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Understanding SOS (Son of Sevenless).

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Key words: Son of Sevenless. Ras. Guanine Nucleotide Exchange Factors. Rac Guanine Nucleotide Exchange Factors. Noonan Syndrome. Dioxin. Carcinogenesis.

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Abbreviations

AhR: Aryl hydrocarbon Receptor

Cdc 25: Cell division cycle 25

CFC: Cardio-Facio-Cutaneous Syndrome

DH: Dbl homology

E3b1: Eps8 SH3 domain binding protein 1

Eps8: EGFR pathway substrate 8

ERK: Extracellular signal-Regulated protein Kinase

Grb2: Growth factor receptor bound 2

FAK: Focal Adhesion Kinase

MAPK: Mitogen Activated Protein Kinase

MEK: MAP ERK Kinase

NS: Noonan Syndrome

PH: Pleckstrin Homology

PTB: Phosphotyrosine Binding

PTPN: Tyrosine-protein phosphatase non-receptor type 1

PxxP: Proline rich domain

REM: Ras Exchange Motif

SOS: Son of Sevenless

TCDD: 2,3,7,8-TetraChloroDibenzoDioxin

TGF: Tumor Growth Factor

Abstract

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6 Son of Sevenless (SOS) was discovered in *Drosophila melanogaster*. Essential for normal eye
7 development in *Drosophila*, SOS has two human homologues, SOS1 and SOS2 (Figure 1C).
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9 The *SOS1* gene encodes the Son of Sevenless 1 protein, a Ras and Rac guanine nucleotide
10 exchange factor. This protein is composed of several important domains. The CDC25 and
11 REM domains provide the catalytic activity of SOS1 towards Ras and the histone fold DH/PH
12 (Dbl homology and Pleckstrin homology) domains function, in tandem, to stimulate
13 GTP/GDP exchange for Rac. In contrast to Ras, there have been few studies that implicate
14 SOS1 in human disease and, initially, less attention was given to this gene. However,
15 mutations in *SOS1* have been reported recently in Noonan syndrome and in type 1 hereditary
16 gingival fibromatosis. Although, there have been very few studies that focus on the regulation
17 of this important gene by physiological or exogenous factors, we recently found that the *SOS1*
18 gene was induced by the environmental toxin, dioxin, and that this effect was mediated by the
19 aryl hydrocarbon receptor (AhR). These recent observations raise the possibility that
20 alterations in the expression of the *SOS1* gene and, consequently, in the activity of the SOS1
21 protein may affect toxicological endpoints and lead to clinical disease. These possibilities,
22 thus, have stimulated much interest in SOS1 recently. In this article, we review the functions
23 of SOS1 and the evidence for its roles in physiology and pathology across species.
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Discovery of Son of Sevenless (SOS)

The work of Bonfini *et al* on drosophila eye development led to the discovery of the SOS (Son of Sevenless) protein [1]. During eye development, a cluster of eight photoreceptor neurons autonomously develops in each ommatidium [2, 3]. An ommatidium contains 8 photoreceptor cells (R) surrounded by support cells and pigment cells. The ommatidium is the functional unit of the drosophila eye and it is composed of a majority of R cells (photosensing cells) with one central R8 cell. These cells are the first to express neuronal markers [4-6]. Within the ommatidium, R8 directly induces a neighboring cell to develop into the R7 photoreceptor neuron. This induction is mediated by Bride of Sevenless protein (Boss), a transmembrane protein localized on the surface of R8 cells. Boss binds and activates sevenless (Sev), a tyrosine kinase receptor expressed by R7 on its cell membrane [7]. These two proteins are essential for R7 cell development and ultimately lead to Ras1 activation [8]. In fact, a genetic screening study in *Drosophila* has shown that seven genes are essential for Sevenless signaling among which are a Ras protein and a protein homologous to *S. cerevisiae CDC25* [9], which is known to promote exchange of guanine nucleotide phosphate bound to Ras [10]. The interaction between Sev and Ras1 is not direct and requires additional factors including Son of Sevenless (SOS). SOS has been found to be a critical member of the signal transduction pathway initiated by the Sevenless receptor [11].

SOS homologues were subsequently discovered and characterized in other species. Essential roles for *let 23* (which encodes a putative tyrosine kinase of the epidermal growth factor (EGF) receptor subfamily) and *SOS* homologues have been documented during vulval development, oocyte maturation and uterine differentiation in *Caenorhabditis elegans* [12, 13]. Several laboratories discovered and characterized two murine homologues of *Drosophila* Son of sevenless, *mSOS1* and *mSOS2* [14-16]. The lack of *mSOS1* protein in mice leads to mid-gestation embryonic death related to placental alterations and potentially associated heart failure [17]. On the other hand, lack of *mSOS2* has not be found to be associated with abnormalities of mouse growth and development [18].

Thus, the *SOS1* gene is essential for normal development in fruit fly, nematode, mouse and man.

Structure and Function of Son of Sevenless 1

Two human SOS homologues, hSOS1 and hSOS2 (Figure 1C), have been identified [19]. These genes map to 2p22-->p16 and 14q21-->q22, respectively, in the human genome. SOS1 is well conserved in drosophila, mice and humans. There is a 30% amino acid homology between hSOS1 and Drosophila SOS, 65% between hSOS1 and mouse SOS1 and a 70% homology between human SOS1 and SOS2 (figure 1A).

The hSOS 1 protein (150 kDa) is composed of 1300 amino acids with several domains and it functions as a Ras and Rac guanine nucleotide exchange factor [20]. Ras proteins are small monomeric proteins from the G protein family, which bind GTP. The conversion of the inactive GDP bound form of Ras to the active GTP bound form is promoted by guanine nucleotide exchange factors like CDC25 and SOS1.

The C-terminal segment of hSOS1 has a proline-rich domain (PXXP). This domain interacts with the SH3 domains (Src Homology 3) of proteins such as Grb2 (Growth factor receptor bound 2, Figure 1B) for Ras and E3B1 for Ras [21].

In its central segment, SOS1 has two domains, REM (Ras Exchanger Motif) and CDC25 (cell division cycle 25). CDC25 activates Ras in yeast and promotes nucleotide exchange on Ras proteins. This catalytic site has a hairpin structure, stabilized by REM alpha helices, forming a hydrophobic pocket. The REM domain contains an activating allosteric site which binds Ras-GTP leading to additional stimulation of the CDC25 domain and potentiating the Ras-GTP exchange stimulated by SOS1.

The N-terminal segment contains two tandem histone folds, the Dbl homology (DH) and the Pleckstrin Homology domain (PH). The two histone folds have two roles. First, they inhibit exchange activity for Rac1 by binding to the PH domain [21] and second, they are responsible for interaction with the cell membrane [22]. The catalytic DH domain allows the activation of Rac, which, classically, is described as a regulator of cell morphology and the cytoskeleton via its connections with actin filaments. The PH domain regulates the DH domain, but the associated mechanisms are still unknown. In addition, both the DH and PH domains inhibit the activation of Ras.

Regulation of the Ras and Rac Pathways by Son of sevenless 1

Although the role of SOS1 as a nucleotide exchanging factor important for the activation of Ras and/or Rac has been explored extensively, the balance of the activation between Ras and Rac is still not well understood.

Ras pathway

Active Ras plays a fundamental role in growth and cellular differentiation and the Ras pathway is involved in the aberrant regulation of malignant transformation. The active form of Ras activates the RAF1/Mitogen-Activated Protein Kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signaling pathway (figure 2A), which is crucial for cell proliferation, transformation, survival and mobility. Overactivation of this pathway is very often described in carcinogenesis [23-26].

Activation of the Ras and MAPK pathways can be initiated by tyrosine kinase receptors (insulin receptor, EGFR, PDGFR) [27]. In addition to tyrosine kinase receptors, B cell, T cell and Monocyte colony-stimulating factor receptors are able to activate Ras through the SOS pathway [28]. When a ligand (for example, EGF or Epidermal Growth Factor) binds to its transmembrane tyrosine kinase receptor (EGFR), it activates the auto-phosphorylation of the receptor. The adaptator protein, Grb2 (Growth factor receptor-bound protein 2) via its SH2 (Src Homology 2) domain, binds this phosphotyrosine (figure 2B) [29, 30]. Grb2 is a small ubiquitously expressed protein (25 kDa), which has a central SH2 domain flanked by 2 SH3 domains. It can interact directly with RTK (EGFR or Hepatocyte Growth Factor Receptor) or indirectly by the intermediate of docking proteins (FRS2 for Fibroblast Growth Factor Receptor signaling or IRS for Insulin Receptor signaling). It can also interact with non-RTK proteins such as Focal Adhesion Kinase [31]. Moreover, Grb2 is highly conserved throughout evolution and it is essential for development. Grb2 knock out mice die at early embryonic stage [29]. The binding of Grb2 to a RTK triggers the recruitment of cytosolic SOS1 to the cell membrane. The SH3 domain of Grb2 binds to the proline-rich C-terminal domain of SOS1, a phenomenon which is a prerequisite for the activation of Ras [32] (figure 2C). Two domains are essential for SOS1 membrane relocalization: the proline-rich C-terminal domain and the N-terminal PH domain, which interacts with the phosphatidic acid that has been generated by phospholipase D2-activation by growth factors [33]. SOS1 relocalization to the cell membrane, enables its interaction with Ras. As previously noted, the

1 interaction with Ras involves two domains of SOS1: the CDC25 domain which has the active
2 site for the nucleotide exchange and the REM domain which contains a recently identified
3 allosteric site responsible for the release of the autoinhibition of the DH-PH domains and
4 which activates the tandem histone folds [34]. Allosteric activation of SOS1 by Ras occurs
5 through a rotation of the REM domain that is coupled to a rotation of a helical hairpin at the
6 SOS1 catalytic site. This motion relieves the steric hindrance of the catalytic site, allowing
7 Ras binding and nucleotide exchange (figure 2C) [35].

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12 The activity of this signaling pathway is ultimately controlled by feedback
13 mechanisms targeting SOS1. A serine/threonine phosphorylation of SOS1 in its C-terminal
14 domain by Mitogen Activated Protein Kinase (MAP Kinase) has been described which may
15 alter its association with Grb2 and inhibit SOS1 function [36-38]. Further, the binding of an
16 antagonist (p27 KIP1, SPROUTY 1 to 4) to Grb2 prevents its binding to SOS1 [39].
17 Oxidative stress also has been shown to inhibit the EGFR signaling pathway by activating the
18 binding of p66(SHC) to EGFR and Grb2 and promoting the dissociation of Grb2 from SOS1
19 [40].
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29 *Rac pathway*

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31 Rac proteins belong to the RHO family. Active Rac forms are also bound to GTP.
32 These proteins play a role in the reorganization of the actin cytoskeleton (figure 2B).
33 Moreover, Rac activates c-Jun N-terminal kinase (JNK) [41, 42] via the recruitment of a
34 scaffold protein POSH (plenty of SH3) which, in turn, activates MKK 4 and 7 and the MAPK
35 pathway [43]. JNK is also involved in cell proliferation, survival, differentiation and
36 apoptosis. Thus, some of the functions of Rac might be redundant with those of Ras.
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42 SOS1 is a Rac nucleotide exchange factor which stimulates the reorganization of the
43 actin cytoskeleton (ruffling membranes, lamellipodia protrusions), cell invasion and
44 migration. The partners of SOS1 in this pathway are different from those involved in the Ras
45 pathway. Upon Rac activation, SOS1 associates with the cytoplasmic EPS8-E3B1 proteins
46 (Figure 2D). The EGFR pathway substrate 8 (EPS8), a substrate of the EGFR kinase, contains
47 three major domains: an N-terminal phosphotyrosine binding (PTB) domain, a central SH3
48 domain and a C-terminal "effector region" domain. The effector domain enables EPS8
49 cellular localization, possibly through its interaction with distinct sets of actin regulatory
50 complexes, and allows Rac activation. The EPS8-SH3 domain binds a PxxP domain of E3B1,
51 the SH3 domain of which binds the PxxP domain of SOS1 [44]. The EPS8-E3B1-SOS1
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complex, linked to actin filaments through EPS8, activates Rac (upon binding with the CDC25 domain) and subsequently, c-JUN N-terminal kinase and MAPK (see above) enabling actin cytoskeleton remodeling. Other membrane receptors that are known to regulate cytoskeleton organization and migration have been shown to regulate Rac through SOS1. For example, integrins activate the phosphoinositide 3-kinase (PI 3-kinase) pathway which disrupts the PH-DH interaction in SOS1 and opens the Rac binding domain [45].

Although the Ras pathway has been clearly characterized, the regulation of the Rac pathway by SOS1 is poorly characterized in spite of the fact that it is critical for the regulation of lamellipodia protrusion, cell migration and invasion. E3B1 is a limiting factor since it binds SOS1 as well as Grb2. The regulation of the EPS8-E3B1-SOS1 and SOS1-Grb2 complexes could involve tyrosine kinase receptors. The p66SHC protein (see above) specifically activates Rac, decreasing the formation of the SOS1-Grb2 complex (and the activation of Ras) and increasing the formation of the SOS1-EPS8-E3B1 complex [46].

Human Disorders Associated With Dysfunctions of Son of sevenless 1

Recent clinical studies have indicated a potential implication of SOS1 in both the Noonan syndrome and hereditary gingival fibromatosis type 1.

Noonan syndrome (NS) and cardio-facio-cutaneous syndrome (CFC)

The NS and CFC syndromes are autosomal dominant disorders characterized by cardiac defects, facial dysmorphism, ectodermal abnormalities and mental retardation. The genetic mutations discovered in these syndromes are associated with abnormal Ras/RAF/MEK/ERK signaling [47].

NS patients exhibit facial dysmorphism (broad forehead, downslanting palpebral fissures, hypertelorism, low-set posteriorly rotated ears and a high arched palate), curly hair, short stature, skeletal anomalies and heart defects. They may also have pulmonary artery stenosis, hypertrophic cardiomyopathy, chest abnormalities, mental retardation and genitourinary anomalies. The incidence of the disease is 1/1000 to 2500 [48, 49]. Sixty per cent of NS cases are sporadic [48]. Mutations of the *PTPN11*, *KRAS*, *RAF1* genes and, recently, the *SOS1* gene were found in patients with NS. Mutations of *PTPN11* are the most frequently reported (50%) and they lead to activation of the Ras/MAPK pathway. These mutations are “gain of function” mutations, which are associated with increased risk of leukemia,

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myeloproliferative disorders and solid tumors [50, 51]. *SOS1* mutations are found in 15 to 20% of NS and are affect in Ras/ERK activation. Patients with *SOS1* mutations exhibit a phenotype composed of curly hair and ectodermal abnormalities. Constant activation of the Ras/ERK pathway is due to mutations in *SOS1* principally in two domains, the Pleckstrin Homology (PH) and the Ras Exchanger Motif (REM) domains, which are not implicated in the exchange activity but rather in the intrinsic inhibitory function of *SOS1*. These mutations lead to the maintenance of *SOS1* in a non-inhibited form. This is the first example of an activating GEF mutation associated with human disease [47, 48, 52].

CFC syndrome resembles NS and is characterized by mental retardation, facial abnormalities, cardiac defects and ectodermal abnormalities (sparse hair, dry and hyperkeratotic skin). Mutations responsible for this syndrome involve the *KRAS*, *BRAF*, *MEK1* and *MEK2* genes. Only a few *SOS1* mutations have been reported [53].

Hereditary gingival fibromatosis type 1

Hereditary gingival fibromatosis type 1 is a rare, autosomal dominant, gingival hypertrophy. It is a benign disease resulting in a progressive, non-hemorrhagic enlargement of the gingival mucosa [54]. This pathology is associated with a *SOS1* mutation in the proline-rich C-terminal domain, which normally allows the recruitment of *SOS1* by Grb2 [54]. Interestingly, the mutation induces constitutive *SOS1* membrane recruitment, spontaneous activation of the Ras/MAPK pathway and, thus, cell proliferation. In gingival fibroblasts, there is an increase of the intensity and the duration of the MAPK signal, which favors the transition from G1 to S phase. The reasons as to why the hypertrophy is confined only to the gingival tissue, remain undetermined [55].

In addition to its participation in gingival fibromatosis, increased expression of *SOS1* has been shown to be necessary for gingival healing. *SOS1* overexpression is caused by a cascade of intracellular signals induced by growth factors and cell adhesion receptor like integrins [56].

SOS1 and carcinogenesis

Besides NS and hereditary gingival fibromatosis type 1, a few other clinical manifestations have been related to *SOS1*. Historically, mutations of *SOS1* have not been reported to be pro-oncogenic in NS [51] but recently this has been challenged. In fact, overexpression of *SOS1* is known to activate the Ras pathway and, thus, to promote both proliferation and differentiation [23]. *SOS1*-Grb2 binding and activation of the Ras/MAPK

1 pathway are promoted by ionizing radiation, which ultimately stimulates growth of human
2 breast cancer [57]. The link with carcinogenesis would seem, thus, to be a propitious area of
3 investigation. Denayer *et al* have shown that NS patients with SOS1 mutations sometimes
4 display tumors including embryonal rhabdomyosarcomas, Sertoli cell testis tumors, or
5 granular cell tumors of the skin [58]. Further, the amplification of Grb2 and SOS1 seems to
6 play a role in the etiology of bladder cancer [59]. A recent study has demonstrated that in
7 African-American men, SOS1 expression is enhanced in prostate cancer epithelial cells [60].
8 Similarly, overexpression of SOS1 has been demonstrated in immortalized human kidney
9 cancer cell lines [61]. Involvement of EGFR and SOS1 has been incriminated, also, in non-
10 small cell lung carcinomas and in some skin cancers. Indeed, Sibia *et al* showed that a
11 transgenic mouse model expressing a dominant form of Son of Sevenless (SOS-F) in basal
12 keratinocytes developed skin papillomas with 100% penetrance and demonstrated a cell-
13 autonomous requirement for EGFR [62]. The role of SOS1 has been highlighted, also, in
14 certain hematological malignancies. For example, in chronic myeloid leukemia, Grb2
15 interacts with the fusion protein, BCR-ABL, and prevents its binding to SOS1 [63]. Finally,
16 Moeller *et al* have shown that p27Kip1, a protein that inhibits the association between Grb2
17 and SOS1, often is reduced in aggressive cancers [64]. All these observations provide
18 additional arguments in favor of the involvement of SOS1 in cancer.
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33 34 **Activation of the Expression of Son of Sevenless 1**

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38 SOS1 is known to play a role in different cellular processes. Its germ-line mutations
39 induce a variety of developmental diseases and SOS1 dysfunction and the overexpression of
40 mutant forms of SOS1 are involved in cancer development. Nevertheless, no transcriptional
41 activator of SOS1 had been discovered until recently. Gene expression data from our
42 laboratory and from that of another group [65], derived from rat hepatocytes, have unraveled
43 the potential regulation of SOS1 by an environmental pollutant, 2,3,7,8-
44 Tetrachlorodibenzodioxin (TCDD). TCDD, which is the most studied dioxin, is a highly
45 persistent contaminant of the environment. It was listed in 1997 as a "human carcinogen" by
46 the International Agency for Research on Cancer (IARC). TCDD is the ligand with the
47 highest affinity of Aryl hydrocarbon Receptor (AhR). The toxic effects of TCDD, including
48 tumor promotion and carcinogenesis, are mediated by the AhR. The AhR is a transcription
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1 factor which induces the expression of target genes, such as those encoding xenobiotic
2 metabolizing proteins.

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4 Our microarray gene expression analysis constituted the first attempt to study the
5 regulation of the previously uncharacterized *SOS1* promoter by the AhR and its ligands in the
6 human hepatoblastoma cell line, HepG2 [66]. We found that the levels of *SOS1* mRNA and
7 protein are up-regulated following treatment with TCDD (RT-PCR, Western blot) and that
8 this process occurs at the transcriptional level. Chromatin immunoprecipitation showed that
9 the AhR binds to the *SOS1* promoter and luciferase gene reporter assays showed activation of
10 the *SOS1* promoter following this binding. Thus, TCDD-mediated transcriptional regulation
11 of *SOS1* is related to the rapid, direct binding of the AhR to the *SOS1* promoter.
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18 We also analyzed the consequences of the *SOS1* transcriptional regulation by the AhR
19 on the Ras and Rac pathways. Although we were not able to observe Rac stimulation upon
20 TCDD treatment, we did find that TCDD stimulates Ras activity (enhancement of Ras-GTP)
21 and subsequent ERK1/2 phosphorylation. *SOS1* is critical not only for this stimulation (as
22 shown by RNA interference experiments blocking *SOS1* induction) but also for dioxin-
23 dependent changes in cell growth. This is the first time that *SOS1* overexpression has been
24 associated with increased activity of the Ras pathway. The AhR is able to bind thousands of
25 environmental pollutants including dioxins, furans and polycyclic aromatic hydrocarbons
26 (tobacco smoke, barbecued food, air pollution) and the induction of *SOS1* by this signaling
27 pathway might be critical for the growth of cancer cells (Figure 3). Although this
28 phenomenon does not require a mutant form of *SOS1*, it is clearly in accord with recent
29 studies that suggest a role for *SOS1* in carcinogenesis [58, 60, 62, 67, 68].
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42 **Conclusion**

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45 *SOS1* was identified in the early 1990's as a member of the Ras signaling pathway. It
46 was quickly identified as a Ras and Rac guanine nucleotide exchange factor. Following the
47 complete characterization of the Ras pathway, subsequent studies have focused on the role of
48 Ras as a significant contributor to numerous tumorigenic processes. Also, recent studies have
49 demonstrated *SOS1* dysfunction in several diseases including cancer or cardio-facio-
50 cutaneous syndrome. Gain of function mutations of *SOS1* stabilize the protein thus
51 stimulating the Ras pathway. One recent study also has suggested that overexpression of the
52 normal protein stimulates the Ras pathway and cell growth [66]. This may result from
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1 exposure to an environmental pollutant and stimulation of a pleiotropic xenobiotic receptor,
2 called AhR. Thus, AhR ligands, which are present extensively in the environment, might
3 stimulate cell growth through stimulation of SOS1 expression. Interestingly, this is not the
4 only study indicating a potential link between SOS1 and the AhR. In *Drosophila*
5 *Melanogaster*, Spineless, the AhR orthologue, regulates the development of the ommatidium
6 and the proper institution of color vision by the retinal mosaic [61]. Color vision requires both
7 R7 and R8 photoreceptor cells. As previously noted, this is an interesting parallel with the
8 fundamental role of *Son of Sevenless* in the development of the R7 photoreceptor neuron.
9 Other observations suggest that SOS1 regulation by the AhR could be relevant *in vivo*. Dioxin
10 has been shown to induce developmental abnormalities including cleft palate. This is due to
11 the disruption of the spatial and temporal expression of EGF and TGF β and the dysfunction of
12 their associated downstream pathways [69-71]. Based upon the recent cancer literature
13 describing gain-of-function effects of SOS1, it appears to us that fruitful areas for future work
14 involve the characterization of SOS1 transcriptional regulation and potential stimuli that could
15 enhance the Ras signaling pathway and physiopathological processes.
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Figure legends

Figure 1: Organization of SOS 1 and Grb2 domains. (A) Representation of SOS1 orthologs of different species, from fruit fly (SOS), nematode (*sos-1*), mouse (mSOS1) and human (hSOS1). SOS1 is composed of 7 different domains, each of which possesses, a particular function. (B) Grb2 is a linker protein whose SH2 domain (Src homology 2) binds phospho-tyrosine in tyrosine kinase receptors and whose SH3 domain binds proline-rich domains (for example, in SOS1). (C) Representation of mSOS2 and hSOS2. The numbers above the SOS proteins correspond to amino acids in the primary structure.

Figure 2: SOS1 activates the exchange of GDP and GTP for Ras and Rac and stimulates their associated signaling pathways. (A) The Ras signaling pathway. (B) The Rac signaling pathway. (C, D) Mechanistic representation of Ras activation (C) or Rac activation (D) signaling pathways by SOS1 (see text).

Figure 3: The Aryl hydrocarbon Receptor (AhR) transcriptionally triggers SOS1 and subsequently activates the Ras signaling pathway in HepG2 cells.

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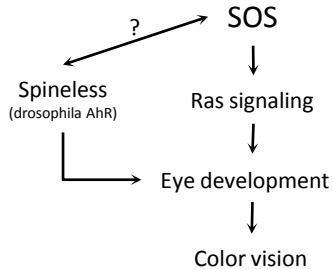
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*Graphical Abstract

Drosophila



Human

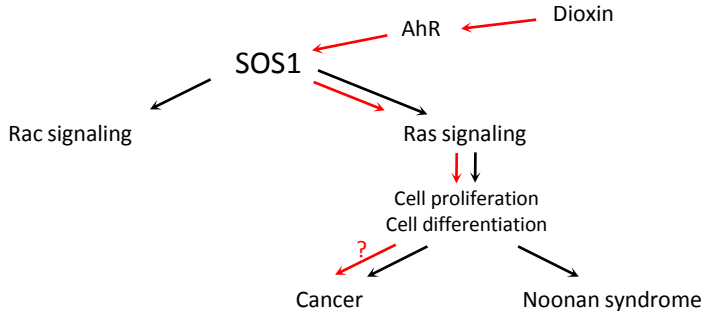


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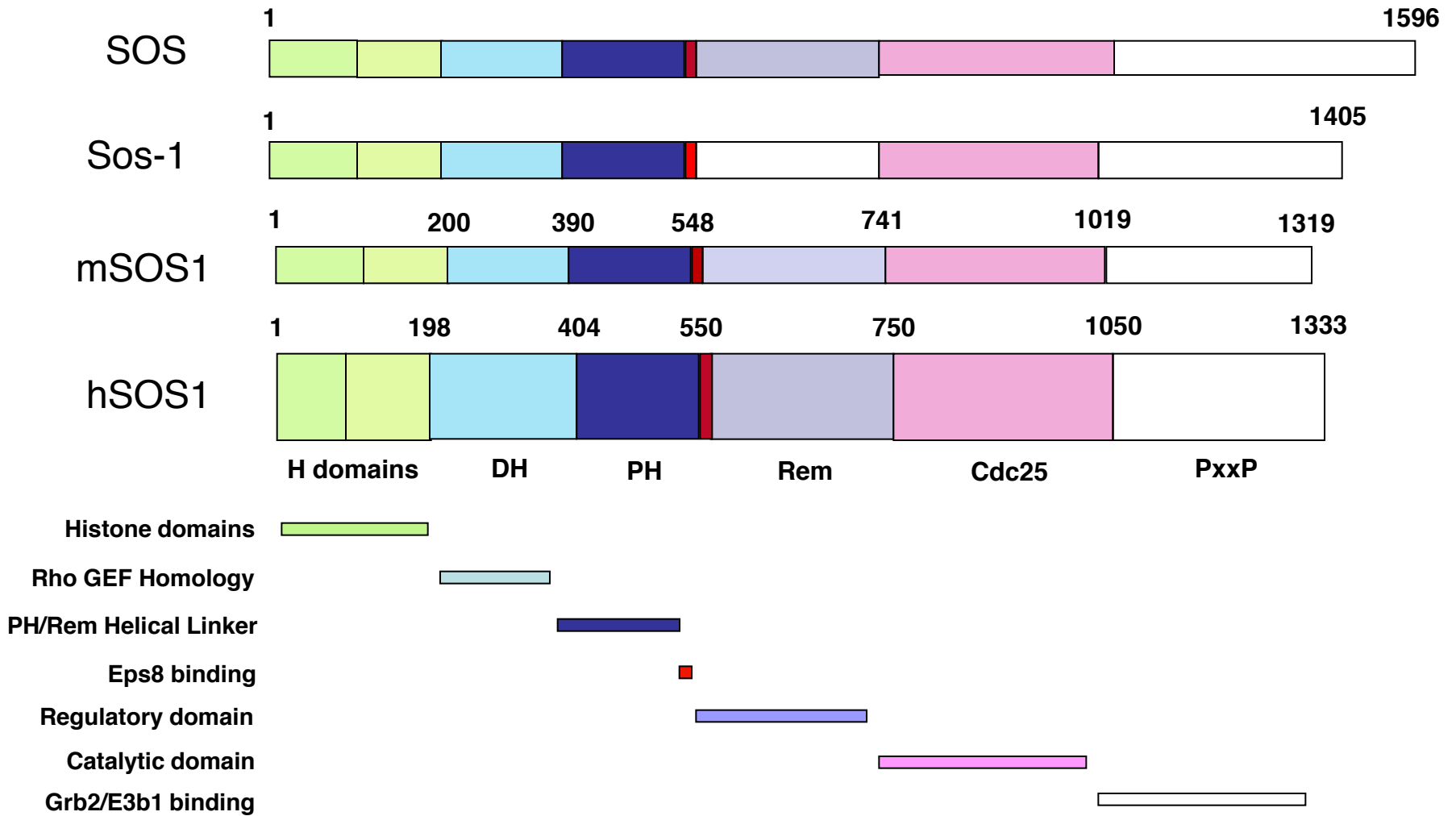


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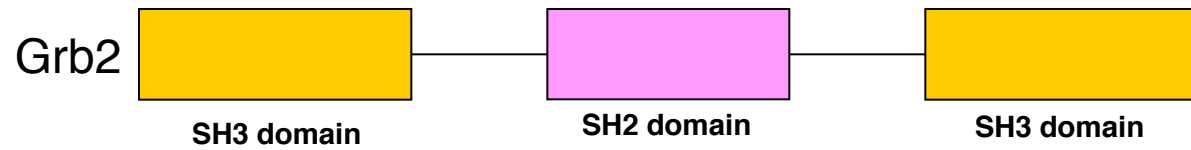


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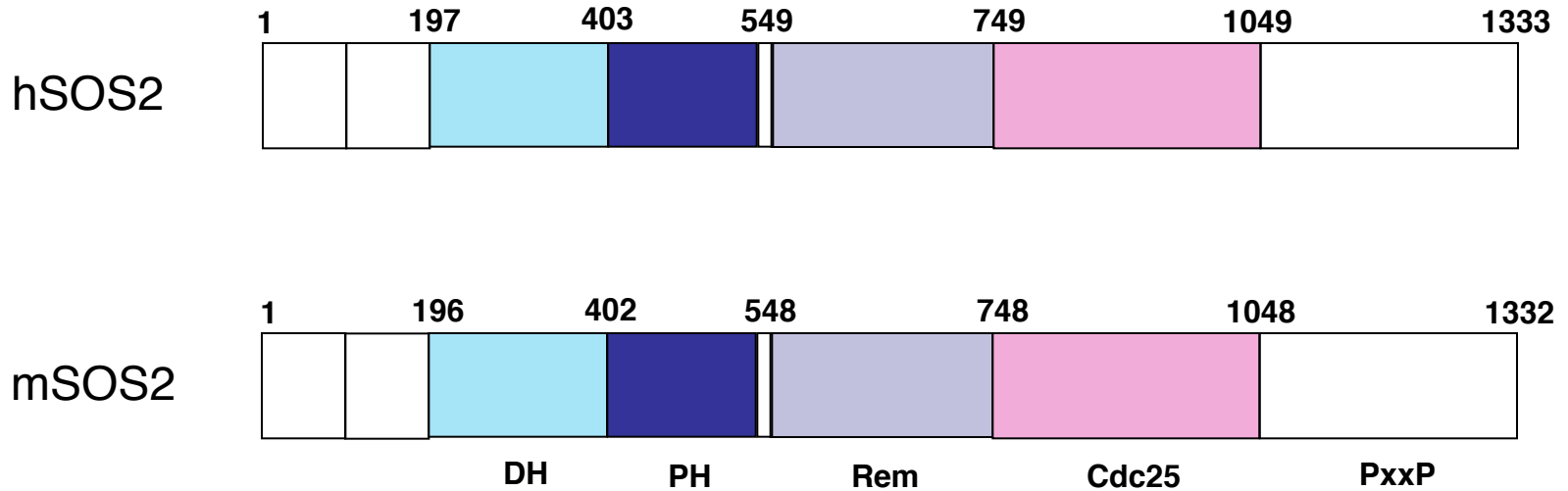


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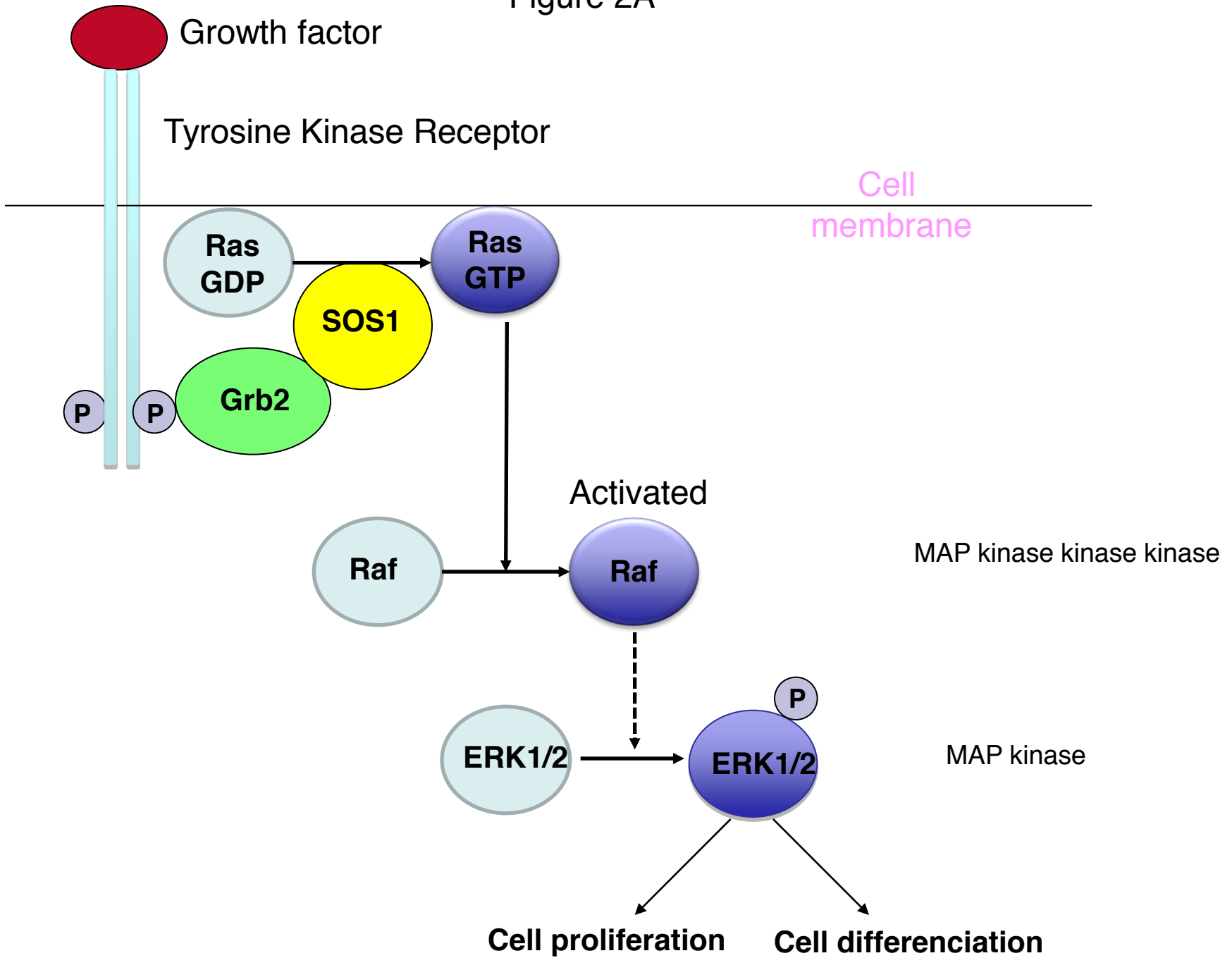


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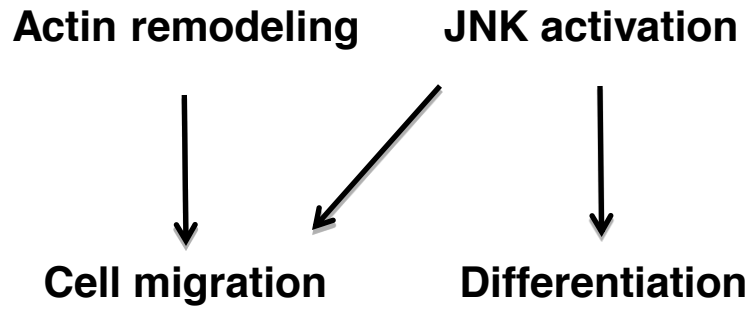
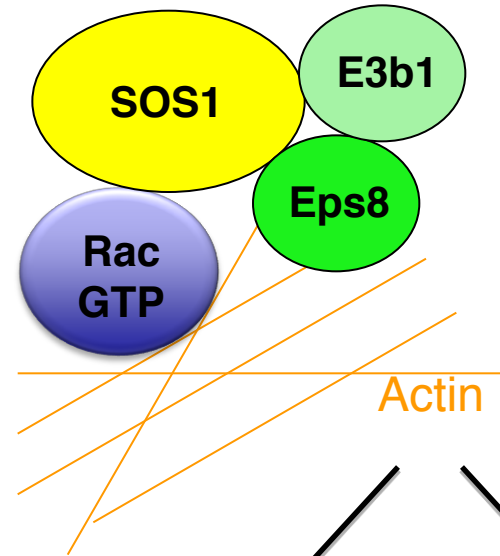
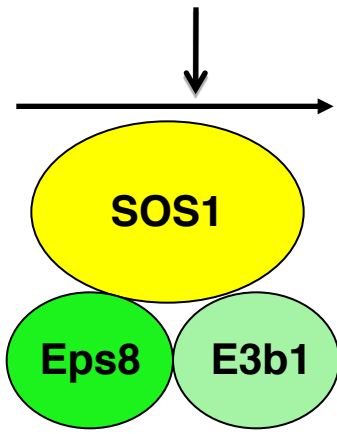
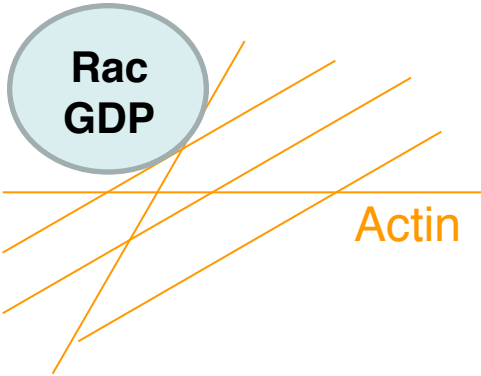
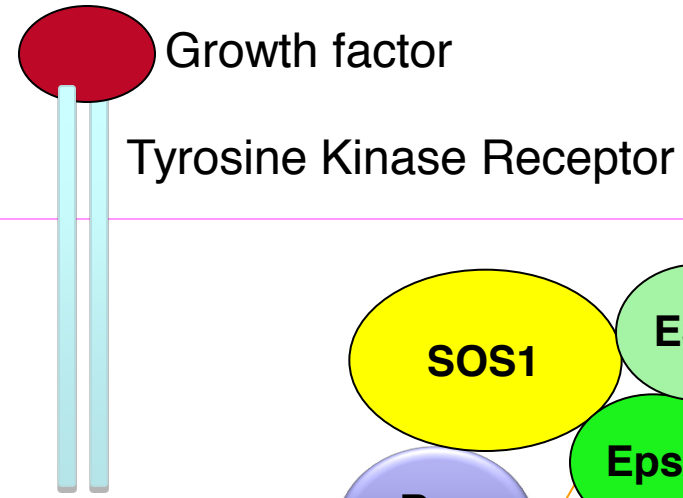


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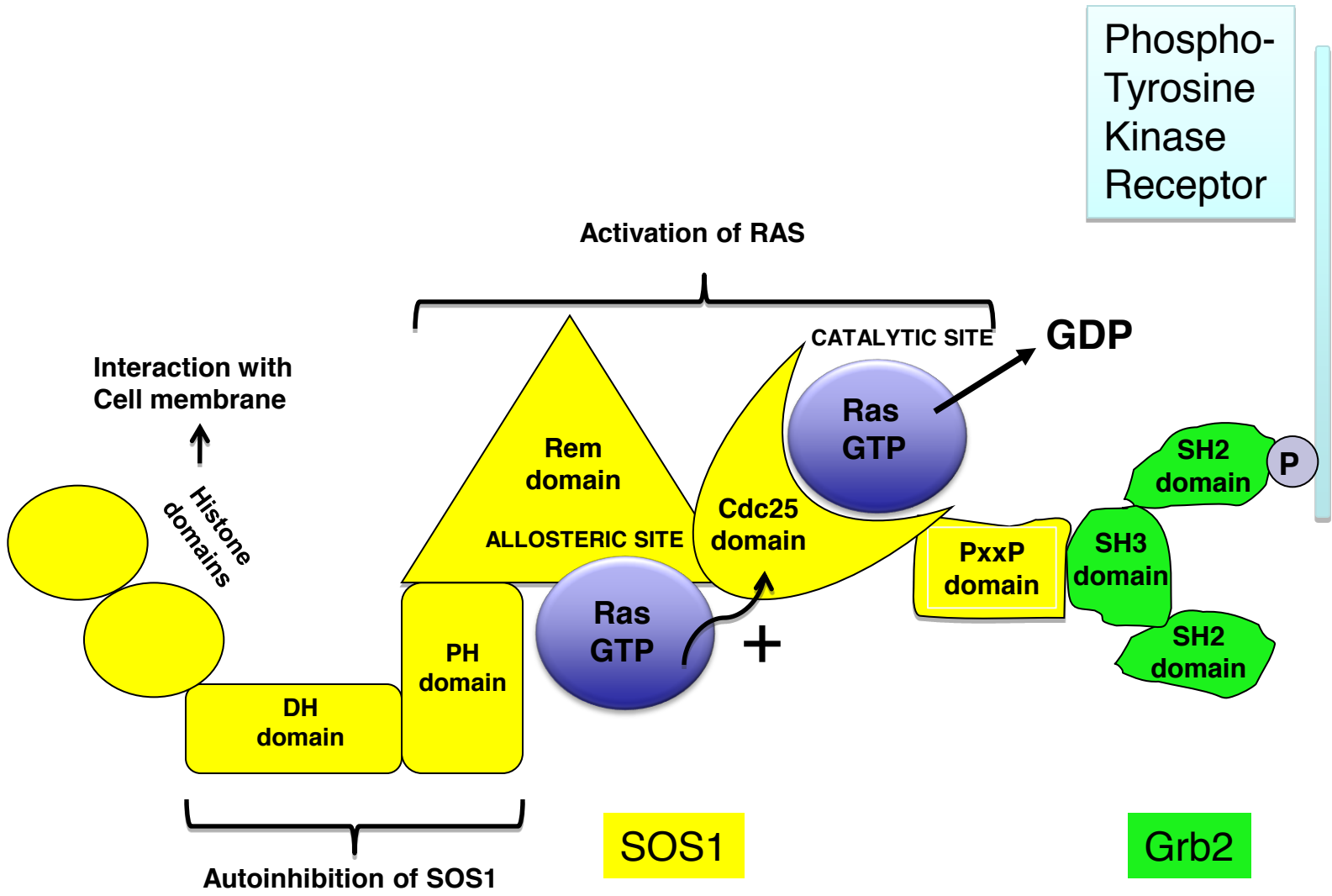


Figure 2D

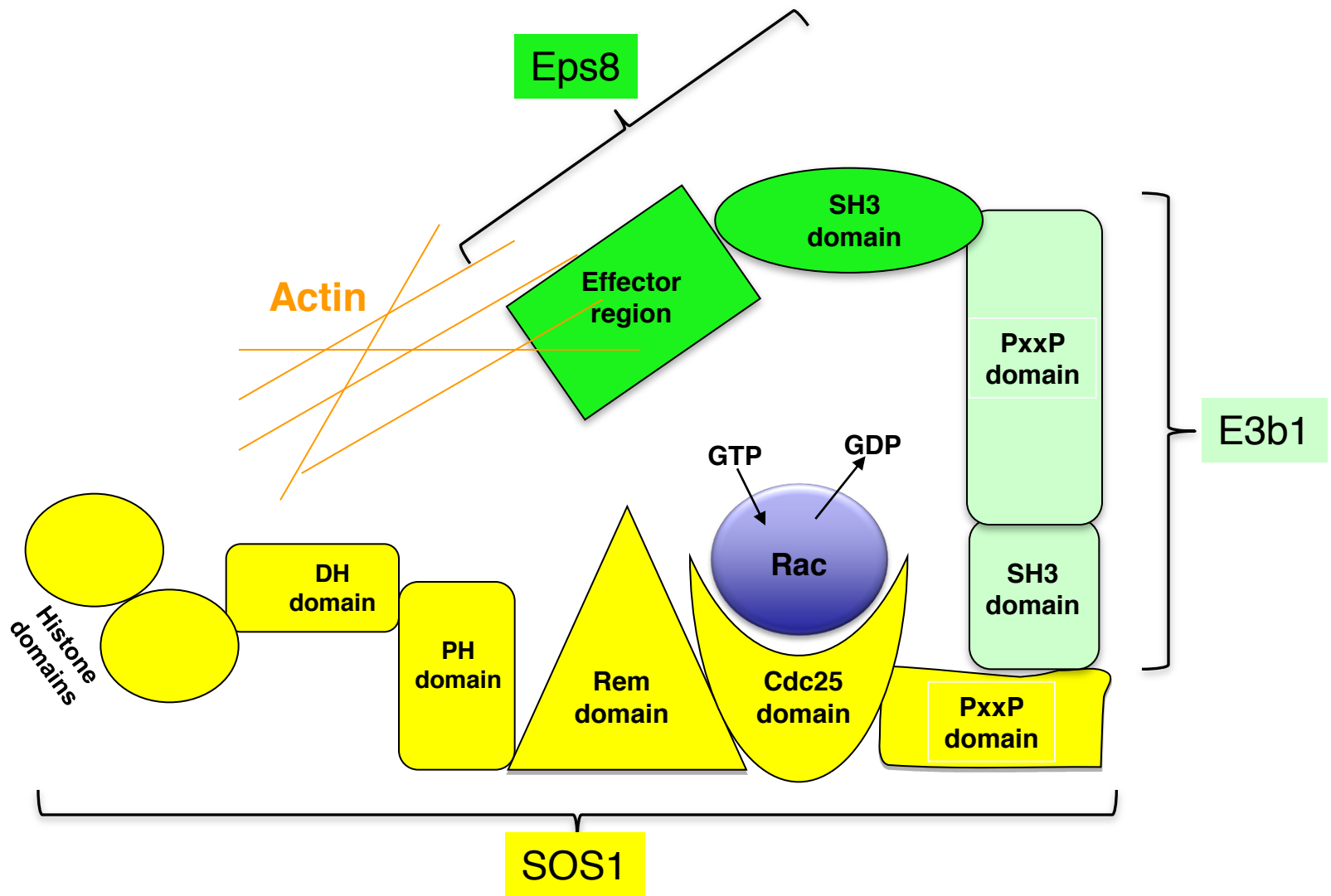


Figure 3

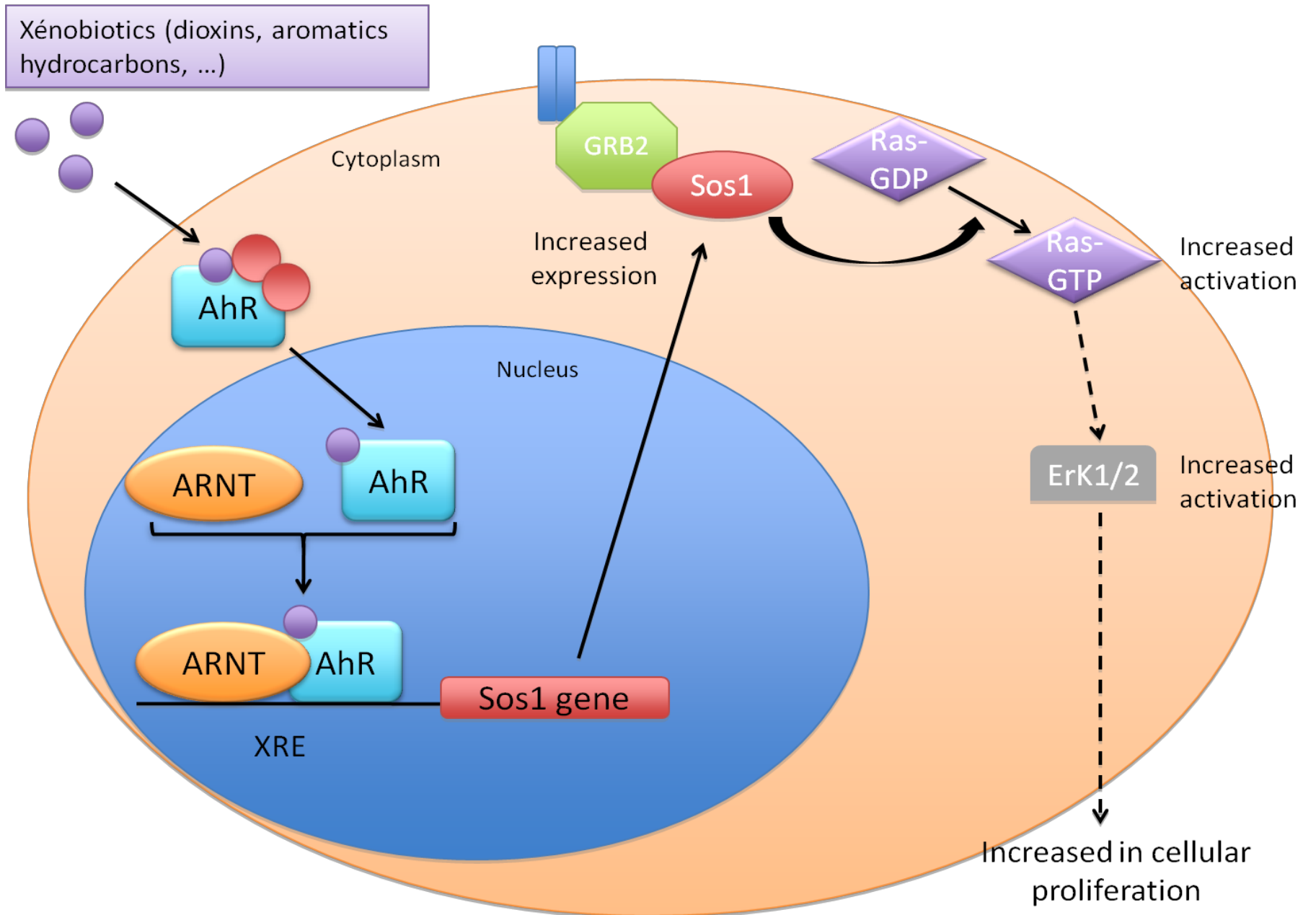


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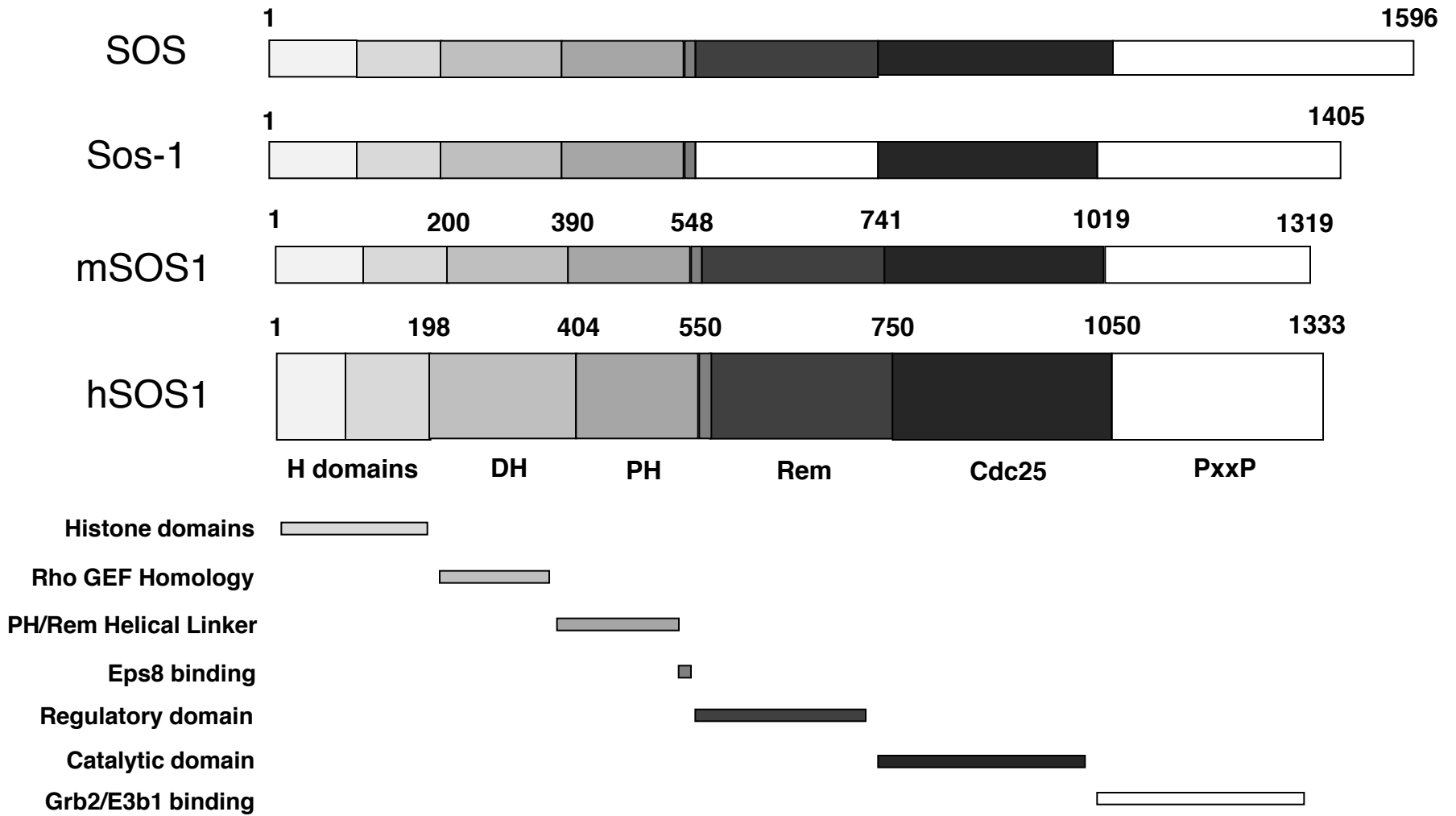


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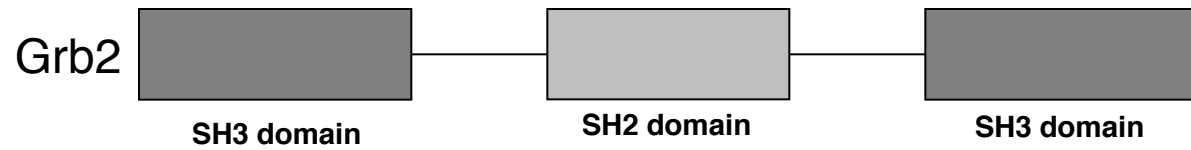


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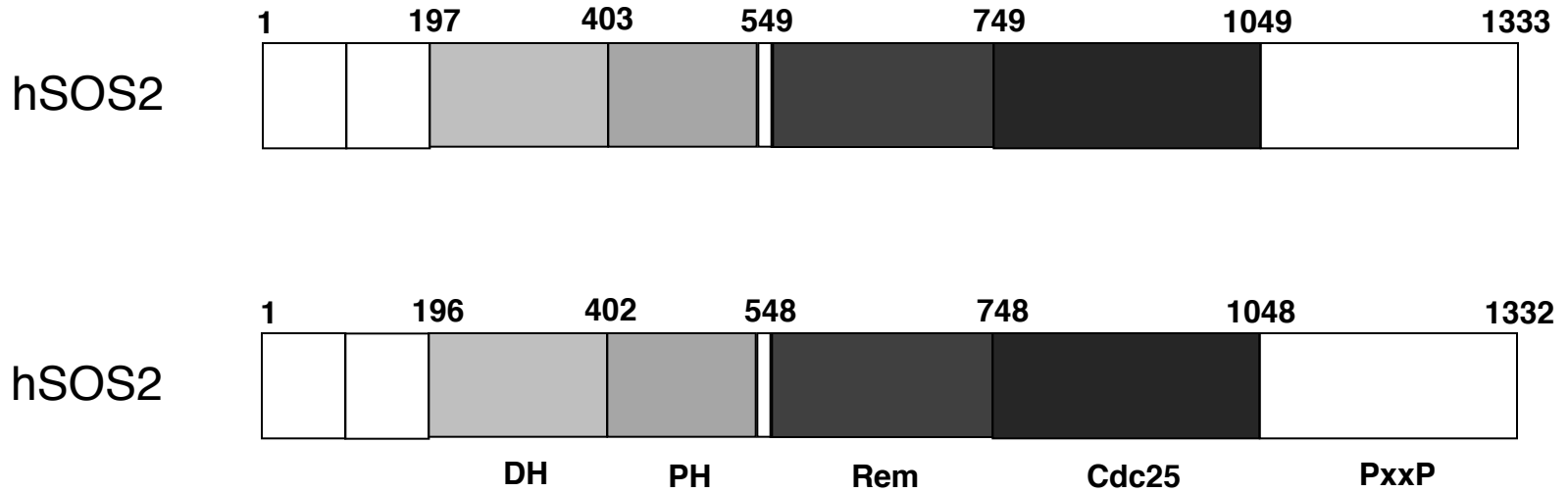


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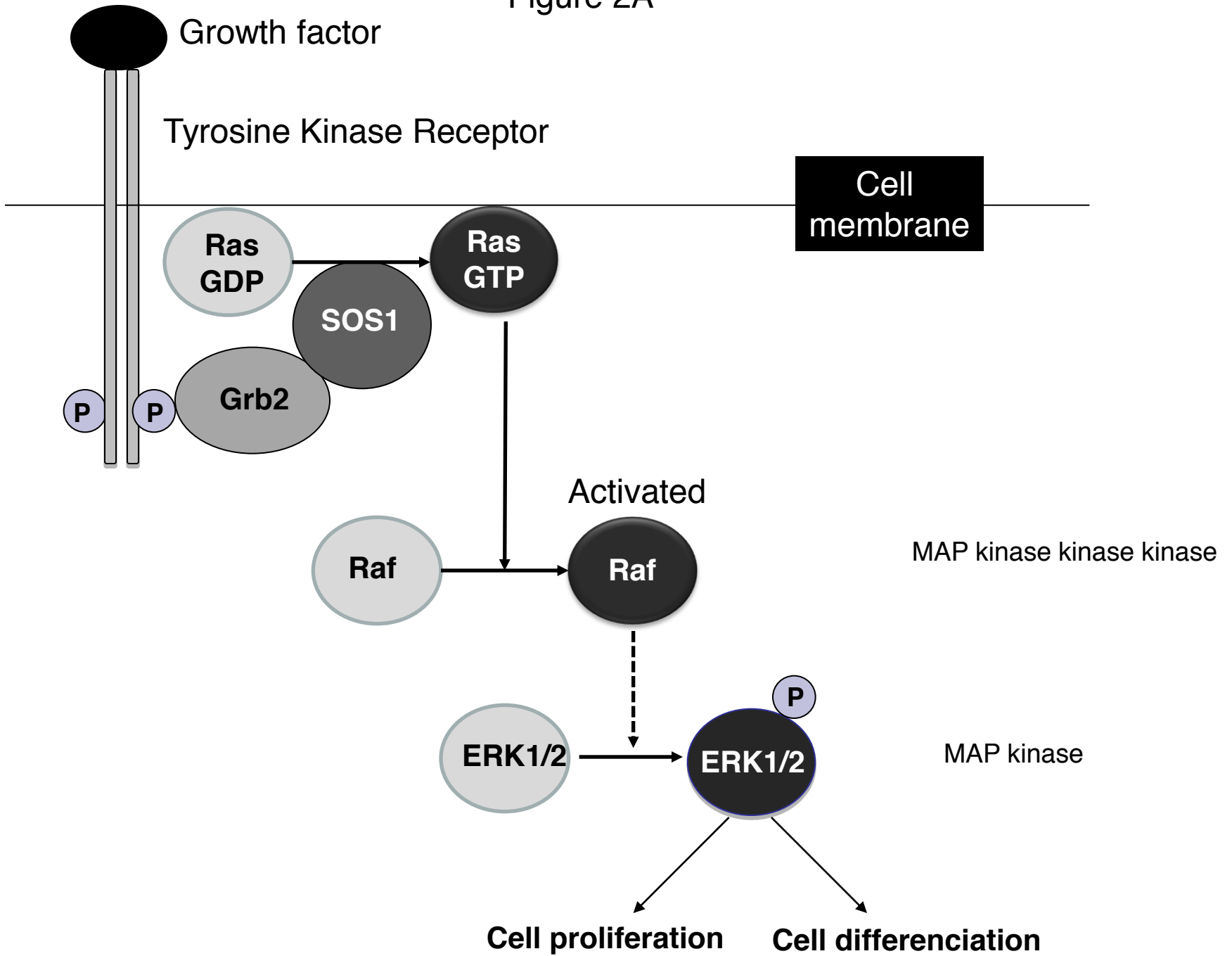


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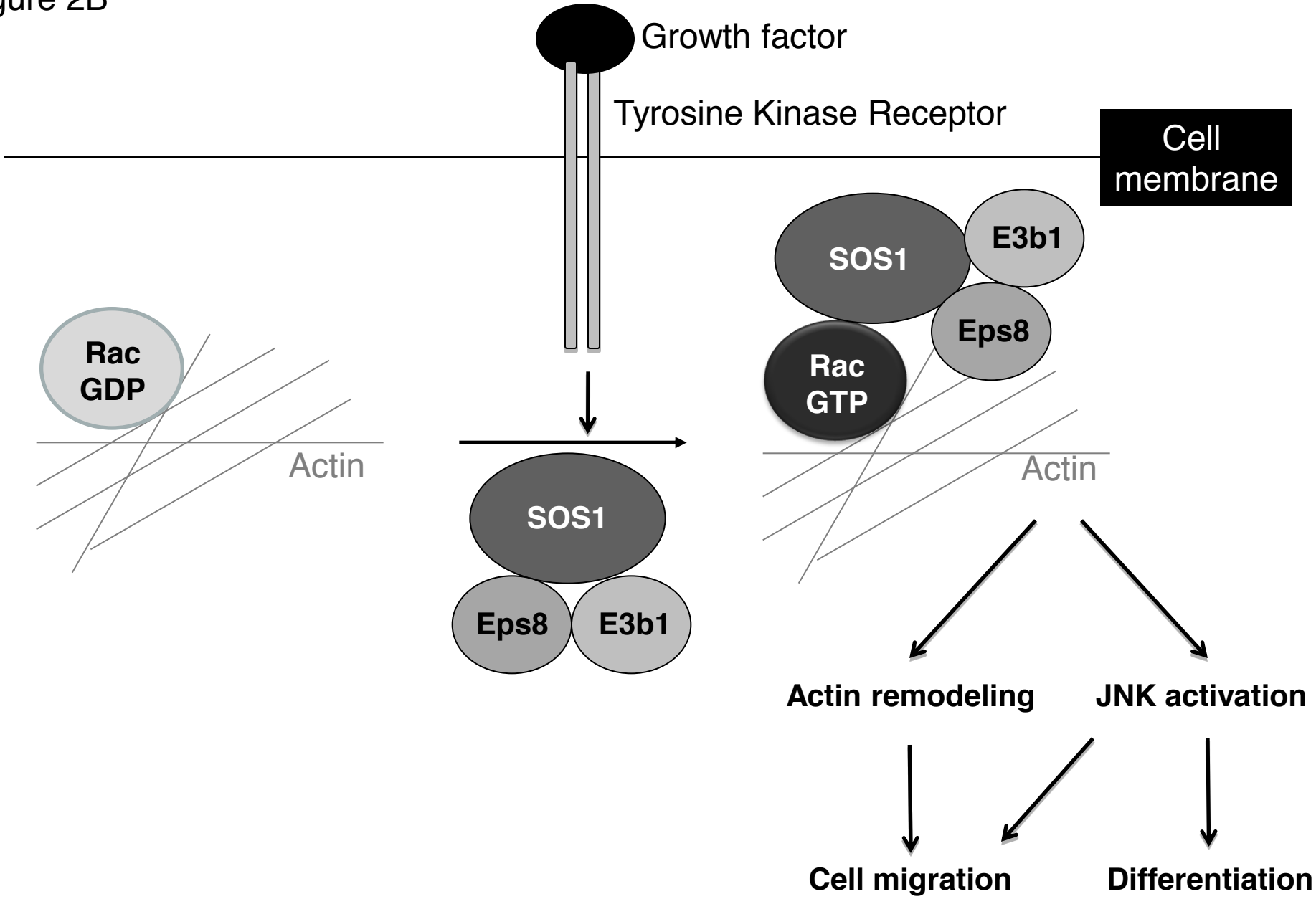


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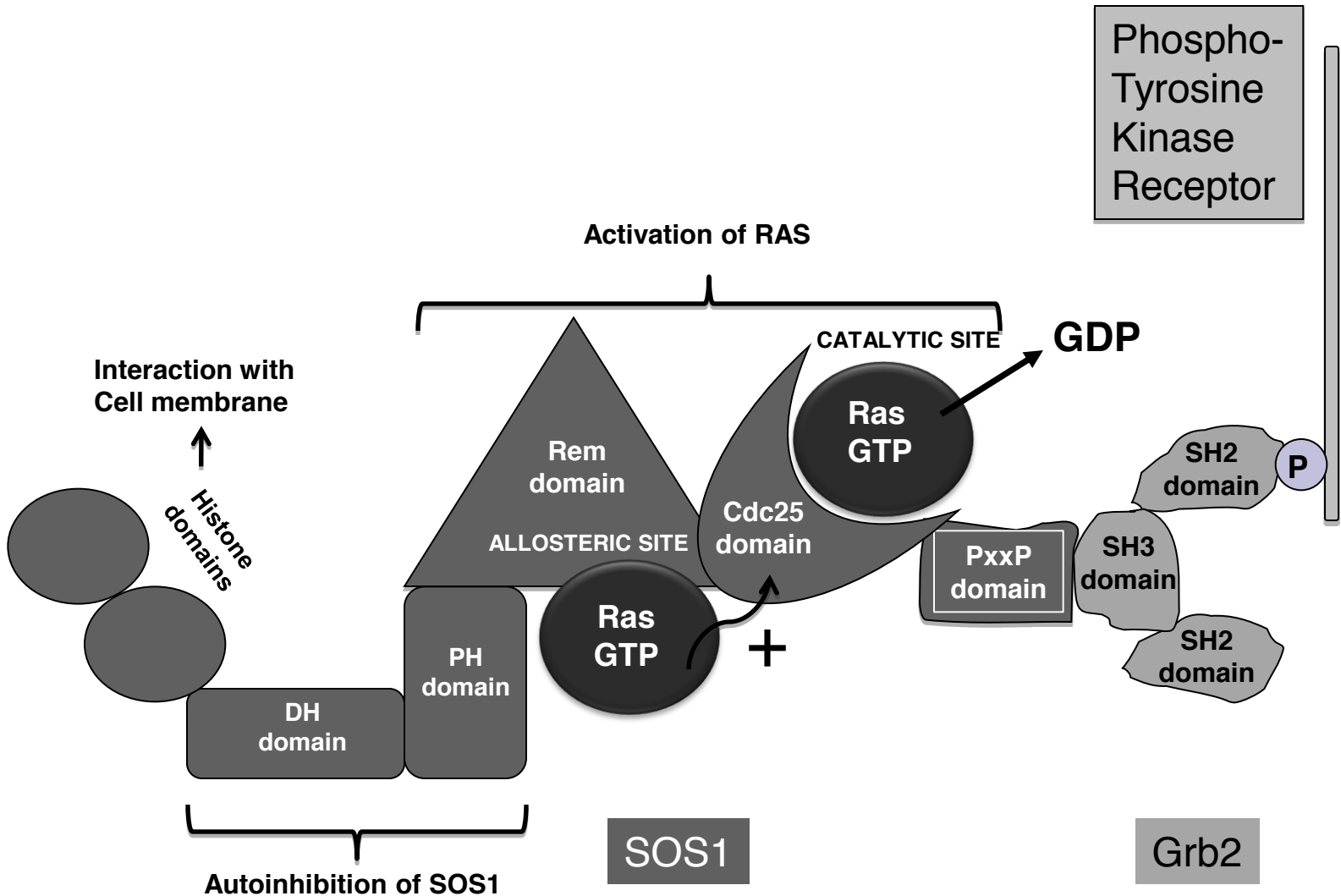


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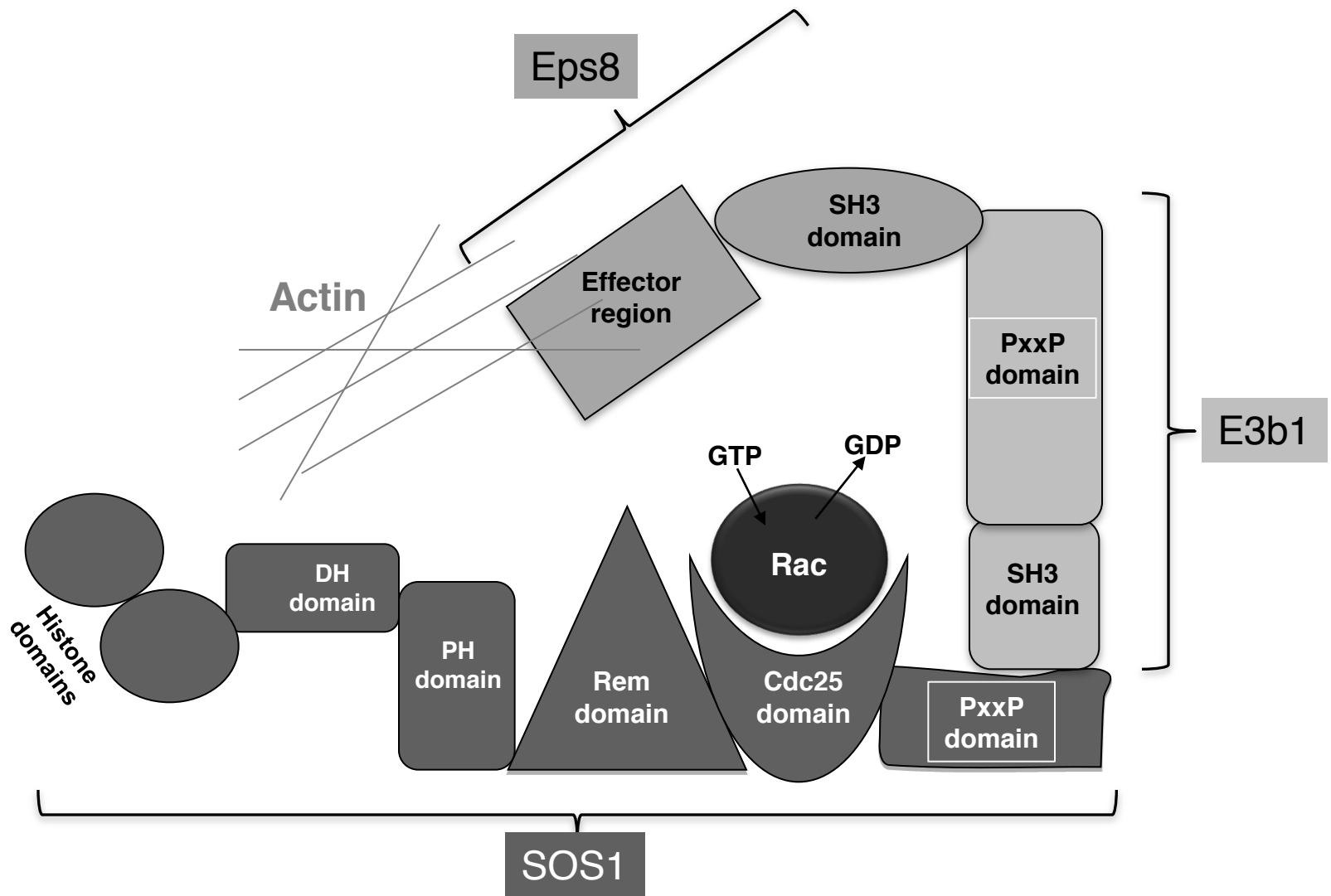


Figure 3

