SHOC2 complex-driven RAF dimerization selectively contributes to ERK pathway dynamics

Isabel Boned del Río, Lucy C. Young, Sibel Sari, Greg G. Jones, Benjamin Ringham-Terry, Nicole Hartig, Ewa Rejnowicz, Winnie Lei, Amandeep Bhamra, Silvia Surinova, and Pablo Rodriguez-Viciani

Edited by Roger J. Davis, Howard Hughes Medical Institute and University of Massachusetts Medical School, Worcester, MA, and approved May 28, 2019

Despite the crucial role of RAF kinases in cell signaling and disease, we still lack a complete understanding of their regulation. Heterodimerization of RAF kinases as well as dephosphorylation of a conserved "S259" inhibitory site are important steps for RAF activation but the precise mechanisms and dynamics remain unclear. A ternary complex comprised of SHOC2, MRAS, and PPI (SHOC2 complex) functions as a RAF S259 holophosphatase and gain-of-function mutations in SHOC2, MRAS, and PPI that promote complex formation are found in Noonan syndrome. Here we show that SHOC2 complex-mediated S259 RAF dephosphorylation is critically required for growth factor-induced RAF heterodimerization as well as for MEK dissociation from BRAF. We also uncover SHOC2-independent mechanisms of RAF and ERK pathway activation that rely on N-region phosphorylation of CRAF. In DLD-1 cells stimulated with EGF, SHOC2 function is essential for a rapid transient phase of ERK activation, but is not required for a slow, sustained phase that is instead driven by palmitoylated H/N-RAS proteins and CRAF. Whereas redundant SHOC2-dependent and -independent mechanisms of RAF and ERK activation make SHOC2 dispensable for proliferation in 2D, KRAS mutant cells preferentially rely on SHOC2 for ERK signaling under anchorage-independent conditions. Our study highlights a context-dependent contribution of SHOC2 to ERK pathway dynamics that is preferentially engaged by KRAS oncopgenic signaling and provides a biochemical framework for selective ERK pathway inhibition by targeting the SHOC2 holophosphatase.

**Significance**

The ERK signaling pathway is hyperactivated in a majority of cancers. However, because it mediates myriad physiological responses, the clinical efficacy of current ERK pathway inhibitors has been severely limited by toxicity. This study uncovers both SHOC2 phosphatase complex-dependent and -independent mechanisms of RAF and ERK activation that are differentially engaged in a context and spatio-temporal-dependent manner. KRAS oncopgenic signaling preferentially depends on SHOC2 dependent-mechanisms, which thus presents a therapeutic opportunity. This study provides a molecular framework for how targeting the SHOC2-holophosphatase regulatory node of the RAF activation process provides a mechanism for selective inhibition of ERK signaling.

Author contributions: I.B.R., L.C.Y., and P.R.-V. designed research; I.B.R., L.C.Y., S. Sari, G.G.J., B.R.-T., N.H., E.R., W.L., and A.B. performed research; I.B.R., L.C.Y., A.B., S. Surinova, and P.R.-V. analyzed data; and I.B.R. and P.R.-V. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1I.B.R. and L.C.Y. contributed equally to this work.
2To whom correspondence may be addressed. Email: p.rodriguez-viciani@ucl.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1902658116/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1902658116
syndrome that cluster around S259 to disrupt the interaction with 14-3-3 (14-17). Furthermore, although RAF1 mutations are rare in cancer, they cluster on residues S257 and S259 (cosmic database).

The precise dynamics and mechanism of S259 dephosphorylation remain unclear (11). We have previously shown that MRAS, a closely related member of the RAS family, upon activation forms a complex with the leucine-rich repeat protein SHOC2 and protein phosphatase 1 (PP1) that functions as a highly specific S259 RAF holophosphatase (18, 19). The importance of the SHOC2-MRAS-PP1 complex (SHOC2 complex) in RAF-ERK regulation is validated by gain-of-function mutations in Noonan syndrome in all three components—SHOC2, MRAS, and PP1—which promote phosphatase complex formation (20–23). On the other hand, the phosphatase PP2A has also been variously implicated in mediating S259 dephosphorylation (24–27), although this was primarily based on the use of okadaic acid and the misconception that it behaves as a specific PP2A inhibitor (28) (in addition to not discriminating between direct or indirect effects). Furthermore, in contrast to its role as a regulatory subunit within a phosphatase complex, other studies have suggested that SHOC2 can function as a scaffold that promotes the RAS–RAF interaction (29–33).

RAF proteins also undergo multiple activating phosphorylation events. Among them, phosphorylation within the charge-regulatory region (N-region) plays a key divergent role among RAF paralogues (11). In CRAF, S338 and Y341 phosphorylation within the S338SY341 motif by PAK and SRC family kinases (SFK) plays a crucial role in regulated activation (34). In contrast, the homologous S259DD motif in BRAF constitutively provides the negative charges required for activity by virtue of acidic D amino acids and constitutive S446 phosphorylation (11, 34, 35). This difference in N-region regulation is believed to account for BRAF having higher basal activity, being the most frequent RAF target for mutational activation in cancer and for BRAF being the initial activator in asymmetric RAF heterodimers (11, 36).

In this study, we have used RNAi and CRISPR to ablate SHOC2 and RAF function, as well as phosphoproteomics to comprehensively characterize the role of the SHOC2 phosphatase complex in RAF and ERK pathway regulation. We have uncovered a selective role for SHOC2 in ERK pathway dynamics, and show that although SHOC2 phosphatase-mediated dephosphorylation of the S259 site is critically required for growth factor-stimulated RAF heterodimerization, there also exist SHOC2-independent mechanisms of ERK activation, which are dependent on N-region phosphorylation of CRAF. Importantly, KRAS oncogenic signaling differentially relies on SHOC2-dependent mechanisms of ERK activation, which provides both a therapeutic opportunity and a mechanism of context-dependent manner.

**Results**

**MRAS and SHOC2 Expression Promotes S365 BRAF/S259 CRAF Dephosphorylation, BRAF-MEK Dissociation, and BRAF-CRAF Dimerization.** To study the role of the SHOC2 complex in the regulation of RAF kinases, we generated an inducible T-REx-293 cell line (T-17 cells), where addition of the tetracycline analog Doxycycline (Dox) leads to expression of active MRAS-Q71L and SHOC2. In these cells, Dox-induced MRAS/SHOC2 expression led to potent S365 dephosphorylation of ectopic TAP6-BRAF that is inhibited in a dose-dependent manner by the serine/threonine phosphatase inhibitor calyculin A (Fig. 1A). To assess possible RAF regions involved in S259 dephosphorylation, transiently transfected BRAF and CRAF mutants were tested for dephosphorylation upon expression of MRAS/SHOC2. Among the mutants tested, only the RBD mutants R89L, BRAF and R89L, CRAF were defective for MRAS/SHOC2-induced S365/S259 dephosphorylation (Fig. 1B and C). Interestingly, when the CRAF R89L RBD mutant was constitutively localized to the membrane by fusion with a RAS membrane-targeting region (CRAF-CAXX R89L), S259 dephosphorylation was efficiently induced by MRAS/SHOC2 expression (Fig. 1C). Taken together, these data suggest that membrane recruitment through interaction with the RBD is required for efficient S259 RAF dephosphorylation.

MRAS/SHOC2 expression levels in T-17 cells did not prove to be tunable because at the lowest Dox concentration that induced expression, there was a maximum effect on MRAS/SHOC2 protein levels and concomitant S365 dephosphorylation (Fig. 1 D and E). When ectopic T6-BRAF in these cells was purified with streptactin beads, MRAS/SHOC2 expression led to a decrease in the amount of MEK bound to T6-BRAF and a concomitant interaction of T6-BRAF with CRAF (Fig. 1 D and E). To further study the specificity of the role for MRAS/SHOC2 on RAF–MEK interactions, GST-pulldown assays were performed after cotransfection of myc-MEK1 with GST-tagged BRAF, CRAF, and KSR1 in HEK293T cells. Under basal conditions, KSR1 bound most strongly to CRAF and only weakly to CRAF (KSR1 > BRAF >> CRAF), and Dox-induced MRAS/SHOC2 expression led to strong dissociation of MEK from BRAF and CRAF but not from KSR1 (Fig. 1F). Taken together, the above data suggest that MRAS/SHOC2-induced S365 BRAF dephosphorylation promotes MEK dissociation from BRAF and BRAF heterodimerization with CRAF.

**SHOC2 Is Required for EGF-Induced S365/S259 Dephosphorylation, RAF Dimerization, BRAF-MEK Dissociation, and Efficient ERK Pathway Activation.** To assess the role of endogenous SHOC2 within the context of growth factor signaling, T-REx-293 cells where SHOC2 expression was stably inhibited by shRNA expression were used to analyze lysates and immunoprecipitates (IPs) of endogenous RAF and RAS proteins in a time course of EGF treatment. EGF-stimulated S365 BRAF dephosphorylation, MEK, ERK, and RSK phosphorylation, but not AKT and EGFR Y1068 phosphorylation, were severely impaired in SHOC2 knockout (KD) cells, consistent with a selective role of SHOC2 in RAF-ERK pathway activation (Fig. 2A).

When immunoprecipitating RAF, MEK can be readily detected in complex with BRAF but not CRAF under basal conditions (37), and higher levels of P-S365 BRAF in SHOC2 KD cells correlate with higher levels of MEK and 14-3-3 bound to BRAF (Fig. 2 A and B). EGF stimulated MEK and 14-3-3 dissociation from BRAF and BRAF binding to CRAF, and this response is strongly inhibited in SHOC2 KD cells (Fig. 2B). EGF-induced BRAF interaction with KSR is also impaired in the absence of SHOC2 (Fig. 2C and SI Appendix, Fig. S1A). In clear contrast, RAF interaction with RAS, as measured on RAS IPs, was not impaired but enhanced in SHOC2 KD cells (Fig. 2B), likely as a result of loss of inhibitory feedbacks (see Discussion).

To extend these observations to other cell lines, a CRISPR/CAS9 strategy was used to completely ablate SHOC2 function in DLD-1 KRAS12117D colon carcinoma cells. EGF-induced dephosphorylation of P-S365/S259 B/CRAF is impaired in SHOC2 knockout (KO) cells (Fig. 2D). Similarly, EGF-stimulated phosphorylation of MEK, ERK, and RSK, but not AKT, is strongly inhibited in SHOC2 KO cells and this response is rescued by reexpression of SHOC2 WT but not SHOC2 mutants defective for interaction with MRAS and PP1, such as D175N or RVxF-SILK (18, 19, 23) (Fig. 2D). SHOC2 E457K disrupts MRAS/PP1 interaction less efficiently (19, 23) and only partially rescues ERK pathway activation by EGF. Therefore, ERK pathway regulation by SHOC2 correlates well with its ability to form a ternary complex with MRAS and PP1.

To analyze RAF interactions in DLD-1 KO cells, endogenous RAF IPs were performed on a time course of EGF stimulation at baseline. When parental DLD-1 cells were treated with EGF, BRAF were defective for MRAS/SHOC2-induced S365/S259 dephosphorylation with dynamics that mirror MEK and 14-3-3 dissociation from BRAF and BRAF dimerization with ARAF and CRAF (Fig. 2 E and F). As seen in T-REx-293 KD cells, SHOC2 KO DLD-1 have higher basal levels of MEK and 14-3-3–bound BRAF complexes. Moreover, EGF-stimulated MEK and 14-3-3 dissociation from BRAF and BRAF heterodimerization...
with CRAF and ARAF are strongly impaired in SHOC2 KO cells (Fig. 2E).

To further validate that the effect of SHOC2 ablation on ERK pathway activation was dependent on its function within an S259 RAF holophosphatase, T6-BRAF WT and S365A mutant (which cannot be phosphorylated and therefore should be insensitive to the phosphatase function of the SHOC2 complex) were stably expressed in parental and SHOC2 KO DLD-1 cells. Expression of BRAF S365A (unlike BRAF WT) leads to higher basal P-MEK and P-ERK levels in both parental and SHOC2 KO cells, consistent with ERK pathway activation by these RAF mutants being insensitive to regulation by SHOC2 (Fig. 2G). When ectopic T6-BRAF was purified from these cells with streptactin beads, T6-BRAF WT displayed higher basal MEK binding in SHOC2 KO cells, whereas no MEK could be detected in complex with T6-BRAF S365A, consistent with a role for S365 dephosphorylation in the regulation of the BRAF-MEK interaction (Fig. 2G).

Taken together, the above results strongly suggest that SHOC2 complex-mediated S259 RAF dephosphorylation is required for 14-3-3 dissociation from RAFs, MEK dissociation from BRAF, and BRAF heterodimerization with ARAF, CRAF, and KSR, but not for RAF binding to RAS (SI Appendix, Fig. S2).

**SHOC2 Is Selectively Required for Early but Not Late ERK Pathway Activation by EGF in DLD-1 cells.** When ERK pathway dynamics were studied in an EGF time course in DLD-1 isogenic cells, MEK, ERK, and RSK phosphorylation was strongly impaired at early time points (2.5–5 min) in SHOC2 KO cells compared with parental cells, whereas little differences were seen between them by 20 min of EGF treatment (Fig. 3A and B). Similar effects were seen on downstream ERK targets sites, such as BRAF T753, CRAF S289/296/301, EGFR T699, and IRS S363/639 feedback sites, as well as RSK targets, such as YB1 S102 (Fig. 3A). No effect was seen in ERK-independent sites on AF6 or RPS6, whereas AKT S473 phosphorylation is enhanced in the absence of SHOC2, consistent with a negative feedback crosstalk upon ERK pathway inhibition (38, 39). This response is reproducibly seen in multiple SHOC2 KO clones tested, ruling out clonal variation (SI Appendix, Fig. S3 A and B) and is completely rescued by reexpression in KO cells of SHOC2 WT but not the MRAS/PP1 interaction-defective SHOC2 D175N (SI Appendix, Fig. S3 C and D).

When other agonists, such as lysophosphatidic acid and FBS were used to stimulate DLD-1 cells, ERK activation was similarly impaired preferentially at early time points in the absence of SHOC2. On the other hand, ERK activation by TNF-α (which is RAS-RAF independent) was completely unaffected (SI Appendix, Fig. S3 E and F). Taken together, these results are consistent with an agonist-dependent bifasic ERK activation response in which a rapid, transient phase requires the SHOC2 complex, whereas a slow, sustained phase is independent of SHOC2 (Fig. 3C).

**Phosphoproteomic Analysis of SHOC2’s Contribution to EGF-Regulated Dynamics.** To further study the contribution of SHOC2 to ERK pathway dynamics in an unbiased manner, a label-free phosphoproteomic approach was used to compare global EGF-regulated phosphorylation in parental or SHOC2 KO DLD-1 cells. The MEK inhibitor Trametinib was also used in parental cells to compare global pharmacological pathway inhibition to genetic SHOC2 inhibition (Fig. 4A).

In total, 7,053 phosphosites were quantified, corresponding to 3,091 inferred proteins. In parental cells that were stimulated with EGF, 89 and 78 phosphosites were found to be significantly regulated at 5 and 20 min, respectively (cutoff: fold-change ± 2, adjusted P < 0.05) (Fig. 4B and Dataset S1). Functional and phosphorylation motif analysis of the inferred proteins in parental cells are shown in SI Appendix, Fig. S4. Pretreatment with Trametinib dramatically reduced EGF-regulated phosphorylation events with only 5 and 10 phosphosites significantly regulated at 5 and 20 min, respectively (94% and 87% inhibition compared with untreated cells) (Fig. 4B). This highlights the crucial role of the ERK pathway in early signaling by EGF either directly or indirectly by providing priming phosphorylation for other EGF-regulated kinases (40).

In SHOC2 KO cells, inhibition of EGF-regulated phosphorylation was significantly more pronounced at 5 than 20 min of EGF treatment (90% vs. 38.5% inhibition, respectively) (Fig. 4B). When the phosphoproteomes of parental and SHOC2 KO cells were compared at either 5 or 20 min of EGF treatment, only 1 phosphosite was significantly changed at 20 min, whereas 26 phosphosites were differentially regulated by EGF in parental but not SHOC2 KO cells at 5 min (21 down-regulated in SHOC2 KO cells vs. 0 in parental cells).

**Fig. 1.** MRAS and SHOC2 expression promotes S365 BRAF/529 CRAF dephosphorylation, BRAF-MEK dissociation, and BRAF-CRAF dimerization. (A) Calyculin A inhibits BRAF S365 dephosphorylation and ERK activation by MRAS-SHOC2 expression. Expression of MRAS L71 and SHOC2 was induced in T-17 cells stably expressing T6-BRAF by 1 μg/mL Dox treatment for 24 h. Cells were incubated with a Calyculin A dose-response for 20 min and lysates immunoprecipitated, as indicated. (B) Intact RBD is required for efficient S365 BRAF dephosphorylation by MRAS-SHOC2 expression. T6-BRAF WT and mutants were transiently transfected into T-17 cells and MRAS-SHOC2 expression induced for 24 h. (C) Impaired MRAS-SHOC2 induced S259 dephosphorylation by R89L RBD CRAF mutation is rescued by constitutive membrane localization. As in B but with T6-CRAF mutants. (D) MRAS-SHOC2 expression stimulates BRAF S365 dephosphorylation, MEK dissociation, and CRAF binding to BRAF. T-17 T6-BRAF cells as in A were treated with different Dox concentrations for 24 h. StrepTactin pull-downs of T6-BRAF and lysates were immunoprecipitated and visualized using a Li-Cor Odyssey scanner. (E) Li-Cor quantification of D. (F) MRAS-SHOC2 expression stimulates MEK1 dissociation from BRAF and CRAF but not KSR1. GST-fusion genes were cotransfected into HEK293T cells, treated with Myc-MEK1 and either empty vector or MRAS-L71 and SHOC2. GST-56k was used as a control. GST pull-downs and lysates were probed as indicated.
SHOC2-Independent Late ERK Activation Requires CRAF. To address the contribution of RAF isoforms to early vs. late SHOC2-dependent and -independent mechanisms of ERK activation, CRISPR was used to knock out the three RAF paralogues in parental DLD-1 cells. In contrast to SHOC2 deletion, ablation of one or any two combinations of RAF isoforms had no significant effect on EGF-stimulated ERK activation (Fig. 5 A and B and SI Appendix, Fig. S5 A–D). However, KD of the remaining CRAF in dual A/B RAF KO cells potently inhibits EGF-stimulated ERK activation (Fig. 5B) and proliferation in colony formation assays (SI Appendix, Fig. S5E). Thus, as observed in other systems (41, 42), there is redundancy among RAF isoforms but RAF function is essential for ERK activation and proliferation of DLD-1 cells. When siRNAs were used to acutely inhibit expression of individual RAF isoforms, transient KD of individual RAF proteins in parental DLD-1 cells had no effect on EGF-stimulated ERK phosphorylation, consistent with the complementation observed in RAF KO cells. In clear contrast, however, CRAF KD (but not ARAF or BRAF) strongly inhibited MEK and ERK phosphorylation in SHOC2 KO cells (Fig. 5 C and D). Similar results were observed in HEK293T cells, although CRAF KD has a modest inhibitory effect in control cells as well (SI Appendix, Fig. S5F). Strong ERK pathway inhibition upon combined SHOC2 and CRAF inhibition correlates with a strong inhibition of proliferation in DLD-1 cells (Fig. 5E). Taken together, these data suggest that, whereas there is redundancy among RAF isoforms in early phase of SHOC2-dependent ERK pathway activation, CRAF is the primary RAF kinase driving sustained ERK activation by EGF in the absence of SHOC2.

SHOC2-Independent ERK Activation Requires Palmitoylation of NRAS and CRAF N-Region Phosphorylation. Previous studies have shown a biphasic HRAS activation response to EGF with a rapid transient phase occurring at the plasma membrane, followed—with a 10- to 20-min delay—by a sustained phase at the Golgi (43, 44), that is strikingly reminiscent of the ERK response observed in this study. Furthermore, HRAS can differentially activate CRAF in some contexts (45, 46). We thus used siRNAs to investigate the contribution of RAS isoforms to ERK activation by EGF.

KD of any RAS protein had no effect on ERK activity in parental DLD-1 cells, consistent with redundancy as observed for RAF isoforms. However, in SHOC2 KO cells, KD of HRAS and NRAS, but not KRAS, significantly impaired EGF-stimulated ERK activation (Fig. 6A). Furthermore, combined KD of HRAS and NRAS inhibited ERK activity more strongly than NRAS/KRAS or HRAS/KRAS combinations in SHOC2 KO cells (SI Appendix, Fig. S6A). Unlike KRAS, NRAS and HRAS are modified by palmitoylation (47) and pretreatment of DLD-1 cells with the palmitoylation inhibitor 2-bromopalmitate
SHOC2 is selectively required for early, but not delayed ERK pathway activation by EGF in DLD-1 cells. (A) Serum-starved DLD-1 parental or SHOC2 KO cells were stimulated with 25 ng/mL EGF for the indicated times. Lysates were probed and visualized by Li-COR. (B) Quantification of P-S365 BRAF, P-S259 CRAF, P-MEK, P-ERK, P-S380 RSK, P-S473 AKT in A (mean ± SD; n = 3), relative to EGF-untreated parental condition. (C) Model of biphasic ERK activation by EGF with an early and transient phase that requires SHOC2 and a delayed, sustained phase that is SHOC2-independent. Based on ref. 1.

To study a molecular mechanism for this selective SHOC2 contribution to 3D growth, lysates of parental and SHOC2 KO DLD-1 cells growing in 2D or suspension (poly-HEMA-coated dishes) were compared. In suspension cells, phosphorylation of AKT and its downstream substrate site S1718 AF6 is strongly impaired [consistent with PI3K/AKT signaling being adhesion-dependent in many cell types (48–50)], but this is unaffected in SHOC2 KO cells (Fig. 7 C–F). Similarly, phosphorylation of FAK and PAK kinases, also known to be regulated by integrin-mediated attachment to the extracellular matrix (48), was similarly down-regulated in suspension in both parental and SHOC2 KO cells, which correlated with decreased phosphorylation of known PAK sites on CRAF (S338) and MEK (S298) (Fig. 7C). In clear contrast, basal ERK signaling, as determined by phosphorylation of ERK and ERK substrate sites on BRAF (T753) and CRAF (S289/290/296), was unaffected in parental DLD-1 cells, but significantly decreased in SHOC2 KO clones only in suspension. A selective inhibition of ERK signaling in cells in suspension upon SHOC2 ablation was also seen in other SHOC2 KO KRAS mutant colorectal cell lines, such as HCT116 (Fig. 7D) and SW480 (Fig. 7E) cells, but not in V600E, dimerization-independent BRAF mutant RKO or HT29 cells (Fig. 7F). Thus, SHOC2 is preferentially required for ERK signaling under anchorage-independent conditions in the context of oncogenic KRAS but not BRAF signaling.

An implication of these observations is that SHOC2-independent mechanisms of ERK activation must predominate under 2D basal growth conditions and that a mechanism similar to that observed in the sustained phase of EGF stimulation involving N-region CRAF phosphorylation by FAK/SRC or PAK kinases (Fig. 6) may also independently operate in the context of anchorage-dependent/2D growth. Consistent with this possibility, treatment of DLD-1 cells growing in 2D with PAK, FAK, and SRC family inhibitors led to decreased CRAF S338 phosphorylation in both parental and

SHOC2 Is Selectively Required for ERK Pathway Activation under Anchorage-Independent Conditions in KRAS Mutant Cells. We have previously shown that SHOC2 is preferentially required for anchorage-independent proliferation in some RAS mutant cell lines (18). We thus set out to use our isogenic DLD-1 system to elucidate a biochemical mechanism for this observation. SHOC2 KO DLD-1 clones had similar growth rates as parental cells in 2D but were impaired in their ability to grow under anchorage-independent conditions in 3D (Fig. 7 A and B). This effect was partially rescued by reexpression of SHOC2 WT, but not the D175N mutant defective for MRAS/PPI interaction (Fig. 7B), and is consistent with a selective requirement for the RAF phosphatase function of SHOC2 for tumorigenic properties in some RAS mutant cells.

(2-BP) selectively reduced ERK activation at 20 min in SHOC2 KO cells (Fig. 6B). These results thus suggest that the SHOC2-independent/CRAF-dependent sustained phase of ERK activity is driven by palmitoylated NRAS/HRAS proteins.

To further investigate additional molecular mechanisms that may be contributing to SHOC2-independent CRAF activation, a panel of kinase inhibitors was tested for their ability to modulate sustained ERK activation. In addition to ERK pathway inhibitors, PAK (FRAXS97), FAK (PF-562271), and SRC family (SU/6656) kinase inhibitors significantly impaired ERK phosphorylation at 20 min of EGF treatment in SHOC2 KO cells (Fig. 6C and SI Appendix, Fig. S6B). Both PAK and SRC are known to phosphorylate the CRAF N-region at S338 and Y341, respectively, whereas FAK has been linked to both SRC and RAC/PAK signaling. Indeed, FAK inhibitors impaired PAK1 phosphorylation and PAK, FAK, and SRC family also impaired CRAF S338 phosphorylation (Fig. 6D). Taken together, these results suggest that N-region phosphorylation in CRAF plays an important role in sustained ERK activation by EGF in the absence of SHOC2. A model summarizing all our data is shown in Fig. 6E.

To study a molecular mechanism for this selective SHOC2 contribution to 3D growth, lysates of parental and SHOC2 KO DLD-1 cells growing in 2D or suspension (poly-HEMA-coated dishes) were compared. In suspension cells, phosphorylation of AKT and its downstream substrate site S1718 AF6 is strongly impaired [consistent with PI3K/AKT signaling being adhesion-dependent in many cell types (48–50)], but this is unaffected in SHOC2 KO cells (Fig. 7 C–F). Similarly, phosphorylation of FAK and PAK kinases, also known to be regulated by integrin-mediated attachment to the extracellular matrix (48), was similarly down-regulated in suspension in both parental and SHOC2 KO cells, which correlated with decreased phosphorylation of known PAK sites on CRAF (S338) and MEK (S298) (Fig. 7C). In clear contrast, basal ERK signaling, as determined by phosphorylation of ERK and ERK substrate sites on BRAF (T753) and CRAF (S289/290/296), was unaffected in parental DLD-1 cells, but significantly decreased in SHOC2 KO clones only in suspension. A selective inhibition of ERK signaling in cells in suspension upon SHOC2 ablation was also seen in other SHOC2 KO KRAS mutant colorectal cell lines, such as HCT116 (Fig. 7D) and SW480 (Fig. 7E) cells, but not in V600E, dimerization-independent BRAF mutant RKO or HT29 cells (Fig. 7F). Thus, SHOC2 is preferentially required for ERK signaling under anchorage-independent conditions in the context of oncogenic KRAS but not BRAF signaling.

An implication of these observations is that SHOC2-independent mechanisms of ERK activation must predominate under 2D basal growth conditions and that a mechanism similar to that observed in the sustained phase of EGF stimulation involving N-region CRAF phosphorylation by FAK/SRC or PAK kinases (Fig. 6) may also independently operate in the context of anchorage-dependent/2D growth. Consistent with this possibility, treatment of DLD-1 cells growing in 2D with PAK, FAK, and SRC family inhibitors led to decreased CRAF S338 phosphorylation in both parental and

(2-BP) selectively reduced ERK activation at 20 min in SHOC2 KO cells (Fig. 6B). These results thus suggest that the SHOC2-independent/CRAF-dependent sustained phase of ERK activity is driven by palmitoylated NRAS/HRAS proteins.

To further investigate additional molecular mechanisms that may be contributing to SHOC2-independent CRAF activation, a panel of kinase inhibitors was tested for their ability to modulate sustained ERK activation. In addition to ERK pathway inhibitors, PAK (FRAXS97), FAK (PF-562271), and SRC family (SU/6656) kinase inhibitors significantly impaired ERK phosphorylation at 20 min of EGF treatment in SHOC2 KO cells (Fig. 6C and SI Appendix, Fig. S6B). Both PAK and SRC are known to phosphorylate the CRAF N-region at S338 and Y341, respectively, whereas FAK has been linked to both SRC and RAC/PAK signaling. Indeed, FAK inhibitors impaired PAK1 phosphorylation and PAK, FAK, and SRC family also impaired CRAF S338 phosphorylation (Fig. 6D). Taken together, these results suggest that N-region phosphorylation in CRAF plays an important role in sustained ERK activation by EGF in the absence of SHOC2. A model summarizing all our data is shown in Fig. 6E.

SHOC2 Is Selectively Required for ERK Pathway Activation under Anchorage-Independent Conditions in KRAS Mutant Cells. We have previously shown that SHOC2 is preferentially required for anchorage-independent proliferation in some RAS mutant cell lines (18). We thus set out to use our isogenic DLD-1 system to elucidate a biochemical mechanism for this observation. SHOC2 KO DLD-1 clones had similar growth rates as parental cells in 2D but were impaired in their ability to grow under anchorage-independent conditions in 3D (Fig. 7 A and B). This effect was partially rescued by reexpression of SHOC2 WT, but not the D175N mutant defective for MRAS/PPI interaction (Fig. 7B), and is consistent with a selective requirement for the RAF phosphatase function of SHOC2 for tumorigenic properties in some RAS mutant cells.
SHOC2 KO cells, but more potently inhibited ERK phosphorylation in the absence of SHOC2 (Fig. 7G).

Taken together, our observations suggest that SHOC2-dependent and CRAF/N-region-dependent mechanism of RAF activation differentially contribute to ERK activation in a context-dependent manner: whereas redundancy makes SHOC2 dispensable for ERK activation in the absence of SHOC2 (Fig. 7), SHOC2 KO cells, but more potently inhibited ERK phosphorylation in the absence of SHOC2 (Fig. 7G).

Taken together, our observations suggest that SHOC2-dependent and CRAF/N-region-dependent mechanism of RAF activation differentially contribute to ERK activation in a context-dependent manner: whereas redundancy makes SHOC2 dispensable for ERK activation in the absence of SHOC2 (Fig. 7), SHOC2 KO cells, but more potently inhibited ERK phosphorylation in the absence of SHOC2 (Fig. 7G).

Discussion

This study highlights a key role for S259 RAF dephosphorylation by the SHOC2 phosphatase complex in regulating the dissociation of attachment to the extracellular matrix KRAS-mutant cells preferentially rely on SHOC2-dependent mechanism for ERK signaling (Discussion and SI Appendix, Fig. S8).

Discussion

This study highlights a key role for S259 RAF dephosphorylation by the SHOC2 phosphatase complex in regulating the dissociation of attachment to the extracellular matrix KRAS-mutant cells preferentially rely on SHOC2-dependent mechanism for ERK signaling (Discussion and SI Appendix, Fig. S8).

Discussion

This study highlights a key role for S259 RAF dephosphorylation by the SHOC2 phosphatase complex in regulating the dissociation of attachment to the extracellular matrix KRAS-mutant cells preferentially rely on SHOC2-dependent mechanism for ERK signaling (Discussion and SI Appendix, Fig. S8).
of 14-3-3 from the N-terminal RAF regulatory region and RAF dimerization. In the absence of SHOC2, EGF-stimulated BRAF-ARAF, BRAF-CRAF, and BRAF-KSR heterodimerization are strongly impaired, whereas RAF interaction with RAS is actually increased (Fig. 2B). This result shows that the RAF–RAS interaction can be uncoupled from RAF dimerization in some contexts and is consistent with a model where coordinate inputs from RAS and the SHOC2 holophosphatase are required for RAF heterodimerization and activation. Increased RAS–RAF interaction in the absence of SHOC2 is incompatible with a role for SHOC2 as a scaffold promoting RAS–RAF interaction as suggested by some overexpression studies (29, 30). Instead, it is consistent with decreased ERK activity in the absence of SHOC2, leading to relief of ERK inhibitory feedbacks, both upstream of RAS and at the level of RAF, such as CRAF S289/296/301 and BRAF T753 that disrupt RAF–RAF interaction (51, 52). Similarly, inhibitory ERK feedback sites on EGFR (T699) and IRS-1 (S636/639) are also inhibited in the absence of SHOC2 and likely contribute to increased AKT phosphorylation upon SHOC2 and ERK pathway inhibition (Fig. 3A and B) (38, 39, 53).

There is controversy around the precise order of the initial steps in the RAF activation cycle and whether S259 dephosphorylation precedes or follows RAS-GTP binding (11). S259A mutation in CRAF promotes association with RAS (54), which can be interpreted to suggest that S259 dephosphorylation may precede RAS binding, possibly by 14-3-3 dissociation facilitating access of the RAF RBD to RAS. However, our studies support an alternative model (SI Appendix, Fig. S2) where RAS-GTP binding to RAF and recruitment to the membrane is independent of, and precedes S259 dephosphorylation by the SHOC2 complex: in a time course where levels of P-S259 RAF and RAF–RAF interaction; see discussion above and

Fig. 6. SHOC2-independent ERK activation requires palmitoylated HRAS/NRAS and CRAF N-region phosphorylation. (A) NRAS and HRAS KO (but not KRAS) inhibit ERK activation in SHOC2 KO cells. SHOC2 KO DLD-1 cells transfected with siRNAs were stimulated with 25 ng/mL EGF for the indicated times. (B) The palmitoylation inhibitor 2-BP reduces sustained ERK activation in SHOC2 KO cells. DLD-1 cells were pretreated with 2-BP (100 μM) for the indicated times before EGF stimulation. (C) PAK, FAK, and SRC family inhibitors (besides RAF and MEK inhibitors) impair sustained ERK pathway activation by EGF in SHOC2 KO DLD-1 cells. Cells were stimulated with 25 ng/mL EGF for 20 min after 30-min pretreatment with indicated kinase inhibitors (and 2-BP). Lysates were probed for ERK activity by Li-COR (mean ± SD) (n = 3–7). Significance is determined using a two-tailed t-test *p < 0.05, **p < 0.01, or ***p < 0.001. See SI Appendix, Fig. S6 for representative experiment. (D) Cells were pretreated with 10 μM PAK (FRAX597), SRC (SU6656), and FAK (PF-562271) inhibitors alone or in combination, 30 min before stimulation with EGF for 20 min. (E) Model of selective contribution of the SHOC2 complex to ERK pathway spatiotemporal dynamics. EGF Receptor activation leads to N/H/K-RAS and MRAS/SHOC2 complex activation at the plasma membrane and an early phase of ERK activation involving A/B/C-RAF isoforms. As a result of intracellular trafficking of palmitoylated proteins (by the constitutive de/reacylation cycle and/or receptor-mediated endocytosis and/or other nonmutually exclusive mechanisms not shown), H/N-RAS travel to endomembrane compartments from where they signal through CRAF to drive sustained ERK pathway activation. Because poly-basic motif-containing KRAS-4B and MRAS (and associated proteins) remain at the plasma membrane, this CRAF is now uncoupled from regulation by the SHOC2 complex, but is instead dependent on N-region phosphorylation by kinases, such as PAK, SFK, and FAK. See Discussion for further details. Membrane anchors represent farnesylation (red) and palmitate (black) groups. S338 and S341 residues in CRAF belong to the N-region. ERK may phosphorylate diverse substrates in different compartments, as shown by different color arrows.

Boned del Rio et al.
Our study has uncovered a selective contribution of the SHOC2 phosphatase complex to ERK pathway dynamics. In activated by EGF (62, 63) as well as by S259 dephosphorylation (56) when the tandem polybasic-motif–containing KRAS-4B is thought to signal exclusively from the plasma membrane (47). The MRAS HVR contains a polybasic motif as a second membrane targeting signal and is thus expected to closely mirror KRAS-4B in its plasma membrane localization, while being refractory to the intracellular trafficking mechanisms of palmitoylated proteins. Indeed, overexpression of YFP/mCherry-fusion proteins in human mammary epithelial cells supports this scenario, as in addition to the plasma membrane, HRAS and NRAS (but not KRAS-4B or MRAS) can be readily detected to colocalize with CRAF at the Golgi and/or other intracellular compartments (SI Appendix, Fig. S7).

We propose a model (Fig. 6E) where upon EGF stimulation, the rapid phase of SHOC2-dependent ERK activation occurs at the plasma membrane, where SHOC2 complex formation upon MRAS activation leads to S259 dephosphorylation on proximal A/B/C-RAF proteins recruited by H/N/K-RAS proteins. In this phase there is redundancy among RAS and RAF isoforms for ERK pathway activation, whereas SHOC2 appears to play an essential, nonredundant role (Fig. 3C). The slow, sustained phase of ERK activation may be driven by internalization of palmitoylated RAF proteins that thereby become spatially segregated from the SHOC2 complex that remains anchored at the plasma membrane by MRAS, alongside KRAS-4B. Internalization may result from intracellular trafficking by the constitutive acylation cycle of palmitoylated proteins and/or receptor-mediated endocytosis and/or other mechanisms operating in a nonmutually exclusive manner (44, 65, 66). From these intracellular
compartments, H/N-RAS proteins signal primarily through CRAF, which is now uncoupled from regulation by the SHOC2 complex but dependent on N-region phosphorylation by kinases such as PAK, SRC, and FAK (directly or indirectly).

A biphasic HRAS activation by EGFR with a slow sustained phase at the Golgi dependent on the acylation cycle (44, 65, 67), as well as different CRAF activation by HRAS (but not KRAS) dependent on endocytosis (45), are both consistent with this model. A similar biphasic ERK response upon G protein-coupled receptor internalization has been linked to phosphorylation of different ERK substrates from spatially distinct signaling platforms (1, 68) and it is likely that SHOC2-dependent and -independent phases of ERK activation are also associated with phosphorylation of ERK substrates at distinct spatial compartments. We also note that similar biphasic kinetics linked to compartment-specific RAS-ERK signaling have been observed during the process of thymocyte selection (66, 69) and future studies should address the role of the SHOC2 complex in immune tolerance.

The contribution of CRAF S259 dephosphorylation and/or dimerization to the slow ERK activation phase remains unclear. We were unable to detect significant S259 dephosphorylation or RAF heterodimerization in the absence of SHOC2, but low levels below the sensitivity of our experimental conditions cannot be ruled out. On the other hand, we note that experimental constraints when analyzing endogenous proteins have not allowed us to measure homodimerization, and S259-independent CRAF homodimerization during the slow, sustained phase remains a distinct possibility. Reports of N-region CRAF phosphorylation promoting relief from autoinhibition and dimerization (70, 71) and of high levels of S335 phosphorylation activating CRAF in the presence of high levels of inhibitory phosphorylation at S43 and S259 (72) support this scenario. It is also worth noting that both SFK and PAK activators, such as RAC and CDC42, are palmitoylated and expected to travel with H/N-RAS during both endocytosis and acylation cycle scenarios of intracellular trafficking, which would thus facilitate N-region phosphorylation of the H/N-RAS bound CRAF at these compartments.

The biochemical mechanisms of SHOC2-independent, CRAF N-region-dependent ERK activation observed in the sustained phase of EGF stimulation in DLD-1 cells appear to operate as well in the context of anchorage-dependent proliferation in 2D (SI Appendix, Fig. S8). Integrin signaling regulates both FAK-SRC and PAK activation and cooperates with RTKs to regulate sustained ERK activation in multiple contexts (73–79). Thus, integrins are well poised to mediate, at least in part, SHOC2-independent ERK activation from sites of attachment to the extracellular matrix.

Redundant SHOC2-dependent and SHOC2-independent/ CRAF-dependent mechanisms of ERK activation under basal 2D conditions are likely to account for the observation that both SHOC2 and CRAF ablation alone are well tolerated, whereas combined inhibition potently inhibits growth (Fig. 5E), as complete inhibition of the ERK response is incompatible with proliferation (8, 41, 42). In clear contrast however, in the absence of adhesion to the extracellular matrix, a key contribution of SHOC2 to ERK activity in KRAS mutant cells is uncovered in 3D (Fig. 7). Basal P3K/AKT and FAK/PAK activation is strongly impaired in the absence of matrix-dependent attachment, which is likely to enhance the dependency on SHOC2-dependent ERK signaling for anchorage-independent growth in RAS mutant cells.

1. B. N. Khodolenko, J. F. Hancock, W. Kolch, Signalling ballet in space and time. Nat. Rev. Mol. Cell Biol. 11, 414–426 (2010).
2. Y. D. Shaoul, R. Segre, The MEK/ERK cascade: From signaling specificity to diverse functions. Biochem. Biophys. Acta 1773, 1213–1226 (2007).
3. K. A. Rauen, The RASopathies. Annu. Rev. Genomics Hum. Genet. 14, 355–369 (2013).
4. D. K. Simanshu, D. V. Nissley, F. McCormick, RAS proteins and their regulators in human disease. Cell 170, 17–33 (2017).
5. A. A. Samatar, P. J. Poulikakos, Targeting RAS-ERK signalling in cancer: Promises and challenges. Nat. Rev. Drug Discov. 13, 928–942 (2014).
6. C. J. Caunt, M. J. Sale, P. D. Smith, S. J. Cook, MEK1 and MEK2 inhibitors and cancer therapy: The long and winding road. Nat. Rev. Cancer 15, 577–592 (2015).
7. G. Bollag et al., Clinical efficacy of a Raf inhibitor needs broad target blockade in Braf-mutant melanoma. Nature 467, 596–599 (2010).
8. R. B. Blasco et al., c-Raf, but not B-Raf, is essential for development of K-Ras oncogene-driven non-small-cell lung carcinoma. Cancer Cell 19, 652–663 (2011).
9. K. L. Jameson et al., IQGAP1 scaffold-kinase interaction blockade selectively targets RAS-MAP kinase-driven tumors. Nat. Med. 19, 626–630 (2013).
10. A. Herrero et al., Small molecule inhibition of ERK dimerization prevents tumorigenesis by RAS-ERK pathway oncogenes. Cancer Cell 28, 170–182 (2015).
11. H. Lavoie, M. Therriens, Regulation of Raf protein kinases in ERK signalling. Nat. Rev. Mol. Cell Biol. 16, 281–298 (2015).
12. G. Tzivion, Z. Luo, J. Avruch, A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. Nature 394, 88–92 (1998).
13. M. M. McKay, D. K. Morrison, Integrating signals from RTKs to ERK/MAPK. Oncogene 30 (2011), 2800–2811.

14. B. Pandit, et al., Gain-of-function RAF mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat. Genet. 39, 1007–1012 (2007).

15. M. A. Razaque et al., Germline gain-of-function mutations in RAF1 cause Noonan syndrome. Nat. Genet. 39, 1013–1017 (2007).

16. T. Kobayashi, Molecular and clinical analysis of RAF1 in Noonan syndrome and related disorders: Dephosphorylation of serine 259 as the essential mechanism for mutant activation. Hum. Mutat. 31, 284–290 (2010).

17. M. Molzan et al., Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. Mol. Cell. Biol. 30, 4689–4711 (2010).

18. L. C. Young et al., An MRRAS, SHOC2, and SCRIB complex coordinates ERK pathway activation with polarity and tumorigenic growth. Mol. Cell 52, 679–692 (2013).

19. R. Procopio-Vieira, E. N. Procopio, A. Burlingame, M. Fried, and F. McCorison, B-Raf as a phosphatase holoenzyme comprised of Shoc2/Scr and the catalytic subunit of PPI1 functions as an ARs effecter to modulate Raf activity. Mol. Cell. 22, 217–230 (2006).

20. V. Cordeddu et al., Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan syndrome with loose anagen hair. Nat. Genet. 41, 1012–1024 (2009).

21. E. M. Higgins et al., Elucidation of MRRAS-mediated Noonan syndrome with cardiac hypertrophy. JCI Insight 2, e91225 (2017).

22. R. M. Zambrano et al., Further evidence that variants in PIP1C1 cause a rasopathy similar to Noonan syndrome with loose anagen hair. Am. J. Med. Genet. A 173, 565–567 (2017).

23. L. C. Young et al., SHOC2-MRRAS-PIK3 complex positively regulates RAF activity and contributes to Noonan syndrome pathogenesis. Proc. Natl. Acad. Sci. U.S.A. 115, E10576–E10585 (2018).

24. O. Rocks, A. Peyker, P. I. Bastiaens, Spatio-temporal segregation of Ras signals: One Ras isoform. JCI Insight 2, 1036–1044 (2018).

25. O. Rocks, A. Peyker, P. I. Bastiaens, Spatio-temporal segregation of Ras signals: One Ras isoform. JCI Insight 2, 1036–1044 (2018).