SHOC2 subcellular shuttling requires the KEKE motif-rich region and N-terminal leucine-rich repeat domain and impacts on ERK signalling

Marialetizia Motta1, Giovanni Chillemi2, Valentina Fodale3,†, Serena Cecchetti4, Simona Coppola5, Silvia Stipo3, Viviana Cordeddu3, Pompeo Macioce4, Bruce D. Gelb6 and Marco Tartaglia1,*

1Genetics and Rare Diseases Research Division, Ospedale Pediatrico Bambino Gesù, Rome, Italy, 2CINECA, SCAI-SuperComputing Applications and Innovation Department, Rome, Italy, 3Department of Hematology, Oncology and Molecular Medicine, 4Department of Cell Biology and Neurosciences, 5Italian National Centre for Rare Diseases, Istituto Superiore di Sanità, Rome, Italy and 6The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

SHOC2 is a scaffold protein composed almost entirely by leucine-rich repeats (LRRs) and having an N-terminal region enriched in alternating lysine and glutamate/aspartate residues (KEKE motifs). SHOC2 acts as a positive modulator of the RAS-RAF-MEK-ERK signalling cascade by favouring stable RAF1 interaction with RAS. We previously reported that the p.Ser2Gly substitution in SHOC2 underlies Mazzanti syndrome, a RASopathy clinically overlapping Noonan syndrome, promoting N-myristoylation and constitutive targeting of the mutant to the plasma membrane. We also documented transient nuclear translocation of wild-type SHOC2 upon EGF stimulation, suggesting a more complex function in signal transduction. Here, we characterized the domains controlling SHOC2 shuttling between the nucleus and cytoplasm, and those contributing to SHOC2S2G mistargeting to the plasma membrane, analysed the structural organization of SHOC2’s LRR motifs, and determined the impact of SHOC2 mislocalization on ERK signalling. We show that LRRs 1 to 13 constitute a structurally recognizable domain required for SHOC2 import into the nucleus and constitutive targeting of SHOC2S2G to the plasma membrane, while the KEKE motif-rich region is necessary to achieve efficient SHOC2 export from the nucleus. We also document that SHOC2S2G localizes both in raft and non-raft domains, and that it translocates to the non-raft domains following stimulation. Finally, we demonstrate that SHOC2 trapping at different subcellular sites has a diverse impact on ERK signalling strength and dynamics, suggesting a dual counteracting modulatory role of SHOC2 in the control of ERK signalling exerted at different intracellular compartments.

†Present address: IRBM Promidis, Pomezia, Rome, Italy.
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Introduction

SHOC2 (also known as SUR-8 and SOC-2) is a widely expressed protein composed almost entirely of leucine-rich repeats (LRRs), and having a region rich in alternating isoleucine and glutamate (or aspartate) residues (KEKE motifs) at the N-terminus. In C. elegans, where it was originally discovered, the protein acts as a positive modulator of intracellular signalling elicited by cell-surface receptor kinases and mediated by LET-60, the homolog of vertebrate RAS sub-family GTPases (1,2). Since LRRs provide a structural framework for protein-protein interactions (3), SHOC2 is believed to function as a scaffold protein linking RAS proteins to downstream signal transducers (4,5). This view has been confirmed by the observation that SHOC2 functions as a regulatory protein of the catalytic subunit of protein phosphatase 1 (PP1C) (6). By binding GTP-bound MAR, SHOC2 promotes PP1C translocation to the membrane, allowing PP1C-mediated dephosphorylation of RAF1 at a major inhibitory residue, Ser259, which is a required event for stable RAF1 binding to activated GTP-bound RAS, RAF1 catalytic activation and efficient transmission of signalling through MEK and ERK proteins (6).

The relevance of SHOC2’s function on RAS-RAF-MEK-ERK (RAS-MAPK, hereafter) signalling has been further highlighted by the discovery that a single missense mutation c.4A>G, p.Ser2Gly) in SHOC2 underlies Mazzanti syndrome (MIM 607721; also known as Noonan syndrome-like disorder with loose anagen hair) (7), a disorder affecting development and growth clinically overlapping Noonan syndrome (MIM 163950), the most common RASopathy (8,9). Similar to other RASopathies, Mazzanti syndrome is characterized by reduced postnatal growth, variable cognitive deficits, congenital cardiac defects, and ectodermal anomalies (7,10). Biochemical and functional studies demonstrated that the disease-causing mutation creates an N-myristoyltransferase (NMT) recognition site driving aberrant N-myristoylation of SHOC2 (25-27), its constitutive plasma membrane targeting, and enhanced ERK activation in a cell type-specific fashion (7).

N-terminal myristoylation is an irreversible form of protein fatty acylation occurring co-translationally in which myristate, a 14-carbon saturated fatty acid, is covalently added to an N-terminal glycine residue of a nascent polypeptide after excision of the initiator methionine residue by methionyl aminopeptidase (11,12). N-myristoylation is a relatively common lipid modification of many membrane-bound signal transducers (13,14). While it contributes to protein anchoring to cellular membranes, the binding energy it provides is relatively weak and not sufficient per se to promote stable binding of proteins to the membrane (15). In N-myristoylated membrane-anchored proteins, a second signal, either a polybasic residue stretch or a palmitoylation recognition motif, is commonly observed (14,16). The former contributes to stabilize protein binding to the membrane via electrostatic interactions with the head groups of acidic phospholipids of the cytoplasmic leaflet of the bilayer (17,18), while the latter favours a strong association with the lipid bilayer by increasing protein hydrophobicity (19,20). Alternatively, membrane interaction of singly acylated proteins can be enhanced by interactions with other membrane bound proteins. Similarly, N-myristoylation does not contain the information required to direct targeting specifically to the cytoplasmic side of the plasma membrane (or to other intracellular membranes), which requires additional events that are usually mediated by membrane-bound proteins and enzymes (13,14).

While growth factor-induced translocation of SHOC2 to the membrane is largely accepted as a required event promoting efficient RAS-mediated ERK activation, we noticed that SHOC2 is uniformly distributed in the cytoplasm and nucleus in resting conditions, and that it translocates to the nucleus following EGF stimulation (7). These observations suggest that SHOC2 may have multiple regulatory roles in intracellular signalling. The structural elements mediating SHOC2 nucleo-cytoplasmic shuttling and the impact of its different subcellular localization on RAS-MAPK signalling have not been characterized yet.

In this study, we explored the mechanisms mediating plasma membrane targeting of the RASopathy-causing SHOC2S2G mutant and EGF-dependent cytoplasm-membrane translocation of wild-type SHOC2, as well as the impact of SHOC2 intracellular mislocalization on ERK signalling. We show that the N-terminal LRRs mediate SHOC2 import into the nucleus and constitutive targeting of myristoylated SHOC2S2G to the plasma membrane, while the C-terminal LRRs do not significantly impact on subcellular compartmentalization. Consistently, we provide evidence that SHOC2’s LRRs are organized into two structurally independent domains that are connected by a flexible hinge constituted by the variable region of LRR 13. We also show that the N-terminal KEKE motif-rich region is required for efficient SHOC2 export from the nucleus. Finally, we demonstrate that while nuclear trapping of SHOC2 is associated with less efficient activation of ERK, its impaired nuclear import results in prolonged activation of the kinase, suggesting a previously unrecognized negative modulatory role of SHOC2 in the control of ERK signalling exerted in the nucleus.

Results

SHOC2 nuclear import is not mediated by conventional nuclear localization sequences

While nucleocytoplasmic trafficking of small proteins is generally achieved by passive diffusion through the nuclear pore complex, larger proteins or protein complexes necessitate active transport (21). Nuclear import generally requires the recognition of a nuclear localization signal (NLS) by the transport machinery, and multiple ‘monopartite’ and ‘bipartite’ NLSs have been identified. The PSORTII (http://psort.hgc.jp) and Wregex (http://wregex.elhubio.es/faces/home.xhtmll) WEB servers were used to scan the SHOC2 sequence to recognize potential NLS motifs. While no putative bipartite NLS was detected, a single monopartite NLS (PGTRKKS at position 79), satisfying the consensus sequence of the Pat7 motif (i.e. PX1-3K/R3-4) was identified. To validate this putative NLS functionality, a SHOC2 mutant (SHOC2P79A/R82G/K83G) carrying multiple substitutions affecting key residues of the motif was generated (Figure 1), and subcellular localization in starved and EGF-stimulated transfected COS-1 cells was analysed by confocal laser scanning microscopy. Similar to what was observed for the wild-type protein, SHOC2P79A/R82G/K83G efficiently translocated to the nucleus following growth factor stimulation and was uniformly distributed in the cytoplasm and nucleus during starvation (Figure 2A, panels a and b). This subcellular distribution pattern was confirmed by cell fractionation experiments (Figure 2B), excluding the role of this motif in mediating nuclear import of the protein and indicating that SHOC2 does not contain a functionally active conventional NLS mediating nuclear import.

Role of KEKE motifs and LRRs in SHOC2’s subcellular localization

SHOC2 is characterized by two distinct regions that are believed to mediate binding of the protein to signalling partners (Figure

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cytoplasmic shuttling, several mutants lacking the KEKE motifs (SHOC2-KEKE) or different subsets of LRRs (SHOC2-LRR2-5, SHOC2-LRR6-10, SHOC2-LRR11-14, and SHOC2-LRR15-19) were generated (Figure 1). The subcellular localization of each mutant was examined by confocal microscopy. Western blot analysis confirmed that all mutants were efficiently expressed, although a relatively reduced level was observed for the SHOC2-KEKE mutant and the cytoplasmic shuttling of several mutants, SHOC2-LRR11-14 and SHOC2-LRR15-19, which lack more C-terminally positioned LRRs, displayed an intracellular distribution that grossly overlapped that of wild-type protein in both serum starved and EGF stimulated cells (Figure 2A, panels f and g). These observations were confirmed using different cell lines (Neuro2A and HEK293) (data not shown). The impaired export from the nucleus of the SHOC2-KEKE mutant and the cytoplasmic localization of the SHOC2-LRR2-5 protein were confirmed by cell fractionation assays (Figure 2B).

Overall, these findings indicate that the first 10 LRRs are required for the transport of SHOC2 from the cytoplasm to the nucleus, while the N-terminal KEKE motif-rich region mediates nuclear export of the protein.

SHOC2 mutants lacking the KEKE motif-rich region and those missing the N-terminal LRRs impact RAS-MAPK signalling differently

Multiple lines of evidence support the view that SHOC2's positive modulatory role on RAS-MAPK signalling is linked to its translocation to the plasma membrane and promotion of RAF1 stable interaction with GTP-bound RAS proteins. To explore the impact of forced nuclear localization and constitutive retention in the cytoplasm of SHOC2 on ERK activation, we analyzed the levels of ERK phosphorylation associated with the overexpression of the SHOC2 mutants lacking different LRR motifs in Neuro2A cells, a line for which the capability of SHOC2 to mediate ERK signalling had previously been demonstrated (7). Following EGF stimulation, overexpression of wild-type SHOC2 resulted in greater ERK phosphorylation compared with what was observed in untransfected cells. As expected, nuclear trapping of the protein resulted in a less efficient activation of ERK (Figure 2C), which is consistent with the required SHOC2-mediated translocation of PP1C to the plasma membrane (or other intracellular membranes) for efficient RAF1 binding to activated RAS, and RAF1 activation. In contrast, impaired nuclear import of SHOC2 was associated with a more sustained activation of ERK (Figure 2C and Supplementary Material, Fig. S2), suggesting a possible involvement of the protein in MAPK signal switch-off, in the nucleus. The specific role of the N-terminal LRRs in this unpredicted effect on ERK phosphorylation was confirmed by the observation that overexpression of SHOC2-LRR11-14 and SHOC2-LRR15-19 resulted in an ERK phosphorylation pattern that was similar in amplitude and duration to that observed in cells transfected with the wild-type protein (Figure 2C and Supplementary Material, Fig. S2). These findings were confirmed by ELK1-driven transactivation assay experiments performed in NIH 3T3 cells used as a readout of MAPK activation (Supplementary Material, Fig. S3).

These data indicate that the specific intracellular localization of SHOC2 differentially impacts RAS-MAPK signalling and that besides the positive role on MAPK signalling associated with the activation of RAF1, SHOC2 might contribute to the switch-off of MAPK signalling, following its translocation to the nucleus.

Dual acylation is not implicated in stable plasma membrane binding of SHOC2^S2G

According to the 'two-signal model' for membrane binding of N-myristoylated proteins, stable membrane association and targeting specificity are generally achieved by either a polybasic residue stretch or a second acylation event (i.e. palmitoylation) (13). Interaction with other membrane-bound proteins might
also stabilize targeting to specific membrane domains. The N- and C-terminal region of SHOC2 contain neither a net positively charged region potentially implicated in phospholipids binding nor cysteine residues, excluding the role of palmitoylation, farnesylation, geranylgeranylation and other lipid anchors as post-translational modifications contributing to stable interaction with the lipid bilayer in the mutant. Similarly, the CSS-Palm v4.0 prediction tool (http://csspalm.biocuckoo.org/) did not identify any consensus motif predicted with high confidence for reversible S-palmitoylation throughout the SHOC2 sequence. A few putative palmitoylation sites (Cys144, Cys238, Cys342 and Cys540) were called with low confidence. To exclude this possibility experimentally, the best candidate site among those was assayed by evaluating the subcellular localization of the SHOC2S2G/C144G double mutant by confocal microscopy in transiently transfected COS-1 cells. Similarly to SHOC2 S2G, the protein was efficiently expressed and exhibited a constitutive plasma membrane localization, indicating that palmitoylation at Cys144 does not occur or does not contribute to stable membrane binding of myristoylated SHOC2 S2G (Figure 3A, panels a and b). A membrane-restricted distribution of the SHOC2S2G/C144G mutant was consistently observed also in cell fractionation assays (Figure 3B).

These analyses strongly suggested that SHOC2S2G targeting to the plasma membrane likely depends on specific interactions with membrane-bound proteins.

Role of KEKE motifs and LRRs in SHOC2S2G subcellular localization

To assess the possible contribution of the LRR motifs in driving plasma membrane targeting of SHOC2S2G, four mutants carrying partial deletions of the LRR domain were generated (Figure 1), and their subcellular localization was examined in transfected COS-1 cells during starvation and following EGF stimulation. Among these mutants, SHOC2S2G/DLRR2-5 and SHOC2S2G/DLRR6-10 proteins displayed constitutive cytoplasmic localization (Figure 3A, panels d and e), establishing the requirement of these LRRs in SHOC2S2G targeting to the plasma membrane. Of note, this distribution pattern overlapped with that observed for the SHOC2D(LRR2-5) and SHOC2D(LRR6-10) mutants, pointing out an equivalent protein-protein interaction-mediated mechanism involving these motifs in the control of both

Figure 2. Subcellular localization of the generated SHOC2 mutants, and their effect on ERK signalling. (A) Localization of the transiently expressed mutants in COS-1 cells during starvation or following growth factor stimulation revealed by confocal microscopy. (B) Localization of the transiently expressed mutants in COS-1 cells cultured in steady state, serum starved and EGF treated conditions, determined by cell fractionation experiments (C, cytoplasm; N, nucleus). (C) Western blot analysis documenting the levels of ERK phosphorylation associated with the overexpression of wild-type SHOC2 or a selected subset of mutants in Neuro2A cells in time-course EGF stimulation experiments.
SHOC2 translocation to the plasma membrane and its import to the nucleus. Consistent with the minor impact of the deletions of LRRs 11–14 and LRRs 15–19 in altering SHOC2 subcellular localization, SHOC2S2G/DLRR11-14 and SHOC2S2G/DLRR15-19 proteins localized constitutively to the plasma membrane (Figure 3A, panels f and g), demonstrating that the C-terminal LRRs are not essential for the constitutive targeting to the membrane of SHOC2S2G, and more generally do not play a major role in controlling SHOC2 intracellular trafficking. The impact of deletions on SHOC2S2G translocation to the plasma membrane was confirmed by cell fractionation assays (Figure 3B).

In sharp contrast with the other SHOC2S2G mutants, deletion of the N-terminal KEKE motifs-rich region unexpectedly resulted in the distribution of the mutant protein more widely throughout the cell (i.e. plasma membrane, cytoplasm and nucleus) (Figure 3A, panel c). The spread and spotted distribution in the cytoplasm of the SHOC2 S2G/DKEKE mutant was suggestive of a non-specific interaction with intracellular membranes, indicating a specific contribution of this region in stabilizing SHOC2S2G targeting to the plasma membrane; on the other hand, its nuclear localization suggested the possibility of defective N-myristoylation of the mutant. To verify this hypothesis, SHOC2S2G/DKEKE’s myristoylation status was evaluated and compared with that of the SHOC2S2G and SHOC2S2G/ALRR2-5 proteins. Differently from SHOC2S2G and the SHOC2S2G/ALRR2-5 mutant, which showed efficient [3H]myristic acid incorporation, N-myristoylation of the SHOC2S2G/DKEKE mutant was reduced (Supplementary Material, Fig. S4), indicating a less efficient recognition by NMT and the requirement of a more ‘extended’ sequence compared to what generally observed in naturally occurring N-myristoylated proteins.

Impaired plasma membrane binding of SHOC2S2G abolishes the gain-of-function effect of SHOC2’s N-myristoylation on MAPK signalling

We originally hypothesized that the RASopathy-causing Ser-to-Gly change at codon 2 in SHOC2 promotes enhanced signalling through the MAPK cascade, driving constitutive targeting of the mutant to the plasma membrane. To further validate this hypothesis, the dynamics of ERK phosphorylation were analysed in time-course experiments in EGF-stimulated Neuro2A cells transiently transfected to express SHOC2 S2G mutants carrying different deletions of the LRR region or lacking the KEKE motif-rich sequence (Figure 3C and Supplementary Material, Fig. S2). In agreement with our previously reported data (7), SHOC2S2G expression was found to promote boosted and sustained EGF-dependent ERK phosphorylation compared to what was observed in cells expressing the wild-type protein.
Phosphorylation of ERK in cells expressing the SHOC2$^{2SG/ALRR11-14}$ and SHOC2$^{2SG/ALRR6-10}$ mutants was similar in amplitude to what observed in cells transfected with wild-type SHOC2 (Figure 3C and Supplementary Material, Fig. S2), providing further evidence for the requirement of plasma membrane targeting of SHOC2$^{2SG}$ for its role in enhancing MAPK signalling following EGF stimulation. On the other hand, sustained ERK phosphorylation was apparent in cells expressing the SHOC2$^{2SG/ALRR6-10}$ and SHOC2$^{2SG/ALRR6-10}$ mutants following stimulation (Figure 3C and Supplementary Material, Fig. S2). This dynamic, which is consistent with the effect of these mutants in promoting prolonged stimulus-dependent ERK phosphorylation, further emphasizes the relevance of nuclear translocation of SHOC2 in switching off MAPK signalling. The levels and dynamics of ERK activation in cells expressing SHOC2$^{2SG/ALRR11-14}$ and SHOC2$^{2SG/ALRR15-19}$ were comparable to those were observed in SHOC2$^{2SG}$ transfected cells, supporting a minor impact of the C-terminal portion of the LRR region in mediating the events controlling SHOC2’s function in RAS-MAPK signalling (Figure 3C and Supplementary Material, Fig. S2), which is in line with its apparently negligible influence on SHOC2 subcellular localization. The milder impact on MAPK signalling of the deletions involving LRRs 11-14 and LRRs 15-19 compared to those affecting LRRs 2-5 and LRRs 6-10 was also confirmed by ELK1 transactivation assay experiments (Supplementary Material, Fig. S3). Finally, a less efficient but sustained ERK activation was observed for the SHOC2$^{2SG/KEKE}$, which would be consistent with the less efficient plasma membrane targeting of this mutant compared to the fully myristoylated SHOC2$^{2SG}$ and the limited EGF-stimulated nuclear translocation of the non-myristoylated SHOC2$^{2SG/KEKE}$ protein (Figure 3C and Supplementary Material, Fig. S3).

SHOC2$^{2SG}$ dynamically associates with lipid rafts

RAS proteins, which are targeted to the plasma membrane by different C-terminal anchors, operate in functionally distinct microdomains (24,25). Among these, the lipid rafts are tightly packed cholesterol/sphingolipid-rich domains, which are more ordered than the surrounding lipid bilayer. These specialized subdomains serve as sorting platforms, reservoirs for inactive signalling proteins or hubs for signal transduction to mediate specific recruitment of proteins, facilitate compartmentalization of signalling in the membrane, or mediate RA signalling tuning (26-28). Localization of RAS in lipid rafts is dynamic. Specifically, palmitoylated HRAS localizes in lipid rafts in its inactive state, and its translocation to cholesterol-independent domains is necessary for efficient activation of the MAPK cascade (27,29). In contrast, NRAS displays an opposite relocation following stimulation (30). This differential spatial localization is likely to account, in part, for the diverse signal outputs promoted by these proteins (31). Since plasma membrane targeting of SHOC2$^{2SG}$ represents a key event in the enhanced and prolonged activation of MAPK signalling, membrane microdomain partitioning of the myristoylated mutant was analysed in lysates of transiently transfected Neuro2A cells starved and stimulated with EGF or left untreated (Figure 4). Sucrose gradient ultracentrifugation was used to separate the detergent-insoluble lipid rafts, which float to low-density fractions of the gradient (32,33), and fractions were then analysed by western blotting to verify the presence of the protein by using flotillin as a lipid raft marker (34,35). In line with previous observations, in starved conditions, HA-RAS was present in the insoluble fractions containing lipid rafts (fractions 4-6) as well as in soluble fractions (fractions 7-9), the latter including proteins located in other membrane domains or in the cytosol (Figure 4, top panel). EGF stimulation of cells resulted in a redistribution of the GTPase from rafts to non-raft domains of the plasma membrane. Consistent with the observed HRAS re-distribution following stimulation, a constitutively activated HRAS mutant, HRAS$^{G12L}$, was observed to localize exclusively in fractions not containing lipid rafts (Figure 4, middle panel). Similarly to what was documented for the wild-type HRAS protein, a dynamic association of SHOC2$^{2SG}$ with lipid rafts was observed, with a translocation of the mutant in cholesterol-independent membrane subdomains following stimulation (Figure 4, bottom panel). We tested two membrane-targeted mutants, SHOC2$^{2SG/KEKE}$ and SHOC2$^{2SG/ALRR11-14}$, and observed that they redistributed to raft and non-raft domains dynamically, similar to that of the myristoylated SHOC2$^{2SG}$ protein (data not shown). These results indicated that deletion of the KEKE motifs or LRRs located at the C-terminus do not impact substantially the interaction with proteins residing in these subdomains of the plasma membrane.

SHOC2’s LRR motifs constitute two structurally recognizable and tandemly arranged domains

Our findings indicated differential roles for the N-terminal and C-terminal LRRs in controlling SHOC2 subcellular localization and modulation of RAS-MAPK signalling, suggesting that these motifs may be organized into two structurally distinct domains. To explore this possibility, a three-dimensional model of the tandemly arranged LRR motifs was built using the SWISS-MODEL automated protein structure homology modelling server. The model showed a similar orientation and curvature of all LRRs 1 to 20, even though a different spacing between LRRs 13 and 14 was apparent (Figure 5A). Molecular dynamics simulations (300 ns) performed to verify the stability of the
ranges structurally and appears to function as a flexible hinge between the two
and LRRs 14–20). (D) Simulations (300 ns) performed to verify the stability of the generated model
served region. (B) Ranged LRR motifs built using the SWISS-MODEL server. (C)
In this study, we dissected the functional and structural organi-
Discussion
LRR 13 was observed to lose its starting secondary structure and
observed (Figure 5C). In this simulation, the variable region of
region of LRR 13, constituting a distinct and stable domain, was
generated model documented well-conserved
bly arranged domains. (A) Three-dimensional model of the tandemly ar-
structure of individual LRRs in their cons-
region. (C) The LRRs appears to be structured into two blocks (LRRs 1–13, and LRRs 14–20). (D) During the simulation, the variable region of LRR 13 re-
ranges structurally and appears to function as a flexible hinge between the two
generated model documented well-conserved β structures of in-
dividual LRRs in their conserved region (Figure 5B; Supplementary Material, Fig. S5). Remarkably, during the simu-
lation, rotation of LRRs 14 to 20, which were structured to form a single block with respect to LRRs 1 to 12 and the conserved region of LRR 13, constituting a distinct and stable domain, was observed (Figure 5C). In this simulation, the variable region of LRR 13 was observed to lose its starting secondary structure and to function as a flexible hinge between the two domains (Figure 5D). LRR 13 showed variability in the length of the β structure in the conserved LRR region, in line with their position at the boundary of the two domains (Supplementary Material, Fig. S5).
Overall, consistent with the experimental data, in silico struc-
tural and molecular dynamics data support the idea that LRRs 1-13 and LRRs 14-20 constitute two structurally independent domains that are connected by a flexible hinge constituted by the variable region of LRR 13. In this structural organization, we expect the presence of residues playing a major role in favouring the flexibility of the linker connecting the two LRR domains and plasticity of their relative orientation. Of note, LRR 13 lacks the leucine residue at the C-terminus of its variable region and the leucine residue at the N-terminus of the conserved region of LRR 14 is replaced by methionine, suggesting a possible structural relevance of the observed amino acid substitutions at these key positions.

Figure 5. SHOC2's LRR motifs constitute two structurally recognizable and tandemly arranged domains. (A) Three-dimensional model of the tandemly arranged LRR motifs built using the SWISS-MODEL server. (B) Molecular dynamics simulations (300 ns) performed to verify the stability of the generated model documenting a very well-conserved β structure of individual LRRs in their conserved region. (C) The LRRs appears to be structured into two blocks (LRRs 1–13, and LRRs 14–20). (D) During the simulation, the variable region of LRR 13 rearranges structurally and appears to function as a flexible hinge between the two domains.

generated model documented well-conserved β structures of individual LRRs in their conserved region (Figure 5B; Supplementary Material, Fig. S5). Remarkably, during the simulation, rotation of LRRs 14 to 20, which were structured to form a single block with respect to LRRs 1 to 12 and the conserved region of LRR 13, constituting a distinct and stable domain, was observed (Figure 5C). In this simulation, the variable region of LRR 13 was observed to lose its starting secondary structure and to function as a flexible hinge between the two domains (Figure 5D). LRR 13 showed variability in the length of the β structure in the conserved LRR region, in line with their position at the boundary of the two domains (Supplementary Material, Fig. S5).
Overall, consistent with the experimental data, in silico structural and molecular dynamics data support the idea that LRRs 1-13 and LRRs 14-20 constitute two structurally independent domains that are connected by a flexible hinge constituted by the variable region of LRR 13. In this structural organization, we expect the presence of residues playing a major role in favouring the flexibility of the linker connecting the two LRR domains and plasticity of their relative orientation. Of note, LRR 13 lacks the leucine residue at the C-terminus of its variable region and the leucine residue at the N-terminus of the conserved region of LRR 14 is replaced by methionine, suggesting a possible structural relevance of the observed amino acid substitutions at these key positions.

Discussion
In this study, we dissected the functional and structural organization of SHOC2 by identifying the domains required for constitutive plasma membrane targeting of the RASopathy-causing SHOC2S2G mutant and those controlling the nuclear import and export of the wild-type protein. We also determined the impact of subcellular mis-localization of the scaffold on ERK signalling and more precisely characterized the SHOC2’s module architecture of its tandemly arranged LRR motifs. Finally, we showed that SHOC2S2G dynamically associates with lipid rafts and translocates to cholesterol-independent domains following stimulation.

SHOC2 is a protein functioning as a positive modulator of the RAS-MAPK signalling cascade (1,2,36). While conflicting data support its ability to bind to multiple members of the RAS subfamily (1,4,6), it is largely accepted that SHOC2’s positive modulatory role on ERK activation is exerted by accelerating stable interaction between RAS and RAF1 (5,6). Consistent with these findings, siRNA-mediated depletion of SHOC2 has been shown to dramatically decrease the extent of stimulus-induced ERK activation (37), and ubiquitination and degradation of SHOC2 has recently been documented to represent an event negatively controlling RAF1 catalytic activation (38). Intriguingly, besides the established role of the scaffold in RAS-MAPK signalling, a still uncharacterized function of SHOC2 in the nucleus has been postulated (7). We excluded the presence of a functionally active NLS in the protein, and confocal microscopy analysis and cell fractioning experiments with cells expressing an opportune generated panel of SHOC2 mutants lacking the N-terminal KEKE motifs-rich region (residues 7–56) or a different subset of the tandemly arranged LRRs allowed us to identify the functional domain of the protein implicated in SHOC2’s nuclear-cytoplasmic shuttling. Specifically, we showed that the first 10 LRRs are required for SHOC2 import into the nucleus, while the N-terminal KEKE motif-rich region of the protein is necessary to achieve efficient nuclear export of the protein, which are in line with our previous observations (7), support a specific role of SHOC2 in the nucleus in response to EGF stimulation. Consistent with our finding, SHOC2 is predicted as a putative nuclear protein by PSORT II (http://psort.hgc.jp/form2.html), and is classified with similar confidence as having a nuclear localization or as a protein shuttling between the cytosol and nucleus by WoLF PSORT (nucl: 19, cyto_nucl: 18.5, cyto: 12) (39).

SHOC2’s positive function in RAS-MAPK signalling has been linked to its ability to bind to PP1C, favouring the translocation of this serine/threonine protein phosphatase to the membrane, where dephosphorylation of Ser259 of RAF1 is required to stabilize RAF1 binding to RAS and proper activation of the kinase (6). Consistent with this view, a virtually invariant amino acid substitution, p.Ser2Gly, promoting SHOC2 myristoylation, and multiple missense mutations affecting Ser259 (or adjacent residues) of RAF1 have been identified as the molecular causes of two clinically related RASopathies, and associated with variably upregulated MAPK signalling (7,40). In the former, myristoylation has been shown to result in constitutive mis-targeting of the scaffold to the plasma membrane; in the latter, loss of Ser259 has been documented to destabilize the autoinhibited RAF1 conformation mediated by 14-3-3 protein binding to pSer259, promoting stable binding of the kinase to GTP-bound RAS (41).
As in our previous observations, we failed to observe a substantial localization of wild-type SHOC2 at the plasma membrane, which is in apparent conflict with the direct role of this scaffold in mediating stable RAF1 interaction with RAS proteins. Notwithstanding this apparent incongruence, the present data confirm the activating role of the Ser2Gly substitution on SHOC2 as well as the dependence of its positive modulating role on
ERK activation and targeting of the protein to the plasma membrane. Our present and previous observations are consistent with a model in which SHOC2 translocation to the membrane in response to factor stimulation is transient and possibly required only as priming for RAF1 activation.

Previous work using red fluorescent protein-(RFP) tagged SHOC2 attempted to characterize the subcellular localization of SHOC2 and the role of the SHOC2<sup>52G</sup> mutant on ERK signalling (37). This group documented a diffuse distribution of the protein in both cytoplasm and nucleus basally, and its translocation to a subpopulation of late endosomes following EGF stimulation, which is in contrast with the present and our previous observations. Galperin and colleagues also reported data not supporting the activating role of the RASopathy-causing mutant on ERK signalling. While the use of a relatively large sized tag (i.e. RFP), might account for the remarkable diverse picture compared with the present data, particularly taking into account the relatively low molecular weight of SHOC2 and the relatively weak interactions characterizing SHOC2 (our unpublished observations), the contrasting data produced using different experimental tools and approaches do require further investigation.

In a subsequent work, the same group utilized an approach based on the generation of truncated RFP-tagged mutants to map the role of individual SHOC2’s structural elements on the subcellular localization of the scaffold (42). In this case, the different generated constructs do not allow a direct comparison of the gathered data with the present findings. It should be considered, however, that the high molecular weight and possible steric hindrance of the tag used for these analyses are expected to dramatically impact on subcellular localization and protein-protein interactions of the generated truncated mutants.

While N-terminal myristoylation is a modification largely used to anchor proteins to intracellular membranes, its binding energy is relatively weak. Stable binding of N-myristoylated proteins to intracellular membranes generally requires additional non-covalent interaction(s) with phospholipids of the cytoplasmic leaflet of the bilayer or other membrane-bound proteins. Similarly, the non-specific hydrophobic interaction mediated by N-myristoylation does not explain the constitutive targeting of SHOC2<sup>52G</sup> to the plasma membrane. SHOC2 does not possess positively charged regions potentially implicated in phospholipids binding nor cysteine residues that might provide additional acylation contributing to membrane binding stabilization. By evaluating the subcellular localization of a series of truncated SHOC2<sup>52G</sup> mutants by confocal microscopy and cell fractioning experiments, we provided evidence showing that the N-terminal LRR motives constituting the first LRR domain are also required for constitutive mis-targeting of myristoylated SHOC2<sup>52G</sup> to the plasma membrane. This finding indicates that this domain has a crucial role in mediating SHOC2’s protein-protein interactions mediating translocation of the protein to different intracellular compartments as well as its dynamic association with lipid rafts.

Consistent with the gathered experimental data, our in silico structural and molecular dynamics analyses support a previously unappreciated organization of SHOC2’s LRRs to constitute two structurally independent domains coupled by a flexible hinge. This structural organization provides a structural framework for the appreciated distinct role of the N- and C-terminal LRRs in mediating protein-protein interactions controlling SHOC2 intracellular localization and modulation of ERK signalling. A systematic analysis of the role of the different residues in the flexibility of the two domains is expected to identify the structure-dynamics determinants governing the hinge role of LRR.
GTG TGG C-3'. The second was obtained utilizing pcDNA6/V5-SHOC2(SG) construct as template and the following primer pair: C144G-Fw 5’-CCA GCA GAG GTG GGA GGT TTA GTA AAT CTC-3’ and C144G-Rv 5’-GAG ATT TAG TAA ACC TCC CAC CTC TGC TGG G-3’.

The SHOC2(AKEKE) and SHOC2(SG/SG) constructs were produced by PCR and cloning of the amplification products into the pcDNA6/V5-HisA expression vector using the following primer pairs: Nhel_AKEKE-Fw 5’-GCT AGC AGG ATT AGT GTG TTA GGA AAA GAA AAG GAC TCC AGT GCC CAA C-3’ and Xho1_SHO2_C2_Rv 5’-CTC GAG TCT GAG CAC CAT GCC AGG ATA TGG ACC-3’, and Nhel_AKEKE_Fw 5’-GCT AGC AGG ATT AGT GTG TTA GGA AAA GAA AAG GAC TCC AGT GCC CAA C-3’ and Xho1_SHO2_C2_Rv, respectively.

Plasmids for expression of SHOC2 mutants with partial deletions of the LRR region (SHOC2(ALRR2-5), SHOC2(ALRR6-10), SHOC2(ALRR11-14) and SHOC2(ALRR15-19) were generated by overlap extension PCR (43). Briefly, for each construct, two PCR products representing the flanking regions of the DNA sequence to be deleted were prepared using the following primer pairs: Nhel_AKEKE-Fw 5’-GCT AGC AGG ATT AGT GTG TTA GGA AAA GAA AAG GAC TCC AGT GCC CAA C-3’ and Xho1_SHO2_C2_Rv (the encompassing the deleted region); Nhel_AKEKE_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’, and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Fw (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region); Nhel_SHO2_C2_Fw, SHOC2(ALRR11-14)_Rv 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(ALRR6-10)_Fw 5’-CA ATC AAA GAG TTG TCA AAA CTC AG-3’, SHOC2_S2G-Rv (the encompassing the deleted region);

Plasmids coding SHOC2(SG) proteins with partial deletions of the LRR region (SHOC2(ALRR2-5), SHOC2(ALRR6-10), SHOC2(ALRR11-14) and SHOC2(ALRR15-19)) were generated by site-directed mutagenesis using pcDNA6/V5-SHO2(ALRR2-5), pcDNA6/V5-SHO2(ALRR6-10), pcDNA6/V5-SHO2(ALRR11-14) or pcDNA6/V5-SHO2(ALRR15-19) vector as template and the following primer pair: SHOC2(SG)-Fw 5’-CAG TCT TGG TCT TGG CAG G-3’, Xho1_SHO2_C2_Rv (the encompassing the deleted region); SHOC2(SG)_Rv, SHOC2(ALRR11-14)_Fw 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’ and SHOC2(SG)_Rv (the encompassing the deleted region); SHOC2(ALRR11-14)_Fw 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’, SHOC2(SG)_Rv (the encompassing the deleted region); SHOC2(ALRR11-14)_Fw 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’, SHOC2(SG)_Rv (the encompassing the deleted region); SHOC2(ALRR11-14)_Fw 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’, SHOC2(SG)_Rv (the encompassing the deleted region); SHOC2(ALRR11-14)_Fw 5’-CTC AAG AGA AAC CAC AAG ACT TG-3’, SHOC2(SG)_Rv (the encompassing the deleted region);

Cell homogenate and protein assay

Neuro2A cells, transfected with the various constructs for 24 h, serum starved overnight and stimulated with EGF for the indicated intervals, were lysed in radioimmunoprecipitation assay (RIPA) buffer, pH 8.0, containing 20 mM NaF, 1 mM Na3 VO4, and protease inhibitors. Lysates were kept on ice (30 min) and then centrifuged at 16,000 g (20 min, 4 °C). Supernatants were collected and their protein concentration was determined by bicinchoninic acid (BCA) assay (44), using bovine serum albumin (BSA) as a standard.

Western blotting

Cell lysates were resolved by 7.5% sodium dodecyl sulphate (SDS)-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). Blots were blocked with 5% non-fat milk powder in PBS containing 0.1% Tween-20 for 1 h and incubated with specific antibodies for 1 h. Primary and secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected by an enhanced chemiluminescence (ECL) detection kit, according to the manufacturer’s instructions. Densitometric analysis of protein bands was performed using Alpha View SA image software (Protein Simple, Santa Clara, CA, USA).

Cell fractionation

In nucleus-cytoplasm fractionation assays, cytoplasmic and nuclear fractions were obtained using the Nuclear Extract Kit. Briefly, transfected COS-1 cells were cultured in the presence of FBS or serum starved overnight and then stimulated with EGF or left untreated. Cells were washed with ice-cold PBS containing phosphatase inhibitors and removed from dish by gently scraping with cell lifter. The cell suspension was centrifuged at 150g (5 min, 4 °C) and the resulting pellet was resuspended in hypotonic buffer supplemented with 5% of detergent. After centrifugation (14,000g, 1 min, 4 °C), the cytoplasmic fraction was collected and the nuclear pellet was solubilized in complete lysis buffer [10% Dithiothreitol (DTT), 1% protease inhibitor cocktail, lysis buffer]. Identical ratios, representing the same percentage of each subcellular fraction, were subjected to SDS-PAGE followed by western blotting, in order to represent the cellular ratio of the SHOC2 distribution in each compartment. To ensure no cross-contamination between the fractions, the same membrane was probed with antibodies to β-Actin (cytoplasmic marker) or YY1 (nuclear marker).

In nucleus-cytoplasm-membrane fractionation assays, transfected COS-1 cells were washed twice with PBS and harvested using buffer A (20 mM Tris- HCl, pH 7.4, 2 mM EDTA, protease inhibitor cocktail). After sample centrifugation (2,000g, 10 min, 4 °C), the supernatant consisting of cytoplasm and membranes was collected and again centrifuged (30,000g, 1 h, 4 °C). The nuclear pellet was resuspended in buffer A containing 1% Triton X-100 and 60 mM octyl-β-D-glucopyranoside (buffer B), passed 10 times through a syringe needle and then centrifuged (16,000g, 30 min, 4 °C). Finally, the cytoplasmic fraction was recovered and the pellet consisting of membranes was solubilized in buffer B. Identical ratios, representing the same percentage of each subcellular fraction, were subjected to SDS-PAGE followed by western blotting, in order to represent the cellular ratio of the SHOC2 distribution in the different cellular compartments. To ensure no cross-contamination between the
fractions, the same membrane was probed with antibodies to β-Actin (cytoplasmic marker), YY1 (nuclear marker) or Na/K-ATPase (plasma membrane marker).

Dual-luciferase assay
ELK transcriptional activity was assessed using the Dual-luciferase reporter assay system. Briefly, NIH 3T3 cells were transfected with the appropriate SHOC2 construct, pFR-Luc reporter plasmid and pFA2-Etk1 fusion trans-activator vector. Renilla luciferase plasmid (pRLTk) was co-transfected as an internal transfection control. After 24 h, cells were serum starved overnight and stimulated with EGF for 6 h or left untreated. Lysates were centrifuged at 12,000 g (1 min, 4 °C) and supernatants were used for measurements of luciferase expression using a Microlite TLX1 luminometer (Dynatech Laboratories, Chantilly, VA).

Isolation of lipid raft-enriched membrane fractions
Lipid raft-enriched membrane fractions were isolated as previously described (45). Briefly, Neuro2A cells were transfected with different constructs for 24 h, starved 4 h, stimulated with EGF for 15 min. Cells were fixed with 3% paraformaldehyde for 30 min at 4 °C and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Cells were then incubated with a specific mouse monoclonal primary anti-phospho-tyrosine antibody. Samples were subjected to SDS-PAGE followed by western blotting or fluorography.

N-myristoylation analysis
COS-1 cells were transfected with SHOC2SGC, SHOC2SGC/ALKKE or SHOC2SGC/ALKKE ΔLRR2-5 construct for 24 h, washed twice with serum-free DMEM and incubated in DMEM with 2% FBS containing [3H]myristoyl (30 μCi/ml) for 5 h. Subsequently, the cells were lysed in ice-cold RIPA buffer supplemented with protease inhibitors, the lysates were centrifuged at 16,000 g (20 min, 4 °C) and the supernatants were used for immunoprecipitation with anti-V5 antibody. Samples were subjected to SDS-PAGE followed by western blotting or fluorography.

Confocal laser scanning microscopy
COS-1 cells (30 × 10^3) were seeded on glass coverslips, transfected with the various constructs for 24 h, serum starved for 16 h and stimulated with EGF for 15 min. Cells were fixed with 3% paraformaldehyde for 30 min at 4 °C and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. Cells were then incubated with a specific mouse monoclonal primary antibody (anti-V5) for 1 h at room temperature, rinsed twice with PBS and incubated with the secondary anti-mouse antibody conjugated with Alexa Fluor 594 for 1 h at room temperature. Alexa Fluor 488 phalloidin dye was used to stain the cortical actin-associated with the plasma membrane. Finally, glass coverslips were mounted on microscope slides using the Vectorshield antifade medium containing DAPI and analysed by a Leica TCS SP2 AOBS apparatus, utilizing excitation spectral laser lines at 405, 488 and 594 nm, tuned with an acousto-optical tunable filter. Image acquisition and processing were conducted using the Leica Confocal Software 2.3 (Leica Lasertechnik, Heidelberg, Germany) and Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA). Signals from different fluorescent probes were taken in sequential scanning mode.

Model generation and simulation protocol
The 3D structure of the native SHOC2 protein was designed using the SWISS-MODEL automated protein structure homology modelling server (http://swissmodel.expasy.org) on template PDB Id: 4MN8 with high resolution 3.06 Å. In order to increase the protein model confidence, we carried out a molecular dynamics simulations with the Gromacs 4.5.6 package (46) and the gromos54a7.ff force field (47). The starting structures were embedded in a dodecahedron box, extending up to 12 Å from the solute, and immersed in SPC water molecules (48). Counter ions were added to neutralize the overall charge with the genion gromacs tool. After energy minimizations, the systems were slowly relaxed for 5 ns by applying positional restraints of 1000 kJ mol\(^{-1}\) nm\(^{-2}\) to the protein atoms. Then unrestrained MD simulations were carried out for a length of 300 ns with a time step of 2 fs. V-rescale temperature coupling was employed to keep the temperature constant at 300 K (49). The Particle-Mesh Ewald method was used for the treatment of the long-range electrostatic interactions (50). LRR motif annotation was performed according to (3) and (51).

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest Statement. None declared.

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