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Review

SOS GEFs in health and disease

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ABSTRACT

SOS1 and SOS2 are the most universal and widely expressed family of guanine exchange factors (GEFs) capable or activating RAS or RAC1 proteins in metazoan cells. SOS proteins contain a sequence of modular domains that are responsible for different intramolecular and intermolecular interactions modulating mechanisms of self-inhibition, allosteric activation and intracellular homeostasis. Despite their homology, analyses of SOS1/2-KO mice demonstrate functional prevalence of SOS1 over SOS2 in cellular processes including proliferation, migration, inflammation or maintenance of intracellular redox homeostasis, although some functional redundancy cannot be excluded, particularly at the organismal level. Specific SOS1 gain-of-function mutations have been identified in inherited RASopathies and various sporadic human cancers. SOS1 depletion reduces tumorigenesis mediated by RAS or RAC1 in mouse models and is associated with increased intracellular oxidative stress and mitochondrial dysfunction. Since WT RAS is essential for development of RAS-mutant tumors, the SOS GEFs may be considered as relevant biomarkers or therapy targets in RAS-dependent cancers. Inhibitors blocking SOS expression, intrinsic GEF activity, or productive SOS protein-protein interactions with cellular regulators and/or RAS/RAC targets have been recently developed and shown preclinical and clinical effectiveness blocking aberrant RAS signaling in RAS-driven and RTK-driven tumors.

1. Introduction

RAS proteins are essential signal transduction regulators controlling signaling pathways that regulate a large variety of biological processes including cell proliferation, differentiation, migration and survival in different tissues and cell types, or at different developmental stages [1,2]. These small GTPases are continuously cycling between inactive (RAS•GDP) and active (RAS•GTP) conformations in a process modulated by negative (GTPase Activating Proteins, RASGAPs) and positive (Guanine nucleotide Exchange Factors, RASGEFs) regulators. Among the main mammalian RASGEF families (SOS, GRF and GRP), SOS1 and SOS2 are the most widely expressed and functionally relevant GEFs regarding to RAS and RAC activation by upstream cellular signals [1,3–6].

The *Son of sevenless* (d*Sos*) gene was first discovered in *Drosophila melanogaster* as a specialized RAS activator acting downstream of the *Sevenless* receptor tyrosine kinase (RTK) in signaling pathways controlling ommatidia development in the fly's compound eye and various SOS homologues were subsequently found in other species, from *Caenorhabditis elegans* up to mammals [7–12].

The two mammalian orthologues of SOS (*SOS1* and *SOS2*) code for highly homologous proteins able to stimulate GTP/GDP exchange on cellular RAS and RAC proteins in the context of multiple different signaling pathways initiated by a great variety of different cellular surface RTKs [1,5,6]. They display a modular structure featuring conserved distribution of specific, functional domains along their N-, middle-, and C-terminal regions [6]. Different intramolecular domain-domain interactions as well as direct interactions between distinct cellular proteins or lipid molecules and individual SOS modular domains mediate the regulatory mechanisms controlling the self-inhibition and the allosteric activation of SOS GEF activity as well as the intracellular homeostasis and stability of the SOS proteins [13–16].

RAS activation by point mutations is known to lead to a variety of pathological alterations including multiple tumor types (sporadic mutations) and various developmental syndromes (inherited mutations) [17–19]. On the other hand, it is also conceivable that other potential, alternative activation mechanisms, including hyperactivation of RASGEFs or dysregulation of other components of RAS-mediated signaling, may also result in physiological or pathological alterations.

The similar protein structures and expression patterns of SOS1 and SOS2 made it initially difficult to ascertain their specific functional properties but most studies suggest a dominant functional role of SOS1 over SOS2 in various physiological and pathological contexts. Initial analyses of constitutive knock-out (KO) mouse strains showed tht SOS1

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constitutive-null animals die during mid-gestation whereas adult SOS2-KO mice are viable and fertile [20–22]. The later use of conditional SOS1-null mutants made it possible to bypass the lethality of homozygous SOS1-null mutations and to analyze the functional specificity and/or redundancy of SOS1 and SOS2 in adults under various physiological or pathological conditions [23,24]. Interestingly, SOS1/2-DKO animals die precociously whereas single SOS1-KO or SOS2-KO mice are viable, suggesting functional redundancy between SOS1 and SOS2 for lymphopoiesis, organismal homeostasis and survival [23]. Furthermore, comparisons between primary mouse embryonic fibroblasts (MEFs) extracted from SOS1-KO and SOS2-KO mice have documented the functional prevalence of SOS1 over SOS2 in control of critical cellular physiological processes including proliferation, migration, inflammation and maintenance of intracellular redox homeostasis [25–27]. Moreover, the use of SOS1/2-DKO mice has demonstrated that SOS1 plays a critical role in BCR-ABL-induced leukemogenesis as well as in homeostasis and chemically-induced carcinogenesis of the skin [26,28,29].

A number of recent studies have also increasingly pointed to the functional implication of SOS GEFs (particularly SOS1) in human tumors and other pathologies. Thus, an important number of gain-of-function genetic alterations or mutations in different SOS1 (and rarely SOS2) domains have been identified in inherited RASopathies such as Noonan syndrome (NS) or hereditary gingival fibromatosis (HGF-1) [30,31], as well as in various sporadic human cancers including, among others, endometrial tumors (UCEC) and lung adenocarcinomas (LUAD) [32–37]. These observations, together with the fact that SOS depletion in KO cell lines and mouse models reduces specific tumorigenic processes mediated by RAS [26,38] or RAC [28,29], and is also associated with increased intracellular oxidative stress and mitochondrial dysfunction [25], support the hypothesis that the SOS1/2 GEFs may constitute relevant biomarkers and/or therapy targets in KRAS-driven (and also EGFR-driven) tumors. This view is reinforced by the seminal demonstration that SOS-mediated activation of WT RAS is critical for the development of RAS-mutant tumors [39–41]. Recent research efforts from multiple academic and industry platforms have focused on developing specific inhibitors capable of blocking either SOS1/2 expression, GEF activity or productive interactions with RAS/RAC targets and other cellular regulators. Fortunately, a number of these SOS inhibitors have proven efficacious in various cell and xenograft models of RAS-driven and RTK-driven tumors, and some of them have even recently reached the clinic [42–52].

In this review, we summarize our current understanding of the structure and regulation of SOS genes and proteins and we also describe various specific functional roles played by the SOS1/2 GEFs in different cells and tissues under physiological, healthy conditions. We also review the variety of genetic alterations recently found in *SOS1* (and rarely *SOS2*) in association with inherited developmental syndromes and sporadic human cancers. Finally, we focus on the potential usefulness of SOS GEFs as markers or therapy targets for RAS-dependent tumors and the recent development of specific SOS inhibitors that may be clinically useful for the management of RAS-dependent and/or RTK-dependent tumors featuring aberrant RTK-SOS-RAS-ERK signaling.

2. The SOS gene family

About three decades ago, studies on the development of the compound eye in *D. melanogaster* led to the discovery of the *Son-of-Sevenless* gene (*dSos*) [7,11,12] and various dSos homologues were later reported in more complex biological systems including the nematode *C. elegans* [9], zebrafish (*Danio rerio*) [53,54], or mouse and human mammalian cells [5,6,8,10].

2.1. Genomic structure and organization

Whereas the *D. melanogaster* and *C. elegans* genomes harbor a single *Sos* gene (7 and 19 exons, respectively), two homologs (*SOS1* and *SOS2*), located in different chromosomes exist in the genomes of zebrafish, mice and humans (https://flybase.org/; https://www.wormbase.org/; https://zfin.org/; www.ensembl.org/). Using the human *SOS* genes as reference for comparison, the ENSEMBL database lists 264 orthologues and 26 paralogues for the *hSOS1* gene and 188 orthologues and 26 paralogues for the *hSOS2* gene.

The zebrafish homolog of *SOS1* (Chr11 position: 45,436,703 - 45,463,766; 15 exons) is located in chromosome 11 whereas its *SOS2* homolog (Chr13 position: 36,638,955 -36,663,358; 23 exons) maps to chromosome 13. On the other hand, the *mSOS1* mouse gene is positioned on chromosome 17 (Chr17 position: 80,393,751 - 80,480,452; 22 exons) and *mSOS2* on chromosome 12 (Chr12 position: 69,583,762 – 69,681,852; 23 exons), whereas the *hSOS1* and *hSOS2* human SOS homologues are located, respectively, on chromosome 2 (Chr2 position: 38,981,396 – 39,124,345; 23 exons) and chromosome 14 (Chr14 position: 50,117,130 – 50,231,578; 23 exons). The overall span of the genomic regions occupied by the *SOS1* and *SOS2* loci varies widely in different organisms but the intron/exon distribution of all these genes is highly conserved, with various specific exons coding for the conserved protein modules that conform the overall, sequential protein domain structure of the SOS1/2 GEFs [5,6] (Fig. 1).

2.2. Non-coding regulatory elements

Focusing only on the non-coding regulatory regions of mammalian *SOS* genes, the Ensembl Regulatory Build database (https://www.ensembl.org/info/genome/funcgen/regulatory_build.html) indicates that the *hSOS1* gene contains one promoter region and several promoter-flanking regions located between exon 1 and exon 2 and between exon 8 and exon 22. In addition, 9 enhancer sites, located between exon 2 and exon 11, as well as 9 CTCF (CCCTC-Binding factor) binding sites distributed from exon 1 to exon 17 are suggested (https://www.ensembl.org/Homo_sapiens/Location/View?db=core;g=ENSG00000115904;r=2:38981396-39124345). Similarly, *mSOS1* displays one promoter region, 6 promoter-flanking regions distributed from exon 1 to exon 11, and 8 enhancer sites mainly located between exon 1 and exon 2. In addition, 7 CTCF sites located between exons 1 and 6 are proposed for *mSOS1* gene (https://www.ensembl.org/Mus_musculus/Location/View?db=core;g=ENSMUSG00000024241;r=17:80393751-80480453) (Fig. 1).

The *hSOS2* gene (chromosome 14) shows some distinctive features in comparison to the *hSOS1* promoter. Two promoters are defined for this gene in the database, one located upstream of the transcription starting site and another one at a 3' site. 4 promoter-flank sites are also described at locations between exon 1 and exon 2. In addition, 4 enhancers (located between exons 13 and 19) and 5 CTCF sites (between exons 1 and 2), are also defined by Ensembl Regulatory Build (https://www.ensembl.org/Homo_sapiens/Location/View?db=core;g=ENSG00000100485;r=14:50117130-50231578). Finally, *mSOS2* gene shows one promotor and four promoter-flank regions located between exon 1 and exon 5 (https://www.ensembl.org/Mus_musculus/Location/View?db=core;g=ENSMUSG00000034801;r=12:69583762-69681852). In addition, one enhancer, located near exon 16, and 5 CTCF sites are also identified (Fig. 1).

In silico analysis using the Transfac database (http://gene-regulation.com/pub/databases.html#transfac) predicts specific binding sites for a variety of transcription factors within defined regions of the promoters of the *hSOS1* and *hSOS2* genes. However, the only direct experimental evidence obtained so far corresponds to the demonstration (by means of ChIP-seq) of the specific binding of the AhR receptor to a defined region of the *hSOS1* promoter [55]. On the other hand, no experimental evidence is yet available in the literature for any transcription factor binding to the *hSOS2* promoter.

The *hSOS1* and *mSOS1* genes contain highly conserved 5'-untranslated regions (5'-UTR) (87 and 46-nt long, respectively, showing

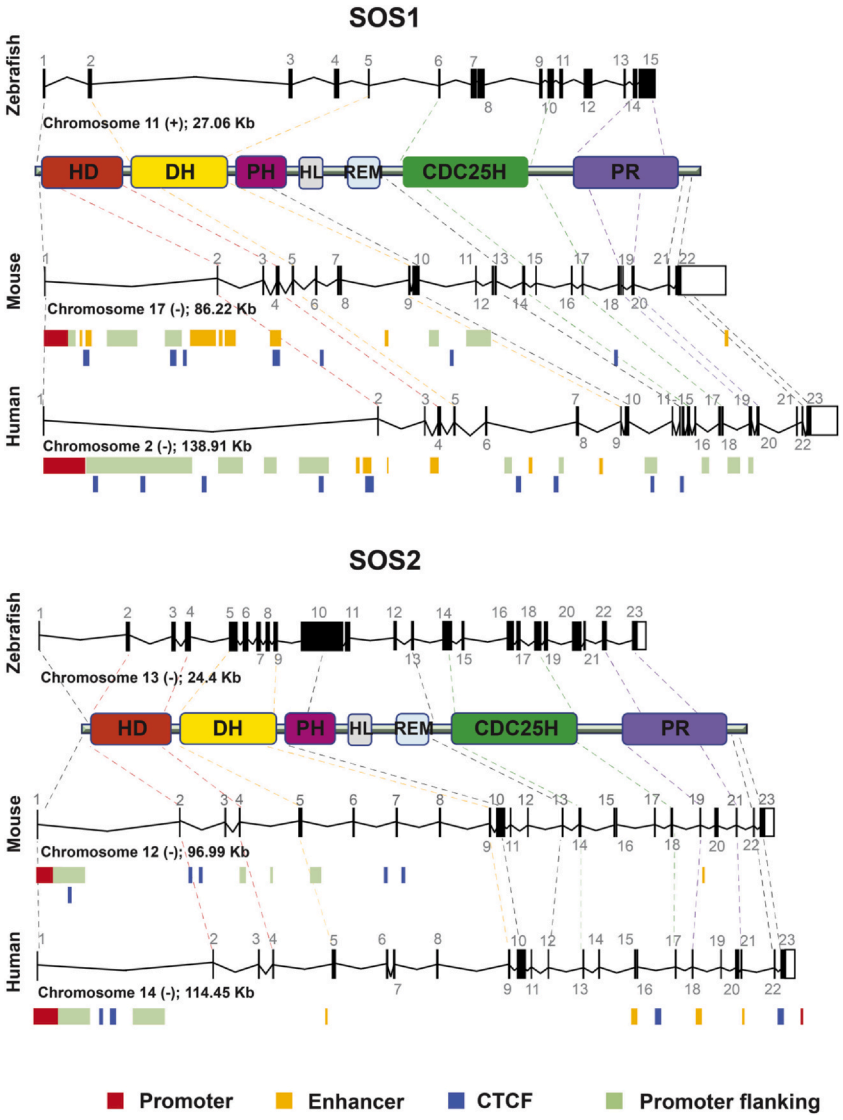

Fig. 1. Genomic structure, organization, and intron/exon distribution of *SOS1* and *SOS2* genes in vertebrate species. Genomic organization of *SOS1* (upper panel) and *SOS2* (lower panel) loci in vertebrate species including zebrafish, mouse and human as indicated. Figure assembled using current data from the Ensembl genome browser database (http://www.ensembl.org/index.html). Chromosomal location, chromosome strand (+ or −) used for transcription, and size (Kb) of the genomic stretches containing the *SOS1* and *SOS2* loci are also indicated for each species. Intron/exon distribution and correspondence of the coding exons (solid vertical boxes) to the conserved modular domains (color coded) of full-length SOS1 and SOS2 proteins is indicated by similarly colored dotted lines in each case. Non-coding regulatory regions in the mammalian *SOS* genes, including 3'-UTR (empty boxes), promoters (red horizontal box), promoter-flanking regions (light green), enhancers (dark yellow) and CTCF binding sites (blue) are also indicated by the corresponding, horizontal colored boxes.

90.2% identity in the first 46 nt sequences). *hSOS1* and *mSOS1* also possess 3'-UTR of 4428 and 4430 nt long, respectively, that share 71.9% identity. On the other hand, the *hSOS2* and *mSOS2* loci possess similar 5'-UTR regions (295 and 251-nt long, respectively, with 74% nt sequence homology in their 251 initial residues) as well as 3'-UTR of 1214 and 1357 nt respectively, sharing 65.9% nucleotide identity in their initial 1214 residues and overall 66.4% similarity if the complete UTR regions are compared. Proposed mRNA secondary structures (likely to participate in translational regulation of the *SOS1* and *SOS2* genes in mice and humans) generated for these UTR sequences by the RNAfold program from the Vienna RNA package (https://www.tbi.univie.ac.at/RNA/) are shown in Supplementary Fig. 1. The significant similarities shown by the primary and secondary structures of the UTR regions of the mouse and human *SOS1* and *SOS2* genes suggest also significant similarities in the mechanisms of transcriptional regulation played by these mRNA sequences in mammalian *SOS* genes.

2.3. SOS gene transcription

Well defined, full-length mRNA transcripts for the *SOS1* and *SOS2* genes have been identified for each of these genes in vertebrate species from fish to mammals (Fig. 1). In addition, the plethora of new sequencing data from many different human cell types and tumors that has been recently deposited in different databases supports the existence of a large number of potential, alternative transcripts or isoforms for both SOS1 and SOS2. Updated summaries of transcript data are available in the Ensembl portal for *hSOS1* (https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000115904;r=2:38981549-39124345) and *hSOS2* (https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000100485;r=14:50117130-50231578).

Computational predictions of alternative splicing events are also updated in the Alternative Splicing Gallery portal (https://brcwebportal.cos.ncsu.edu/asg/index.php?lookupType=match&lookupValue=ENSG00000115904) and for *hSOS2* (https://brcwebportal.cos.ncsu.edu/asg/index.php?lookupType=match&lookupValue=ENSG00000100485). However, direct experimental evidence supporting the real existence of many of the predicted, alternatively spliced mRNA species (as well as corresponding, potentially altered protein products) or non-coding RNAs, is scarce or nonexistent in most cases and, therefore, a comprehensive analysis in this regard is certainly warranted at this time. Our laboratory has described the identification in some human tissues (but not in corresponding mouse tissues) of two hSOS1 isoforms (Isf I and Isf II, differing only by the presence in Isf II of an extra 15-amino acid sequence located within the C-terminal proline-rich motif) that display very different biochemical and biological potencies [56]. In addition, the immunoblot detection (in addition to the full length SOS1 protein band) of a number of

smaller molecular weight bands is also suggestive of the existence of alternatively spliced forms of SOS1 in different tissues or developmental stages [57].

2.4. Genetic polymorphisms

The NCBI database for single nucleotide polymorphisms (SNPs) lists thousands of genomic polymorphisms (including del, delins, ins, mnv and snv) associated to *hSOS1* (https://www.ncbi.nlm.nih.gov/snp/?term=SOS1) and *hSOS2* (https://www.ncbi.nlm.nih.gov/snp/?term=SOS2) that may give rise to multiple structural or functional consequences (intron or exon variants, etc) depending on their location on coding or non-coding regions and are classified based on their clinical consequences as benign, likely-benign, likely-pathogenic, pathogenic, or of uncertain significance. Interestingly, only 44 pathogenic or likely pathogenic SNPs in *hSOS1*, mainly associated to RASopathies like NS and HGF-1 or sporadic tumors like lung adenocarcinoma, uterine corpus endometrial carcinoma or glioblastoma multiforme and 4 SNPs in *hSOS2* (associated with NS) are identified in this database.

2.5. Physiological expression patterns

SOS1 and SOS2 appear to be ubiquitously expressed, as the presence of specific RNAs or proteins for those genes is detectable in practically all human cells, organs and tissues tested. However, the actual levels of mRNA and proteins for *hSOS1* and *hSOS2* differ markedly depending on the specific organ or tissue analyzed.

Based on transcriptomic analyses across all major organs and tissue types in the human body, the Consensus dataset of the Human Protein Atlas database indicates that the highest mRNA expression levels reported for *hSOS1* (https://www.proteinatlas.org/ENSG00000115904-SOS1/summary/rna) and *hSOS2* (https://www.proteinatlas.org/ENSG00000100485-SOS2/summary/rna) are detected in the parathyroid gland, and variable levels of quantitatively significant mRNA expression levels are also detected in all other organs and solid tissues, with hematological cells and tissues showing the lowest relative levels of RNA expression for these two RASGEFs. Focusing only on blood cell types, these consensus transcriptional profile analyses show that the relative *hSOS1* mRNA expression levels are highest in natural killer (NK) and CD4/8-positive cells whereas this expression is almost absent in basophils (https://www.proteinatlas.org/ENSG00000115904-SOS1/blood). In contrast, *hSOS2* mRNA is highly expressed in both basophils and NK cells (https://www.proteinatlas.org/ENSG00000100485-SOS2/blood).

Interestingly, recent, highly sensitive, single cell transcriptome data from different mouse organs and tissues (Tabula Muris database, https://tabula-muris.ds.czbiohub.org/) or from specific mouse and human lung cell types (https://research.cchmc.org/pbge/lunggens/genequery_dp.html?spe=HU&tps=pnd1&geneid=SOS1; https://research.cchmc.org/pbge/lunggens/genequery_dp.html?spe=HU&tps=pnd1&geneid=SOS2) identify endothelial cells as the specific cell type expressing the highest level of *hSOS1* mRNA.

The datasets for SOS1 and SOS2 protein expression levels (based on immunological detection methods are necessarily less quantitatively accurate than the corresponding mRNA datasets for the same tissues due to background signals and/or the partial specificity of some of the antibody reagents but still roughly similar profiles of protein expression are observed in most cases. Interestingly, in contrast to the mRNA profile datasets, the relative levels of hSOS1 and hSOS2 proteins detected in blood cells and tissues are in the same quantitative range than those found in solid tissues (https://www.proteinatlas.org/ENSG00000115904-SOS1/tissue; https://www.proteinatlas.org/ENSG00000100485-SOS2/tissue) or cells (https://www.proteinatlas.org/ENSG00000100485-SOS1/cell; https://www.proteinatlas.org/ENSG00000100485-SOS2/cell). Interestingly, an elegant quantitative proteomic analysis of the core components of the EGFR-MAPK pathway across different cell types has recently revealed that the absolute abundance of SOS1 and SOS2 proteins (2000-5000 copies per cell) is far lower than that of most other core proteins (50000-70000 copies per cell) in the EGFR-MAPK pathway, suggesting that the low-abundance of SOS GEFs may serve as a regulatory bottleneck in this pathway [58].

2.6. Expression in pathological contexts

The alterations of RNA and protein levels of SOS1 and SOS2 that are expressed in a variety of human tumors and cell lines are updated and quantitated in a timely fashion in the Protein Atlas database (https://www.proteinatlas.org/ENSG00000115904-SOS1/pathology; https://www.proteinatlas.org/ENSG00000100485-SOS2/pathology). An overview of the normalized, baseline RNA expression measurements (RNA-seq data and NX normalized units) carried out in a wide collection of tumors affecting different organs shows variable but relatively similar ranges of *SOS1* mRNA levels in most cell lines analyzed, with some lines of myeloid origin showing the highest relative levels of expression (https://www.proteinatlas.org/ENSG00000115904-SOS1/cell#rna). Likewise, roughly similar levels of *SOS2* transcripts are also quantitated in the same cell line collection, with the highest levels detected in some lines of lymphoid origin (https://www.proteinatlas.org/ENSG00000100485-SOS2/cell#human). On the other hand, proteomics analysis of a large variety of tumor cell lines detects the highest hSOS1 protein expression in CoCM-1 cells (https://www.proteomicsdb.org/proteomicsdb/#protein/proteinDetails/58554/expression) whereas CAL-27 oral squamous carcinoma cells are the ones exhibiting highest hSOS2 protein expression (https://www.proteomicsdb.org/proteomicsdb/#human/proteinDetails/Q07890/expression).

The KERIS database, which collects data about the altered transcriptional profiles of specific genes among different inflammatory diseases and conditions (http://igenomed.org/immune/index) documents that *hSOS1* expression is significantly upregulated in whole blood cell extracts of pediatric patients with acute community-acquired *Staphylococcus aureus* infection [59] and in patients with Acute Respiratory Distress Syndrome (ARDS)/Acute Lung Injury (ALI) and Sepsis; in contrast, the *hSOS1* mRNA levels are very significantly downregulated in patients who suffered severe burns or physical trauma (http://www.igenomed.org/immune/gene?search=SOS1. Interestingly, the database shows a very different pattern of altered expression for *hSOS2* under similar pathological conditions. In contrast to *hSOS1*, the expression of the *hSOS2* gene shows measurable upregulation following *S. aureus* infection, burn, trauma and sepsis (http://www.igenomed.org/immune/gene?search=SOS2), but very strong reduction in patients with ARDS/ALI http://www.igenomed.org/immune/gene?search=SOS2).

Regarding other pathological conditions, transcriptomic analysis from whole blood samples has detected significant downregulation of *hSOS1* gene expression upon acute ethanol exposure [60] and, curiously, a recent report has also identified *hSOS1* as a significantly enriched component of a hub of protein products from the RAS-ERK pathway that are linked to development of human inguinal hernia [61].

2.7. miR-mediated regulation of expression

Post-transcriptional regulation of the expression of *SOS1* and *SOS2*, mediated by a great variety of specific miRNAs has also been reported under physiological and/or pathological conditions in many different cell types (Supplementary Table 1). As detailed in the miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/search.php#target) or the DIANA-TarBase v8 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex/), the evidence supporting the regulatory role of many different miRNAs on the expression of *SOS1* (http://mirtarbase.mbc.nctu.edu.tw/php/search.php?org=hsa&kw=SOS1&opt=target; (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex&miRNAs%5B

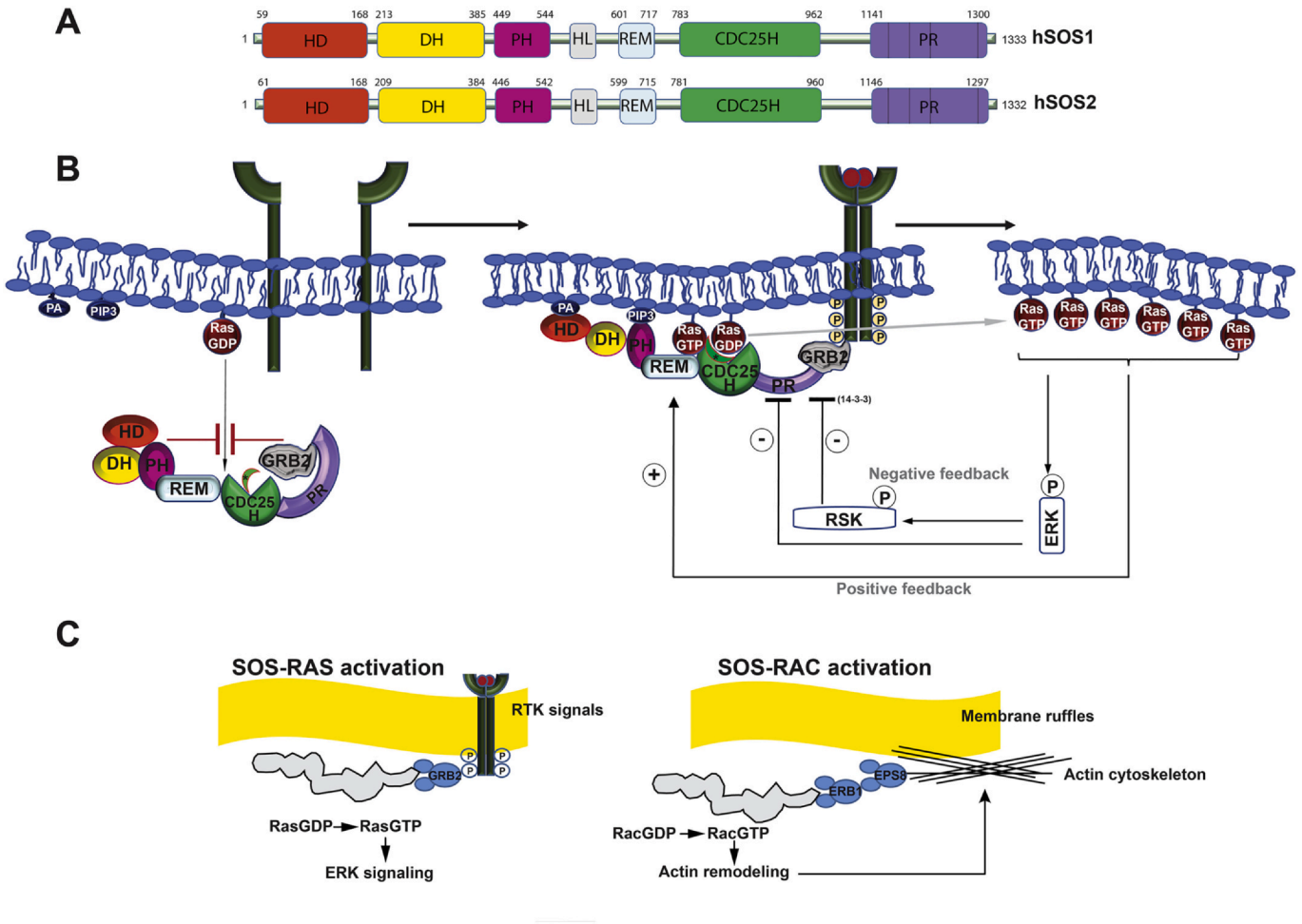

Fig. 2. Structure, conformation and regulation of SOS proteins.
(A) Primary structure of human SOS1 and SOS2 proteins. HD: Histone-like Domain; DH: Dbl Homology; PH: Pleckstrin Homology; HL: Helical Linker; REM: Ras Exchange Motif containing the allosteric site, CDC25H: homologous to Cell Division Cycle; PR: Proline-Rich, containing several SH3-binding motifs (vertical lines). (B) In resting conditions, the N-terminal DH-PH tandem and the C-terminal PR region mediate independent mechanisms maintaining SOS in an auto-inhibited state. Self-inhibition is relieved through recruitment of SOS proteins to the plasma membrane facilitated by the interaction of the HD and PH domains with membrane phospholipids and the interaction of the C-terminal PR region with SH3 domains of adaptor proteins like GRB2 that are also complexed through SH2 domains to pTyr residues of agonist-stimulated RTKs. Sequential, coordinated action of the REM allosteric site (activated by RAS•GTP) and the CDC25H catalytic site (reorienting its helical hairpin, represented here by a red crescent shape, to facilitate GDP/GTP exchange) creates a positive feedback loop of SOS activation. RAS•GTP accumulation at the membrane is also responsible for activation of downstream RAS signaling and triggering of a negative feedback regulatory loop blocking the association of SOS with GRB2 and inhibiting SOS function through ERK and RSK2 action. (C) The intermolecular interaction of the SOS PR domain with adaptor proteins such as GRB2 and E3B1 determines their recruitment to the specific subcellular locations where activated SOS GEF proteins meet and activate their specific RAS or RAC targets. GRB2:SOS interaction leads to RAS activation in the inner side of the membrane. E3B1-EPS8-SOS interaction leads to activation of RAC1 molecules in actin filaments of membrane ruffles.

%5D=&genes%5B%5D=SOS1&sources%5B%5D=1&sources%5B%5D=7&sources%5B%5D=9&publication_year=&prediction_score=&sort_field=&sort_type=&query=) or *SOS2* (http://mirtarbase.mbc.nctu.edu.tw/php/search.php?org=hsa&kw=SOS2&opt=target; http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex&miRNAs%5B%5D=&genes%5B%5D=SOS2&sources%5B%5D=1&sources%5B%5D=7&sources%5B%5D=9&publication_year=&prediction_score=&sort_field=&sort_type=&query=) range from simple theoretical predictions of interaction between different miRNAs and defined regions, mostly belonging to the 3'-UTR, of the *SOS1* and *SOS2* genes, to direct experimental reports showing that increased expression of many different miRNAs correlates with reduced *SOS1* or *SOS2* expression in a large variety of normal and/or tumoral cell types and conditions.

A great variety of different miRNAs have so far been identified that are able to modulate either *SOS1* or *SOS2* mRNA expression in different, normal or tumoral cell types (Supplementary Table 1). The bulk of experimental evidence identify specific sequences within the 3'-UTR of the *hSOS1/2* genes as the targets recognized by the different binding miRNAs identified, although, at least in theory, some other regions of the *SOS1* and *SOS2* genes may also be recognized for binding by specific miRNAs. Packing of the miRNAs into exosomes may be relevant for their SOS1/2 regulatory functions as indicated by a report showing that macrophage-derived *miR-155*-containing exosomes suppress fibroblast proliferation during cardiac injury through its interaction with *hSOS1* [62].

Although most published studies on miRNAs targeting *SOS1/2* expression have focused on their inhibitory effects on different tumor-derived cell lines (Supplementary Table 1), miR-mediated down-regulation of SOS1 expression is also functionally relevant in non-tumoral contexts such as the reduction of fibroblast proliferation upon cardiac injury [62], the reduction of fibrogenic activity of human gingival fibroblasts [63], or the induction of cytoxicity by silver nanoparticles in human dermal fibroblasts [64].

Despite the high homology existing between the 3'-UTR sequences of *SOS1* and *SOS2*, it is relevant to notice that the sets of miRNAs reported to bind to either gene are very different among themselves, suggesting a high degree of specificity for the mechanisms of miRNA-mediated regulation of the expression of the *SOS1* and *SOS2* genes. Indeed, only *miR-148a* has been reported to target both the *SOS1* and *SOS2* genes, although binding to different, non-homologous regions of the 3'-UTR of the two genes [65] (Supplementary Table 1).

2.8. Drug-mediated Regulation of Expression

A wide array of natural compounds or drugs previously known to exhibit antitumor activity have also been recently reported to alter the expression levels, and consequently the intracellular biological activity, of the SOS1 and/or SOS2 RASGEFs. Most of these compounds cause reduced expression levels of SOS1 protein in a variety of tumor cell types, although in some cases they are also reported to inhibit SOS2 protein expression (Supplementary Table 2). Of note, carcinogenic environmental pollutants such as 2,3,7,8-tetrachlorodibenzodioxin show the opposite effect, triggering increased expression of SOS1 in HepG2 cells [55].

3. SOS Protein structure and regulation

Analyses of the primary amino acid sequences [1,5,6,13,66] and the three-dimensional (3D) structures available for SOS1 and SOS2 proteins in various databases including the Protein Data Bank (https://www.rcsb.org) have provided the basis for our current understanding of the structural and conformational changes involved in the regulation of the cellular functions of these SOS proteins [5,13–16].

3.1. Modular domain structure

The primary structure of the SOS1/2 proteins displays a sequential, linearly organized modular structure featuring conserved distribution of specific functional domains containing protein motifs that possess specific biochemical properties and binding affinities for various intracellular proteins or lipids. The overall structure of SOS proteins can be divided into three distinct tiers corresponding to their C-terminal, central and N-terminal regions, and the regulation of the GEF activity of these proteins originates from the collective operations and interactions mediated by the modular domains within those regions [13–16,67] (Fig. 2A).

In the native cytosolic state the guanine nucleotide exchange activity of SOS is auto-inhibited through independent blockade mechanisms mediated by the N-terminal and the C-terminal regions, and its GEF activity only becomes activated upon membrane recruitment [68]. Different intra-molecular domain-domain interactions, as well as direct inter-molecular interactions between distinct cellular proteins or lipid molecules and various individual SOS modular domains mediate the multilayer regulatory mechanisms that control the processes of self-inhibition and the release of the blockade of the catalytic activity of SOS, as well as the intracellular homeostasis and stability of these key RAS activator proteins [13,15] (Fig. 2B).

Basically, the N-terminal region is responsible for maintenance and release of auto-inhibitory mechanisms as well as subcellular membrane location of the SOS proteins. The central region contains the domains responsible for allosteric modulation and catalytic activity functions controlling the activation (GTP loading) of RAS proteins. The C-terminal domain contains several proline-rich (PR) motifs responsible for various subcellular protein-protein interactions (PPI) that are critical for fine regulation of the processes leading to intracellular activation of RAS or RAC proteins, and subsequent downstream signaling. The PR domain also exerts an auto-inhibitory role over the central catalytic module under native, unstimulated cellular conditions (Fig. 2B).

3.2. The Amino-terminal Region of SOS

The initial N-terminal stretch of ~550 amino-acids in SOS proteins contains three well-defined structural domains: the Histone-like Domain (HD), the Dbl Homology domain (DH), the Pleckstrin Homology domain (PH) and the Helical Linker (HL) (Fig. 2A). Together, these 3 domains act cooperatively modulating SOS GEF auto-inhibition as well as the firm attachment of full-length SOS proteins to the inner side of plasma membrane and the subsequent release of their initial, native auto-inhibition.

The HD domain (~110 aa´) contains two tandem folds with homology to histone H2A [69] and plays the dual (negative) regulatory role of directly participating in the occlusion of the allosteric site in the central region, and also stabilizing the basal inhibitory conformation of the DH-PH tandem module (~350 aa´ located immediately before the catalytic module) that prevents SOS GEF activation [70] (Fig. 2B). The HD has also been recently shown to interact with the CSN3 subunit of the COP9 signalosome [71] which functions in the ubiquitin-proteasome pathway [72] suggesting a role of this domain as regulator of the stabilization and intracellular homeostasis of SOS1 protein.

Consistent with the recognized roles of the DH domains typically found in other GEFs as activators of proteins the RAC/RHO/CDC42 family [73], the SOS DH domain has also been implicated in coupling RAS activation to the activation of RAC, although the exact mechanistic details still remain unclear [13,66,74].

Both the HD and the PH domains have been shown to bind to specific lipids in membranes, an essential step in the release of SOS GEF autoinhibition [75]. The HD has the ability to interact with negatively charged membrane phospholipids through a conserved region (similar to the H2A DNA-binding surface) possessing positive electrostatic potential [76]. On the other hand, the PH domain (Fig. 2) binds to phosphoinositol phosphates with higher affinity for PIP_3 than for PIP_2 [77].

3.2.1. SOS N-term-mediated auto-inhibition and release

Indeed, the HD and the DH-PH unit are conformationally coupled [70] and all their concerted lipid interactions are critical for growth factor-driven SOS membrane targeting and subsequent RAS activation. Crystal structure analyses have shown that the DH and PH domains together are integrated into a structural module constituting a functional unit that directly participates in control of SOS intramolecular inhibition and its interaction with the cell membrane [70,76,78]. Besides favoring SOS1 anchorage to the cell membrane [77], the DH-PH tandem exerts a negative allosteric control by reducing the catalytic activity of SOS [79].

Crystallographic and biochemical analyses of a truncated SOS construct containing all but the C-terminal PR domain of SOS showed that, under native conditions, the SOS DH-PH modular tandem blocks the allosteric binding site (where RAS•GTP binds to the REM domain) thus suppressing the GEF activity of the SOS catalytic module [79] (Fig. 2B). This inhibitory effect is relieved through the interaction of different N-terminal domains with membrane phospholipids [70,75,78,80]. The simultaneous binding of the PH domain with membrane phosphoinositol phosphates, together with the electrostatic interactions between the positively charged HD surface and negatively charged membranes, releases the inhibitory conformation of SOS resulting in a productive reorientation of the protein at the membrane and increased accessibility to RAS binding (Fig. 2B). The inducible association of HD with membranes contributes to the catalytic GEF activity of SOS by forcing that domain to adopt a conformation that destabilizes the self-inhibitory state (Fig. 2). Thus, the HD plays a critical role in controlling the catalytic output of SOS by coupling membrane recruitment to the release of self-inhibition [78].

Consistent with the negative regulatory role of the SOS N-terminal region, several reports have described germline gain-of-function mutations in this region (mostly located in the DH-PH tandem domain)

associated to ~13-20% of patients with Noonan Syndrome (NS) [31], an autosomal dominant developmental disorder characterized by hyperactivation of the RAS-RAF-MEK-ERK pathway [31,81,82]. In any event, it should be emphasized that the regulatory mechanisms of the N- and C-terminal regions of SOS protein are independent of each other and full autoinhibition requires both the N- and C-terminal inhibitory modes [15].

3.3. SOS catalytic and allosteric domains

The central region of the SOS proteins (approximately, residues 550 to 1050) located between the Helical Linker (HL) (~30-40 aa´) and the C-terminal (PR) region (about 300 aa´), constitutes the catalytic core of the SOS GEF proteins [6,13,16]. The catalytic module, frequently designated as SOScat [83] contains two distinct domains: the REM (Ras Exchange Motif) housing the allosteric site, and the CDC25H domain (homologous to Cell Division Cycle 25, a RASGEF in yeast) containing the catalytic site with specific ability to act on targets of the Ras subfamily of proteins. The sequence of the CDC25H domain is highly conserved from fungi up to mammals whereas the sequence of the REM domain is more variable across evolution [84] (Fig. 2A,B).

3.3.1. The CDC25H catalytic domain. SOS GEF activity promotes nucleotide release

Analysis of the crystal structure of 1:1 RAS:SOScat complexes provided the basic clues to understand the mechanisms whereby the SOS GEFs promote exchange of guanine nucleotides on RAS targets, indicating that the SOS proteins induce a wide opening of the active site of RAS, displacing bound nucleotide and thus facilitating its release from RAS [13,85].

The structure of 1:1 RAS:SOScat crystals showed that the SOScat partner of the complex interacts with, and stabilizes, the nucleotide-free form of RAS *via* an interface involving the Switch I and Switch II regions of RAS [85,86] where Switch II provides the main anchoring point for SOS. In this structure, a helical hairpin element protruding from the main body of the CDC25H domain gets inserted between the Switch I and Switch II of RAS, acting as a molecular wedge prying open the active site of RAS and forcing the release of any previously bound the nucleotide (Fig. 2B). In this context, the SOS GEF activity of the CDC25H domain does not impose any preference on whether RAS is reloaded with GTP or GDP. Once the nucleotide bound to RAS is released by the GEF, RAS is reset by GTP binding driven by the higher intracellular concentrations of GTP over GDP.

3.3.2. The REM domain. SOS allosteric activation and positive feedback loop

In addition to the above mechanisms mediated by CDC25H, the study of crystals containing a 2:1 complex of RAS and SOScat [83] led to the discovery that the GEF activity of the CDC25H domain is also allosterically activated by RAS•GTP through the action of the neighboring REM domain. The structure of these crystals revealed that nucleotide-free RAS was bound to the active site in the CDC25H domain as in the 1:1 RAS:SOScat complexes, but that a second RAS•GTP molecule was bound to a distal site wedged between the REM and CDC25H domains (Fig. 2B). Comparing the structure of isolated SOScat to that of the RAS-SOScat complexes clarified the mechanism of this allosteric activation. In the native, RAS-free state of SOS proteins, the helical hairpin of CDC25H is tilted towards the active site of SOS thereby constricting the site where SOS is supposed to engage Switch II of nucleotide-free RAS. This suggests that RAS binding to the active site of SOS requires the helical hairpin to be pulled back. In this regard, the allosteric binding of RAS•GTP to SOS facilitates the rotation and opening of the helical hairpin, thus freeing the catalytic site to bind RAS through the switch II [87,88].

The sequential, coordinated action of the REM and CDC25H domains through the above described mechanisms creates a positive feedback loop of SOS activation [14] whereby the binding of an active RAS on the allosteric REM site of a native, RAS-free SOS molecule generates an allosterically activated SOS CDC25H that then produces active RAS•GTP, which in turn returns to the allosteric REM site of the minimally active allosteric RAS-devoid SOS, thereby activating SOS. This loop couples with the membrane-binding-mediated release of autoinhibition in cells [14,89–91] to activate SOS in such a way that only a small fraction of active SOS is needed to produce active RAS•GTP [79,89–91]. In this way, once a single molecule of SOS is allosterically activated by RAS•GTP at the membrane, hundreds of RAS molecules can then be processively activated by that SOS molecule [13]. Thus, RAS is by itself an essential determinant of SOS regulation [79].

3.4. Carboxy-terminal Segment of SOS

The approximately 300 aa´-long region of the C-terminal region of SOS proteins has an overall disordered structure adopting a left-handed polyproline type II helix conformation [15,92] that complicates crystallization and purification of full length SOS proteins. Specifically, the sequence of this carboxyl-terminal PR region features four *bona fide* Proline-Rich motifs (PΨΨPPR) as well as other imperfectly matching SH3-minimal binding sites (ΨPXΨP) [93] with ability to bind to SH3 (Src Homology 3) domains present in the sequence of intracellular adaptor proteins such as GRB2 or E3B1 [66]. Under native, unstimulated conditions, this adaptor-free C-terminal tail exerts an autoinhibitory effect (independent of that produced by the N-terminal region) on the catalytic GEF activity of native SOS proteins [94]. It is precisely the intermolecular interaction of the SOS PR domain with different adaptor proteins, particularly GRB2 and E3B1, which determines the release of that auto-inhibition through their recruitment to the specific subcellular locations where the activated SOS GEF proteins meet and activate their RAS (*via* GRB2 interaction) or RAC (*via* E3B1 interaction) targets [1,5,6,13,66,74] (Fig. 2).

The possibilities of modulation of SOS GEF activity by interacting intracellular proteins are not limited to GRB2 and E3B1. Besides these two proteins, the SOS proteins are also able to interact through their C-terminal region with a large number of SH3-containing adaptors or scaffold proteins including SHC, NCK, p130Cas, EZRIN, SPROUTY, BCR-ABL, GPCRs, LAT or 14-3-3 [13,95,96]. Updated record of all possible partners capable of interacting with SOS1 or SOS2 in intracellular protein complexes are kept in the Biogrid (https://thebiogrid.org/112537/summary/homo-sapiens/sos1.html; https://thebiogrid.org/112538/summary/homo-sapiens/sos2.html) and String (https://string-db.org/) databases. Likewise, updated data concerning the multiple different posttranslational modifications that SOS proteins may undergo and thus alter their participation in those complexes [97] updated data for SOS1 and SOS2 is also found in the Phosphosite database https://www.phosphosite.org/proteinAction.action?id=5000&showAllSites=true; https://www.phosphosite.org/proteinAction.action?id=8108&showAllSites=true). The great variety of potential binding partners and the many posttranslational modifications that may alter those interactions offer multiple regulatory possibilities to modulate the processes of RAS or RAC activation by SOS1/2 in different biological contexts.

Interestingly, alternatively spliced SOS1 isoforms affecting the structure/disposition of some of the individual PR motifs have been found in humans that show different SH3 binding ability, specificity and biological potency [56,98]. The existence of alternative SOS1/2 isoforms, together with the availability of many different possible intracellular adaptor/scaffold binding partners, suggests the existence of multiple layers of fine regulation of the process of RAS and RAC activation by SOS in different biological and cellular contexts, as well as modulation of the cross-talk between RAS and RAC activation pathways.

3.4.1. SOS-RAS activation pathway and negative regulatory feedback loop

Early mutagenesis and structural studies provided most of the mechanistic insights explaining the interaction of GRB2 with SOS [99]. Upon stimulation of cell surface receptors by upstream agonists, the SH3-mediated SOS1-GRB2 complexes can be recruited from the cytosol to the inner surface of the plasma membrane through the interaction of the SH2 domains of GRB2 with phosphotyrosine residues of the activated RTKs [1,5,6,13]. Such translocation facilitates the CDC25H domain of SOS1 to trigger GDP-GTP exchange on the membrane-bound RAS molecules [6] and subsequent activation of the RAS-ERK pathways. The overall level of SOS GEF activation at the membrane is balanced by the reversible binding kinetics of the SOS:GRB2 interaction [100] which can be modulated by the alternative binding of GRB2 to many other potentially competing intracellular protein partners. Once SOS reaches its location at the membrane, it remains there until being actively removed by membrane endocytosis [100] (Fig. 2B,C).

The activity of this GRB2-SOS-RAS signaling pathway is ultimately controlled by feedback mechanisms targeting SOS1. In particular, phosphorylation of specific Ser/Thr residues of the C-terminal region of SOS by ERK and RSK kinases is known to alter its association with GRB2 and inhibit SOS1 function, thus constituting the main mechanism responsible for the negative feedback of the RAS activation pathway [101–106] (Fig. 2B,C). Furthermore, binding of antagonists such as p27KIP1 or SPROUTY1-4 to GRB2 also prevents its binding to SOS1 [107]. Oxidative stress has also been shown to inhibit EGFR-SOS-RAS signaling by activating the binding of p66SHC to EGFR and GRB2 and promoting the dissociation of GRB2 from SOS1 [108].

3.4.2. SOS-RAC activation pathway

The differential activity of SOS over RAS or RAC targets *in vivo* appears to be mostly mediated by mutually exclusive interactions with either GRB2 or E3B1 adaptor proteins [74]. For SOS to activate RAC, SH3-mediated SOS1-E3B1 complexes are recruited to actin filaments found within membrane ruffles (where RAC is preferentially localized) in an EPS8-dependent manner (through the interaction of an EPS8 SH3 domain with a PXXP domain of E3B1) [109] facilitating the DH domain of SOS1 to catalyze GTP-GDP exchange within RAC [110]. Once linked to actin filaments through EPS8, the EPS8-E3B1-SOS1 complex can activate the resident RAC molecules and trigger subsequent downstream signals activating c-JUNK and other members of the MAPK cascade, thus enabling actin cytoskeleton remodeling [5,111] (Fig. 2B,C).

The mechanisms regulating the pathway of RAC activation by SOS1 through formation of the E3B1-EPS8-SOS complex are still poorly understood although they are essential for the control of lamellipodia protrusion and cellular migration or invasion. E3B1 is certainly a critical limiting factor as it binds SOS1 as well as GRB2. It is also known that p66SHC specifically activates RAC by reducing formation of the SOS1-GRB2 complex (activating RAS) and increasing the formation of the SOS1-EPS8-E3B1 complex that specifically targets RAC [112]. It was also reported that phosphorylation of the C-terminal region of *hSOS1* on Y1196 is sufficient to elicit its RACGEF activity in response to the activation of various receptor and non-receptor TKs, and contribute to BCR-ABL-induced leukemogenesis [28].

3.4.3. C-terminal PR-mediated SOS autoinhibition

Although the main role for the PR domain seems to be the recruitment of SOS to activated receptors *via* binding to the SH3 domain of GRB2 [113,114], there is ample evidence suggesting an additional inhibitory role for this domain. On one side, constitutive membrane targeting (using lipid tethers) of SOS proteins significantly strengthens RAS activation in transfected cells [115]. On the other, truncated SOS constructs lacking the C-terminal domain are able to bypass GRB2-mediated membrane recruitment and act as potent RAS activators [93,116], indicating that the C-terminal region of hSOS1 downregulates the intrinsic RASGEF activity of this protein and its deletion leads to enhanced RAS-signaling and transforming activity [93,94]. Consistent with this notion, nonsense mutations causing premature stop codons that abolish the C-terminal PR domain have been found in hyperplastic syndromes such as HGF-1 [6,117,118] and are also known to promote oncogenic transformation [116]. Furthermore, several cancer-associated mutations detected in *hSOS1* also elicit truncations in the C-terminal region that may contribute to human cancer [100,119].

Taken together, the available data suggest that the PR domain accounts not only for the recruitment of SOS to the plasma membrane but also for modulation of its intrinsic RASGEF activity. Indeed, the first two canonical GRB2 binding sites in the C-terminal region of hSOS1 have a critical role for this regulation [93]. The dual role of GRB2 (recruitment of SOS to the plasma membrane upon TKRs stimulation and downregulation of the SOS RASGEF activity under basal conditions) has relevant physiological implications, suggesting that the C-terminal region of SOS has the ability to independently inhibit RAS binding to SOS, probably by blocking the access of RAS to either the allosteric or catalytic site of SOS [15] (Fig. 2B).

In any event, it is apparent that complete self-inhibition of SOS requires allosteric inhibitory modes of both the N- and the C-terminal regulatory modules, and that these N- and C-terminal inhibitory functions are independent of each other (Fig. 2B). The multiple possibilities available in theory for membrane interactions with a great variety of distinct lipids or activated RTKs suggests the possible existence of multiple, alternative regulatory mechanisms capable of releasing either the N- and/or the C-terminal inhibitory conformations and the subsequent allosteric activation of SOS in cells [15].

4. SOS GEFs in physiology

4.1. Functional specificity/redundancy and hierarchy of action

There is ample consensus indicating that the members of the SOS family of GEFs are the most universal and functionally significant GEF activators of the RAS GTPases [1,5,6,16]. Whereas SOS1 and SOS2 are widely expressed in practically all cells, tissues and organs analyzed, the pattern of expression of the two other main families of mammalian RASGEFs (GRF1-2 and GRP1-4) is much more limited, with the GRFs being preferentially expressed in the central nervous system and the GRPs preferentially expressed in hematopoietic cell lineages [1,4,5,16,91,120].

Despite the overall evidence supporting a dominant functional role of the SOS GEFs over other GEFs with regards to the process of activation of RAS GTPases in metazoan cells, there are still many relevant mechanistic questions to be clarified concerning the functional specificity or redundancy of the two SOS family members, or their hierarchy of action on their many different, potential target GTPases. These questions are difficult to address because they are concerned with two co-expressed, highly homologous SOS1/2 isoforms with proven ability to activate, at least *in vitro*, all members of the RAS family and some RHO family members, particularly RAC1. These difficulties are further compounded by the frequent co-expression, in many different cell types, of both SOS1/2 isoforms together with other GEFs [121,122].

The phenotypic analysis of various animal models pertaining loss-of-function or gain-of-function of SOS genes in flies, worms, zebrafish and rodents along metazoan evolution, as well as studies of exogenously expressed or mutagenized SOS molecules in different mammalian cell lineages, have started to provide initial, relevant answers to many of these general functional questions. All these studies firmly document that the SOS1/2 GEFs play central, critical roles in the process of activation of RAS GTPases in eukaryotic cells.

Regarding target specificity, there is extensive experimental evidence, arising from a wide variety of cellular settings, documenting the ability of the SOS GEFs to activate not only all canonical members of the RAS subfamily (HRAS, NRAS and KRAS), but also the RAC1 members of the RHO subfamily [28,66,74,123,124]. As previously

mentioned, the capacity of the modular SOS proteins to act as GEFs for both RAS and RAC proteins is based on their ability to differentially interact with specific intracellular adaptor proteins such as GRB2 or E3B1, respectively [1,5,6,13,66,74].

Regarding hierarchy of action, most reports support the functional predominance of SOS1 over SOS2 in a majority of biological contexts tested, with SOS2 playing secondary, complementary roles that are more easily detected in experimental systems where SOS1 is absent [23,25,26,28]. However, in some cases a functional predominance of SOS2 over SOS1 has been reported, and even a hierarchical requirement for SOS2 regarding mutant RAS-driven transformation has also been clearly shown. In particular, a hierarchical requirement for SOS2 regarding mutant RAS-driven transformation has been recently claimed in view of the experimental evidence showing that RTK-SOS2-WT RAS signaling, but not allosteric SOS2 activation, is a critical mediator of PI3K signaling in the context of mutant RAS [125,126].

All in all, despite the considerable functional knowledge so far accumulated [1,4–6,66], we still do not know most of the specific mechanistic details accounting for the multilayered and multifaceted regulatory circuits modulating the specific action(s) of SOS1 or SOS2 in various cellular contexts as well as in different physiological and pathological processes. Progress in this regard will not be easy but it is of vital importance, especially now that the SOS GEFs have started to be considered as valuable therapy targets for human developmental and/or tumoral pathologies.

4.2. Role in organismal developmental

The critical role played by SOS GEFs in developmental processes as well as in overall organismal survival and homeostasis is supported by phenotypic analyses and observations in different animal models along metazoan evolution, ranging from flies to mammals (Supplementary Table 3). As previously mentioned, SOS was initially discovered as an essential regulator/contributor/component of the neural development pathway responsible for formation of the *D. melanogaster* compound eye that are initiated by the *Sevenless* RTK [7,11,12]. Interestingly, those early studies showed also that direct mutagenic inactivation of this locus by irradiation or treatment with ethylmethanesulfonate gave rise to recessive lethal SOS null mutants, demonstrating its essential role not only for neuronal development but also for larval survival [7,11,12].

In the nematode *C. elegans*, the previously known *let-341* gene has also been shown to encode SOS1 and to play a critical role in the process of vulval development initiated by the upstream *let-23* RTK in this worm [9]. Regarding organismal development, RNAi-mediated silencing of *SOS1* caused low penetrance lethality (8%) and the surviving animals escaping early larval lethality displayed slow growth and scrawny body morphology [9]. Interestingly, a gain-of-function SOS1 mutation mapping to the DH domain was later found and shown to suppress the defective vulvar phenotype in specimens harboring let-23 (EGFR) loss-of-function mutations, thus highlighting the known autoinhibitory role exerted by this domain over the overall GEF activity of SOS1 [127].

The process of meiotic maturation of stage II oocytes of the amphibian *Xenopus* has also proven to be a useful system to analyze functional activities of signaling molecules participating in RTK-RAS signaling pathways [128,129]. Interestingly, microinjection of a peptide coding for the SOS1 PH domain was shown to trigger Germinal Vesicle Breakdown in this system suggesting that, unlike PH domains from other cellular proteins, isolated SOS1 PH domains retain functional specificity and the ability to productively interact with RAS-mediated signaling pathways participating in the GVBD process [130].

During zebrafish development, it has been shown that a signaling complex containing Nostrin along with SOS1 and RAC1 is necessary for proper vascular development during developmental angiogenesis [131]. Furthermore, consistent with GWAS studies of chronic kidney disease patients that identified an association of *SOS2* with the disease, knock-down of *SOS2* in zebrafish embryos was also shown to cause abnormalities of embryonic kidney development (involving changes of glomerular gene expression and renal tubule morphology) [132].

Regarding loss-of-function mammalian animal models, the initial analysis of constitutive knockout (KO) murine strains for *SOS1* gene showed that *SOS1* is essential for intrauterine development, with constitutive-null-animals dying during mid-gestation due to defects of the embryos [22] and/or placental development [20]. In contrast, adult SOS2-constitutive-KO mice were perfectly viable and fertile, with no evident phenotypic abnormalities [21]. Interestingly, the SOS1 null mutations found in SOS1-constitutive-KO mice are the only embryonic lethal mutations identified among all different RASGEFs in mammals, suggesting a dominant role of SOS1 over SOS2 and the rest of mammalian RASGEFs regarding embryonic development.

The difficulties initially imposed on SOS1 functional analysis in adult animals by the embryonic lethality of the homozygous SOS1-KO null alleles were bypassed by using a conditional, floxed SOS1-null allele generated in the Samelson's NCI lab to characterize the functional significance of *SOS* genes during thymocyte development [24] using a Cre recombinases expressed under the control of T-cell specific promoters. Using Samelson´s $Sos1^{fl/fl}$ mice, our group generated tamoxifen-inducible SOS1-KO mice expressing the SOS1 null mutation throughout the adult body upon TAM administration [23], and subsequent cross-breeding with constitutive SOS2-KO mice colony [21] allowed generation of comparable sets of WT, SOS1-KO, SOS2-KO and SOS1/2-DKO mice sharing the same genetic background, thus facilitating phenotypic comparisons and allowing functional conclusions regarding the SOS1/2 GEFs in a variety of biological contexts. Interestingly, whereas single SOS1-KO and SOS2-KO mice are perfectly viable, the SOS1/2-DKO mice died precipitously, suggesting some degree of functional redundancy for SOS1 and SOS2 with regards to adult organismal homeostasis and survival [23]. The availability of this inducible, loss-of-function system in mice has opened avenues to characterize specific function of SOS1 and/or SOS2 in a variety of biological contexts including different cell lineages as well as various physiological and pathological or tumoral processes. Furthermore, some recent reports have also described the use of modern CRISPR/Cas9 technologies to generate SOS1-devoid or SOS2-devoid [125,126,133–135] tumor cell lines that are instrumental for characterization of specific contributions of SOS1/2 to different cancer signaling pathways or for the search of new SOS agonists and antagonists.

Mouse models involving gain-of-function have also been instrumental for functional characterization of SOS gene products. Thus, transgenic mice developing upon expression of a dominant form of SOS (K5-SOS-F) in basal keratinocytes [136] have been useful to characterize EGFR- and SOS1-dependent signaling pathways in skin tumors [137,138]. Adult mice heterozygous for a dominant $SOS1^{E846K}$ mutation in the CDC25H domain have also been used as a valid model for NS studies [139]. On the other hand, no animal models exhibiting SOS2 gain-of-function mutations have yet been reported in the scientific literature.

4.3. Roles in specific cell lineages

The functional specificity or redundancy of the SOS1/2 GEFs has also been analyzed in a wide range of non-tumor and tumor cell lines cells using loss-of-function or gain-of-function approaches. This section summarizes available reported data on functional roles played by SOS1/2 under physiological conditions in a variety of specific cell lineages and tissues (Supplementary Table 4).

4.3.1. SOS1/2 Role in progenitor and embryonic stem cells

Analysis of the two initial, independently developed, constitutive SOS1-KO mouse strains reported in the literature, indicated that SOS1 is essential for intrauterine development, with homozygous null animals dying in mid-gestation [20,22]. Whereas the earlier report [22]

attributed the deaths of the mice to yolk sac and embryonic heart defects, detailed analysis of the KO animals generated by Qian et al. demonstrated that their embryonic lethality is due to impaired development of the trophoblastic and spongio-trophoblastic lineages of the placental labyrinth [20]. Consistent with this, more recent studies involving the use of $SOS1^{-/-}$ mESCs derived from Wang's KO strain confirmed that SOS1-GRB2 interaction is essential to control embryonal stem cell fate, driving the embryonic stem cells toward the primitive endoderm, an essential, clearly extraembryonic lineage [67]. Interestingly, whereas SOS2-KO mice do not show any alteration of intrauterine development [20,21], SOS2 silencing in $SOS1^{-/-}$ mESCs resulted in inhibited priming and differentiation of these cells to primitive endoderm, suggesting the possibility of some partially redundant functional role of SOS2 regarding these developmental processes [67].

4.3.2. SOS1/2 function(s) in mesenchymal lineages

Regarding fibroblasts, the main stromal cell lineage, analysis of primary MEFs cultures derived from the initially generated SOS1-KO mouse strains documented the critical role of SOS1 in growth factor signaling initiated by upstream RTKs like the EGFR [20,22]. Further analysis of SOS1-KO fibroblasts, which retain normal SOS2 expression, showed that $SOS1^{-/-}$ cells (unlike $SOS1^{+/-}$ cells) are resistant to transformation by upstream tyrosine kinase oncogenes and suggested that SOS1 participates in short-term and long-term RAS-ERK signaling whereas SOS2-dependent signals are predominantly short-term [20]. More recently, detailed comparisons among WT, SOS1-KO, SOS2-KO an SOS1/2-DKO primary MEFs sharing the same genetic background have demonstrated a critical, dominant role of SOS1 over SOS2 in control of essential critical cellular processes including proliferation, migration, inflammation or maintenance of intracellular redox homeostasis [23,25,27]. Interestingly, whereas single SOS2-KO fibroblast cells did not show measurable defects in any of the above mentioned cellular processes, the defective phenotypes of DKO cells were routinely stronger than in single SOS1-KO cells, suggesting some partial ability of SOS2 to functionally contribute to those cellular processes under normal conditions, or at least in the absence of SOS1 [25,26].

The critical functional role played by SOS1 (but not SOS2) in adipocytic cell lineages was initially substantiated in studies that identified (for the first time) a negative regulatory mechanism acting on SOS1 GEF activity. Interestingly, after insulin stimulation of 3T3L1 adipocytes, and once maximum levels of RAS•GTP are reached, hormone treatment causes also marked hyperphosphorylation of SOS1 and subsequent decrease (by 50%) of cellular GRB2-SOS1 complexes. This indicates that this SOS1-dependent negative feedback mechanism is an essential component of the physiological process of desensitization after insulin hormone stimulation of those cells [140]. The functional involvement of SOS1 in modulation of adipocytic differentiation is further supported by a report showing that the endocytic DAB2 adaptor protein determines the commitment of pre-adipocyte cell populations through a mechanism involving the disassembly of GRB2-SOS1 complexes associated with clathrin-coated vesicles in those pre-adipocytic cell types [141].

Regarding muscle cell lineages, SOS1 was initially shown to be an essential component of a signaling complex (SHC-GRB2-SOS-EGFR) mediating hydrogen peroxide-induced activation of the RAS-ERK pathway in mouse vascular smooth muscle cells [142]. In the same cell type, SOS1 has also been shown to modulate RAC1-dependent processes of cellular migration involving the formation of podosomes and dorsal ruffles [143]. Furthermore, SOS1 was also shown to participate in the process of laminin-induced activation of RAC1 in mouse cell types involved in skeletal muscle contraction [144].

Finally, regarding bone-related cell lineages, SOS1-mediated signals have been shown to participate in osteoblast differentiation [145] and upregulated *SOS1* mRNA levels have been reported in rat osteoblasts exposed to spaceflight as compared to those in the ground [146].

4.3.3. Sos1/2 function in hematological lineages

The functional role(s) of SOS1/2 GEFs in T cell development and signaling has been studied and characterized in detail. Initial studies documented the critical role of SOS1 in early T-cell development, during DN2/DN3 transition, and demonstrated that SOS1 is required for preTCR- but not TCR-stimulated developmental signals [24]. In particular, SOS1 deletion led to a partial block at the DN-to-DP transition, whereas positive and negative selection performed under TCR-mediated strong stimulatory conditions, remained intact upon SOS1 depletion.

Further functional analysis of other RASGEFs during thymocyte development has shown that, although SOS1 is the dominant RASGEF at pre-TCR stages, GRP1 becomes more functionally prevalent at later stages of thymocyte maturation [23,90,147,148]. This functional interplay/redundancy between SOS1 and GRP1 during positive and negative selection steps may constitute a safeguard, adaptive mechanism ensuring appropriate responses to possible variations of physiological environmental conditions.

Although SOS1 is clearly the leading physiological SOS player regarding pre-TCR selection steps, a partial, limited contribution of SOS2 to these processes cannot be excluded based on the observation of an almost complete disappearance of the thymus and a dramatic reduction of thymocyte counts in SOS1/2-DKO animals as compared to single-KO mice. Furthermore, absolute counts of mature B and T cells in spleen and peripheral blood (PB) were unchanged in single-KO mutants, while significantly reduced in SOS1/2-DKO mice [23].

Regarding RTK-RAS-dependent signaling in T cells, the study of murine strains undergoing *Lck-Cre* or *CD4-Cre*-driven depletion of floxed SOS1 alleles has confirmed that SOS1 is essential for ERK activation downstream of the pre-TCR at the DN3 stage, but becomes progressively less important as T cells maturation proceeds. Consistent with separate observations in Jurkat and peripheral T cells, analysis of SOS1/2-DKO mice shows that TCR-mediated ERK activation in peripheral CD4+ T cells is independent of SOS1 and SOS2 [23,24,90,149–151]. In contrast, SOS1/2-DKO CD4+ T cells show impaired IL-2-mediated ERK activation as well as activation of PI3K/AKT signaling associated to subsequent impairment in T-cell migration upon TCR and IL-2 stimulation [149,152], an observation also seen in Jurkat and primary CD4+ T cells [153–155]. SOS1 also mediates amplification of TCR-induced ERK phosphorylation upon IL-7 and IL-15-induced priming of human T cells isolated from PB [156]. Interestingly, in primary human T cells, RASGRP1 regulates transient ERK activation but sustained ERK phosphorylation depends on both RASGRP1 and SOS [157] and SOS1 depletion results in a loss of adhesion capacity that has been directly related to reduced activity of RAC1 and RHOA and decreased integrin-mediated signaling [158]. Finally, SOS1 acts as a scaffold to nucleate oligomerization of the T cell adaptor protein LAT (Linker for Activation of T cells) *in vivo* in T cells but, interestingly, the GEF activity of SOS1 and the SOS1-dependent oligomerization of LAT are separable functions *in vivo* [151].

Comparatively less is known about functional role(s) of SOS1 and SOS2 in B lymphocytes. Analysis of SOS-KO mice showed reduced percentage of total bone marrow (BM) precursors in single-KO animals, but a dramatic depletion of B-cell progenitors was specifically detected in SOS1/2-DKO mice. Likewise, absolute counts of mature B cells in spleen and PB were almost unchanged in single-KO mutants but very significantly reduced in SOS1/2-DKO mice, suggesting functional redundancy between SOS1 and SOS2 for development and maturation of B lymphocytes in mice [23].

Regarding participation in B-cell signaling pathways, early studies indicated that ERK phosphorylation upon BCR stimulation is mainly regulated by RASGRP1/3, rather than by SOS in DT40 B cells [90,159]. However, more recent studies have reported that both SOS1 and SOS2 downregulation leads to reduced RAS- and p38-mediated signaling in different lines of B cells after BCR or EGFR stimulation [65,154,155]. Consistently, SOS1 GEF over-activation resulting from transfection of

SOS1cat constructs, results in increased RAS activation upon EGFR stimulation in DT40 and RAJI B cells [90].

Regarding inflammation-related cell populations such as macrophages, it has been shown that LPS induces lysosomal degradation of SOS1 (and subsequent inhibition of ERK activation) in a DOK3-dependent manner in BM-derived macrophages (BMM) [160]. SOS1 has also been shown to regulate BMM podosome assembly and macrophage invasive capacity [161]. More recently, a study using murine peritoneal macrophages and the P388D1 mouse macrophage cell line has demonstrated that weak CD40-mediated signal in macrophages activates NRAS via SOS1/2 and regulates ERK activation and IL-10 production [162]. Finally, skin wound-repair in combined SOS1/2-DKO mice showed markedly reduced migration of macrophages to the injury site [26]. SOS1/2 signaling plays a key role in determining the responsiveness of neutrophils in regions of inflammation. Thus, studies using SOS1/2-DKO mice have shown that the SOS GEFs play significant roles in regulation of pro-inflammatory responses, in primed, but not basal neutrophils, upon GM-CSF1 and TNFα stimulation [27,163]. Strikingly, single SOS1 deletion has no effect on neutrophil recruitment upon skin injury stimulation, whereas SOS2 absence increases the capability of neutrophils to migrate to the inflammatory site. However, combined deletion of SOS1 and SOS2 results in almost complete blockade of the migration of neutrophils (and also macrophages) to the site of injury [26]. In NK cells, SOS1 appears to be essential to establish NKG2D-mediated immunological synapse [164]. On the other hand, SOS2 has been proposed to participate in the activation of basophils upon IgE-stimulation [165].

Although no significant changes of the levels of early erythroid precursors were observed in SOS1/2-deficient mice [23], treatment of primary human CD34+ cells with a SOS-SH3 inhibitor showed that SOS-mediated signaling is essential for EPO-mediated erythroid differentiation by reducing MASL-1 expression [166]. In fact, *SOS1* appears as an enriched gene during BM-derived mesenchymal stromal cells-regulated proliferation and differentiation of CD34+ erythroid cells [167]. Consistent with this, SOS1 gain-of-functions occurring in NS patients cause an increase in the number and survival of CD34+ cells [168]. Direct involvement/participation of SOS1 and SOS2 protein complexes in the process of EPO-induced ERK activation in human F-36P erythroid cells [169] or mouse Ba/F3 cells [170] has also been reported.

Finally, the functional relevance of SOS proteins acting as RACGEFs in platelets has been documented by the demonstration that protein-protein interaction between SOS and PI3K-SYK is crucial for fibrinogen-induced lamellipodia and filopodia formation in this particular cell type [171]. Moreover, a recent study have demonstrated that NSC-658497 SOS1 inhibition significantly inhibited GPVI-mediated platelet adhesion, dense granule secretion and integrin activation [172].

Consistent with our observations indicating that the embryonic lethality of SOS1-KO mice is due to defective development of the trophoblast layers of the placental labyrinth [20], recent reports have documented substantial roles of SOS1 in endothelial and vascular development [173,174]. Indeed, the critical role of SOS GEFs in angiogenesis is also supported by *in vivo* and *ex vivo* assays in SOS1/2-DKO mice that showed significantly reduced levels of the steady-state density of dermis blood vessels as well as markedly reduced capacity of formation of new blood vessels in the skin and in aortic ring explants from SOS1/2-DKO mice [26]. Interestingly, independent studies of different endothelial cell systems from mouse, human or zebrafish origin have identified SOS1 as an essential mediator of RAC1 activation by different agonists and subsequent for induction of specific responses in those cell types [131,175,176].

4.3.4. SOS1/2 functional role(s) in epithelial cells

The critical functional role played by SOS GEFs in epithelial cell lineages was evident from their initial discovery in primitive metazoan developmental systems dependent upon EGFR-like initiated signals such as those leading to formation of the compound eye in flies [7,11,12] or the vulval organs in nematodes [9,127]. Consistent with this, an initial report characterizing mechanisms of EGFR involvement in mouse skin development demonstrated that expression of a dominant SOS transgene caused significant hyperproliferation of the basal keratinocyte compartment leading to hyperplasia and papilloma formation [136]. Conversely, analysis of the skin of SOS KO mice has shown that single SOS1 depletion (but not SOS2 depletion) significantly reduced keratinocyte proliferation in the epidermis *in vivo,* but this defect was significantly worsened when both SOS1 and SOS2 were concomitantly absent in DKO mice, suggesting a predominant role of SOS1 (and a possible secondary adjuvant role of SOS2) in control of keratinocyte proliferation [26]. On the other hand, studies on normal foreskin human keratinocytes have shown that SOS1 appears to be totally dispensable for the process of E-cadherin-induced RAC1 activation during the establishment of cadherin junctions among these cells in the skin [177].

The role of SOS1 acting downstream of the EGFR to activate RAS-ERK proliferative signaling in epithelial cell lineages has also been demonstrated in epithelial cells of the intestine where EGFR-SOS1-RAS growth signals are opposed and dampened through the action of another GEF, GRP1, to modulate normal homeostasis of the intestinal epithelium [178]. Likewise, FGF2-mediated dampening of SOS1-derived (proliferative) signals appears to be an important mechanism in processes of repair of intestinal epithelial damage occurring in various gut pathologies [179].

The importance of the EGFR-SOS1 signaling axis is further demonstrated by experiments showing that the EGFR uses SOS1 to drive constitutive activation of NFκB in various epithelial cancer cells [180] or that SOS1-mediated signaling seems also essential during epithelial-to-mesenchymal transition in tubular renal cells [181].

Regarding lung epithelia, shRNA-mediated silencing of SOS1 in human bronchial epithelial cells has demonstrated the critical role of SOS1 for proper cell migration and tight junction formation during lung airway morphogenesis. Introduction of a SOS1 gain-of-function mutant (N233Y) in immortalized tracheobronchial epithelial cells results in increased RAS-ERK signaling, cell proliferation, and colony and tumor formation, further supporting the relevant role of SOS1 in RAS-ERK activation in lung epithelia [182]. In contrast, cyclic stretch-mediated ERK activation in alveolar epithelial cells appears to be independent of SOS-mediated signals in this particular cell type [183].

4.3.5. SOS functional role(s) in neural cell lineages

The functional involvement of SOS GEFs in neural cell lineages was already evident from its initial discovery as an essential regulator of the development of the neuronal R7 photoreceptor cells in the *Drosophila* compound eye [12,184]. Further studies in *Drosophila* also demonstrated that SOS signaling, involving specifically its RAC1 GEF activity, is essential for axon guidance to interpret midline repulsion clues, that regulating which axons cross the midline [185–187]. SOS has also been identified in *Drosophila* glial cell lineages as an essential upstream RAC1 GEF activator required for proper glial responses to axonal injury, during post-injury responses involving glial engulfment of axonal debris in this fly nervous system [124]. Consistently, the introduction of magnetic nanoparticles carrying bound SOScat domain has been recently proposed as a potential therapy approach in Parkinson's disease, to guide neurite regeneration in human dopaminergic neurons [188].

Multiple reports have also described the expression and functional interactions of SOS GEFs with other signaling molecules and binding partners in a large variety of neuronal cell types. For example, in Schwann cells, neuregulin ligands (members of the EGF family of ligands) have been reported to induce rapid association of SOS with the HER2/HER3 receptor complex [189]. Likewise, analysis of murine brain extracts, as well as isolated neurons and nerve terminals, have identified the endocytic protein intersectin as a major binding partner of SOS1 [190]. Studies using chicken embryonic tissues and cell

cultures have also shown that PKCε-dependent HRAS activation and subsequent regulation of neuronal differentiation and highly specialized functions encompasses the recruitment the SOS1 (and neurofibromin) in the lipid rafts of embryonic neurons [191]. Studies on Huntington´s disease using transfected neuro2A cells have also shown that mutant huntingtin exhibits higher affinity than GAB1 for, and replaces it, in the GRB2-SOS-GAB1 complexes that are normally found in healthy neural cells as essential mediators of sustained RAS activation [192].

Although the SOS1/2 GEFs appear to be ubiquitously expressed, there are quantitative differences of their expression in different cellular and subcellular locations, suggesting functional specificity of one of the other SOS isoform at the spatial or temporal level, or under different neural contexts. For example, very distinct intensities of expression of SOS1 and SOS2 are detected in different layers and cell-subpopulations of the retina in rats and humans [193]. Furthermore, it has also been reported that SOS1, but not SOS2, is found in the post-synaptic density fraction of the rat forebrain, suggesting a dominant role of SOS1 in the regulation of RAS-ERK signals participating in synaptic transmission and its regulation at post-synaptic sites [194]. Likewise, SOS2 has been shown to be dispensable for NMDA-induced ERK activation and LTP induction in cortical neurons, implying that SOS1 is the main regulator of this neuronal functions [195]. In contrast, SOS2 appears significantly upregulated in a population of neurons located at the subgranular layer of the hippocampus after traumatic brain injury, suggesting a potential role of SOS2 at least upon brain insult [196]. On the other hand, SOS1 and SOS2 exhibit functional redundancy in the process of RIT-mediated differentiation of neuronal pheochromocytoma PC6 cells that involves formation of TRKA-SHC-SOS complexes [197]. Interestingly, in PC12 cells, SOS1 and SOS2 are both crucial for RAS activation upon NGF-stimulation, but they seem to be largely dispensable for RAC activation under the same conditions [198].

5. SOS GEFs in pathology

5.1. Gain-of-function mutations in pathology

Classical studies along the last 30 years have demonstrated that the activation of RAS proteins by means of point mutations causes a variety of pathological alterations including multiple types of tumors (sporadic mutations) as well as inherited developmental syndromes ("RASopathies") (germline mutations). In addition, experimental evidence has recently suggested that other potential, alternative mechanisms of RAS activation, including hyperactivation of cellular GEFs or deregulation of other components of RAS signaling pathways, may also lead to development of physiological or pathological alterations [2,5,6,199].

In particular, an ample collection of recent reports on SOS gene products have started to identify a variety of molecular mechanisms including (i) gene amplifications, (ii) gain-of-function mutations, or (iii) overexpression of *SOS* gene products that result in GEF hyperactivation and subsequent hyperactivation of RAS signaling in different biological contexts. Importantly, many of these molecular alterations have been found In association with various pathologies including RASopathies (SOS germline alterations), cancer (somatic alterations) or other non-cancer pathologies [5,6,82,200]. It is precisely this specific association of SOS1/2 molecular alterations with specific tumoral and non-tumoral pathologies which underlies current academic and industry efforts at determining whether these molecular alterations of SOS GEFs may constitute meaningful biochemical biomarkers or therapeutic targets.

5.2. SOS in RASopathies and developmental syndromes

The RASopathies encompass a defined group of hereditary developmental syndromes that are caused by germline mutations affecting genes encoding components or regulators of the RAS-ERK pathway. They represent one of the most prevalent groups of human malformation syndromes and include a variety of different clinical syndrome entities including neurofibromatosis (NF1), Costello syndrome (CS), Legius (LGSS), Leopard (LPRD), cardiofaciocutaneous syndrome (CFC), Noonan (NS) and other NS-like disorders. As the common underlying biochemical phenotype shared by all the RASopathies is hyper-activation of the RAS-ERK pathway, it is not uncommon to observe overlapping phenotypic features among these syndromes including, among others, facial dysmorphology, cardiovascular disease, and musculoskeletal anomalies or mental disorders [31,81,82,200]. These pathogenetic mechanisms may include functional alteration of RAS-GTPases, RASGAPs, RASGEFs, kinases, scaffolding or adaptor proteins, ubiquitin ligases, phosphatases, and pathway inhibitors [82,200,201]. Remarkably, among all mammalian RASGEFs, only SOS1 and SOS2 have been found altered in RASopathies, further supporting the predominant functional role of the SOS1/2 GEFs over other RASGEFs in mammalian cells.

5.2.1. SOS1/2 in Noonan syndrome

The most common RASopathy is NS, an autosomal dominant condition whose features may include distinctive facial appearance, short stature, broad or webbed neck, congenital heart defects, bleeding problems, skeletal malformations, as well as physical and neurodevelopmental delays and cognitive deficits [31,82,202,203].

NS is genetically heterogeneous and may be caused by a mutation in any of several genes coding for different components of RAS signaling pathways and can be classified into subtypes (NS1 through NS12) (OMIM database, https://omim.org/phenotypicSeries/PS163950) according to the responsible gene (https://rarediseases.info.nih.gov/diseases/10955/noonan-syndrome). *PTPN11* is responsible for NS1 and is the most frequently mutated gene in NS (~50% of cases). *SOS1* is the second most mutated gene (~16.5% of familiar and sporadic cases) and its mutational activation is the cause of the NS4 subtype. More than 60 NS-related mutations leading to various levels of constitutive hyperactivation of SOS1 have been identified in different domains of the *hSOS1* gene. Most of them are missense mutations, although small deletions, small insertions and small indels have been also reported [31,204] (Fig. 3). Moreover, specific NS Ras mutants (I24N, T50I, V152G and D153V) have been reported to deregulate allosteric SOS autoactivation [205].

The phenotype of NS4 caused by *SOS1* mutations lies within the NS spectrum although a certain prevalence of cardiac defects and ectodermal abnormalities has been were initially noted [31,206].

Consistent with the overlapping phenotypic features exhibited by different RASopathies, there are also reports of rare SOS1 mutations detected in patients with related RASopathies, such as CS/CFC syndrome [207] or NS-like/multiple giant cell lesion syndrome [208].

In addition, some NS patients bearing *SOS1* mutations have also been reported to develop various types of tumors including, paraspinal tumors [209], pediatric colorectal cancer [210], embryonic rhabdomyosarcoma, Sertoli cells tumor or granular skin tumors [211,212], melanoma [201] or acute lymphoblastic leukemia [213], thus highlighting the predisposition and interplay existing between these developmental syndromes and cancer.

Although the initial screenings did not detect mutations in *SOS2* gene in NS patients [202], more recent studies have detected the presence of missense activating mutations in 7 specific residues of the DH domain of SOS2 that are responsible for maintaining this GEF in an autoinhibited conformation and appear to favor predisposition to lymphatic complications (Fig. 3) [30,82,214,215] in a small percentage of NS subjects with marked ectodermal involvement, thus defining the NS9 subtype of this syndrome (https://omim.org/phenotypicSeries/PS163950).

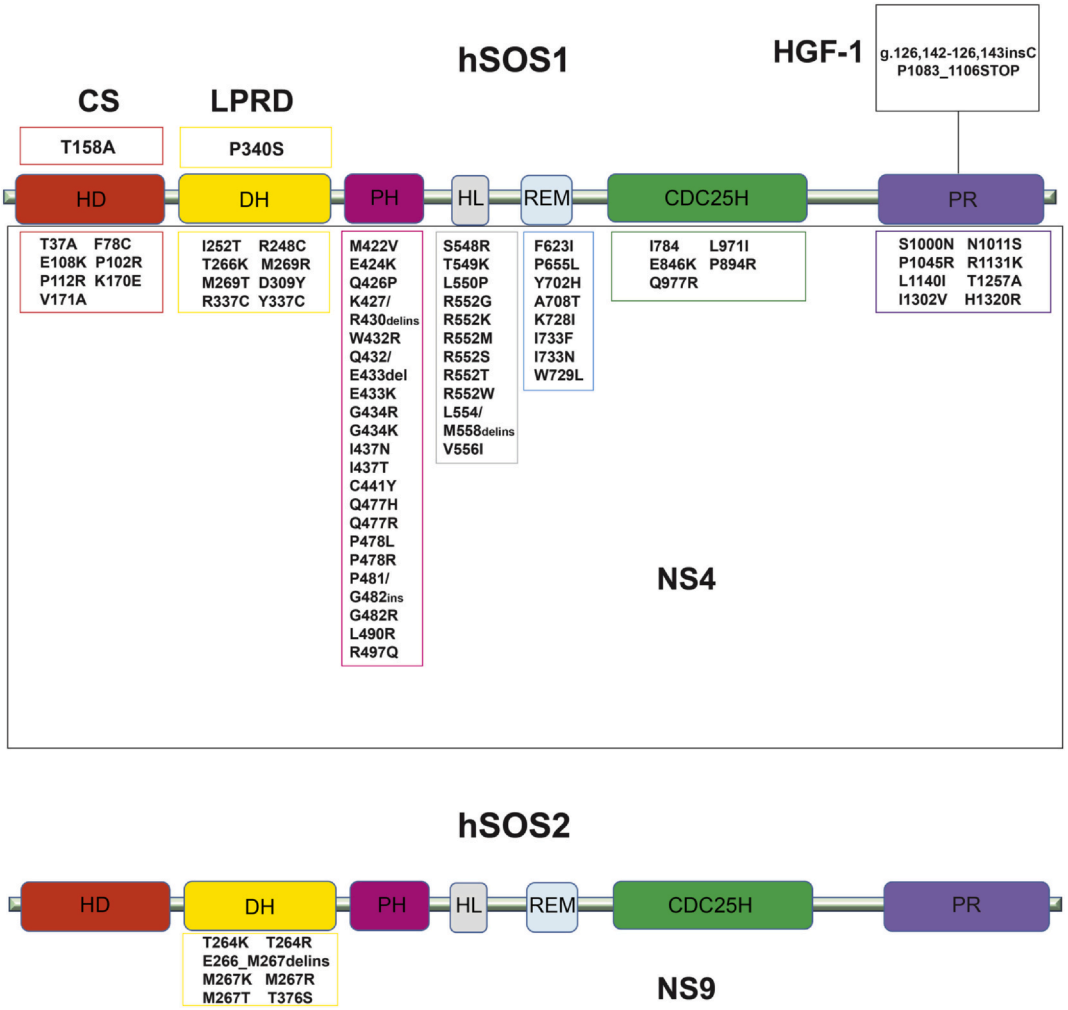

Fig. 3. SOS mutations in RASopathies.
Schematic representation of mutations affecting the indicated domains of hSOS1 or hSOS2 that have been reported in different inherited RASopathies including NS4: SOS1-related Noonan syndrome; NS9: SOS2-related Noonan syndrome; HGF-1: Hereditary gingival fibromatosis; CS: Costello syndrome; LPRD: Leopard syndrome.

5.2.2. SOS1/2 in hereditary gingival fibromatosis (HGF-1) and related syndromes

HGF is a rare, autosomal dominant condition characterized by benign, uncontrolled gingival overgrowth. Large-scale genetic analyses of affected individuals identified a causal mutation involving a single cytosine insertion in exon 21 of the *SOS1* gene (3248_3249insC) introducing a frameshift and creating a premature stop codon that lead to a shortened SOS1 protein having a 22 amino-acid missense addition at the C-terminus and missing almost completely the C-terminal PR domain. The loss of the PR region results in loss of autoinhibition and constitutive activation of SOS1 and subsequent RAS signals that provide a likely pathogenic mechanism for this malformation [118,216]. It is unclear why the overgrowth of tissue is seen only in the gums, but histological analysis identified increased fibroblast numbers (over 30%) in the gingiva of SOS1 HGF patients than in normal counterparts. Furthermore, cultures of HGF fibroblasts showed significantly higher rates of proliferation and levels of RAS-ERK activation than in normal human fibroblasts [117]. Interestingly, *miR335-3b*, which is capable of directly targeting *SOS1* gene, has shown antifibrotic potential in assays using normal human gingival fibroblasts [63]. Nevertheless, HGF displays genetic heterogeneity, and mutations in genes other than *SOS1* are also likely to be involved, as suggested by reports analyzing different cohorts of familial and non-familial gingival overgrowth patients [217,218].

SOS1 mutations have also been found in other syndromes clinically related to HGF such as pure mucosal neuroma syndrome, an autosomal dominant neurocutaneous disorder where different frameshift mutations affecting exon 20 of the C-terminal portion of SOS1 (c.3266dup, c,3248dup and c.3254dup) as well as a heterozygous *SOS1* exon 20 mutation (c.3255_3265del) have been recently described, thus defining a clinical phenotype distinct from MENS2B and characterized by notable gingival hypertrophy associated to arytenoid neuromas which are a recognized feature of the SOS1 mutations [219,220]. Likewise, transcriptional analysis of fibrous epulis samples, a type of hyperplastic lesions with tumor-like appearance predominantly located in the gingival or alveolar mucosa, show significantly elevated levels of *SOS1* expression [221].

Interestingly, in hereditary hyperplastic gingivitis, an autosomal recessive condition found predominantly in farmed silver foxes that is analogous to the HGF occurring in humans, *SOS2*, but not *SOS1*, has been found to be upregulated and proposed as a candidate gene for this disease [222].

5.3. SOS in sporadic cancers

The 3 canonical RAS family members (KRAS, NRAS and HRAS), as well as upstream RTKs such as the EGFR or downstream cytoplasmic kinases like RAF and PI3K, account for the majority of cancer driver genes identified during the past 3 decades as undergoing activating somatic mutations in a wide variety of human tumors types sharing the

dysregulation of RTK-RAS-ERK or RTK-RAS-PI3K signaling pathways as a common mechanistic feature [32,33,223,224]. However, as more and more tumor samples have been profiled in recent years, many new mutations (unnoticed in the earlier screens) affecting genes coding for other components of RAS-ERK pathways have now been reproducibly found (in smaller proportions) in some specific tumor types, indicating that they have real pathophysiological relevance for the development of such tumors [32,33,223]. Indeed, this has been the case for many *SOS1* mutations that were initially found as germline mutations in RASopathies [31,82,225] and have been more recently reported as somatic mutations in different sporadic tumor types.

Earlier genomic screenings of different tumor types did not report any *SOS* gene alterations [226–229], and other studies started identified recurrent *SOS1* gene alterations in germline RASopathies [31,82,225]. However, more recent studies have started to uncover *SOS1* gene alterations as rare but reproducibly present in many sporadic tumors including, in particular, lung adenocarcinoma (LUAD), uterine corpus endometrial carcinoma (UCEC), urothelial bladder cancer (BLCA), lung squamous cell carcinoma (LUSC), liver hepatocellular carcinoma (LIHC), acute myeloid leukemia (AML), lower grade glioma (LGG) and cutaneous melanoma (SKMC) [32,33,37,230]. The list of *SOS1* gene alterations found in specific association with different human tumor types includes some scarce amplifications resulting either from copy number variations or upregulated expression, as well as more frequent somatic mutations, particularly base substitutions, causing amino acid changes or premature terminations in specific functional domains of SOS1 that have been recognized as gain-of-function genetic mutations capable of hyperactivating SOS1, and subsequently RAS-ERK signaling, in those tumors [33] (Fig. 4).

An updated catalog of *SOS1* somatic mutations detected in many different tumor types can be found in the COSMIC database (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=SOS1#genome-browser), where about one third of tumor samples tested are reported to present mutations in *SOS1* including missense substitutions (~39%) followed by synonymous substitutions (~7%) and nonsense, truncating substitutions (~2.5%). The most frequent substitution found is C > T (~23%) although, frameshift insertions and deletions in *hSOS1* have been also detected with much lower frequencies (< 2%).

It is also relevant to mention that, except for endometrial and LUAD tumors, most *SOS1* somatic mutations reported in different cancer types are not currently classified as canonical cancer driver genes according to present criteria and standards, based mostly on frequency of detection (https://www.intogen.org/search?gene=SOS1). However, given the central position and regulatory role played by SOS1 GEF in RAS signaling pathways [33,223], these gain-of-function mutations are likely to be significant components of the pathophysiological mechanisms driving development or maintenance of those tumors, and this has been confirmed by many recent reports (see below). The consideration of these SOS1 mutations as potentially oncogenic alterations is supported by the initial discovery of SOS mutations as the driving cause in pathological conditions like NS and HGF-1 [30,31,117]. In addition, this notion is further reinforced since the same recurrent functional mutations have also been more recently identified in small percentage of otherwise RAS-pathway driver-negative LUAD and UCEC tumors (thus labeled as “non BRAF-non RAS” (NBNR) and “non-EGFR” tumors) [32,33]. These observations underscore the notion that low-frequency mutations of components of RAS signaling pathways that might otherwise have gone statistically unnoticed in the past (such as the SOS1 gain-of-function mutations mentioned here), can be significantly relevant for tumor development based on their capacity to hyperactivate RAS signaling in those tumors.

Many recent reports in the scientific literature document the various genetic alterations (including somatic mutation, amplification or differential expression) undergone by *SOS1* genes in different tumor types. Regarding somatic mutations, although early studies found *SOS1* mutations only very rarely in screenings of primary tumors [230,231] or cohorts of specific tumor types [232,233], more recent reports have started to document the specific association of SOS1 mutations with a number of different cancer types, including LUAD otherwise devoid of mutations in RTKs, RAS or RAF genes [32,182], NBNR thyroid carcinomas [234], mucosal neuromas without MEN2B-associated RET mutations [220], melanomas devoid of NRAS or BRAF mutations [201]. A recent review of genomic data from 9125 tumors profiled in the TCGA database has confirmed the detection of rare, potentially oncogenic *SOS1* mutations identifying recurrent (hotspot) mutations (A90V/T; N233Y/S) and other activating SOS1 mutations (M269I/V; G434R; R552S/K/G/M; E846K) in 1% of LUAD and 1% of UCEC independent of subtype, as well as in lower percentages of several other cancer types including BLCA, LUSC, LIHC, AML, LGG and SKMC [33] (Fig. 4).

Rare polymorphic SOS1 variants have also been found in patients with AML including two *SOS1* (F494L and I610T) variants which were heterozygously found in *SOS1* exon 11 [235].

The amount of available cellular SOS1 is also relevant for tumor development. It has been shown that the stability of SOS1-EPS8-E3B1 complex is critical for the metastatic ability of ovarian carcinoma cells as shown by the observation that deficiency of at least one of the members of this complex correlates directly with the loss of metastatic capacity of ovarian tumors [36,236]. Genomic alterations involving amplification of *SOS1* gene have also been reported in specific tumor types. Thus, early Northern and WB studies identified SOS1 overexpression in different tumor cell lines originated from renal, bladder or prostate, carcinomas [57,237–239]. More recently, significant copy number variations of *SOS1* have been detected in isolated tumor cells and extracellular DNA from a cohort of LUAD patients [240] and comparative genomic hybridization has also identified a large amplification (> 15 copies) of the *hSOS1* gene in a patient with high-grade glioma that was negative for BRAF mutations [35].

Several other reports underscore the diagnostic or prognostic value of *SOS1* gene alterations in different tumor types, further supporting the relevance and pathophysiological role of SOS1 alterations in cancer. For example, SOS1 appears to be differentially expressed in breast tumors from African-Americans (AA) as compared with Caucasian women [241], and this differential expression is especially marked in "quadruple negative" (QNBC) breast cancers of AA women [242], suggesting a possible contribution of this overexpression to the poor outcome in AA patients. Likewise, a comprehensive transcriptomic analysis that detected the upregulation of specific gene set including *SOS1* in a large cohort of tumor samples from women with locally advanced cervical cancer (LACC) identified the overexpressed genes as potential new therapeutic targets which could be relevant for the clinical outcome of LACC patients [243]. *SOS1* was also the most frequently upregulated gene detected by microarray profiling in a cohort of Egyptian bladder cancer patients, suggesting its potential usefulness as a molecular biomarker of target for these patients [244]. *SOS1* has also been shown to be part of a set of 10 differentially expressed, cross-talk genes from important signaling pathways involved in hepatocellular carcinomas (HCC) that have been proposed to predict HCC prognosis and also provide potential biomarkers or therapeutic targets for HCC [245]. *SOS1* has also been identified as a member of a network of 14 differentially expressed genes involved in inflammatory-mediated mechanisms in colorectal cancer (CRC) that has been postulated as a potential prognostic marker for this tumoral disease [246]. The significantly upregulated levels of circulating RNA for *SOS1* detected by means of next generation sequencing in the plasma of melanoma patients have also been validated as a potential novel RNA-based biomarker for diagnostic use [34]. Finally, a recent report has also demonstrated that enhanced expression of SOS1 is predictive of poor prognosis in uveal malignant melanoma patients [37].

In sharp contrast to SOS1, and despite the similar expression patterns of the two SOS family members, there are significantly fewer reports describing specific associations of *SOS2* gene alterations with sporadic tumors (https://www.intogen.org/search?gene=SOS2). In

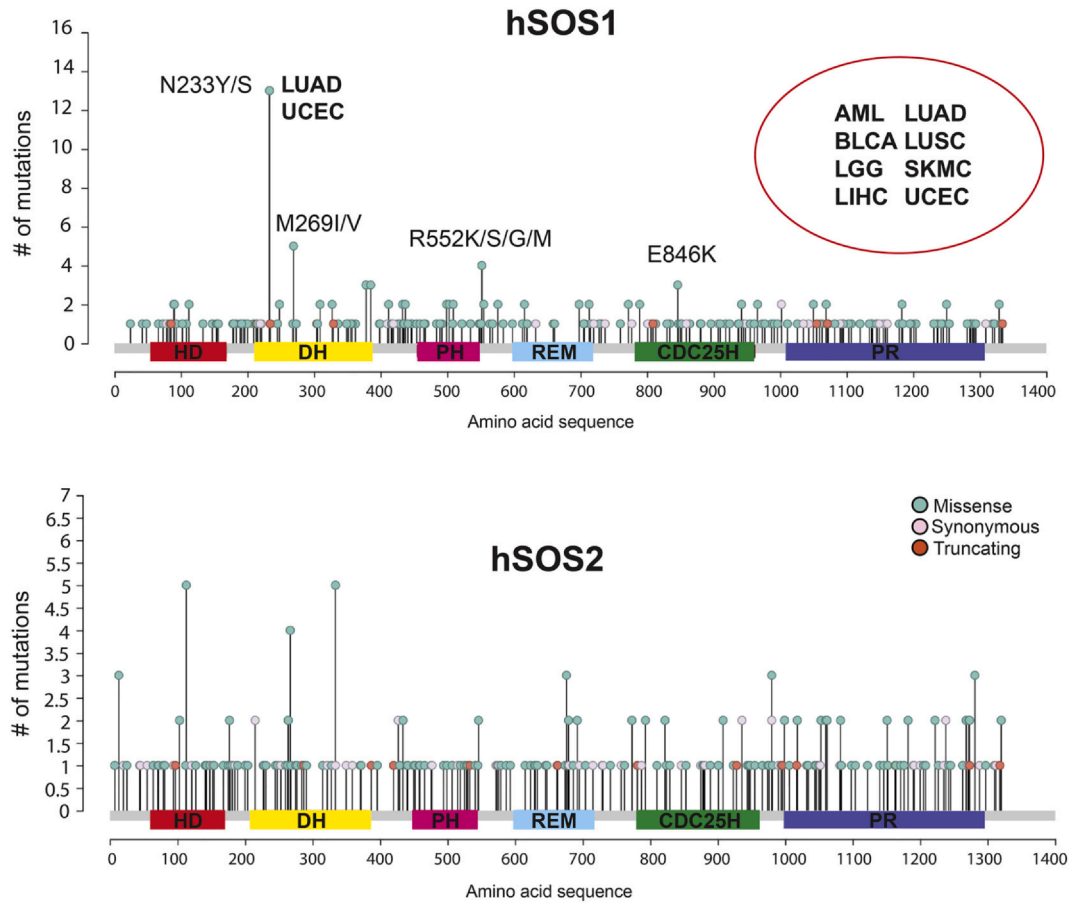

Fig. 4. SOS mutations in cancer.
Schematic representation of different somatic mutations affecting the indicated domains of hSOS1 (https://www.intogen.org/search?gene=SOS1) or hSOS2 (https://www.intogen.org/search?gene=SOS2) that have been identified by the intOGen cancer mutation browser in different sporadic cancers including AML (Acute Myeloid Leukemia), BLCA (Urothelial Bladder Cancer), LGG (Lower Grade Glioma), LIHC (Liver Hepatocellular Carcinoma), LUAD (Lung Adenocarcinoma), LUSC (Lung Squamous Cell Carcinoma), SKMC (Cutaneous Melanoma) and UCEC (Uterine Corpus Endometrial Carcinoma) as indicated. Missense (blue), synonymous (pink) and truncating (red) mutations are represented here. Location of recurrent mutations detected at higher frequency is indicated. The higher frequency of recurrent N233Y/S mutations in *hSOS1* allows for their consideration as *bona fide* oncogenic drivers in LUAD and UCEC.

particular, direct exome sequencing has detected the presence of somatic missense *SOS2* mutations in 3.1% of gallbladder carcinomas samples tested [247]. Similarly, exome sequencing of desmoplastic melanomas characterized by a high mutation burden did not find the typical BRAF or NRAS mutations occurring in regular melanomas, but frequent activating mutations in components of RAS-ERK pathways including, in particular, SOS2 [248]. Finally, recent analysis of gene expression profiles in the TCGA database has reported *MuD*-dependent upregulation of *SOS2* expression in cohorts of TCGA glioblastomas (GBM), and a correlation between high expression of the two genes and longer survival of proneural GBM patients [249]. An updated catalog of somatic mutations in the *SOS2* gene detected in many different tumor types can be found in the COSMIC database, where ~8% of *SOS2* mutated samples correspond to synonymous substitutions, ~38% correspond to missense mutations and ~4% correspond to nonsense substitutions (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=SOS2#genome-browser).

6. SOS GEFs in the clinic

6.1. SOS as biomarkers and therapy targets

Despite their low frequency in comparison to other oncogenic drivers, the presence of distinct (inherited or sporadic) gene alterations of *SOS1* or *SOS2* in specific RASopathies [30,31] and sporadic tumors [210,211,213,250] supports the use of RNA or protein probes for these genes as potentially useful biomarkers for diagnosis or prognosis of all those diseases. In fact, a number of recent reports have already documented the clinical prognostic value of the differential expression of SOS1 for specific subsets of patients with QNBC, LACC, BLCA, HCC, CRC, melanoma or uveal melanoma [34,37,241–243,245,246].

In addition, the SOS GEFs are also being increasingly considered as worthy therapy targets based on the fact that all inherited RASopathies and sporadic cancers harboring gain-of-function *SOS1/2* gene alterations share the dysregulation of RTK-RAS-ERK signalling pathways as a common, underlying physiopathological mechanism. Indeed, the central position played by SOS GEFs in control and regulation of RAS activation and signalling suggests that SOS GEFs may be considered as potential therapy targets not only in the reduced percentage of tumors bearing recurrent, gain-of-function SOS mutations, but also in other more frequent RAS-dependent tumors (lacking SOS mutations but driven by mutations in EGFR, RAS, RAF, etc), where blockade of SOS action may be of therapeutic benefit.

Based on frequency and overall clinical impact, the use of SOS1 as a therapy target may be especially relevant in the case of NS-related and HGF-related malignancies, as well as in sporadic cancers driven by any oncogenic mutation causing activation of the RAS signalling pathway. This is so because, although oncogenic RAS proteins are constitutively activated (not needing, in theory, the action of upstream GEFs to become GTP-loaded), seminal research from Bar Sagi´s laboratory [39,40]

has demonstrated that the cross-activation of WT RAS (which is SOS-dependent) by oncogenically mutated RAS is of critical importance for tumorigenic development in mutant RAS-driven tumors. Consistent with those observations, our analyses of single and double SOS1-KO and SOS2-KO mouse strains have demonstrated that SOS1 depletion significantly reduces BCR-ABL-induced leukemogenesis [28] and DMBA/TPA-induced skin tumors [26], thus confirming the critical role of SOS1 in those tumorigenic processes.

The clinical usefulness of targeting SOS is further supported by a report highlighting the unique dependence on SOS1 of KRAS(G12D)-induced leukemogenesis by showing that SOS1 deletion ameliorates KRAS-induced mieloproliferative neoplasm phenotypes and prolongs survival of KRAS G12D/+ mice [29]. Likewise, siRNA-based genetic targeting of *SOS1* and *SOS2* (as well pharmacological targeting of SHP2) has been shown to enhance efficacy of MEK inhibition in KRAS-amplified gastroesophageal models *in vitro* and *in vivo* [38,251–253]. It has also been reported that deficiency of any individual component of the SOS1-EPS8-E3B1 complex correlates directly with loss of metastatic capacity of ovarian tumors [36].

Furthermore, analysis of primary cells derived from single and double SOS1-KO and SOS2-KO mice has shown reduced cell proliferation rates and increased intracellular oxidative stress and mitochondrial dysfunction [23,25], as well as remarkable phenotypic similarities between RAS-less and SOS-less cells [254,255]. These observations at the cellular level also support the notion of SOS1/2 proteins as meaningful therapy targets in KRAS-driven (and also EGFR-driven) tumors since, according to them, it is not unreasonable to expect that the inhibition of SOS1/2 GEFs activity by specific inhibitors may, on one side inhibit tumor progression and, on the other, increase intracellular ROS production and alter mitochondrial metabolism thus triggering programmed cell death in the treated tumors. These encouraging, initial observations have fostered the many different efforts currently ongoing in industry and academy to develop specific inhibitor of SOS1/2 GEFs as an approach to block hyperactivation of the RAS pathway in tumors.

6.2. Targeting aberrant RAS signaling in tumors

The RTK-SOS-RAS-ERK pathway is the most frequently altered signaling pathway in human tumors and activating RAS mutations are found in about 25% of all cancers types. KRAS is the single most frequently altered gene (~9% across samples), followed by BRAF (~7%) and EGFR (~4%), and smaller percentages of activating mutations are reported for other participants in the pathway including the NRAS (~2%) and HRAS (~1%) oncogenes [33,43,256]. The most recent estimate of RAS mutation data in cancer databases cites overall frequencies of 14.3% for KRAS, 3.1% for NRAS and 1.3% for HRAS [256].

Whereas a number of clinically effective drugs against EGFR- and BRAF-driven tumors are already in use [257–259], much less success has been achieved regarding mutant RAS-driven tumors after almost four decades of intensive research [260–264]. Indeed, although the RAS oncoproteins were frequently deemed as “undruggable” in recent years [265,266], recent breakthroughs have started to challenge that perception with the development of new anti-KRAS inhibitors that are currently being tried under different stages/phases of clinical testing [42,43] (Supplementary Table 5).

Although great expectations are now focused on the novel drugs that target specific RAS oncogenic mutations such as the G12C mutation [42,267–270], the array of promising new therapies directed at inhibiting or attenuating oncogenic RAS signaling in tumors is not limited to direct RAS oncoprotein inhibitors. Indeed, the recent recognition of the critical functional role played by non-mutated, WT RAS proteins in the context of mutant RAS cancers [39,41] has also paved the way for additional, new therapeutic strategies focused on either targeting various downstream effectors of RAS signaling [271] or disrupting the interaction of RAS proteins with upstream signaling molecules, including RTKs like the EGFR [258,259], the scaffolding phosphatase SHP2 [38], or, particularly, the upstream SOS1/2 activating GEFs [42,43]. The following section highlights recent developments regarding novel approaches to downregulate RTK-RAS-ERK oncogenic signaling by specifically targeting the SOS1/2 GEFs with novel compounds capable of directly blocking the intrinsic GEF activity of SOS proteins or impairing the functional interactions between RAS and SOS within the SOS:RAS complex.

6.3. Inhibiting SOS function and SOS-RAS interactions

Different approaches to SOS inhibition, including efforts (i) to physically remove SOS from the tumoral context, as well as attempts to block (ii) the intrinsic GEF enzymatic activity of SOS proteins or (iii) the functionality of SOS:RAS interactions, have been tried in recent years in the context of attempts to target or attenuate oncogenic signaling in tumors harboring altered RTK-RAS-ERK signaling pathways [272] (Supplementary Table 5).

6.3.1. SOS GEF depletion/removal

The initial proof of concept of the efficacy of targeting SOS in tumors was provided by experimental reports where physical elimination of SOS1 resulted in significant impairment of tumor development. Specifically, depletion of SOS1 in KO mouse strains has been shown to result in significant reduction of skin tumor formation [26] and leukemogenesis [28,29]. Consistently, silencing of *SOS1* by means of siRNA or shRNA also produces significant therapeutic benefits in gastroesophageal tumors [38]. Interestingly, the miRNAs modulating SOS expression can be chemically modified in order to increase its activity, thus raising the possibility of using them as therapeutical agents in different pathological contexts. This is the case, for example, with chemically modified miR-143 (miR-143#12), which shows increased affinity for *hSOS1* and displays measurable anti-cancer effects on bladder cancer upon systemic or intravesical treatment [273] or on colon carcinoma DLD-1 cells [274] (Supplementary Table 1).

Physically impairing, blocking or displacing SOS proteins from interaction with their physiological intracellular protein partners or correct subcellular locations has also proven to be therapeutically beneficial. For example, transfected VPS8 has been shown to directly bind SOS1 and inhibit SOS1-RAS signaling initiated by peptide growth factors including EGF [275].

6.3.2. High molecular weight (HMW) inhibitors of SOS protein-protein interactions (PPI)

The use of HMW reagents (natural or synthetic) aimed at blocking or impairing PPI between SOS GEFs and their physiological RAS targets or signaling partners has also proven to be an effective approach to inhibit correct SOS-RAS complex formation and subsequent downstream oncogenic signaling. Early experiments showed that permeable synthetic orthosteric peptide mimetics are able to disrupt the interaction between SOS-αH with the switch I loop of RAS resulting in significant blockade of SOS-RAS interaction and efficient reduction of RAS-ERK activation upon stimulation [276]. Likewise, hydrocarbon-stapled, stabilized α-helices of SOS1 (SAH-SOS1) were shown to directly bind and inhibit WT and mutant forms of KRAS [277]. More recently, the introduction of d-amino acids in the sequence of these peptides has improved their affinity and specificity, giving rise to reagents able to specifically bind to KRAS (G12C) and induce selective apoptosis in cells harboring this RAS mutation [278]. Finally, another HMW agent, the *Bacillus pumilus* ribonuclease Binase has been shown to directly interact with endogenous KRAS and inhibit downstream ERK signaling by reducing RAS interaction with its GEF SOS1 [279].

Approaches blocking the interaction of SOS with its physiological, intracellular signaling partners may also provide effective ways to inhibit oncogenic RAS signaling. For example, a synthetic analog of UCS15A, a potent inhibitor of proline-rich ligand-mediated PPI (PPLI) produced by *Streptomyces* species, has been reported to block GRB2-

SOS1 interaction and also inhibit MEK activity *in vivo* without causing morphological changes to treated cells [280]. More recently, the efficacy of other synthetic covalent inhibitors of GRB2:SOS1 interaction has also been proven *in vivo*, resulting in migration inhibition and cell death in breast cancer cells [281]. Interestingly, RMC-4550, an aminopiperidine-derived small-molecule used as an allosteric SHP2 phosphatase inhibitor has also been shown to disrupt the SHP2-GRB2-SOS1 complex module and decrease oncogenic RAS-ERK signaling and cancer growth by disrupting SOS1-mediated RAS•GTP loading in RAS-driven cancers [253].

6.3.3. Small-molecule SOS inhibitors active against RAS-driven tumors

So far, the most promising approaches to modulating or downregulating SOS activity in tumors in a clinical setting appear to derive from the rational design of small-molecule inhibitors able to target directly its intrinsic RASGEF enzymatic activity [282] or its functional, dynamic interaction with RAS targets [42,272] (Supplementary Table 5).

In this regard, Zheng´s laboratory provided a solid proof of concept for rational design of small-molecule inhibitors targeting RASGEF enzymatic activity by using *in vitro* GEF assays and a high throughput screening exchange assay platform to identify benzopyran-based molecules and other compounds capable of directly targeting the GEF enzymatic activity of SOS proteins by binding to specific residues in its catalytic region [282].

On the other hand, most screenings for small-molecule inhibitors have focused on targeting the RAS:SOS complex, searching for inhibitors able to block (i) the formation or stabilization of this complex or (ii) some of its internal conformational and functional dynamic interactions between the SOS and RAS partners [88,272,283]. In this regard, when evaluating the outcome of this type of screenings it is relevant to ascertain (i) which one of the three possible regions of the complex (RAS moiety, SOS moiety or the RAS:SOS interphase) is bound by the small-molecules identified, (ii) the chemical nature of these compounds, and (iii) whether or not the binding to the complex is reversible or irreversible. The commonly accepted wisdom suggests that the most efficacious inhibitors from the clinical point of view are covalent inhibitors binding irreversibly to specific mutant RAS proteins in the complex [42,284,285].

Very different chemical structures have been identified over the years in the multiple screenings performed by various laboratories in search of small molecules able to inhibit SOS-RAS functional interactions within the SOS:RAS complex. For example, as a result of some of the early screenings, various D-arabinose-derived compounds [286] as well as different andrographolide derivatives [287] or bisphenol compounds [288] were identified that were capable of inhibiting SOS1-mediated nucleotide exchange in the complex by binding to a site near to switch-II region of RAS proteins. Interestingly, bisphenol A has been recently demonstrated to induce increase of SOS1 expression and proliferation in human uterine leiomyoma cells [289]. Likewise, other screens have identified structurally different Indole- and sulfonamide-derived compounds that bind to the same pocket site in the SOS:RAS complex and specifically inhibit SOS-mediated RAS activation [290,291].

Covalent compounds that act irreversibly are the most potent inhibitors able to restrain SOS:RAS interaction. Consistent with this view, a X-ray crystallography-based analysis of an extensive collection of compounds binding to the RAS:SOS complex led to the discovery of three distinct fragment binding sites on this complex. Interestingly, the compounds binding in the inner regions of SOS or in the SOS:RAS interface did it reversibly and were not sufficiently potent inhibitors. In contrast, the NEM-based compounds binding in the RAS part of the complex were shown to produce covalent modification of Cys118 and result in effective inhibition of the exchange of GDP for GTP in the WT and mutant (G12C and G12V) RAS proteins [284].

Interestingly, small-molecule compounds have also been reported that bind to the RAS:SOS complex and increase the rate of SOS-mediated nucleotide exchange. X-ray crystallography analysis reveals that these compounds bind in a hydrophobic pocket in the CDC25H domain of SOS that is adjacent to the Switch II region of RAS in the SOS:RAS complex [292,293]. Mechanistically, these SOS1 agonists elicit biphasic modulation of ERK phosphorylation and simultaneous inhibition of AKT in treated cells [133,294–297], further highlighting how a SOS pocket may be exploited to modulate RAS signaling.

The most clinically effective *in vivo* inhibitors initially identified belonged to the wide family of compounds capable of binding irreversibly in a previously unrecognized switch II pocket to oncogenic RAS^{G12C} mutant proteins that were initially discovered in Shokat´s laboratory [298] and later developed and improved refined further in his own lab and a number of other laboratories and pharma companies [42,285]. Different mutation-specific compounds targeting G12C (including ARS853, AMG510, 2C07, MRTX849, etc) have produced successful *in vivo* results, demonstrating their antitumor activity and therapeutic benefit in various preclinical and clinical tests [45,46,48,50,299,300].

Furthermore, while the search remains active for inhibitors targeting other specific RAS oncogenic mutations, like G12D or G12V, recent reports have described the discovery of novel small-molecule inhibitors capable of selectively inhibiting the functional interaction of SOS1 with all different RAS isoforms, thus making it possible to simultaneously target all active and inactive forms of KRAS *in vivo* [42] (Supplementary Table 5). Mechanistically, one of these new direct inhibitors (BI-2852) has been shown to bind with nanomolar affinity to a newly named switch I/II pocket of RAS in both the ON and OFF states, thus being able to block interaction with all activating partners and effectors, as well as inducing nonfunctional dimers of KRAS [47,49,52]. In contrast, other recently described inhibitors (including BAY293, BI-3406, BI-170192, etc) have been shown to bind directly to the SOS partner of the RAS:SOS complex and to be able inhibit the growth of multiple, different KRAS G12 and G13 mutant tumor cell lines [44,51] (NCI trial NCT04111458). The possibility of future development of inhibitors able to discriminate between SOS1 and SOS2 may also offer additional therapeutic flexibility and possibilities to the arsenal of small molecules regarding *in vivo* antitumor action.

7. Concluding remarks

We summarized in this review a collection of experimental evidence that together support the identification of SOS1 and SOS2 as the most universal and widely expressed family of GEF activators of the members of the RAS family of proteins (HRAS, NRAS, KRAS) in metazoan cells that are also able to activate RAC1 in certain contexts. In this regard, it is evident that the SOS GEFs play a central regulatory role in RTK-RAS/RAC-ERK signaling in all metazoan cells.

We described initially the very high homologies exhibited by SOS1 and SOS2 with regards to the structural and regulatory aspects of their encoding genes and protein products. However, despite those homologies, most functional studies have demonstrated the functional predominance of SOS1 over SOS2 in most physiological and pathological contexts, although some residual, partial functional redundancy was still observable in different experimental settings.

Interestingly, a series of activating, gain of function mutations, occurring preferentially in *SOS1* genes, have been found in association with various inherited RASopathies and with different sporadic cancers. The fact that these pathological conditions share the dysregulation of RAS-ERK signaling as a common, underlying mechanistic feature indicates that SOS GEF function is critical, not only for regulation of physiological RAS signaling in normal cells but also for pathological, oncogenic signaling in tumors.

In this regard, the specific association of SOS1 mutations with distinct RASopathies and/or tumors, as well as the observation that SOS1 depletion blocks carcinogenesis in animal models, support the

consideration of SOS1 as a potential marker and/or therapy target in those pathological conditions. On the other hand, the central regulatory role played by SOS GEFs in RAS signaling, together with the seminal demonstration that SOS-mediated activation of WT RAS is critical for tumorigenic development in mutant RAS-driven tumors, expands the usefulness of SOS GEFs as potential therapy targets. In this regard, this potential usefulness would apply not only to the low percentage of RASopathies and tumors bearing gain-of-function SOS mutations but also to all RAS-mutant tumors or RTK-dependent tumors that depend on the activation of WT RAS by SOS for their development.

Happily, a number of small-molecule, specific SOS inhibitors have been recently described and shown high promise in preclinical and clinical tests, thus raising hope that the possible modulation of SOS function by means of these inhibitors may help with the clinical management of RAS-driven and even RTK-driven tumors. Furthermore, given the partial functional redundancy exhibited by SOS1 and SOS2 in different biological settings, it is likely that the eventual development of specific SOS1 or SOS2 inhibitors may significantly enrich the possibilities of clinical intervention for the management and therapy of different types of RAS-dependent tumors.

Exciting times certainly lie ahead to try and prove the anticancer effectiveness of small-molecule SOS1 and/or SOS2 inhibitors used alone or in combination with other inhibitors acting at different points of the RAS-ERK pathway or other oncogenic pathways.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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