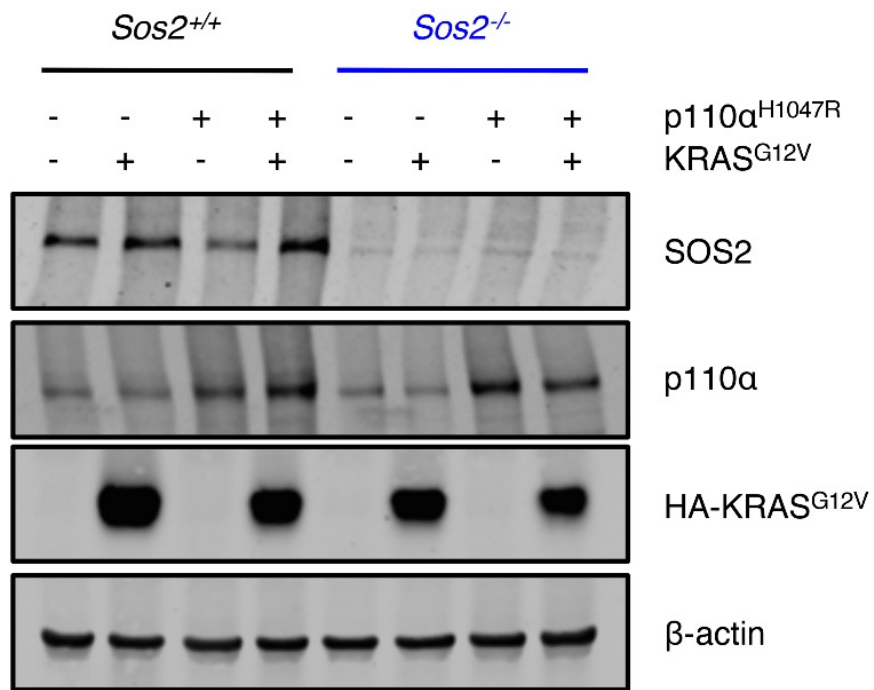
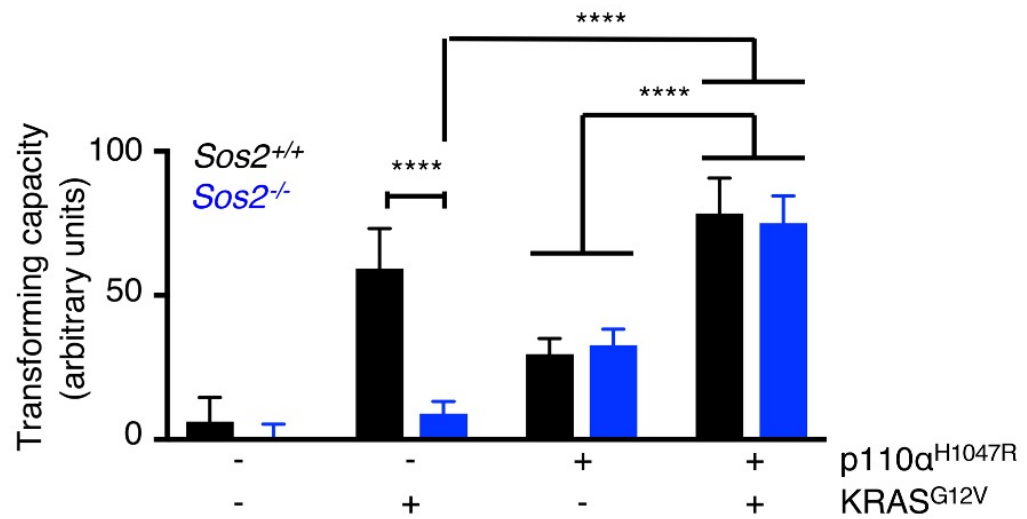
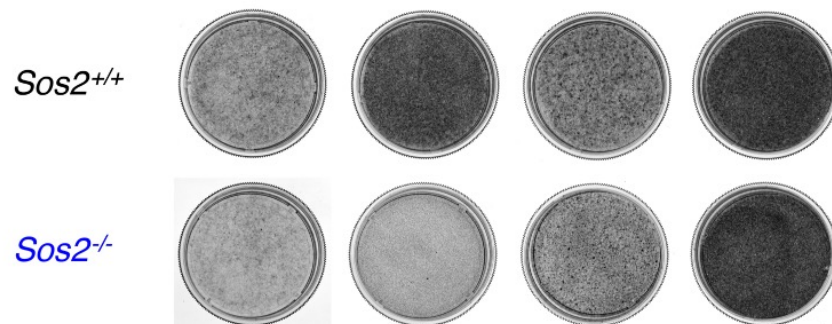
A**B****C**

Fig. 2-6: Activated PI3K (p110 α) cooperates with mutant KRAS to transform *Sos2*^{-/-} MEFs.

(A) *Sos2*^{+/+} and *Sos2*^{-/-} MEFs were transduced with lentiviruses expressing KRAS^{G12V} +/- p110 α ^{H1047R}. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for SOS2, p110 α , HA (KRAS^{G12V}), or β -actin. Blots are representative of three independent experiments. (B) *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing KRAS^{G12V} +/- p110 α ^{H1047R} were assessed for loss of contact inhibition by focus forming assay (stained dishes below, quantified above). Images are representative from three independent experiments. See Fig. 2-S6 for 10 $\times$ images of cells from (B).

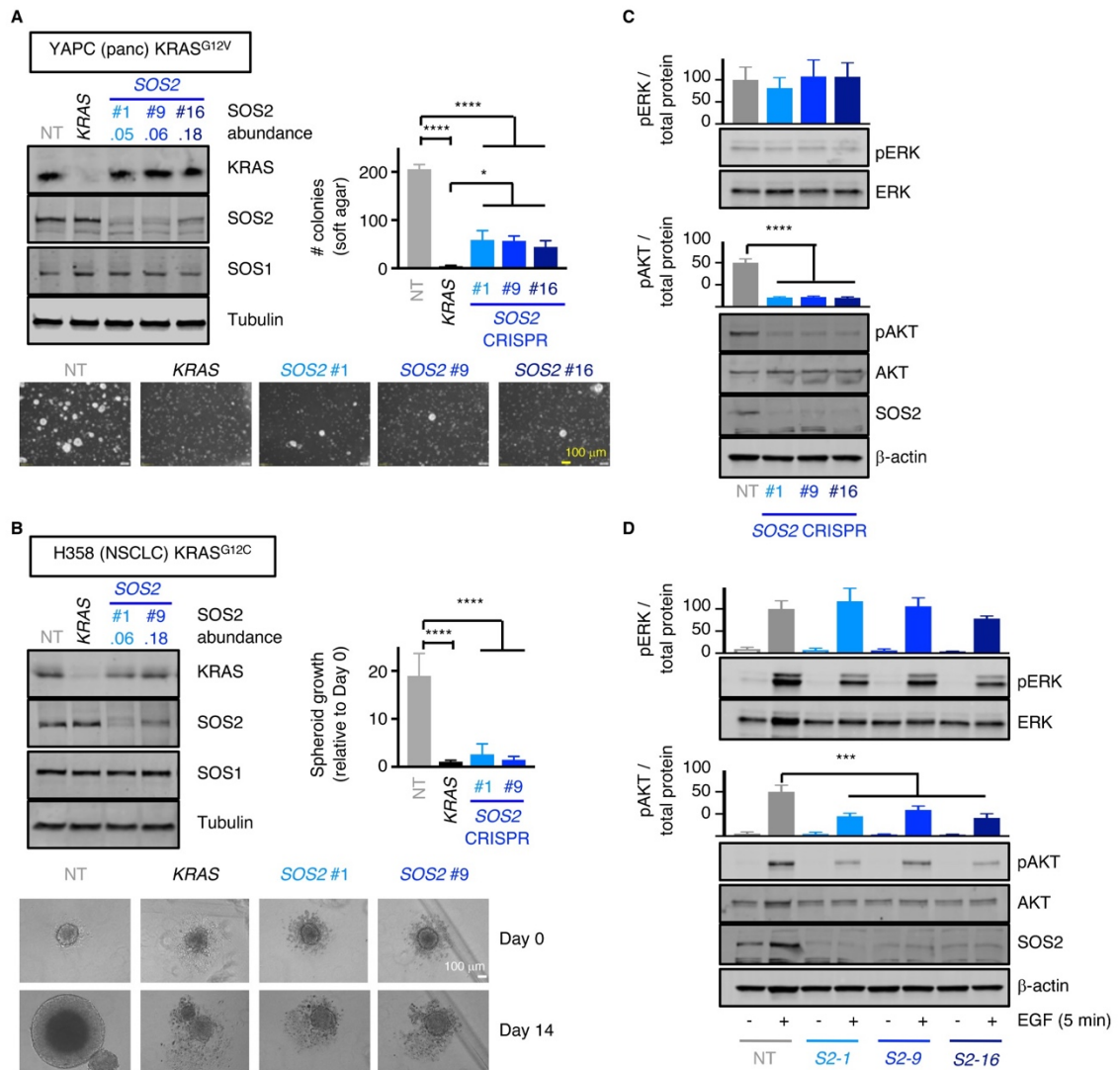


Fig. 2-7: SOS2 critically mediates transformation of *KRAS* mutant tumor cells.

(A) YAPC pancreatic cancer cells (harboring a *KRAS*^{G12V} mutation) were transduced with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT), an sgRNA targeting *KRAS*, or one of three different sgRNAs targeting *SOS2*. WCLs were analyzed by Western blotting with antibodies specific for *KRAS*, *SOS2*, *SOS1*, or tubulin (left). The *SOS2* protein abundance relative to the NT sgRNA control in the *SOS2* CRISPR samples is given. Cells were assessed for colony growth in soft agar 21 days after plating to assess anchorage independent growth (right), and 10× images showing transformed colonies growing in soft agar were taken (bottom; scale bars, 100 μm). (B) H358 NSCLC cells (harboring a *KRAS*^{G12C} mutation) were transduced with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT), an sgRNA targeting *KRAS*, or one of two different sgRNAs targeting *SOS2*. WCLs were analyzed by Western blotting with antibodies specific for *KRAS*, *SOS2*, *SOS1*, or tubulin (left). The *SOS2* protein abundance relative to the NT sgRNA control in the *SOS2* CRISPR samples is given. Cells were assessed for anchorage independent growth by cancer spheroid assay (right) and cancer spheroid growth 16 hours after plating (day 0) or 14 days later (10× images below; scale bar, 100 μm). (C and D) YAPC cells from A were either lysed actively cycling (C) or starved overnight and then stimulated with EGF 100 ng/mL for 5 minutes (D) prior to lysis. Multiplex Western blotting for pERK1/2, ERK1/2, pAKT (Ser⁴⁷³), AKT, and β-actin was performed on a LI-COR Odyssey machine. Quantification of pERK1/2 and pAKT (Ser⁴⁷³) abundance versus a weighted average of total proteins (ERK1/2, AKT, and β-actin) are shown above. All data are mean ± SD from three independent experiments; all blots and images are representative of 3 independent experiments. * P<0.05, *** P<0.001, ***** P<0.0001 by ANOVA using the Tukey's method to correct for multiple comparisons.

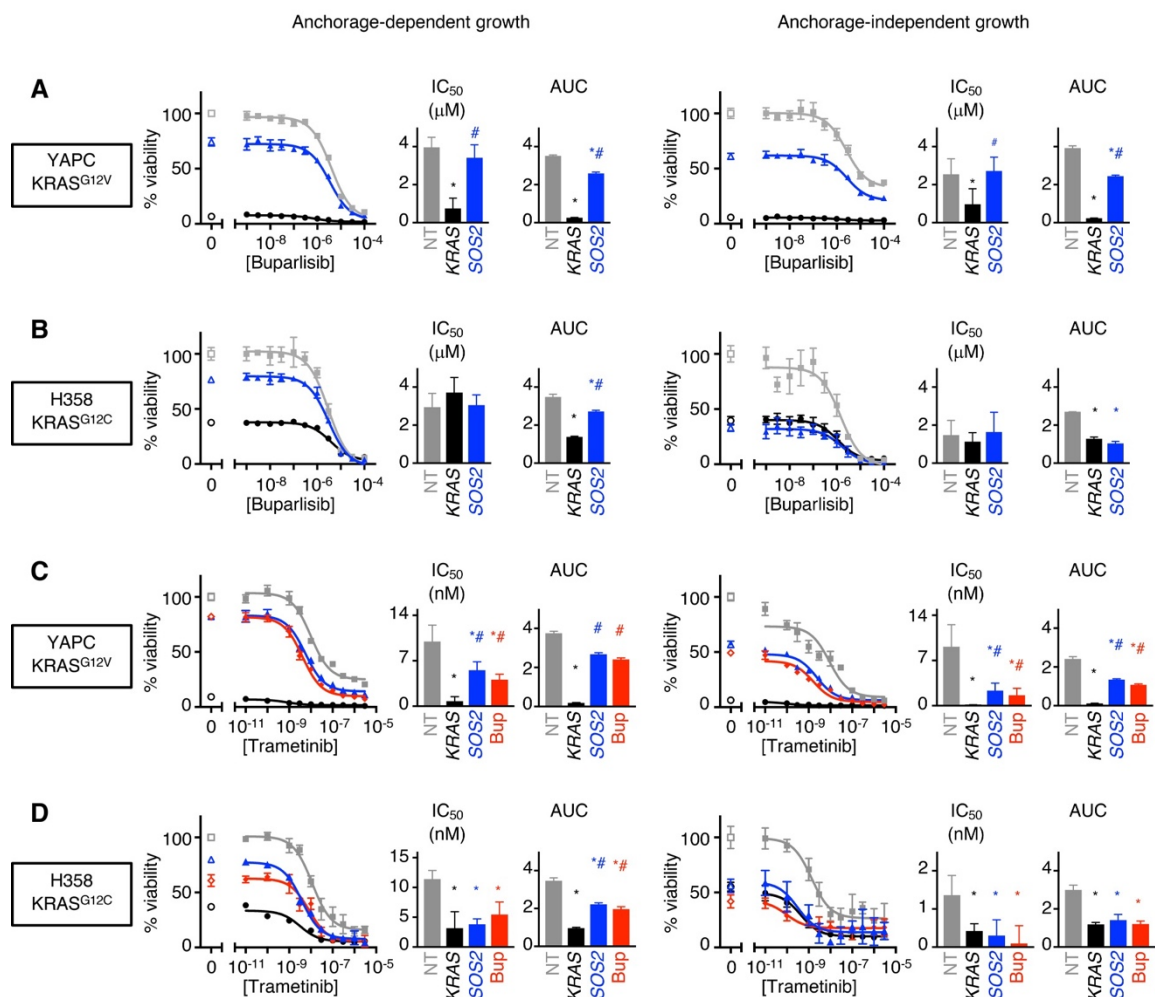


Fig. 2-8: *SOS2* deletion synergizes with MEK inhibition to revert the transformed phenotype of *KRAS* mutant tumor cells.

(A to D) *KRAS* mutant YAPC pancreatic cancer cells (A and C) or H358 NSCLC cells (B and D) transduced with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT), an sgRNA targeting *KRAS*, or an sgRNAs targeting *SOS2* were seeded onto either tissue-culture treated 96-well plates to assess anchorage-dependent growth (left) or low-attachment 96-well plates to assess anchorage-independent growth (right). Cells were treated with the indicated concentrations of the PI3K inhibitor buparlisib (A and B) or the MEK1/2 inhibitor trametinib (C and D) for five days, and cell number was assessed. For (C) and (D), NT cells were either treated with the indicated concentration of trametinib alone or in the presence of 100 ng/mL buparlisib. IC₅₀ values and AUC measurements are shown. Data are mean $\pm$ SD for three independent experiments, presented relative to vehicle-treated controls. * P<0.05 (versus NT), # P<0.05 [versus *KRAS* deletion. NT (grey squares), *KRAS* deleted (black circles), *SOS2* deleted (blue triangles), NT + 100 ng/mL buparlisib (red diamonds)] by ANOVA using the Tukey's method to correct for multiple comparisons.

SUPPLEMENTARY MATERIALS

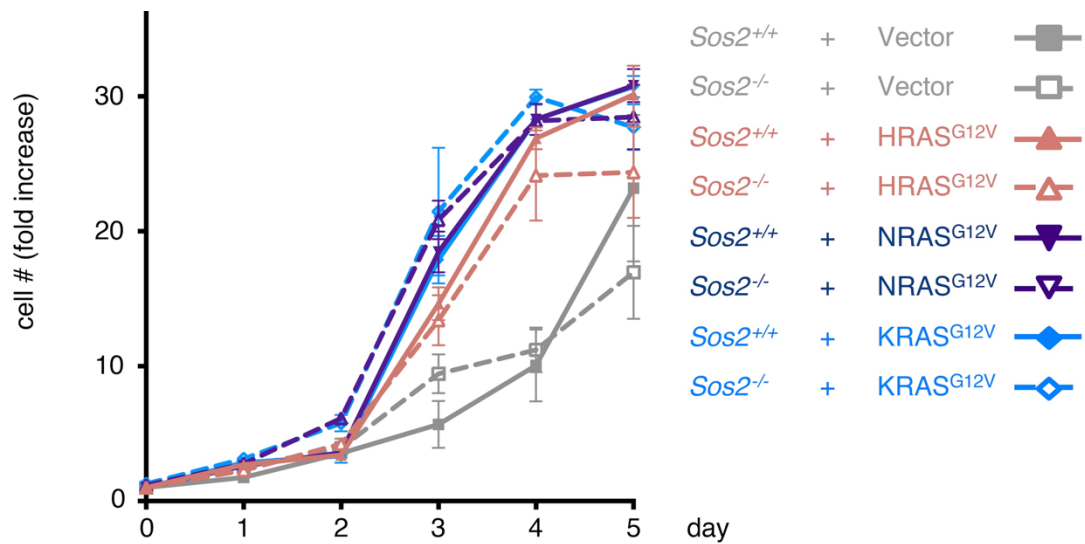


Fig. 2-S1. *Sos2* deletion does not alter cellular proliferation in MEFs expressing oncogenic RAS.

Overlay of proliferation data from Fig. 1B. WT MEFs are shown as solid lines with closed symbols, *Sos2*^{-/-} MEFs as dashed lines with open symbols. Vector control (gray squares), HRAS^{G12V} (salmon triangles), NRAS^{G12V} (purple inverted triangles), KRAS^{G12V} (blue diamonds). Data are representative from three independent experiments.

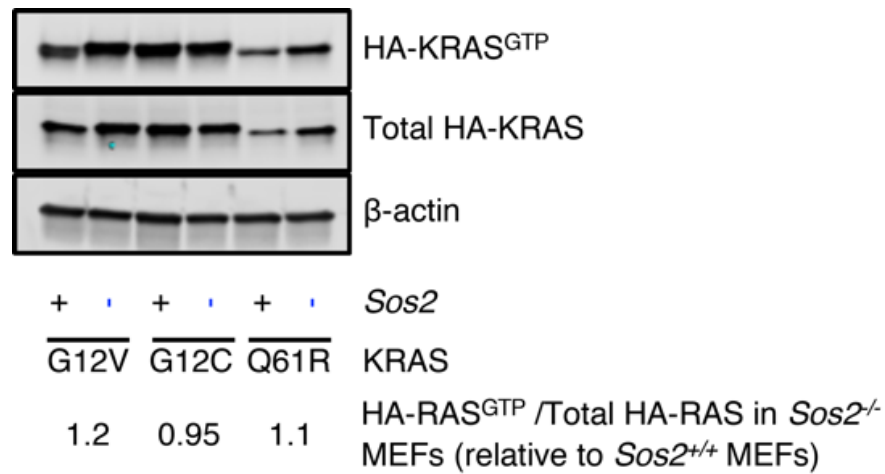


Fig. 2-S2. *Sos2* deletion does not alter GTP loading of mutant KRAS in MEFs.

Western blotting for activated HA-KRAS^{GTP} from GST-RBD pulldowns (top) or for total mutant HA-KRAS and β-actin from WCLs (below) from *Sos2*^{+/+} (+) or *Sos2*^{-/-} (-) MEFs expressing KRAS^{G12C}, KRAS^{G12V}, or KRAS^{Q61R}. A quantification of HA-RAS^{GTP} / total HA-RAS in *Sos2*^{-/-} MEFs for each mutant KRAS protein, relative to the level of HA-RAS^{GTP} / total HA-RAS in the corresponding *Sos2*^{+/+} MEFs, is shown. Blots are from three independent experiments. Data are mean ± SD from three independent experiments.

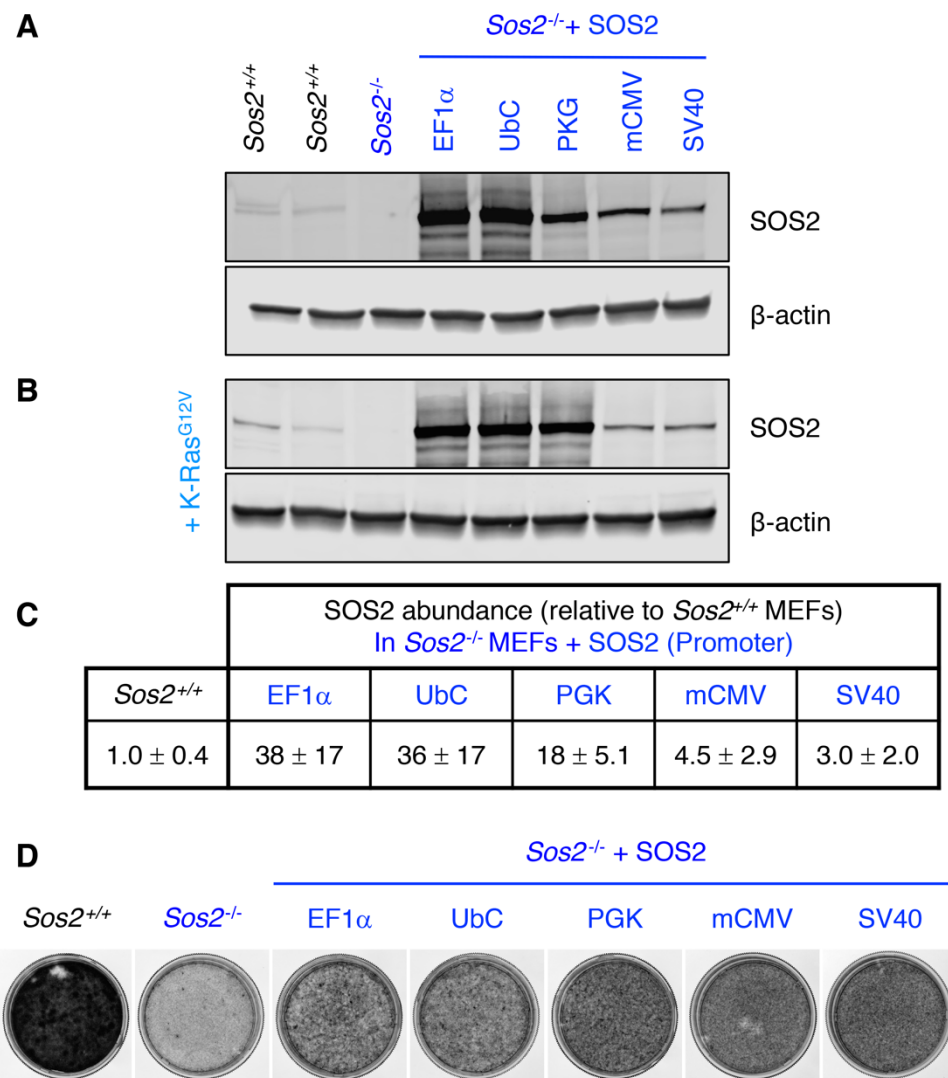


Fig. 2-S3. Introduction of SOS2 into *Sos2*^{-/-} MEFs using different promoters to drive *Sos2* expression.

(A-B) *Sos2*^{-/-} MEFs **(A)** or *Sos2*^{-/-} MEFs expressing K-Ras^{G12V} **(B)** were transduced with lentiviruses expressing WT SOS2 driven by one of five different promoters. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for SOS2 or β -actin and compared to duplicate WCLs from *Sos2*^{+/+} MEFs. Blots are representative of three independent experiments. **(C)** Quantitation of SOS2 abundance in *Sos2*^{-/-} MEFs expressing WT SOS2 driven by the indicated promoters as compared to SOS2 abundance in *Sos2*^{+/+} MEFs. Quantitation is from three independent experiments. **(D)** Stained confluent to assess loss of contact inhibition in K-Ras^{G12V}—expressing MEFs from **B**. Data are representative from three independent experiments.

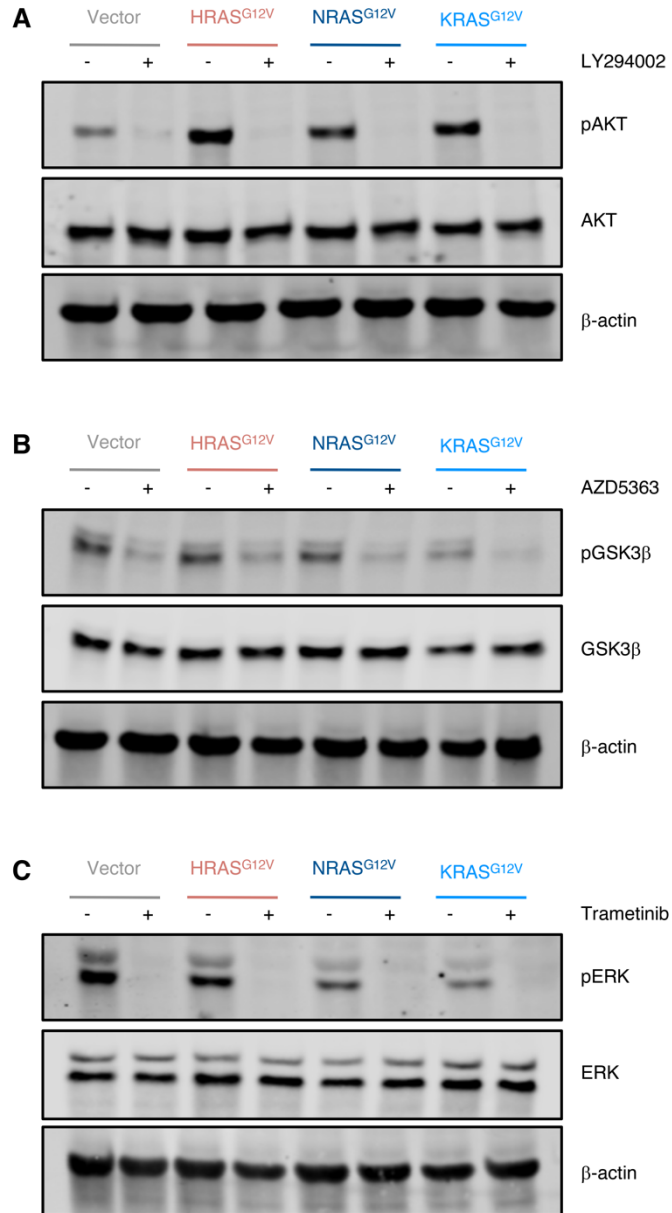


Fig. 2-S4: Inhibitor treatment of MEFs expressing mutant H-, N-, and KRAS blocks their corresponding downstream signaling pathways.

(A-C) *Sos2*^{+/+} MEFs expressing the indicated mutant RAS isoforms were treated for 2 hours with (A) 30 μ M LY294002, (B) 10 μ M AZD5363, or (C) 1 μ M trametinib prior to lysis. WCLs were analyzed by multiplex Western blotting for pAKT (Ser⁴⁷³), AKT, and β -actin (A); pGSK3 β , GSK3 β , and β -actin (B); or pERK1/2, ERK1/2, and β -actin (C) on a LI-COR Odyssey machine. Blots are representative of three independent experiments.

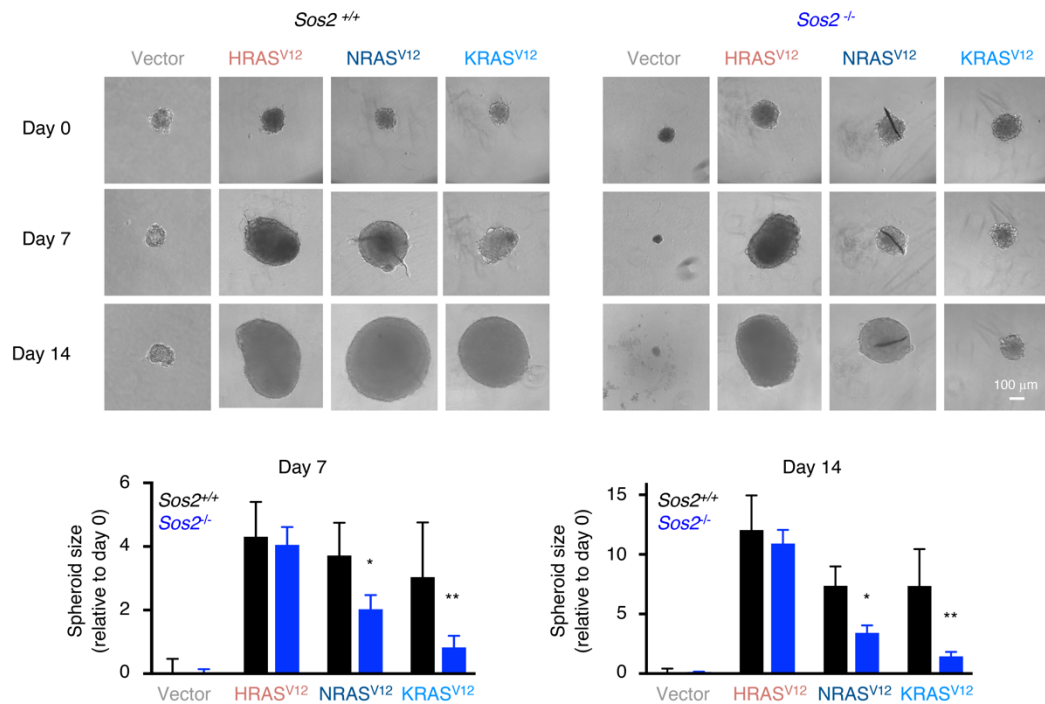


Fig. 2-S5. There is a hierarchical requirement for SOS2 in promoting mutant RAS-driven cancer spheroid growth.

Sos2^{+/+} or *Sos2*^{-/-} MEFs expressing the indicated mutant RAS isoforms were seeded in low-attachment 96-well round-bottomed plates to assess cancer spheroid formation. Images of spheroids were taken 16 hours after plating (Day 0) and again seven and fourteen days later. Cell number was assessed using CellTitre-Glo 2.0 and are expressed relative to the cell number in parallel plates at Day 0. Data are mean $\pm$ SD from three independent experiments. *P<0.05; ** P<0.01 by ANOVA using the Tukey's method to correct for multiple comparisons.

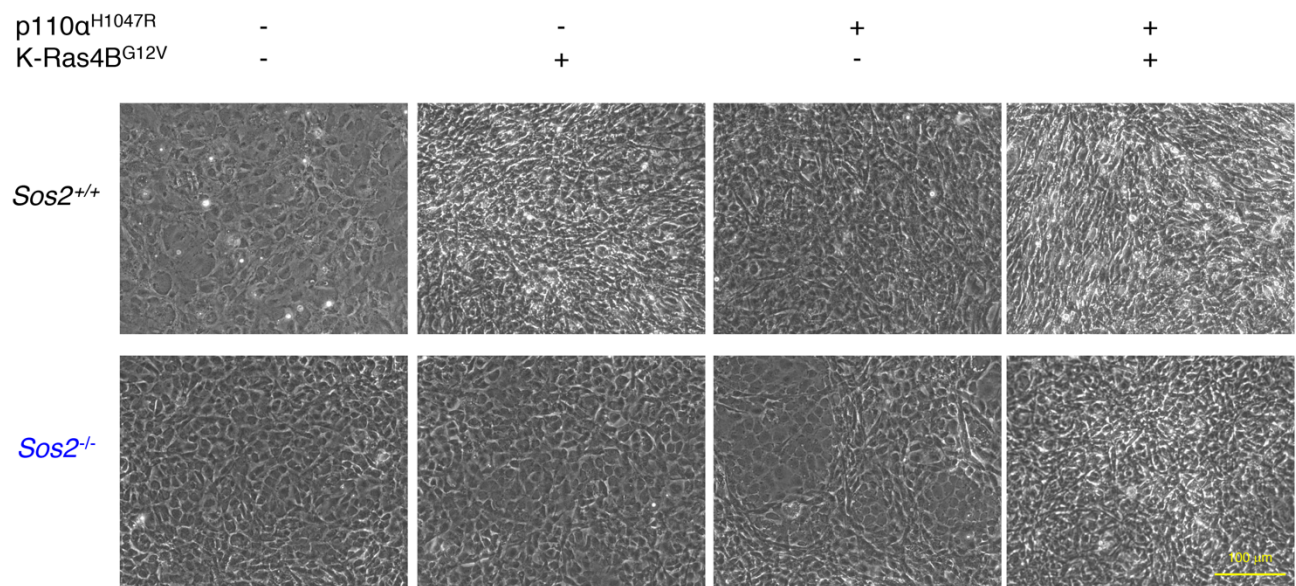


Fig. 2-S6. Activated PI3K (p110 α) cooperates with KRAS^{G12V} to transform *Sos2*^{-/-} MEFs.

10 × images of post-confluent *Sos2*^{+/+} and *Sos2*^{-/-} MEFs expressing KRAS^{G12V} +/- p110 α^{H1047R} (from **Fig. 6**) showing that combined expression of oncogenic KRAS^{G12V} + p110 α^{H1047R} can transform *Sos2*^{-/-} MEFs. Data are representative from three independent experiments.

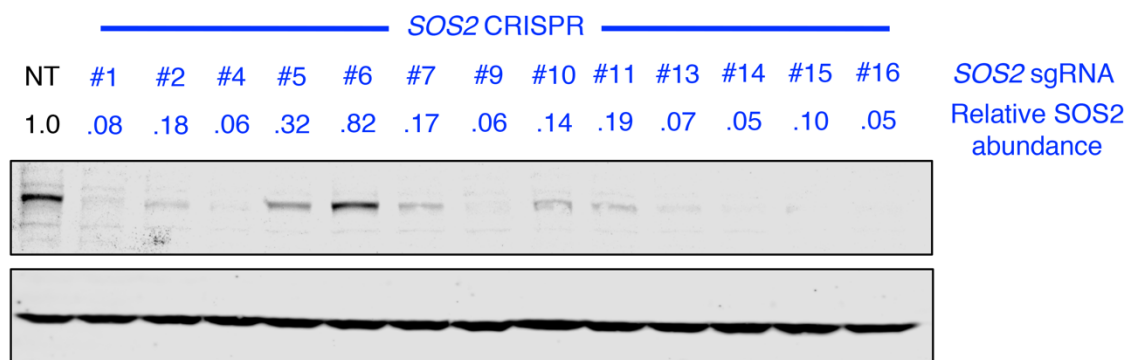


Fig. 2-S7. Deletion of SOS2 using CRISPR/Cas9.

293T cells were transduced with lentiviruses expressing Cas9 and either a non-targeting sgRNA (NT) or one of sixteen different sgRNAs targeting *SOS2*. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for SOS2 or β -actin. Cells transduced with SOS2 sgRNA #3, #8, and #12 did not survive well post infection and selection in multiple experiments and were excluded from any further analysis. The SOS2 protein abundance relative to the NT sgRNA control in the *SOS2* CRISPR samples is given. Blots are representative of three independent experiments.

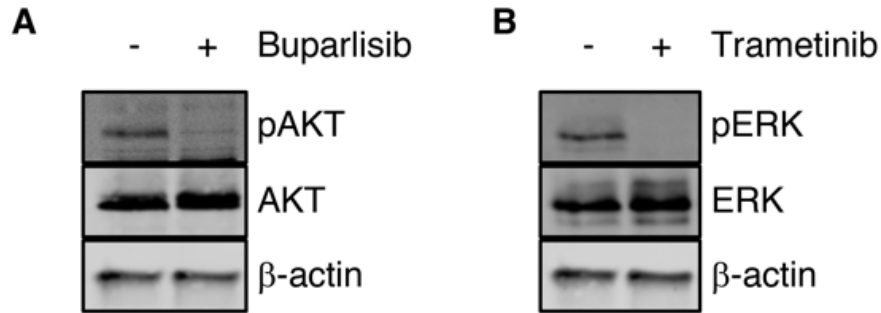


Fig. 2-S8. Inhibitor treatment of YAPC cells blocks their corresponding downstream signaling pathways.

(A-B) YAPC pancreatic cancer cells (harboring a KRAS^{G12V} mutation) transduced with lentiviruses expressing Cas9 and a non-targeting sgRNA were treated for 2 hours with **(A)** 10 μ M buparlisib or **(B)** 1 μ M trametinib prior to lysis. WCLs were analyzed by multiplex Western blotting for pAKT (Ser⁴⁷³), AKT, and β -actin **(A)**; or pERK1/2, ERK1/2, and β -actin **(B)** on a LI-COR Odyssey machine. Blots are representative of three independent experiments.

Table 2-S1. sgRNA sequences used in this study.

sgRNA target	Sequence
Non-targeting (NT)	CCATATCGGGGCGAGACATG
<i>KRAS</i>	TCATTGCACTGTACTCCTCT
<i>SOS2</i> #1	GAATCGGTGCCAAACATGAA
<i>SOS2</i> #2	GCCAATTTGTCTGCGTCAGT
<i>SOS2</i> #3	GGCCGACATGGCTCTACCTC
<i>SOS2</i> #4	ACTGGCACCAGCATAAGACG
<i>SOS2</i> #5	GTTCAACCGTCTGAACTTGT
<i>SOS2</i> #6	GTGACCTCGTAAAGTACCTG
<i>SOS2</i> #7	AGCCGAGTCTGGCCATGATT
<i>SOS2</i> #8	AGCAGATTGTGCATCAGCAA
<i>SOS2</i> #9	GAGAACAGTCCGAAATGGCG
<i>SOS2</i> #10	GTCCACCTCCACCAATTGAA
<i>SOS2</i> #11	AGGCCTAACACAGGCCGACA
<i>SOS2</i> #12	ACCTACTGACGCAGACAAAT
<i>SOS2</i> #13	TCTACCTCAGGTACTTTACG
<i>SOS2</i> #14	TTGGCACCGATTCTTGTCAA
<i>SOS2</i> #15	TGTCCTTCCACGTCTTATGC
<i>SOS2</i> #16	AATTATGCATGGCCCAGCCA

CHAPTER 3: 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 tumors. 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 tumor 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 signaling 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 signaling. *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.

RAS IN CANCER

The RTK–RAS pathway is among the most commonly mutated pathways in cancer(168). The three RAS genes, *HRAS*, *NRAS*, and *KRAS*, are the most commonly mutated gene family, with mutations found in around 30% of human tumors(218). Within the RAS gene family, *KRAS* is the most frequently mutated, accounting for 85% of RAS-driven cancers(218), including 95% of pancreatic cancers(130), 42% of colon cancers(91), and 20-30% of lung cancers(235), which are the top three causes of cancer-related death in the United States(135; 218). *H*- and *NRAS* mutations are common in other cancer types, including bladder cancers and skin cancers, respectively(228). 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(58; 132; 235; 273). Targeting other RTK pathway members has also had limited success in RAS-driven cancers, due to high toxicities and resistance arising from disruption of feedback mechanisms in the RTK–RAS–RAF pathway (39). Together, these difficulties in developing treatments for RAS-driven tumors 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 signaling 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(27; 198; 325); in *KRAS*-mutant tumors loss of heterozygosity (LOH) at the *KRAS* locus correlates with increased tumor

growth and shortened overall survival(318). These data suggest that in *KRAS*-mutant tumors, wild-type *KRAS* may have a tumor suppressor role(65; 95; 170), a hypothesis which has been supported by observations *in vitro*(6; 66) and *in vivo* with mouse models(6; 283; 324).

In contrast, the two non-mutated RAS family members may play a tumor-promoting role in *RAS*-mutant tumors. In *RAS*-mutated cancer cell lines, wild-type RAS isoforms promote mutant RAS-driven proliferation and transformation (15; 105; 177; 317). Wild-type RAS isoforms may contribute to oncogenic signaling through their ability to activate effector pathways that the mutant isoform does not strongly activate, making the cellular outcome a product of signaling by wild-type and mutant RAS(33). 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(25). SOS1 can also be activated by allosteric binding of RAS-GTP(185), 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 tumors. 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 tumors.

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(260). 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 signaling to anchorage-dependent proliferation of RAS-mutant cancer lines by Young et al. (317).

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 to 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(199). 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 (Fig. 3-1). 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(129). 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 μ m 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(124). 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 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 (Fig. 3-2, bottom), which did not alter 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 cell expressing mutated *HRAS*, *NRAS*, or *KRAS* (Fig. 3-2, 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 (Fig. 3-2, 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* deletions significantly reduced transformation, as we had previously observed in *KRAS* mutated YAPC pancreatic cancer cells(260).

To confirm 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* (Fig. 3-3, 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 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/PKI3K signaling 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 (Fig. 3-2). In contrast, AKT phosphorylation was not reduced by *SOS2* deletion in RD cells, perhaps due to their already high levels of AKT phosphorylation (Fig. 3-2, middle) and consistent with previous reports where AKT phosphorylation was shown to be independent of WT RAS

in RD cells (317). To determine whether the decreased AKT phosphorylation we observed was due to reduced RTK signaling, we assessed EGF-stimulated PI3K/AKT signaling 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 (Fig. 3-4).

We further assessed whether *SOS2* was required for RTK-stimulated WT RAS activation in *HRAS* mutated (T24) and in *KRAS* (H23) mutated cancer cells (Fig. 3-5) using RAS binding domain (RBD) pulldowns after overnight serum deprivation. In *KRAS* mutated cancer cells, we found that *SOS2* 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(32; 217). 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 four hours after plating. As expected, oncogenic *RAS* deletion reduced cell 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 (Fig. 3-6A). These data are consistent with the differential requirement for *SOS2* to support transformation of *KRAS* mutated cancer cells (see Figs. 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 (Fig. 3-6B).

Taken together, these results demonstrate the differential dependence on *SOS2* for *RAS*-dependent transformation originally established in MEFs (260) 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 tumors. It also indicates that promising drug targets like *SOS2* (124), RTKs (72; 183; 221; 278), or Shp2 (82; 181; 243), 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 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 signaling 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 the 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 (Fig.7A) (105; 106; 172; 293; 312). *BRAF* and *KRAS* mutations are generally mutually exclusive in cancer (cBioPortal)(37; 87), 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)(37; 87). In fact, *PIK3CA* mutations and *HRAS* or *KRAS* mutations co-occur in cancers more frequently than would be expected by random mutation (cBioportal)(37; 87), 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 (312)(Fig. 3-7A), 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, mutant HRAS-expressing cells were more sensitive to MEK inhibition than mutant KRAS-expressing cells(260). 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 signaling help explain why *SOS2* deletion specifically affects transformation by KRAS, but not HRAS. We previously showed that at the level of effector signaling, *Sos2* deletion reduced RTK-dependent AKT phosphorylation in MEFs expressing all mutant RAS isoforms(260), 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 signaling in promoting RAS-driven transformation ($KRAS \geq NRAS > HRAS$) 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 signaling than cells expressing mutated NRAS or HRAS. Furthermore, KRAS-driven transformation could be rescued in *Sos2*^{-/-} MEFs by introduction of an activated PI3K catalytic subunit,

suggesting that *Sos2* deletion specifically reduced PI3K–AKT signaling to block oncogenic transformation.

Based on our previous results(260) and those of others, we propose a working model of SOS signalling in mutant KRAS-driven cancers (Fig.7B). 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(185), relieving SOS1 autoinhibition (269) and thereby activating a mutant KRAS^{GTP}–SOS1–wild-type RAS positive feedback loop that helps drive cell proliferation (136). In parallel, constitutive RAF–MEK–ERK signalling causes multiple ERK- and RSK-dependent negative feedback phosphorylation events (36; 54; 67-69; 117; 142; 164; 193; 236; 247) including phosphorylation of SOS1 that result in both ERK-dependent dissociation of the Grb2/SOS1 complex(54; 67; 142; 164) and RSK-dependent cytoplasmic sequestration of SOS1 by 14-3-3(69; 247). 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(247), 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(260). 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(260). 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(84). 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(30; 31; 45; 108; 116) which has been proposed as a mechanism of regulation for RAS signalling or (ii) by activation of Rac/p110 β 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 (25; 210; 257). 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 (128). In primary MEFs, combined deletion of SOS1 and SOS2 is required to reduce RTK-stimulated RAC activation(174). Furthermore, activating mutations in the DH domain of SOS2 are associated with Noonan Syndrome, indicating the functional importance of this domain in SOS2 (55;

311). 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(260). These results indicate that *SOS2*-dependent PI3K signaling 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 tumors that have wild-type PI3K/PTEN signaling, since expression of an activated p110 α 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 (Figs. 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(260). 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 nonessential amino acids, and 1% penicillin-streptomycin.

Production of recombinant lentiviruses. Lentiviruses were produced by co-transfecting MISSION lentiviral packaging mix (Sigma) into 293T 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 $\mu\text{g/mL}$. All cell lines were selected with 4 $\mu\text{g/mL}$ 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. 48 hours post-infection, cells were selected in 4 µg/mL Puromycin. Ten days after selection, cells were analyzed 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 minutes at 4°C and spun at 10,000 RPM for 10 minutes. Clarified lysates were boiled in SDS sample buffer containing 100 mM DTT for 10 minutes 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 Ser⁴⁷³ (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 minutes 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 minutes. GST-RBD bound to glutathione-sepharose beads (Millipore) were used to isolate RAS-GTP from lysates by rotating incubation for one hour 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 minutes 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 hours after plating to account for any discrepancies in plating, and then every 48 hours for 7 days. Data were analyzed 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 hours 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 CellTiter 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-bottomed (130) plates (1-1000 cells/well), cultured for 7-10 days, and scored for the formation of cancer spheres(129). 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(124).

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.

FIGURES

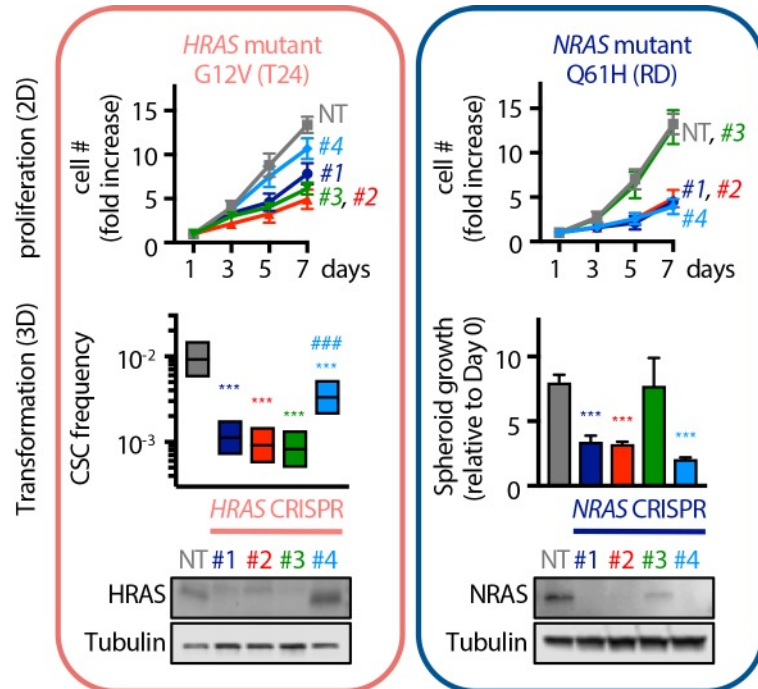


Fig. 3-1. Mutant RAS is required for 2D and 3D growth in *HRAS* and *NRAS*-mutant cancer cells.

(A) *HRAS* (G12V) mutant T24 bladder cancer cells were transduced with lentiviruses with 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 analyzed 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 $\pm$ 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.

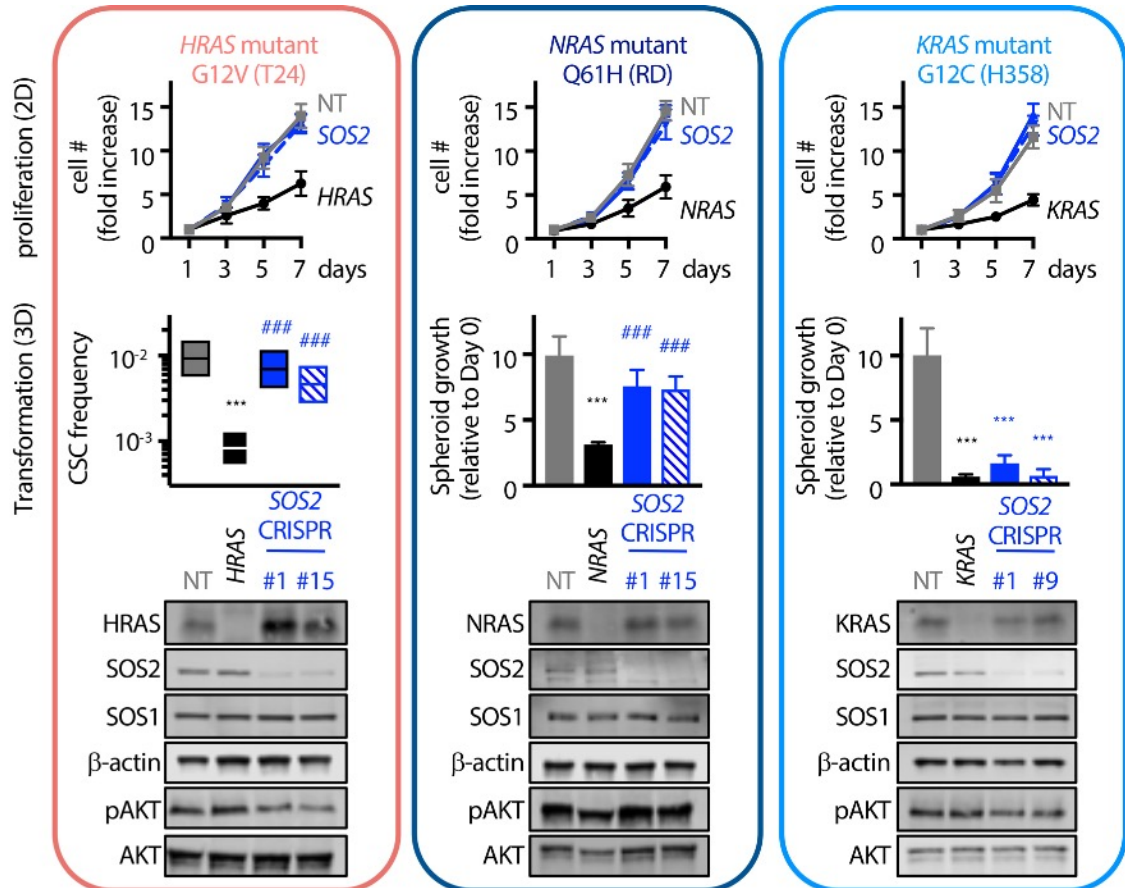


Fig. 3-2. *KRAS*-mutant cancer cells depend on *SOS2* for anchorage-independent, but not anchorage-dependent, growth.

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 (lite 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 analyzed by Western blotting with antibodies specific for *SOS1*, *SOS2*, *HRAS*, *NRAS*, *KRAS*, β -actin, pAKT, and AKT (bottom). Data are mean $\pm$ 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 mutant *RAS* deletion.

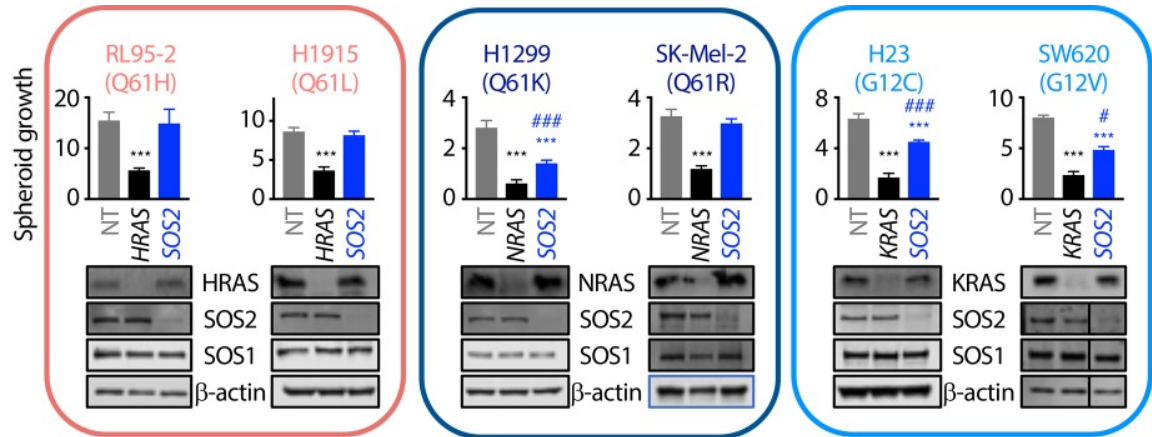


Fig. 3-3. There a differential requirement for SOS2 to promote anchorage-independent growth in *RAS*-mutant cancer cell lines.

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 analyzed 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 3 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.

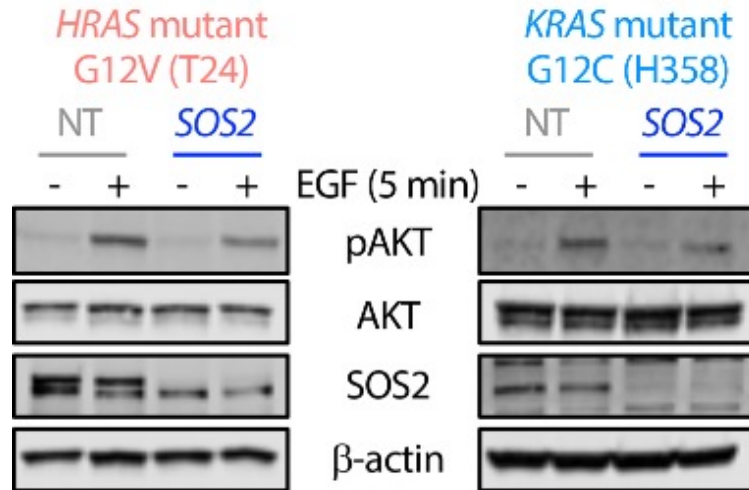


Fig. 3-4. SOS2 is required for full RTK-stimulated AKT phosphorylation in *RAS* mutated cancer cells.

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 minutes prior to lysis. Multiplex Western blotting for pAKT (Ser⁴⁷³), AKT, SOS2, and β -actin was performed on a LI-COR Odyssey machine.

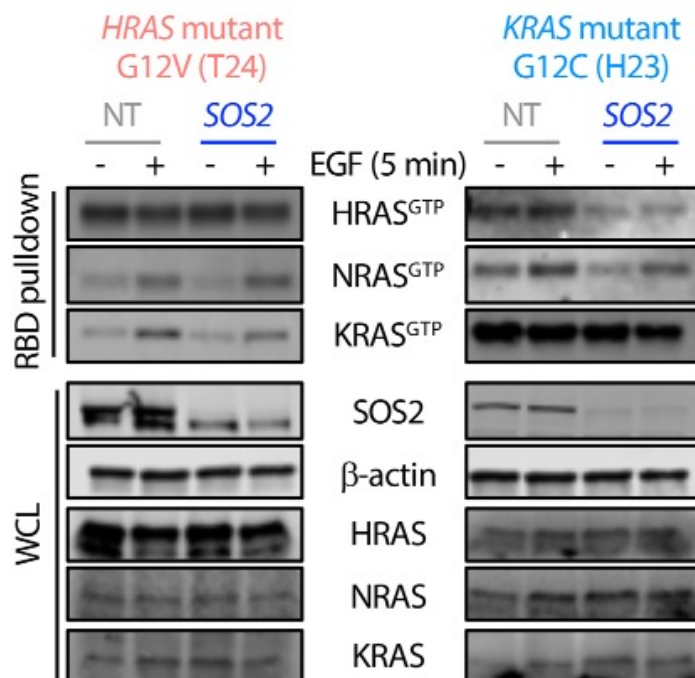


Fig. 3-5. SOS2 is required for full RTK-stimulated WT RAS activation in *RAS* mutated cancer cells.

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 minutes prior to lysis. Lysates were subjected to GST-RAS binding domain pulldowns, 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.

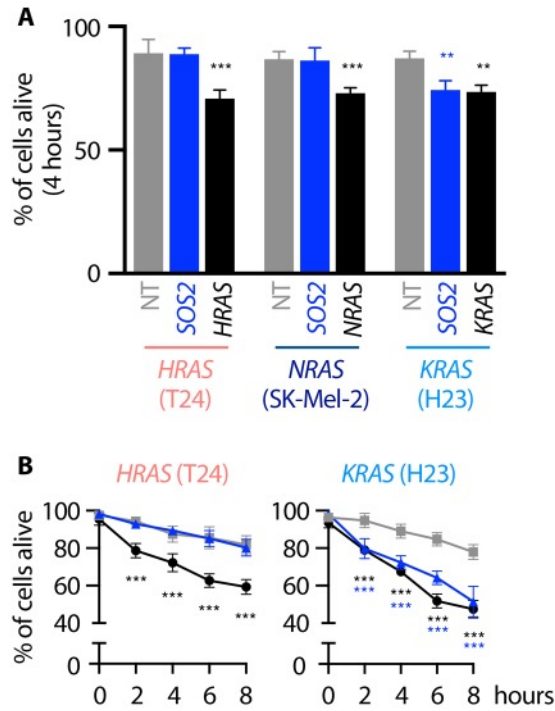


Fig. 3-6. SOS2 is required for resisting anoikis in *KRAS*-mutated cancer cells.

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 four hours (A) or for 0, 2, 4, 6, or 8 hours (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.

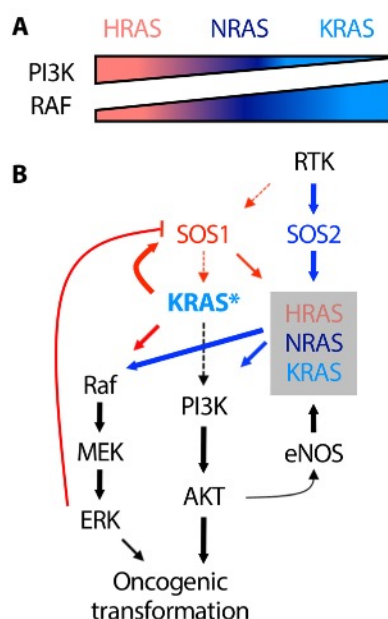


Fig. 3-7. An integrated working model of SOS signaling in RAS mutated cancer cells.

(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 signaling in mutant KRAS-driven cancers.

TABLES

Table 3-1. 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 adenocarcinoma

CHAPTER 4: Preliminary findings on the role of SOS1 in oncogenic RAS-driven anchorage-dependent and anchorage-independent growth

INTRODUCTION

Thus far our studies have focused on SOS2 due to its requirement in KRAS-driven cancer cells and the high frequency of *KRAS* mutations. However, understanding the role of SOS1 in RAS-mutant cancer is important both in fully understanding how RasGEFs contribute to oncogenic RAS signaling and because SOS1 inhibitors have already been developed and are currently moving towards early clinical trials (119; 121). Understanding the role SOS1 plays in RAS-driven cancer is key to using these drugs effectively, for predicting effective combination therapies, and for identifying potential resistance mechanisms. This chapter includes our studies to date on the role of SOS1 in RAS-driven cancers, including evaluating its role in proliferation and transformation, investigating the molecular mechanisms of its contribution, and determining the role of SOS1 in signaling in the context of mutant RAS. Our results concur with previous studies on the role of SOS1 in proliferation in mice (11; 175), mouse embryonic fibroblasts (MEFs) (174), and *KRAS*-mutated cancer cells (136; 316). We extend these results to systematically evaluate the effects of *SOS1* deletion on proliferation and transformation in immortalized MEFs expressing mutant RAS and *RAS*-mutated cancer cells of all isoforms. Our findings on both SOS1 and SOS2 reinforce the need to screen for therapeutic targets in cancer with anchorage-independent (3D) growth assays, as both *SOS1* and *SOS2* deletion affect 3D growth differently than 2D growth, in such a way as changes their potential as therapeutic targets.

SOS1 is necessary for mouse development (79; 229), although not for survival of adult mice, where SOS2 compensates for *Sos1* deletion (11). *Sos1* conditional knockout in adult mice decreases thymocyte proliferation (11; 154) and keratinocyte proliferation,

alters hair follicle and blood vessel integrity in the skin, and decreases adipose tissue (175). Double knockout of *Sos1* and *Sos2* worsened these effects and blocked wound repair and the neutrophil inflammatory response, suggesting that SOS1 is not fully responsible for proliferation and normal signaling in these cells (175). In primary MEFs, *Sos1* knockout decreased proliferation and viability and increased mitochondrial oxidative stress. *Sos1* deletion also altered cell morphology, leading to a flat morphology and increased cell perimeter not seen in wild-type or *Sos2* single knockout cells (174). *Sos1* knockout delays tumor initiation, growth and progression in a carcinogen-mediated mouse-model of HRAS-driven skin cancer (175), indicating that SOS1 may play an important role in RAS-driven oncogenesis.

SOS1 can participate in mutant RAS-driven oncogenic signaling through multiple potential mechanisms. In two well-established mechanisms, SOS1 contributes through the activation of wild-type RAS, which plays an important role in mutant RAS-driven cancer (325). First, SOS1 can be allosterically activated by RAS (186), which increases its ability to activate wild-type RAS. This mechanism enhances proliferation in *KRAS*-mutated pancreatic cancer cells (136). Second, SOS1 can also participate in canonical RTK-mediated activation of wild-type RAS, which can then signal in parallel with constitutively active mutant RAS (317). SOS1 may also contribute to activation of mutant RAS. Oncogenic *RAS* mutations significantly reduce GTPase activity, but do not completely prevent it, and thus oncogenic RAS mutants may rely on GEFs for GTP reloading after hydrolysis. In addition to acting as a GEF on wild-type or mutant RAS, SOS1 may also contribute to downstream signaling independently of its RasGEF activity, either through alternative catalytic activity, such as its ability to activate the small

GTPase Rac (128), or as a scaffold for receptors and other signaling components (123; 150).

Here, we used immortalized *Sos1^{fl/fl}* and *Sos1^{fl/fl}Sos2^{-/-}* MEFs and RAS-mutated cancer cell lines to systematically investigate the role of SOS1 in mutant RAS-driven proliferation and transformation for all RAS isoforms. We found that SOS1 is required for proliferation with and without mutant RAS, but only partially required for RAS-driven transformation, regardless of RAS isoform. Using established SOS1 mutant constructs, we showed that full proliferation requires both its GEF activity and its ability to be allosterically activated by RAS. We also investigated the contribution of SOS1 to downstream RAS effector pathway signaling in the context of mutant RAS and found that *Sos1* deletion alone did not affect AKT or ERK activation, suggesting that its effects on proliferation and transformation are mediated through a different pathway. These results indicate that SOS1 plays an important role in mutant RAS-driven proliferation and transformation through its ability to accept positive feedback from active RAS and activate more RAS in turn. They provide a preliminary understanding of the mechanism behind the contribution of SOS1 to RAS-driven cancers and suggest new directions to pursue in future studies.

MATERIALS AND METHODS

Cell culture. RD cells and immortalized MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM); YAPC cells were maintained in Roswell Park Memorial Institute medium (RPMI); 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 nonessential amino acids, and 1% penicillin-streptomycin.

RAS construct cloning. HA-HRAS^{G12V} was PCR amplified from pBabePuro and cloned into EcoRI/BamHI digested pCDH-CMV-MCS-EF1-Puro (System Biosciences) using the GeneArt Seamless Cloning and Assembly Kit (Invitrogen). To clone in wild-type KRAS and wild-type NRAS, pCDH-HA-HRAS^{G12V} was digested with BamHI/NotI to remove HRAS but maintain the HA tag, and wild-type KRAS or NRAS were PCR amplified and cloned in using GeneArt. Oncogenic point mutations (NRAS^{G12V} and KRAS^{G12V}) were then introduced by site-directed mutagenesis.

Production of recombinant viruses. Lentiviruses were produced by co-transfecting MISSION lentiviral packaging mix (Sigma) into 293T cells using TransIT-Lenti transfection reagent (Mirus). CRE retroviruses were produced by calcium phosphate transfection of MCSV-IRES-GFP (YFP) or YFP-IRES-CRE vectors into Phoenix-Eco cells using TransIT-Lenti transfection reagent (Mirus) or 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 $\mu\text{g/mL}$. MEFs were selected with 150 $\mu\text{g/mL}$ Hygromycin (Invitrogen), 4 $\mu\text{g/mL}$ Puromycin (Invitrogen), or 2.5 $\mu\text{g/mL}$ Blasticidin (Invitrogen). YAPC cells were selected with 6 $\mu\text{g/mL}$ Puromycin (Invitrogen). RD and T24 cells were selected with 4 $\mu\text{g/mL}$ Puromycin (Invitrogen).

CRISPR-mediated gene targeting. A non-targeting (NT) single guide RNA (sgRNA), a KRAS-targeted sgRNA, an HRAS-targeted sgRNA, an NRAS-targeted sgRNA, and the three confirmed SOS1-targeted sgRNAs were each cloned into

pLentiCRISPRv2 as previously described (249). sgRNA sequences are given in supplemental table S1. Lentiviruses were produced as described above. 48 hours post-infection, cells were selected in 4 µg/mL Puromycin. Ten days after selection, cells were analyzed for KRAS, HRAS, NRAS, and/or SOS1 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 minutes at 4°C and spun at 10,000 RPM for 10 minutes. Clarified lysates were boiled in SDS sample buffer containing 100 mM DTT for 10 minutes 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-β-actin (Sigma AC-15; 1:5,000), anti-HA (Santa Cruz sc-805; 1:1000), 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 (Thr³⁰⁸ [Cell Signaling 2965; 1:1000] or Ser⁴⁷³ [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).

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) to assess cell number. Cell number was assessed 24 hours after plating to account for any discrepancies in plating, and then every 48 hours for 7 days. Data were analyzed as an increase in luminescence over Day 0.

For competition assays, *Sos1^{fl/fl}Sos2^{-/-}* MEFs were infected with constructs as indicated before infection with retrovirus expressing MCSV-IRES-GFP (YFP) or YFP-IRES-CRE. After four days, cells were trypsinized and suspended in PBS with 1% bovine serum albumin. The percent of cells expressing YFP was measured by flow cytometry using a BD Accuri C6 flow cytometer. Cells were plated in a 3T1 protocol. The percentage of YFP positive cells was assessed every three days as the cells were passaged.

Low-density proliferation assays were performed as described by Drosten et al (70). Briefly, cells were plated at $5\text{--}10 \times 10^3$ cells per 10-cm dish and allowed to form colonies over two weeks. Plates were fixed with methanol and stained with crystal violet (Alfa Aesar). Colonies were scored using ImageJ (254).

Transformation Studies. For assessing loss of contact inhibition, cells were seeded at 10^5 cells per 6-cm dish (focus forming assays). 10× images of focus forming assays were taken 14-21 days after seeding, and then the dishes were stained with 1% bromophenol blue (153). Stained focus forming assay dishes were scanned on a Biorad Chemidoc Imaging System and cell density was quantified using Image Lab software.

To assess anchorage-independent growth, cell growth was measured in soft agar or spheroids. For growth in soft agar, cells were seeded in 0.32% Nobel agar at 2×10^4 cells per 35-mm dish. Soft agar colonies were counted 21-28 days after seeding. 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 hours 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 CellTiter 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-bottomed plates (130) (1-1000 cells/well), cultured for 7-10 days, and scored for the formation of cancer spheres (129). 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 (124).

RESULTS

To systematically investigate the role of SOS1 in RAS-driven proliferation, transformation, and signaling, we used immortalized mouse embryonic fibroblasts (MEFs) with either *Sos1^{fl/fl}* or *Sos1^{fl/fl}Sos2^{-/-}* (260). We used lentiviral constructs to express HA-tagged HRAS^{G12V}, NRAS^{G12V}, or KRAS4B^{G12V} (hereafter referred to as KRAS) in both MEF lines (Fig. 4-1A). By expressing CRE in these cell lines, we generated all combinations of SOS1 and SOS2 expression: wild-type (WT), *Sos1^{-/-}*, *Sos2^{-/-}*, and the

double knockout (DKO). Deletion of either *Sos* gene did not affect the expression of the other (Fig. 4-1A).

SOS1 is required for RAS-driven proliferation regardless of isoform

To determine the effect of *Sos1* on RAS-driven proliferation, we assessed cell number increases over time in the MEF lines from Fig. 4-1A. We found that SOS1 is required for proliferation in cells expressing all RAS isoforms, in contrast to SOS2, which is not necessary for proliferation (Fig. 4-1B), as we previously demonstrated (259; 260). Knocking out both *Sos1* and *Sos2* led to a small reduction in cell number increase relative to *Sos1* knockout alone, except in cells expressing HRAS^{G12V}, indicating that SOS2 may be able to compensate for loss of SOS1 to a small extent.

SOS1 is partially required for RAS-driven transformation regardless of isoform

We assessed transformation in MEFs through two assays for three-dimensional (3D) anchorage-independent growth. The first assay measures loss of contact inhibition. Non-transformed cells form monolayers on tissue-culture-coated plates. Transformed cells are able to continue proliferating when in contact with other cells, allowing them to grow past a monolayer and form foci that are detectable when fixed and stained (Fig. 4-1C). The second assay measures anchorage-independent growth by assessing the ability of cells to form colonies in soft agar. Non-transformed cells are unable to proliferate in soft agar and remain as a single cell suspension, while transformed cells proliferate to form colonies that can be counted (Fig. 4-1D). *Sos1*^{-/-} MEFs expressing oncogenic RAS mutants demonstrated reduced anchorage-independent growth relative to wild-type MEFs, indicating that SOS1 is partially required for RAS-driven transformation. In contrast to the previously established hierarchical pattern of SOS2 dependence, SOS1

was required for transformation equally by all three RAS isoforms (Fig. 4-1C-D). *Sos1* deletion did not fully prevent anchorage-independent growth for any RAS isoform compared to the extent that *Sos2* deletion impaired anchorage-independent growth in mutant KRAS-expressing cells. Knocking out both *Sos* genes did not enhance their individual effects on anchorage-independent growth, with the double knockout matching the more severe single knockout for each isoform. These results imply that SOS1 serves a different role in RAS-driven transformation than SOS2 does.

We next tested whether our results in MEFs could be replicated in RAS-mutated cancer cell lines. We chose three cell lines, T24 (bladder cancer, HRAS^{G12V}), RD (rhabdomyosarcoma, NRAS^{Q61H}), and YAPC (pancreatic adenocarcinoma, KRAS^{G12V}), that we had previously used to investigate the effect of *SOS2* deletion (259; 260). We used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) to delete *SOS1* or the mutant RAS isoform in each cell line to achieve long-term deletion for the duration of the transformation assays, which assess growth for two to four weeks. We tested several candidate single-guide RNAs (sgRNAs) targeting *SOS1* from a genome-wide CRISPR/Cas9 screen (199) to identify three sgRNAs that targeted nonoverlapping regions of *SOS1* and effectively deleted *SOS1* (Fig. 4-1E). Each cell line was infected with a non-targeting (NT) sgRNA, an sgRNA targeting the mutant RAS isoform, or one of the three sgRNAs targeting *SOS1* (#1, #2, or #8). The infected cells underwent selection for 10 days after infection to allow for CRISPR-Cas9-mediated gene deletion. Deletion of the mutant RAS isoform prevented anchorage-independent growth in each cell line, confirming their dependence on mutant RAS. Deletion of *SOS1* reduced anchorage-independent growth in all three cell lines, consistent

with the equal requirement of SOS1 for anchorage-independent growth across isoforms seen in the MEFs. We also assessed the effect of *SOS1* deletion on proliferation in YAPC cells, where *SOS1* deletion significantly limited cell number increase over time, consistent with the proliferation results in MEFs.

SOS1 GEF activity and allosteric activation by RAS are both required for full proliferation

Two models for SOS contribution to mutant RAS signaling have been proposed in the literature, both involving SOS-mediated activation of wild-type RAS. In the first model, allosteric activation of SOS by mutant RAS increases its GEF activity, allowing it to activate more wild-type RAS (136; 186). In the second, RTK-mediated activation of wild-type RAS through SOS works in parallel to constitutive mutant RAS signaling to activate pathways necessary for proliferation and transformation (317). We previously established that allosteric activation of SOS2 is not necessary for KRAS-driven transformation and that SOS2 contributes to KRAS-driven transformation through RTK-mediated activation of wild-type RAS and PI3K (259; 260). To determine the contributions of allosteric activation and GEF activity to the role of SOS1 in proliferation, we use previously identified point mutations to generate three mutant constructs: SOS1^{F929A}, which lacks GEF activity (104), and SOS1^{W729E} and SOS1^{LA/RE}, which are both unable to be allosterically activated by RAS (186; 241; 269). We assessed the ability of each construct to rescue proliferation in double knockout MEFs with a low-density proliferation assay. Cells were plated at a low density and allowed to proliferate to form colonies (Fig. 4-2A). Colonies were counted and normalized as a percentage of colonies formed by *Sos2*^{-/-} MEFs (Fig. 4-2B). Colony numbers in WT, *Sos1*^{-/-}, *Sos2*^{-/-}, and DKO cells paralleled the results from the cell number increase assay. *Sos2*^{-/-} cells were

infected with activated EGFR (EGFR^{L858R}) and mutant RAS isoforms, along with wild-type SOS1, SOS2, and SOS1 mutant constructs prior to infection with CRE. As expected, activated EGFR did not rescue proliferation in the absence of SOS, since EGFR is upstream of SOS in the pathway. The three mutant RAS isoforms were able to partially rescue proliferation to similar levels, but were not able to fully compensate for the absence of SOS1, emphasizing the importance of SOS1 in mutant RAS-driven proliferation, despite being upstream in the canonical pathway. Wild-type SOS2 rescued proliferation in the double knockout to match the *Sos1* knockout, as expected. Likewise, wild-type SOS1 fully rescued proliferation to the same levels as the wild-type and *Sos2*^{-/-} cells. GEF-dead SOS1^{F929A} failed to fully rescue proliferation and mirrored the *Sos1*^{-/-} cells, indicating that the GEF activity of SOS1 is necessary for its major role in proliferation. The two feedback-resistant mutants, SOS1^{W729E} and SOS1^{LA/RE}, partially rescued proliferation to a greater extent than the GEF-dead mutant, indicating that while GEF activity is required for proliferation, positive feedback from active RAS also contributes. Overall, MEF proliferation was severely reduced without GEF and feedback competent SOS1, and other effectors rescued to a limited extent.

Like many cell lines, MEFs proliferate more readily in the presence of neighboring cells. To account for potential confounding factors from the lack of nearby cells in the low-density proliferation assay, we assessed proliferation in MEFs expressing SOS constructs using a novel growth competition assay. *Sos1*^{fl/fl}*Sos2*^{-/-} MEFs, expressing SOS1 or RAS constructs as indicated, were infected with retroviruses expressing either YFP (control) or YFP-IRES-CRE (Fig. 4-3A). After four days, the percentage of successfully infected cells was assessed using flow cytometry, measured as the

percentage of YFP positive (YFP⁺) cells. Mock-infected cells from the same cell population were mixed with the infected cells to standardize the YFP⁺ percentage across conditions and the YFP⁺ percentage of the standardized cells was checked with flow cytometry. The mixed cells were initially plated at a density of $10^5/20\text{ cm}^2$ and passaged in a 3T1 protocol for 9 days. The percentage of YFP⁺ cells was measured every three days as the cells were passaged. Over the course of nine days, cells infected with the YFP control were able to proliferate and compete with uninfected cells, as expected, leading to a stable percentage of YFP⁺ cells (Fig. 4-3B, top row). In contrast, cells infected with YFP-IRES-CRE did not proliferate enough to compete with uninfected cells due to deletion of *Sos1*, leading to a drop in the percentage of YFP⁺ cells (Fig. 4-3B, second row). Expression of wild-type SOS1 rescued the decrease in percentage of YFP positive cells, as expected (Fig. 4-3B, third row). Expression of mutant KRAS did not rescue proliferation, consistent with the requirement for SOS1 in RAS-driven proliferation (Fig. 4-3B, bottom row) and with the results from the low-density proliferation assay. The inability of mutant RAS to rescue proliferation suggests that although the MEFs used here were not expressing mutant RAS, MEFs expressing mutant RAS would likely behave similarly.

We used the competitive growth assay to compare the ability of SOS1 and SOS2 constructs to rescue proliferation (Fig. 4-3C). We found that, as shown in the low-density proliferation assay, feedback-resistant SOS1 (W729E) partially rescued proliferation. GEF-dead SOS1 and SOS2 were unable to rescue proliferation in this assay, suggesting that further studies on the non-GEF-related contributions of SOS1 are needed to confirm their role in proliferation. Wild-type SOS2 and feedback-resistant SOS2 rescued

proliferation to the same extent, consistent with our finding that allosteric activation of SOS2 is not necessary in KRAS-driven transformation and wild-type RAS activation (260). SOS2 rescued proliferation to the same extent as feedback-resistant SOS1, implying a connection between the requirement for SOS1 but not SOS2 in proliferation and the ability of SOS1 to participate in the allosteric feedback mechanism. In order to confirm these results in cancer cell lines, we have developed CRISPR-resistant mutant constructs of *SOS1* and *SOS2*. We introduced a silent mutation to the NGG PAM motif recognized by the corresponding sgRNA, allowing us to infect cancer cells with a given construct of *SOS1* or *SOS2* and then use CRISPR/Cas9 to delete the endogenous wild-type copies. Cells infected with CRISPR-resistant *SOS1* maintained SOS1 expression after infection with Cas9 and the corresponding *SOS1* sgRNA, while cells infected with wild-type SOS1 did not (Fig. 4-4A). CRISPR-resistant *SOS1* also rescued anchorage-independent growth in *KRAS*-mutated cancer cells infected with a *SOS1* sgRNA (Fig. 4-4B). We will use these tools to express GEF-dead and feedback-resistant SOS constructs in cancer cells and confirm our results from immortalized MEFs.

***Sos1* deletion did not alter RTK-stimulated AKT or ERK phosphorylation**

To parallel our SOS2 signaling findings, we investigated the effects of *SOS1* deletion and *SOS* double knockout on ERK and AKT phosphorylation downstream of epidermal growth factor (EGF) stimulation. We used immortalized MEFs expressing KRAS^{G12V} with the indicated *Sos* genotypes and serum-starved them overnight before stimulation with 100 ng/mL of EGF. Protein lysates were collected at the indicated time points and Western blotted for SOS1, SOS2, phosphorylated AKT (T308 and S473), total AKT, phosphorylated ERK, total ERK, and β -actin (Fig. 4-5). As we previously reported,

Sos2 deletion significantly reduced EGF-stimulated AKT phosphorylation at both sites, especially in long-term time points, but did not affect ERK phosphorylation. *Sos1* deletion, on the other hand, did not affect either AKT or ERK phosphorylation. Double knockout of both *Sos* genes did not further decrease AKT phosphorylation compared to *Sos2* deletion alone, but did block ERK phosphorylation, unlike either single knockout. The same effects were found in cells expressing mutant HRAS and mutant NRAS (Fig. 4-6), consistent with our previous results showing that effects of *Sos2* deletion on ERK and AKT signaling were consistent across RAS isoforms (259; 260).

Discussion

RAS genes are mutated in ~30% of human tumors (226; 273), many of which respond poorly to currently available therapies. RAS-driven proliferation and transformation is still dependent on upstream signaling, despite constitutive activation of mutant RAS. SOS1 plays a role in RAS-driven proliferation (136) and SOS1 inhibitors have been developed to target RAS-driven cancers, but the mechanism of SOS1 contribution to RAS-driven proliferation and transformation is not fully understood. Here we showed that SOS1 is required for proliferation and partially required for transformation driven by mutant HRAS, NRAS, and KRAS. The requirement for SOS1 in proliferation includes both its GEF activity and its allosteric RAS binding site, in line with previous studies (136). We also show that *SOS1* deletion alone does not affect RTK-mediated PI3K/AKT or Raf/MEK/ERK activation in the context of mutant RAS, indicating that further studies are needed to identify the effector pathways mediating the effects of SOS1 on proliferation and transformation.

Previous studies have demonstrated that SOS1 is required for proliferation in primary MEFs (174) and tumor growth and progression in a mouse skin cancer model (175). Multiple mechanisms have been proposed for SOS contributions to oncogenic RAS signaling. Two mechanisms involve SOS activation of wild-type RAS, which has been shown to contribute to oncogenic signaling in the context of mutant RAS (85; 325). In the first model, RAS-GTP increases SOS1 activity through binding to an allosteric site on SOS1 (186). Allosteric binding of active RAS to SOS1 creates a positive feedback loop from constitutively active mutant RAS to wild-type RAS that can promote proliferation in *KRAS*-mutated cancer cells (136). Our findings using SOS1 mutant constructs indicate the importance of this model in SOS1 contributions to proliferation. In the second model, RTK-dependent activation of wild-type RAS through SOS supplements basal mutant RAS signaling and promotes G2 checkpoint integrity (90) and proliferation in *RAS*-mutated cancer cells (317). The two models can cooperate and the allosteric activation of SOS1 by active RAS has been shown to support prolonged RAS/ERK activation downstream of the T-cell and B-cell receptors (61; 154; 241) and potentiate EGF-stimulated signaling to downstream effector pathways (22). Further studies on the contributions of SOS1 to RTK-stimulated and basal wild-type RAS activation will be necessary to draw conclusions on the relevance of the second model. However, our previous studies indicate that RTK-stimulated wild-type RAS activation is dependent on SOS2 in the context of mutant RAS, but that basal wild-type RAS activation is not. These observations suggest that SOS1 contributes to wild-type RAS activation primarily through allosteric activation by mutant RAS independent of RTK stimulation (260).

SOS1 may contribute to oncogenic signaling through activation of mutant RAS. Preliminary results from bioluminescence resonance energy transfer (BRET) assay for RAS activation (206; 280) showed that activation of mutant RAS isoforms is significantly reduced in the absence of SOS1 (unpublished data). We previously showed that *SOS2* deletion does not affect GTP-loading of mutant RAS (Fig. 2-S2, (260)), implying that SOS1 can provide adequate GEF activity for mutant RAS on its own. Different RAS mutants rely on GEFs to maintain activation to different extents, correlated with their different GTPase activities. G12 and G13 substitutions block catalysis by RasGAPs, but do not necessarily interfere with the intrinsic GTPase activity. On the other hand, Q61 directly participates in catalyzing the hydrolysis reaction, and so substitutions may reduce the already low intrinsic activity. In KRAS, Q61 mutants have lower GTPase activity than G12 or G13 mutants, potentially making them less dependent on GEF activity to maintain their active state (126). In our SOS2 studies, KRAS Q61 mutants had a reduced dependence on SOS2 for transformation (260). The experiments presented here used G12V mutants, with the exception of NRAS^{Q61H} mutant RD cells. RD cells required SOS1 for full transformation, and future studies will use other Q61 mutants to confirm this finding. If SOS1 primarily contributes through activation of mutant RAS, we would expect cells expressing Q61 mutants to have a reduced dependence on SOS1 for proliferation and transformation compared to G12 and G13 mutants.

On the other hand, if SOS1 contributes primarily through activation of wild-type RAS, we would not expect a differential dependence on SOS1 between different RAS mutants based on their dependence on RasGEFs for activation. Instead, differences may arise due to different effects on wild-type RAS activation between mutants. For example,

KRAS^{G13D} cells have increased wild-type RAS activation downstream of EGFR signaling due to strong interactions between KRAS^{G13D} and NF-1 that block wild-type RAS inactivation. KRAS^{G13D} cancer cells depend on this wild-type RAS activation and thus respond to EGFR inhibition (192). If SOS1 contributes primarily through wild-type RAS activation, KRAS^{G13D}-expressing cells may be more sensitive to *SOS1* deletion than cells expressing G12 and Q61 mutants. Further studies on mutant RAS activation in the presence and absence of SOS1 and potential differences in SOS1 dependence between mutants will be necessary to determine whether SOS1 primarily contributes through mutant RAS or wild-type RAS activation. Our findings also indicate that SOS1 may contribute to proliferation to a limited extent through RasGEF-independent functions, potentially through its identified roles as a scaffold (150) or as a RacGEF (128). We have developed CRISPR-resistant SOS1 constructs to confirm these results in RAS-mutated cancer cells.

The PI3K/AKT and Raf/MEK/ERK pathways are generally considered to be the major effectors of oncogenic RAS signaling. However, we have shown that SOS1 deletion alone does not affect RTK-mediated activation of either pathway in cells expressing mutant RAS. Thus, the contributions of SOS1 to proliferation and transformation must be mediated either through basal RAS signaling independent of RTK stimulation or RTK signaling to different pathways. Further studies are needed to define the role of SOS1 in downstream effector pathway signaling in the context of mutant RAS. We also confirmed our previous finding that SOS2 is necessary for RTK-mediated AKT phosphorylation, but like *SOS1*, *SOS2* deletion alone had no effect on RTK-stimulated ERK phosphorylation. Combined *SOS1* and *SOS2* deletion blocked RTK-

stimulated ERK phosphorylation in cells expressing all mutant RAS isoforms. These results indicate that the RTK signals are transduced through SOS to the Raf/MEK/ERK pathway and that either SOS1 or SOS2 is capable of providing the upstream signal. Previous studies have suggested that PI3K requires stronger upstream signals for activation than Raf, potentially explaining why either SOS1 or SOS2 can compensate for loss of the other in ERK phosphorylation, but only SOS1 cannot compensate for loss of SOS2 in AKT phosphorylation. SOS1 and SOS2 are both low abundance proteins compared to upstream activators and RAS, indicating that they may serve as a “bottleneck” in the pathway, where small changes in SOS activity have significant impact on signal transduction (262). ERK and the ERK target RSK negatively regulate SOS1, but not SOS2. Thus, moderate increase in signaling to ERK by RTK stimulation or constitutive RAS activation could plausibly lower SOS1 activity below the threshold needed for PI3K activation in the absence of SOS2 while remaining above the threshold needed for Raf activation. SOS2 does not undergo the same negative regulation, and thus could remain capable of providing sufficient signal, in line with our signaling observations. A low level of RTK-stimulated AKT phosphorylation remains in SOS2 and double knockout cells, likely from direct activation of PI3K by RTK (38; 238), since the deletion of *SOS1* in addition to *SOS2* does not further decrease AKT phosphorylation. Future studies will include signaling, proliferation, and transformation assays with SOS1 constructs unable to be negatively regulated by ERK and RSK and RNA-seq studies with pathway analysis to investigate the effect of *SOS1* deletion on other pathways that may mediate its effects on proliferation and transformation.

Multiple SOS1 inhibitors are in development as therapeutics for RAS-driven cancers (119; 121). Our results support pre-clinical indications that SOS1 is an effective therapeutic target in RAS-mutated cancers across isoforms. However, our finding that SOS1 is only partially required for anchorage-independent growth suggests that combining SOS1 inhibition with other therapies will be necessary to achieve fully effective treatment. Further studies on the mechanisms of SOS1 contribution to oncogenic RAS signaling will allow for prediction of successful combination therapies and potential mechanisms of resistance.

FIGURES

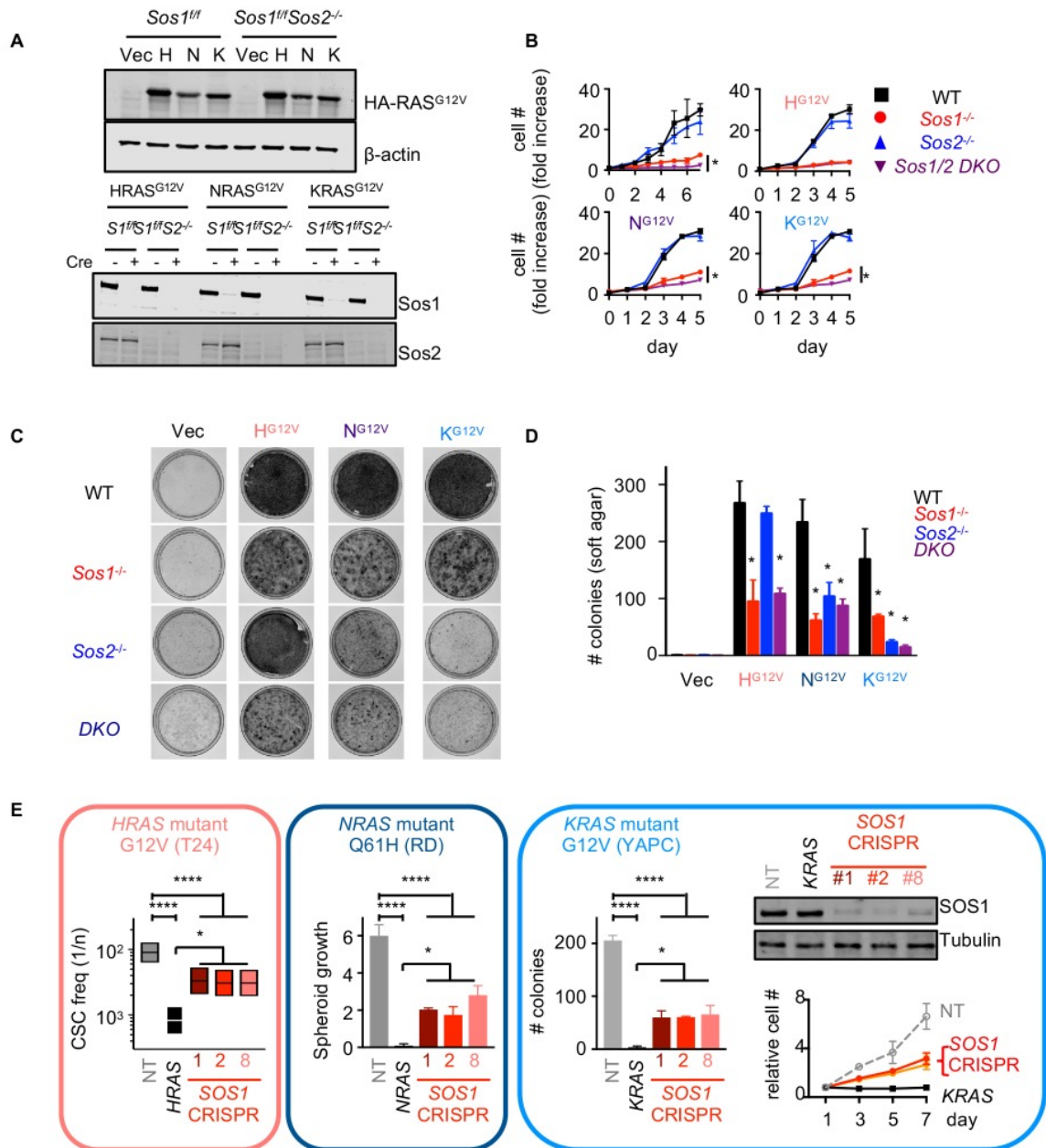


Fig. 4-1. SOS1 is required for mutant-RAS-driven anchorage-dependent (2D) proliferation and anchorage-independent (3D) transformation in cells expressing oncogenic RAS family isoforms.

(A) *Sos1^{ff}* and *Sos1^{ff} Sos2^{-/-}* MEFs were transduced with lentiviruses expressing either empty vector (Vec) or the indicated HA-tagged mutant RAS isoform. Indicated cells were also transduced with retrovirus expressing CRE. Whole cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for HA (for RAS^{G12V}), SOS1, SOS2, or β -actin to assess total protein. (B-D) *Sos1^{ff}* and *Sos1^{ff} Sos2^{-/-}* MEFs were assessed for (B) proliferation in 2D culture plates, (C) loss of contact inhibition by focus forming assay, and (D) anchorage-independent growth by colony growth in soft agar. (E) HRAS (G12V) mutated T24 bladder cancer cells (salmon), NRAS (Q61H) mutated RD rhabdomyosarcoma cells (dark blue), or KRAS (G12V) mutated YAPC pancreatic cancer cells (light blue) were transduced with lentiviruses expressing Cas9 and either a non-targeting (NT) sgRNA, an sgRNA targeting the mutant RAS, or one of three sgRNAs targeting SOS1. Cells were selected for ten days before being assessed for 3D transformation or 2D proliferation (YAPC bottom). WCLs were analyzed by Western blotting with antibodies specific for SOS1 and tubulin (YAPC top). Data are means $\pm$ SD from three independent experiments. Blots and images are representative images of independent experiments. * $P < 0.05$, **** $P < 0.001$ by ANOVA using the Tukey's method to correct for multiple comparisons.

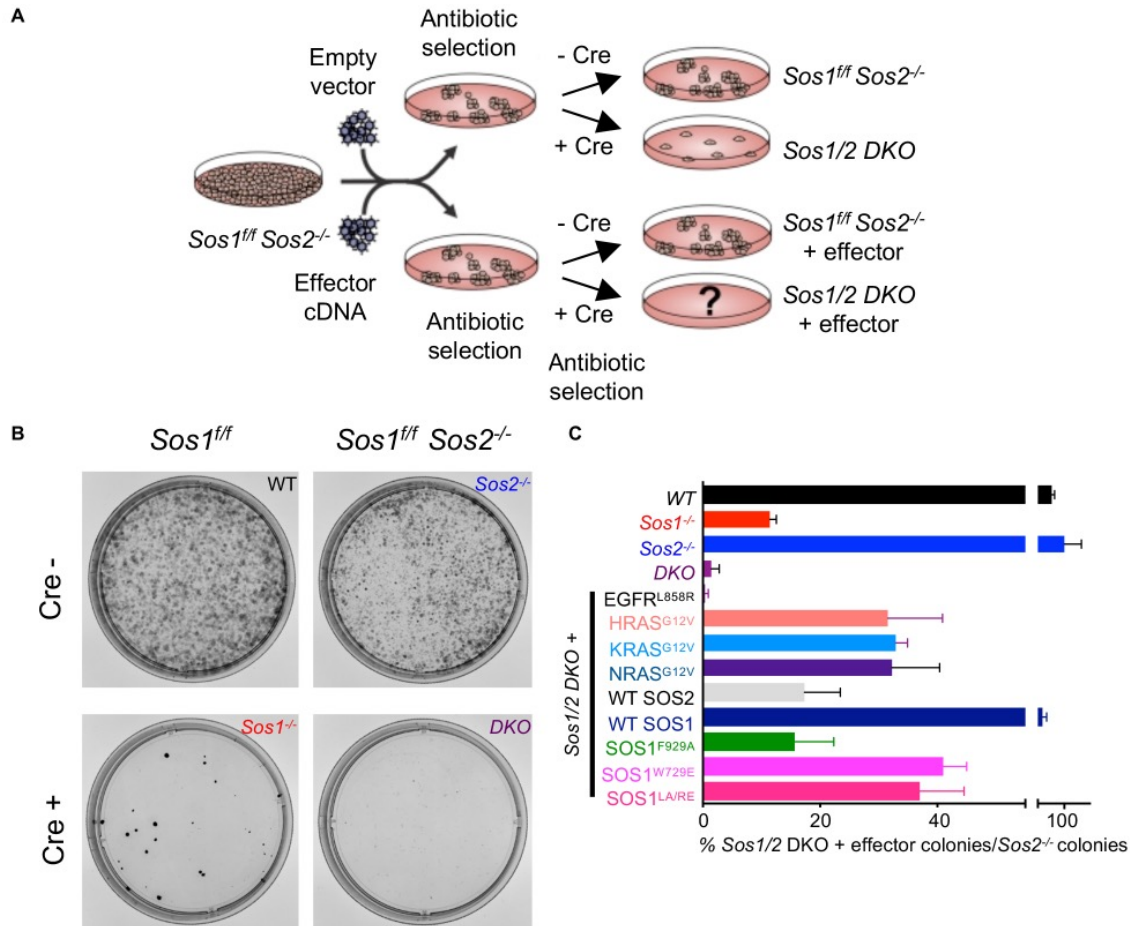


Fig. 4-2. SOS1 GEF-activity and its allosteric RAS-GTP binding site are both required for proliferation.

(A) *Sos1^{ff}Sos2^{-/-}* MEFs were transduced lentiviruses expressing the indicated effectors or the empty vector and selected before being transduced with retrovirus expressing CRE (diagram modified from Drosten et al. (70)). (B) MEFs were assessed for proliferation using a low-density proliferation assay and (C) quantified as a percentage of the colonies formed by *Sos1^{ff}Sos2^{-/-}* MEFs.

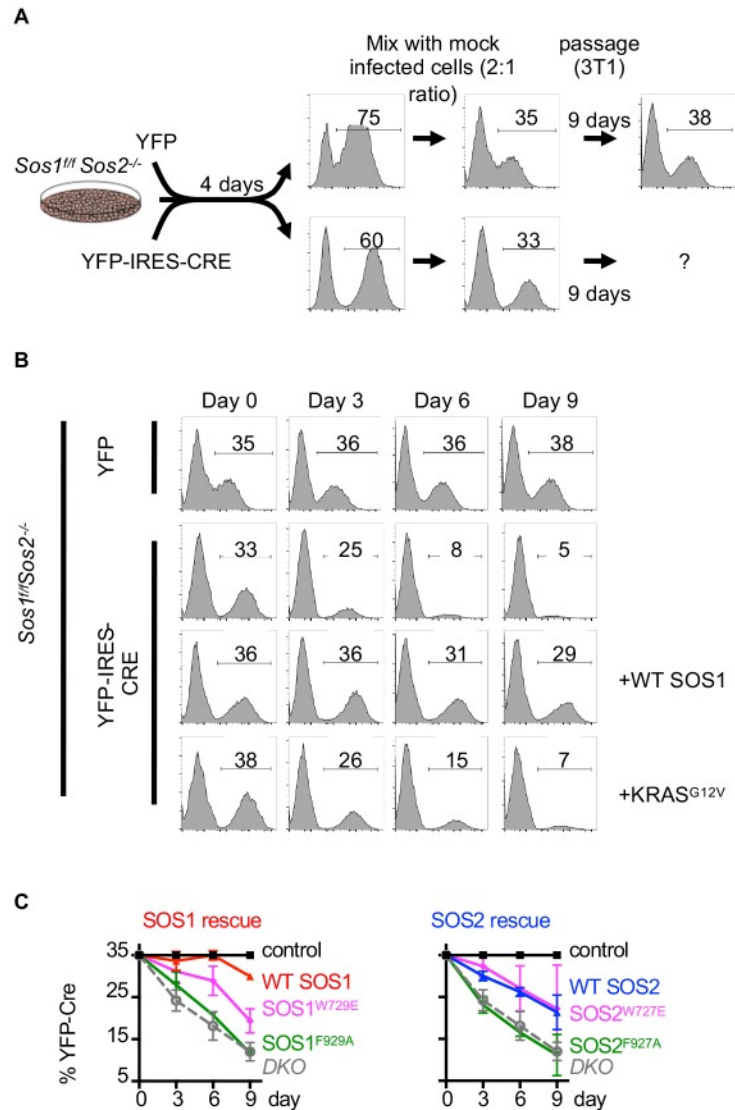


Fig. 4-3. A competitive growth assay confirms the requirement for SOS1 GEF-activity and its allosteric RAS-GTP binding site.

(A) *Sos1^{fl/fl}Sos2^{-/-}* MEFs expressing either an empty vector or a SOS rescue construct were transduced with either a control YFP retrovirus or YFP-IRES-CRE. Four days following infection, cells were assessed for the percentage of YFP⁺ cells by flow cytometry and then mixed with non-infected cells to a standard ratio of 2:1 non-infected:YFP⁺. Cells are then passaged under a 3T1 protocol for 9 days and (B) the percentage of YFP⁺ cells is assessed at each passage and (C) plotted. Data are means $\pm$ SD from three independent experiments.

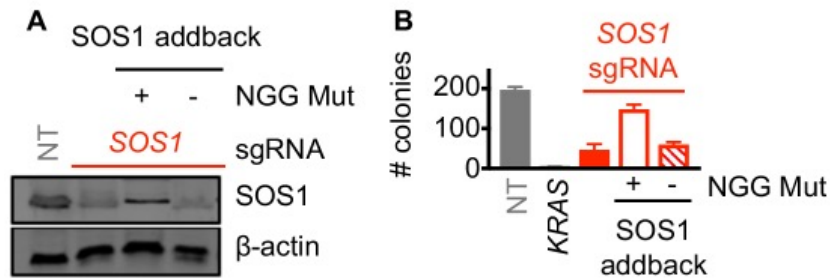


Fig. 4-4. Silent mutation of the NGG PAM motif in SOS1 restores SOS1 expression and KRAS-driven transformation following SOS1 deletion by CRISPR/Cas9.

(A) YAPC cells were infected with lentiviruses expressing the NGG PAM motif mutant SOS1 or wild-type SOS1 as indicated. Cells were also infected with lentiviruses expressing Cas9 and either non-targeting (NT) or SOS1 sgRNAs. Lysates were analyzed by Western blotting with an antibody specific for SOS1 or β -actin. (B) YAPC cells expressing Cas9, the indicated sgRNA, and the indicated SOS1 construct were assessed for colony growth in soft agar to assess anchorage-independent growth. Data are means $\pm$ SD from three independent experiments.

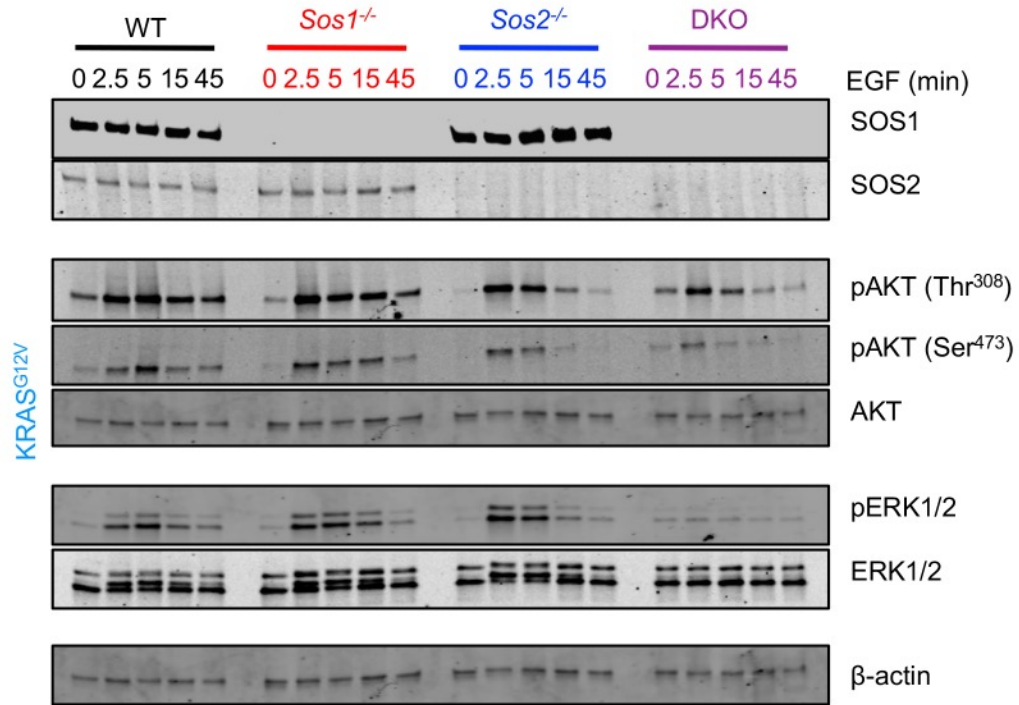


Fig. 4-5. *Sos2* deletion reduces EGF-stimulated AKT phosphorylation, whereas combined *Sos1/2* deletion is required to reduce ERK phosphorylation in MEFs expressing oncogenic KRAS^{G12V}.

Sos1^{fl/fl} (WT), *Sos1*^{fl/fl} + CRE (*Sos1*^{-/-}), *Sos1*^{fl/fl} *Sos2*^{-/-} (*Sos2*^{-/-}), and *Sos1*^{fl/fl} *Sos2*^{-/-} + CRE (DKO) MEFs expressing KRAS^{G12V} were placed in serum-free media overnight and then stimulated with EGF (100 ng/ml) for the indicated times. WCLs were analyzed by multiplex Western blotting for SOS1, SOS2, pAKT (Thr³⁰⁸), pAKT (Ser⁴⁷³), AKT, pERK1/2, ERK1/2, and β-actin on a LI-COR Odyssey machine. Blots are representative of three independent experiments.

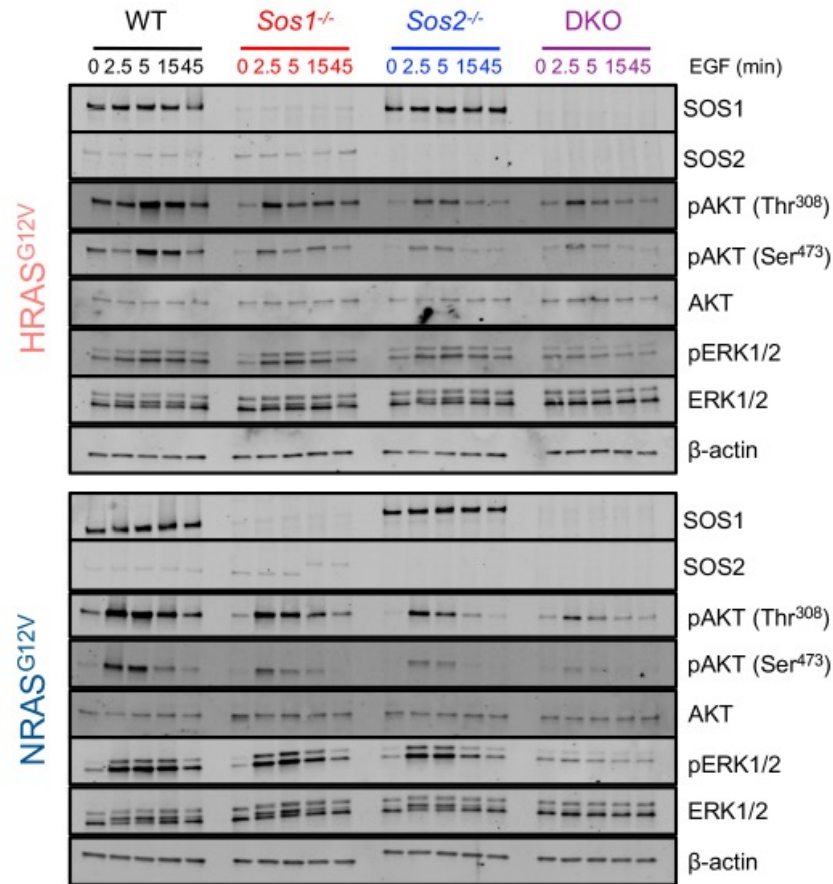


Fig. 4-6. *Sos2* deletion reduces EGF-stimulated AKT phosphorylation, whereas combined *Sos1/2* deletion is required to reduce ERK phosphorylation in MEFs expressing oncogenic HRAS^{G12V} or NRAS^{G12V}.

Sos1^{flf} (WT), *Sos1^{flf}* + CRE (*Sos1^{-/-}*), *Sos1^{flf} Sos2^{-/-}* (*Sos2^{-/-}*), and *Sos1^{flf} Sos2^{-/-}* + CRE (DKO) MEFs expressing the indicated mutant RAS isoform were placed in serum-free media overnight and then stimulated with EGF (100 ng/ml) for the indicated times. WCLs were analyzed by multiplex Western blotting for SOS1, SOS2, pAKT (Thr³⁰⁸), pAKT (Ser⁴⁷³), AKT, pERK1/2, ERK1/2, and β-actin on a LI-COR Odyssey machine. Blots are representative of three independent experiments.

CHAPTER 5: Conclusion

Our studies aimed to determine the roles of SOS1 and SOS2 in RAS-mutated tumors, the mechanisms of their contributions, and their potential as therapeutic targets. Using an immortalized MEF model system and RAS-mutated cancer cell lines, we have systematically investigated the effects of *SOS1* and *SOS2* deletion on proliferation and transformation driven by all three RAS isoforms. We have integrated our current results to propose a working model of SOS signaling in RAS-mutated cancer and found that both SOS1 and SOS2 have the potential to serve as effective therapeutic targets in different subsets of RAS-mutated cancers.

SOS2 CONTRIBUTIONS TO RAS-DRIVEN CANCER

SOS2 is differentially required for RAS-driven transformation

The requirement for SOS1 alone in development and proliferation has led to the perception that SOS2 does not have a non-redundant function in cell signaling (11; 79). We demonstrated that SOS2 is not required for proliferation, as previously shown, but is hierarchically required for RAS-driven transformation in both MEFs and cancer cells. Cancer cells and immortalized MEFs expressing mutant KRAS require SOS2 for anchorage-independent growth, while mutant HRAS-expressing cells do not and mutant NRAS-expressing cells have an intermediate requirement. RasGEFs have been previously proposed as targets in RAS-driven cancers (21), but the focus has been on SOS1 due to its role in proliferation (136; 175). Our findings show that SOS2 has a unique role in RAS-driven cancer and is also a potential therapeutic target.

SOS2 is required for RTK-stimulated wild-type RAS activation and PI3K signaling for all RAS isoforms

We found that SOS2 is required for full RTK-stimulated wild-type RAS activation in cells expressing any mutant RAS isoform. Wild-type RAS activation by SOS2 required its GEF activity but not the ability to be allosterically activated by RAS-GTP. These findings are consistent with the proposed model of SOS contribution to mutant RAS signaling through RTK-mediated activation of wild-type RAS in parallel with constitutive signaling from mutant RAS (317). While SOS2 may have an allosteric RAS-binding site homologous to the site found in SOS1 (186), its ability to participate in this positive feedback is unconfirmed. Margarit et al. (186) originally discovered the SOS1 allosteric site in their crystal structure of the catalytic domain of SOS1, which bound a RAS-GTP analogue in a 2:1 ratio, rather than the expected 1:1 ratio. Analysis of the structure revealed the secondary RAS binding site. Allosteric interaction of RAS with SOS2 has been inferred based on sequence homology. SOS2 allosteric binding of RAS could be investigated by assessing binding ratios, for example through co-immunoprecipitation after cross-linking, but ultimately a crystal structure of SOS2 in the presence of RAS-GTP would be needed to provide full confirmation that SOS2 contains a homologous allosteric binding site. However, our results suggest that even if SOS2 is structurally capable of binding RAS at the allosteric site, the feedback may not be physiologically relevant.

Our investigation of downstream effector signaling revealed that SOS2 is required for RTK-stimulated PI3K/AKT signaling in the context of mutant RAS, but not necessary for Raf/MEK/ERK signaling, regardless of isoform. The effect of SOS2 deletion alone on PI3K/AKT signaling could be due to colocalization of PI3K with SOS2 and not with

SOS1, or SOS1 signaling alone could be insufficient for PI3K activation downstream of RTK stimulation. Some studies have suggested that PI3K activation requires stronger signaling from RTK than Raf activation (84). SOS1, but not SOS2, is negatively regulated by ERK and RSK (46; 54; 223). Thus, in the context of strong Raf/MEK/ERK signaling from mutant RAS and RTK-stimulation, SOS1 activity may be diminished. The limited activity may provide a sufficient signal for Raf activation but not for PI3K, making SOS1 unable to compensate for the loss of SOS2 in PI3K/AKT signaling, consistent with our observations. This model is also supported by preliminary data showing that in MEFs without mutant RAS, *SOS2* deletion has no effect on RTK-stimulated AKT phosphorylation, potentially due to the lower Raf/MEK/ERK signaling in the absence of constitutively active RAS allowing SOS1 to retain more activity.

The requirement for SOS2 in KRAS-driven transformation is linked to its role in PI3K signaling

SOS2 is necessary for full RTK-mediated PI3K signaling in cells expressing any mutant RAS isoform, but only fully required for KRAS-driven transformation. Using pharmacological inhibitors of PI3K and AKT, we demonstrated that the requirement for SOS2 in KRAS-driven transformation is due to the sensitivity of *KRAS*-mutated cells to decreases in PI3K signaling. We found *HRAS*-mutated and *NRAS*-mutated cells are not as sensitive to PI3K or AKT inhibition as *KRAS*-mutated cells, leading to their hierarchical response to SOS2 deletion. The differential sensitivity to PI3K inhibition correlates with their relative ability to activate PI3K, where KRAS activated PI3K poorly compared to HRAS, with NRAS in the middle (293; 312) (Figure 3-7). We also showed that *HRAS*-mutated cells are more sensitive to MEK inhibition than *NRAS*-mutated or *KRAS*-mutated cells, again correlating with their relative abilities to activate MEK, which are inverse to

their abilities to activate PI3K (293; 312). We found that constitutively active PI3K rescued KRAS-driven transformation in the absence of SOS2, confirming the connection between the effects of *SOS2* deletion on PI3K activation and KRAS-driven transformation. Our findings are consistent with previous studies that have proposed that wild-type RAS contributes to oncogenic signaling by activating effectors that the mutant RAS activates poorly (33). PI3K is often associated with cell survival in cancer cells (32; 59; 78; 171). Resistance to anoikis, or anchorage-independent programmed cell death (217), is correlated with increased Raf/MEK/ERK and PI3K/AKT signaling (62) and is necessary to allow cells to grow in a transformed, anchorage-independent manner. We demonstrated that *SOS2* deletion prevents KRAS-mutant cells from resisting anoikis. These results indicate that SOS2 contributes to KRAS-driven transformation by mediating RTK-dependent PI3K activation through wild-type RAS, supplementing PI3K/AKT signaling to allow for cell survival.

***SOS2* deletion synergizes with MEK inhibition to block anchorage-independent growth in *KRAS*-mutated cancer cells**

Combined MEK and PI3K inhibition is an established therapeutic strategy to limit tumor growth and promote apoptosis in *KRAS*-mutated cancer cells and in vivo models (77; 270). Unfortunately, high toxicities limit the application of this combination in patients (39). Multiple studies have demonstrated that RTK inhibition indirectly blocks PI3K signaling in *KRAS*-mutated cancer cells (72; 183; 302), but resistance arises through activation of alternative RTKs (221; 278; 292). We found that SOS2 serves as an alternative target for indirect PI3K inhibition. While no SOS2 inhibitors have been developed, we showed that *SOS2* deletion synergized with MEK inhibition to the same extent as an intermediate dose of a clinically-used PI3K inhibitor did.

Taken together, our findings on the role of SOS2 in RAS-driven proliferation and transformation are consistent with the proposed model of SOS contribution to RAS-driven cancer through RTK-dependent activation of wild-type RAS in parallel with constitutive mutant RAS signaling (317). SOS2 is particularly responsible for PI3K activation in the context of mutant RAS, potentially due to the requirement for stronger upstream signaling in PI3K/AKT activation compared to Raf/MEK/ERK activation and the reduction in SOS1 activity by negative feedback. The dependence on SOS2 for PI3K signaling leads to the requirement for SOS2 in specifically KRAS-driven transformation, since *KRAS*-mutated cells are more sensitive to decreases in PI3K signaling than *HRAS*-mutated and *NRAS*-mutated cells are. We demonstrated that targeting SOS2 is a potential therapeutic strategy for indirect inhibition of PI3K, which has been shown to synergize with MEK inhibition to limit tumor growth in *KRAS*-mutated cancer models (72; 182; 221). These studies used RTKs as targets for indirect inhibition of PI3K. SOS2 inhibition has two main advantages over RTK inhibition for indirectly blocking PI3K signaling: first, it is a common pathway intermediate downstream of all RTKs, limiting the development of common resistance mechanisms; and second, SOS2 is not necessary for normal cell functions, suggesting that its inhibition will have very low toxicity in patients. Our results indicate that SOS2 is a viable potential therapeutic target in *KRAS*-mutated, PI3K wild-type cancers, which represent a majority of RAS-driven tumors.

SOS1 CONTRIBUTIONS TO RAS-DRIVEN CANCER

SOS1 is required for proliferation and partially required for transformation in RAS-mutated cells

Consistent with previous studies, we found that SOS1 is required for proliferation in MEFs (175; 229). We extended these studies to demonstrate that this requirement for

SOS1 in proliferation holds true in both immortalized MEFs and human cancer cells expressing oncogenic RAS mutants. We also found that SOS1 is partially required for RAS-driven transformation for all three RAS isoforms. The partial requirement for anchorage-independent growth suggests that SOS1 inhibition alone may not be enough to successfully treat RAS-mutated tumors. These findings emphasize the importance of assessing targets for RAS-driven cancer in 3D anchorage-independent growth conditions in addition to conventional 2D growth assays. *KRAS*-mutated cancer cell lines previously thought to be independent of KRAS for maintenance based on 2D assays were found to remain dependent on KRAS for anchorage-independent growth (242), indicating that cancer cells may require different signals for 2D and 3D growth. Use of 3D assays is important both to reveal previously unappreciated targets, as in our SOS2 studies, and to accurately assess when potential targets may be required in 2D conditions but not in 3D conditions, as we observed with SOS1, which may limit their efficacy as therapeutic targets.

SOS1 GEF activity and allosteric activation by RAS are required for full proliferation

We found that proliferation requires both the GEF activity of SOS1 and its ability to be allosterically activated by RAS, consistent with the proposed model of SOS1 contributing to mutant RAS-driven proliferation through allosteric feedback from mutant RAS to wild-type RAS through SOS1 (136). Future studies will assess the mechanisms of SOS1 contributions for RAS-driven transformation and confirm our proliferation results in RAS-mutated cancer cell lines using CRISPR-resistant SOS mutant constructs.

Future studies will also include assessing the role of SOS1 in the activation of wild-type and mutant RAS. We found that *SOS2* deletion blocks RTK-stimulated wild-

type RAS activation, but not basal wild-type RAS activation, suggesting that SOS1 may activate wild-type RAS independently of RTK signaling, potentially through its allosteric activation by mutant RAS. Preliminary BRET assays for mutant RAS activation have shown that mutant RAS activation significantly decreases in the absence of SOS1, suggesting that SOS1 may also contribute through activation of mutant RAS. Future studies will determine the relative importance of these potential SOS1 contributions to proliferation and transformation.

Multiple drugs inhibiting SOS1 have been developed and moved forward toward clinical trials (119; 121). While the most advanced SOS1 inhibitor has been presented as a pan-RAS inhibitor for mutant RAS (121), our proposed model indicates that SOS is primarily involved in the activation of wild-type RAS. Understanding whether SOS1 contributes primarily to wild-type RAS activation or mutant RAS activation will be key to determining what treatments will synergize with SOS1 inhibition to increase its efficacy. If SOS1 primarily contributes by activating wild-type RAS, SOS1 inhibition will likely synergize with inhibition of the pathways activated by mutant RAS, similar to the synergy between *SOS2* deletion and MEK inhibition. On the other hand, if SOS1 primarily contributes through activation of mutant RAS, SOS1 inhibition would more likely synergize with inhibition of the pathways activated by wild-type RAS. In either case, combination of SOS1 with an inhibitor in the same pathway would likely be ineffective due to relief of negative feedback on the pathways activated by the other type of RAS (317). Additionally, if SOS1 contributes primarily through mutant RAS activation, SOS1 inhibition could then be used to enhance the efficacy of current KRAS^{G12C} inhibitors, which bind GDP-bound mutant KRAS to lock it in the inactive

state. If SOS1 inhibition decreases the activation of mutant RAS, a higher proportion of the mutant RAS will be GDP-bound and available for the drug to act on, increasing its efficacy. Our future studies will include investigating the activation of mutant and wild-type RAS in *SOS1* knockout cells and using SOS1 constructs to determine the SOS1 activities necessary for RAS activation.

***SOS1* deletion does not affect RTK-stimulated ERK or AKT phosphorylation**

We showed that *SOS1* deletion has no effect on RTK-mediated signaling through either of the two primary pathways in RAS-driven oncogenic signaling. For PI3K/AKT signaling, we propose that PI3K activation may require a stronger signal from RTK than SOS1 alone is able to provide due to negative feedback on SOS1 by ERK and the ERK target RSK (46; 54; 84; 223). As discussed earlier, SOS2 is not affected by this feedback, and is therefore capable of providing adequate signaling to PI3K in the absence of SOS1, while SOS1 is not able to compensate for the absence of SOS2. Neither *SOS1* nor *SOS2* deletion alone affects RTK-stimulated ERK phosphorylation, but we found that deletion of both *SOS1* and *SOS2* blocked ERK phosphorylation. This finding again agrees with the previous studies suggesting that the Raf/MEK/ERK pathway can be activated by weaker signals from RTK than PI3K requires (84), allowing SOS1 or SOS2 alone to provide sufficient signaling, but requiring at least one SOS to transduce the signal. Future studies will use mutant SOS1 constructs that block feedback from ERK and RSK to confirm the role of negative feedback in SOS1 regulations that our current findings suggest.

Our current findings on the effects of *SOS1* deletion on signaling provide insight into the contributions and regulation of SOS1 in the context of mutant RAS. However,

they do not provide answers for what pathways mediate the requirement for SOS1 in RAS-driven proliferation and transformation. The lack of significant changes in RTK-stimulated Raf/MEK/ERK or PI3K/AKT signaling in the absence of SOS1 implies that SOS1 must work either through basal signaling driven by mutant RAS rather than RTK stimulation or through other RAS effector pathways. Our findings show that in the absence of RTK stimulation, wild-type RAS activation is not affected by *SOS2* deletion. These findings suggest that SOS1 mediates this basal wild-type RAS activation, potentially through activation by allosteric feedback from mutant RAS, although further studies are necessary for confirmation. In addition to determining the contributions of SOS1 to basal, non-RTK-mediated signaling, we will also investigate other effector pathways that could be affected by *SOS1* deletion. With the multitudes of potential RAS effector pathways, many of which have established contributions to cell proliferation and survival, we propose a broad approach for our future studies. We will conduct RNA-seq experiments on wild-type, *SOS1* knockout, and *SOS2* knockout RAS-mutated cancer cell lines with pathway analysis to determine any changes in pathway activation in the absence of SOS1 or SOS2. These experiments will allow us to investigate the contributions of SOS1 and SOS2 to signaling in a broad and unbiased way, adding to our understanding of their role in RAS signaling and potentially revealing new strategies for combination therapies.

Overall, we have demonstrated that both SOS1 and SOS2 are potential therapeutic targets to limit growth and circumvent common resistance mechanisms in RAS-driven cancers. Our findings also emphasize the importance of screening for therapeutic targets in anchorage-independent 3D growth conditions to reveal potential targets that may be

obscured by conventional anchorage-dependent 2D assays. Based on our preliminary results, SOS1 inhibition may partially block tumor growth driven by all RAS isoforms, but will likely need to be used in combination with other therapies to achieve effective tumor regression. Our findings indicate that SOS2 may be more successful than SOS1 inhibition in *KRAS*-mutated cancers, but will have little effect in *HRAS*-mutated tumors or tumors with constitutively activated PI3K signaling. *KRAS* mutations are found in 85% of *RAS*-mutated cancers, so effectively targeting these cancers has the potential to significantly increase patient survival. Thus, we propose that efforts should be made to develop SOS2 inhibitors in parallel with SOS1 inhibitors.

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