Cell

Interaction Domains of Sos1/Grb2 Are Finely Tuned for Cooperative Control of Embryonic Stem Cell Fate

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http://dx.doi.org/10.1016/j.cell.2013.01.056

SUMMARY

Metazoan evolution involves increasing protein domain complexity, but how this relates to control of biological decisions remains uncertain. The Ras guanine nucleotide exchange factor (RasGEF) Sos1 and its adaptor Grb2 are multidomain proteins that couple fibroblast growth factor (FGF) signaling to activation of the Ras-Erk pathway during mammalian development and drive embryonic stem cells toward the primitive endoderm (PrE) lineage. We show that the ability of Sos1/Grb2 to appropriately regulate pluripotency and differentiation factors and to initiate PrE development requires collective binding of multiple Sos1/Grb2 domains to their protein and phospholipid ligands. This provides a cooperative system that only allows lineage commitment when all ligand-binding domains are occupied. Furthermore, our results indicate that the interaction domains of Sos1 and Grb2 have evolved so as to bind ligands not with maximal strength but with specificities and affinities that maintain cooperativity. This optimized system ensures that PrE lineage commitment occurs in a timely and selective manner during embryogenesis.

INTRODUCTION

Metazoan signaling proteins frequently contain multiple modular domains, each with individual binding or enzymatic functions. A comparison of eukaryotic proteomes suggests that linking diverse domains in new combinations has yielded novel cellular functions required for metazoan evolution (Jin et al., 2009; Lim and Pawson, 2010). For example, the juxtaposition of distinct interaction domains, as in the case of adaptor proteins, can form new signaling pathways (Howard et al., 2003), whereas combining interaction and catalytic domains can target an enzyme to specific substrates (Mayer et al., 1995) and facilitate intramolecular interactions that regulate catalytic activity (Filippakopoulos et al., 2008; Sicheri et al., 1997). Furthermore, a protein containing several interaction domains can be coupled to multiple inputs that act either separately or synergistically to regulate signaling (Carnegie et al., 2009; Prehoda et al., 2000).

Although the individual functions of specific domains are well understood, we are more poorly informed about those properties of interaction and catalytic domains that allow them to act cooperatively in mediating complex biological processes, such as the timing and specificity of cell-fate decisions in the mammalian embryo. Determining the physiological relevance of the multidomain architecture of signaling proteins requires that they are studied in a proper biological context. To this end, we have exploited the critical roles of Son of Sevenless 1 (Sos1), a guanine nucleotide exchange factor (GEF) for the Ras GTPase, and its upstream SH2/SH3 adaptor Grb2 in directing the differentiation of murine embryonic stem cells (mESCs) into primitive endoderm (PrE; Cheng et al., 1998). During early mammalian development, the inner cell mass (ICM) differentiates into epiblast, which gives rise to the embryo proper, and PrE, an essential extraembryonic lineage. PrE differentiation is induced by fibroblast growth factor 4 (FGF4; Feldman et al., 1995) and its cognate receptor tyrosine kinase (RTK) FGF receptor 2 (FGFR2; Arman et al., 1998), which in turn transmits a phosphotyrosine (pTyr)-based signal through Grb2 to Sos proteins and thus to the Ras-Erk pathway (Cheng et al., 1998; Hamazaki et al., 2006). This signal initiates PrE differentiation by creating a metastable state of the ICM, wherein expression of the pluripotency factor Nanog is heterogeneous

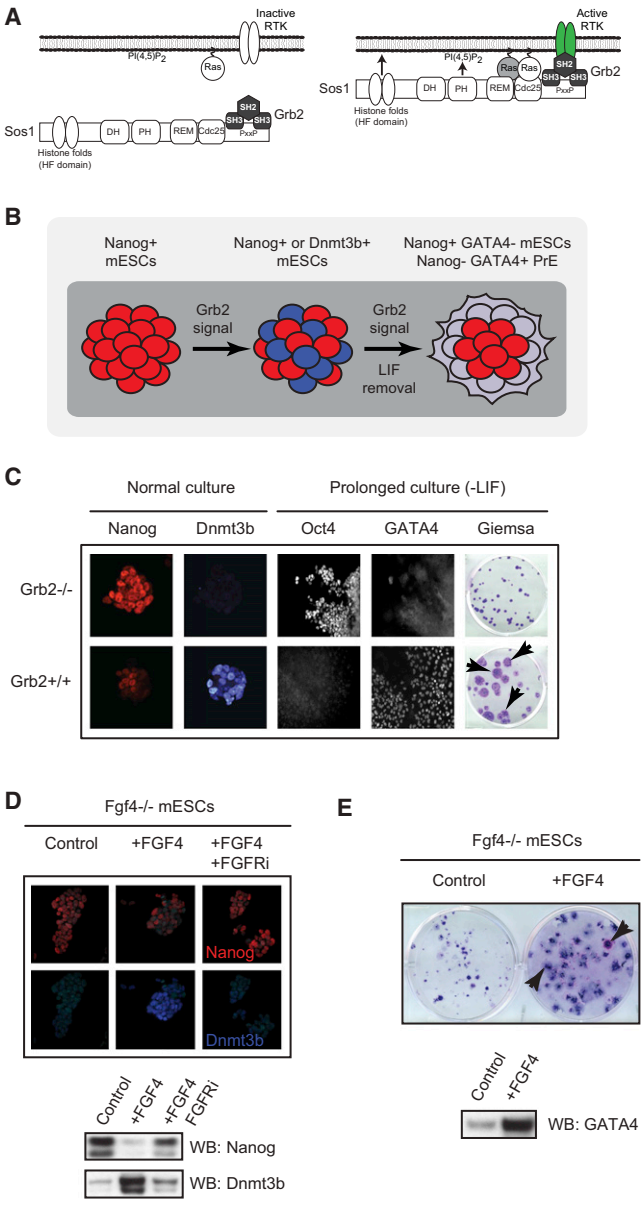


Figure 1. FGF4-Grb2 Signaling Generates a Metastable, "Primed" mESC State and Drives PrE Lineage Commitment

(A) Model of Sos1 activation; allosteric Ras (gray) and substrate Ras (white) occupy different binding sites on the Sos1 RasGEF module.

(B) Model of PrE differentiation from mESCs. Grb2 signaling induces segregation of mESCs into Nanog^{+} (red) and Dnmt3b^{+} (blue) subpopulations and subsequent differentiation of the PrE lineage (GATA4^{+}, purple).

(C) Grb2$^{-/-}$ and WT mESCs stained for Nanog, Dnmt3b, Oct4, or GATA4 expression or giemsa stained.

(D) Nanog and Dnmt3b staining (top) and immunoblot analysis (bottom) of Fgf4$^{-/-}$ mESCs. FGF4 (100 ng/ml) and FGFRi (SU5402;10 μM) were added for 48 hr prior to analysis.

(E) Giemsa staining (top) and GATA4 immunoblot analysis (bottom) of Fgf4$^{-/-}$ mESCs following prolonged culture in the presence or absence of FGF4.

See also Figures S1 and S2.

(Chazaud et al., 2006), a phenomenon we refer to as "priming." In a similar vein, mESCs transit through a metastable primed state of low Nanog expression prior to lineage commitment (Chambers et al., 2007). Downregulation of Nanog allows for upregulation of transcription factors, such as GATA6, that drive expression of genes required for PrE differentiation, including GATA4 (Morrisey et al., 1998; Soudais et al., 1995).

Metastable priming is lost in Grb2$^{-/-}$ embryos and mESCs (Chazaud et al., 2006; Hamazaki et al., 2006) but can be regenerated by expression of a chimeric protein in which a truncated Sos1 polypeptide lacking the proline-rich C terminus that binds the Grb2 SH3 domains is fused to the Grb2 SH2 domain (Cheng et al., 1998). This Sos1-Grb2 chimera is directly recruited to pTyr sites through its intrinsic Grb2 SH2 domain, circumventing the requirement for Grb2 itself in early embryonic development. In addition to the C-terminal Grb2-interaction motifs, Sos1 has a Cdc25 domain that catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on Ras, preceded by a *R*as *e*xchanger *m*otif (REM), which together form a RasGEF module that binds both substrate Ras and a second, allosteric Ras molecule (Margarit et al., 2003; see Figure 1A). The binding of Ras-GTP to the allosteric site activates the Cdc25 domain, thereby creating a positive feedback loop that sensitizes Sos1 to an initial burst of Ras-GTP (Boykevisch et al., 2006). Sos1 also has a DH-PH cassette, of which the PH domain binds to phospholipids such as phosphatidylinositol-(4,5)-bisphosphate (PI(4,5)P_2; Chen et al., 1997; Kubiseski et al., 1997). At the N terminus, Sos1 possesses a histone folds (HF) domain with a basic surface that binds membrane phospholipids (Yadav and Bar-Sagi, 2010).

In the absence of growth factor stimulation, Sos1 is autoinhibited by intramolecular interactions involving the DH-PH module and HF domain (Gureasko et al., 2010; Sondermann et al., 2004). Human SOS1 mutations from patients with Noonan Syndrome (NS), a familial disorder caused by activating mutations in the Ras-Erk pathway, drive signaling by disrupting the autoinhibited conformation of SOS1 or increasing membrane recruitment (Roberts et al., 2007; Tartaglia et al., 2007). Physiological Sos1 activation following growth factor stimulation occurs at the membrane through intermolecular interactions, in which the C-terminal tail and the REM, PH, and HF domains bind external ligands, thereby relieving autoinhibition and driving Sos1 into an active configuration (reviewed in Findlay and Pawson, 2008). Whether these protein-protein and protein-lipid interactions are all required for Sos1 regulation in a biological context and, if so, what functional advantage this confers remain uncertain.

We have used the regeneration of PrE differentiation in Grb2$^{-/-}$ mESCs by the Sos1-Grb2 chimera as a system to explore how the multiple protein- and phospholipid-interaction domains of Sos1 and Grb2 regulate a key developmental cell-fate decision. Our results indicate that these domains are optimized not for maximal ligand affinity but to cooperatively determine the timing and selectivity of cell-fate specification. These data suggest a central mechanism through which the acquisition of complex domain architectures could be advantageous to multicellular organisms.

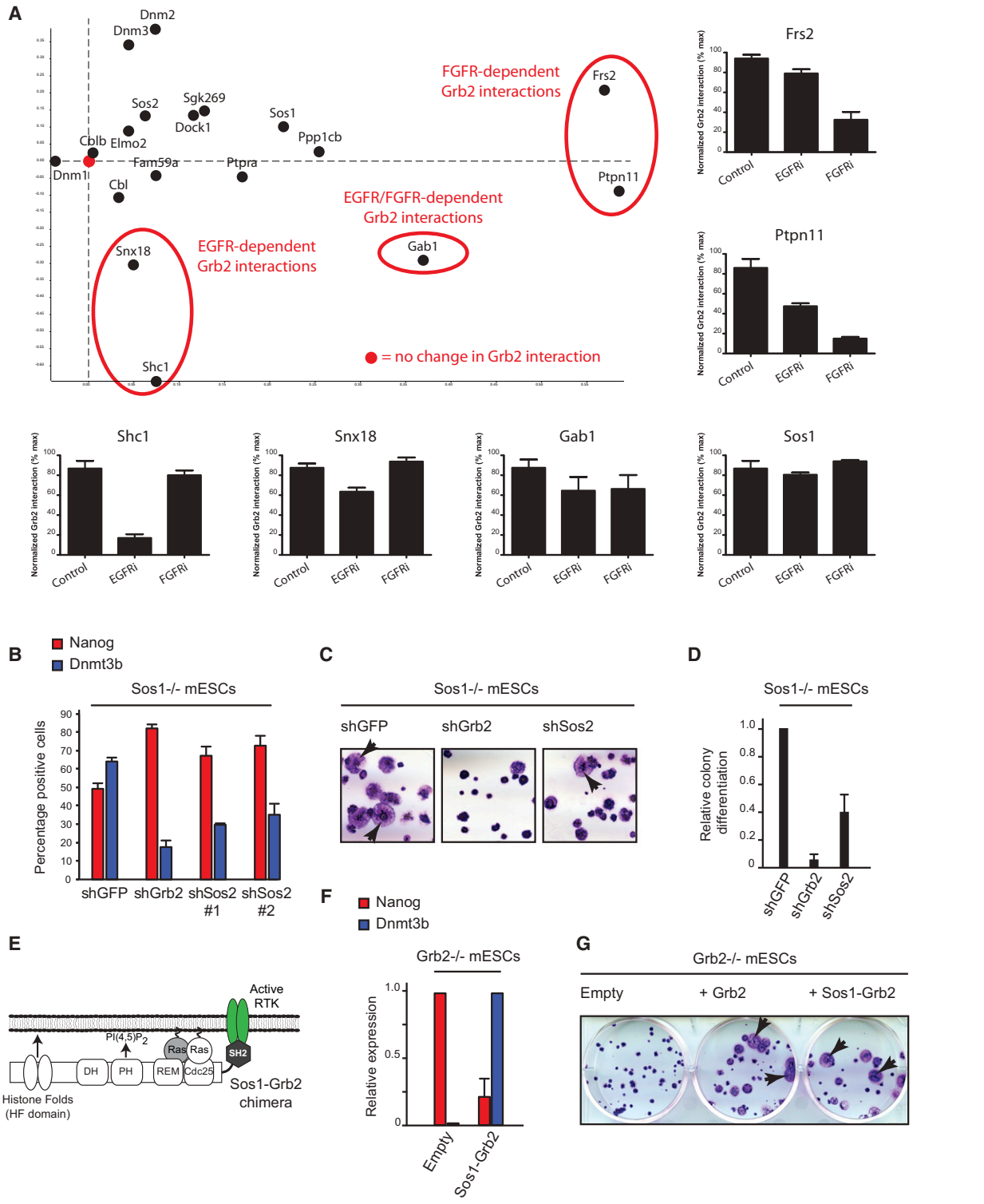


Figure 2. Identification of Grb2-Signaling Complexes that Regulate PrE Differentiation

(A) PCA plot of Grb2-interacting proteins quantified by SWATH-MS. The positions of the circles on the plot represent the relative behaviors of individual proteins (see Table S1). Graphical data are average ± standard error of the mean (SEM) (n = 4).

(B) Cellomics analysis of Nanog/Dnmt3b expression in lentiviral shRNA-infected Sos1$^{-/-}$ mESCs. Data shown are average ± standard deviation (SD) (n = 3).

(C) Giemsa staining of lentiviral shRNA-infected Sos1$^{-/-}$ mESCs.

(D) Quantification of differentiated colonies in lentiviral shRNA-infected Sos1$^{-/-}$ mESCs relative to shGFP control. Data shown are average ± SD (n = 3).

(legend continued on next page)

RESULTS

Average Domain Complexity Has Increased from Yeast to Higher Eukaryotes

The increased presence of multidomain proteins in metazoans compared to yeast (Rubin et al., 2000) suggests that increasing domain complexity has been selected for during evolution. To quantify this within eukaryotes, we plotted the cumulative distribution of the number of domains per protein across a range of proteomes (Figure S1A available online). The cumulative distribution span is shortest within the *S. cerevisiae* proteome and increases in length throughout metazoans (Figure S1A; average domains per protein, yeast = 1.9, human = 3.1), confirming that domain complexity increases throughout eukaryotic evolution.

For any given signaling protein, it seems logical that acquiring more domains (domain "accretion"; Koonin et al., 2000) would elevate its connectivity, providing a fitness advantage. A particular instance of domain accretion is given by comparing the RasGEFs Cdc25p and SOS1, which activate the Ras GTPase in yeast and human, respectively. Cdc25p contains only the Cdc25 catalytic core and an SH3 domain (Figure S1B), whereas human SOS1 consists of the REM-Cdc25 catalytic core, with additional HF, DH, and PH domains, as well as proline-rich motifs that connect to the SH2/SH3 adaptor protein Grb2. To explore the biological importance of the various protein- and lipid-interaction domains of Sos1 and Grb2, we investigated their roles in the differentiation of mESCs to the essential PrE lineage (Figure 1B).

A Physiological Assay for Grb2-Sos1 Signaling: The Grb2-Sos1 RasGEF Complex Generates a Metastable mESC State Primed for PrE Lineage Commitment

Wild-type (WT) mESC colonies display a heterogeneous Nanog expression pattern, characteristic of the primed state, whereas Grb2$^{-/-}$ mESCs are homogeneously Nanog positive (Figure 1C). In WT mESCs, Nanog heterogeneity is accompanied by induction of the early PrE differentiation marker DNA methyltransferase 3b (Dnmt3b; Hirasawa and Sasaki, 2009), whereas Grb2$^{-/-}$ mESCs have constitutively low Dnmt3b levels (Figure 1C). We therefore examined whether Grb2 signaling generates a distinct subpopulation of primed mESCs. On average, 47% of WT cells were Nanog^{+}, 52% were Dnmt3b^{+}, and 1% were both Nanog^{+} and Dnmt3b^{+} (Figure S2A). By contrast, Grb2$^{-/-}$ mESCs were 95% Nanog^{+}/Dnmt3b^{-}, with the remaining 5% either Dnmt3b^{+} or Nanog^{+}/Dnmt3b^{+}. We also confirmed that autocrine FGF4 production is required for lineage priming. Indeed, Fgf4$^{-/-}$ mESCs were largely Nanog^{+}/Dnmt3b^{-}, and priming was restored by incubation with FGF4 and reversed by the FGFR inhibitor SU5402 (Figure 1D). These data suggest that mESCs are primed for differentiation by segregating Nanog^{+} or Dnmt3b^{+} subpopulations in a fashion that depends on FGF4-Grb2 signaling.

Differentiation of primed cells to the PrE lineage, as monitored by the expression of GATA4/6 PrE markers and formation of a differentiated colony morphology, relies on Grb2 signaling both in vitro and in the embryo (Chazaud et al., 2006; Figure 1B). Grb2$^{-/-}$ mESC colonies failed to differentiate PrE, as judged by expression of the pluripotency marker Oct4, failure to induce GATA4/6 PrE markers, and retention of an undifferentiated colony morphology (Figures 1C and S2B). Similarly, Fgf4$^{-/-}$ mESCs failed to induce GATA4 and retained an undifferentiated morphology, which was rescued by addition of FGF4 (Figure 1E). Following the creation of the primed mESC state, prolonged FGF4-Grb2-dependent signaling is therefore required for PrE lineage commitment.

To elucidate the biochemical mechanisms by which Grb2 transmits the prodifferentiative FGF4 signal in mESCs, we used a proteomics technique termed *S*ingle *W*indow *A*cquisition of all *Th*eoretical spectra *M*ass-*S*pectrometry (SWATH-MS; Gillet et al., 2012) to quantify changes in Grb2-interacting proteins following blockade of either FGFR or epidermal growth factor receptor (EGFR) kinase activity in mESCs (Figure S3A). We employed principal-component analysis (PCA) of quantitative SWATH data to identify subsets of proteins that behaved similarly in their interaction with Grb2 following RTK inhibition (Figure 2A). This revealed that the scaffold protein Frs2 and the tyrosine phosphatase Ptpn11 associate with Grb2 in mESCs in a fashion that was selectively suppressed by the FGFR inhibitor SU5402, whereas binding of Grb2 to Shc1 and Snx18 was primarily lost upon treatment with the EGFR inhibitor AG1478 (Figure 2A). As tyrosine phosphorylation of Frs2 and Ptpn11 creates binding sites for the Grb2 SH2 domain (Hadari et al., 1998; Kouhara et al., 1997), Frs2 and Ptpn11 appear to be the major upstream docking proteins for Grb2 in FGF-stimulated mESCs. We also identified a number of known Grb2 SH3 domain effectors, including Sos1, Sos2, Gab1, Cbl, and Cblb (Figure 2A). Interaction of Grb2 with Sos1 was largely unperturbed by RTK inhibition, suggesting that the stoichiometry of the Grb2/Sos1 complex is not significantly altered by EGFR or FGFR activity in mESCs. We went on to confirm that Sos proteins are required for PrE differentiation. Sos1$^{-/-}$ embryos and mESCs differentiate normally (Wang et al., 1997), likely as a result of increased Sos2 expression (Figure S3B). However, silencing Sos2 expression in Sos1$^{-/-}$ mESCs inhibited priming (Figures 2B and S3C) and PrE differentiation (Figures 2C, 2D, S3D, and S3E), indicating that Sos1/2 act as effectors of Grb2 during PrE lineage specification.

Synthetic Biology Reveals that Multiple Sos1/Grb2 Ligand-Binding Domains Cooperate in PrE Lineage Commitment

To address the roles of the various Sos1 interaction domains in PrE specification, we examined mESC lineage priming and commitment induced by the Sos1-Grb2 chimera (Figure 2E). Expression of Sos1-Grb2 restored Grb2$^{-/-}$ mESCs to a

(E) Diagram of the Sos1-Grb2 chimera.
(F) Nanog and Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing Sos1-Grb2. Data shown are average ± SD (n = 3).
(G) Giemsa staining of Grb2$^{-/-}$ mESCs expressing Grb2 or Sos1-Grb2.
See also Figure S3 and Table S1.

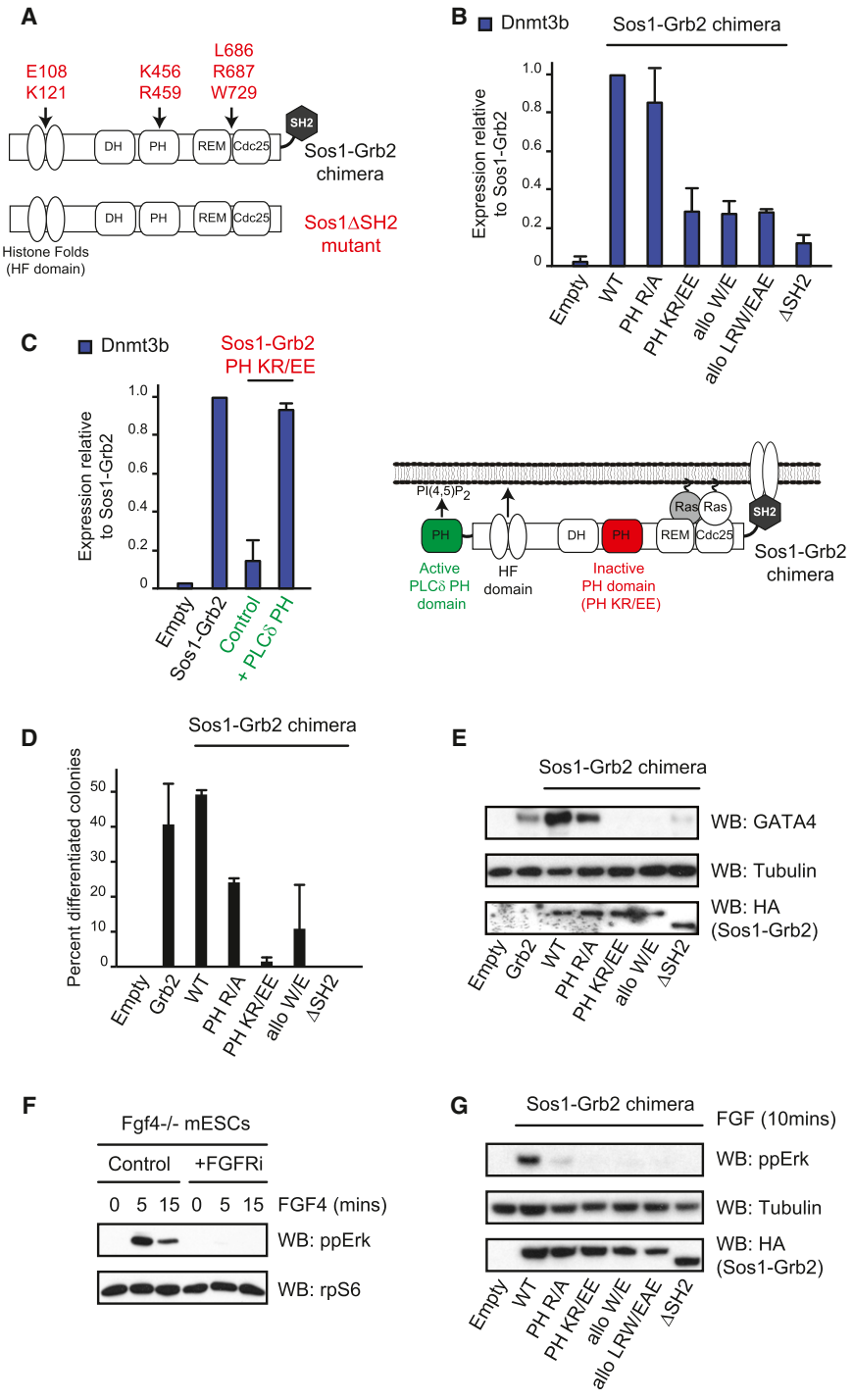


Figure 3. Sos1 Integrates Diverse Membrane Signals via Modular Domains to Control PrE Differentiation

(A) Sos1-Grb2 mutations. E108/K121 = HF domain membrane interaction surface; K456/R459 = PH domain PI(4,5)P_2-binding site; L686/R687/W729 = allosteric Ras-binding site.

(B) Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2. Data shown are average ± SD (n = 3).

(C) A PLCδ PH domain was tethered to the Sos1-Grb2 KR/EE PH mutant (right). Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing Sos1-Grb2 WT, KR/EE PH mutant, or PLCδ PH-tethered KR/EE PH mutant (left) are shown. Data are average ± SD (n = 3).

(D) Quantification of differentiated colonies from Grb2$^{-/-}$ mESCs expressing Grb2, WT Sos1-Grb2, or mutant Sos1-Grb2. Data shown are average ± SD (n = 3).

(E) GATA4 levels in Grb2$^{-/-}$ mESCs expressing Grb2, WT Sos1-Grb2, or mutant Sos1-Grb2.

(F) ppErk levels in Fgf4$^{-/-}$ mESCs following FGF4 stimulation (100 ng/ml) in the presence or absence of FGFRi (SU5402, 10 μM).

(G) ppErk levels in Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2 following 10 min stimulation with 100 ng/ml aFGF.

See also Figure S4.

Nanog^{-}/Dnmt3b^{+} primed state (Figures 2F and S4A) and rescued PrE differentiation (Figure 2G). Therefore, we expressed ligand-binding domain mutants of Sos1-Grb2 (Figure 3A) in Grb2$^{-/-}$ mESCs and assessed their relative abilities to induce cell-fate change.

Binding of the Sos1 PH domain to membrane phospholipids such as PI(4,5)P_2 is important for RasGEF signaling in vitro (Gureasko et al., 2008). To test the physiological relevance of such phospholipid recognition, we mutated the Sos1 PH domain to disrupt its interaction with PI(4,5)P_2 (Figures 3A and S4B). WT Sos1 PH domain bound to soluble PI(4,5)P_2 with a dissociation constant (K_D) of 29 ± 3.4 μM (Figure S4C). An R459A PH mutant (Kubiseski et al., 1997, henceforth PH R/A) increased the K_D for PI(4,5)P_2 to 114 ± 12.6 μM, whereas a combined K456E/R459E substitution (Chen et al., 1997; henceforth PH KR/EE) further increased the K_D for PI(4,5)P_2 to > 230 μM (Figure S4C). The PH R/A mutation had little effect on the ability of Sos1-Grb2 to rescue induction of Dnmt3b expression in Grb2$^{-/-}$ mESCs (Figure 3B), consistent with its modest effect on the Sos1 PH-PI(4,5)P_2 interaction. However, the PH KR/EE variant markedly interfered with the rescue of Dnmt3b expression by Sos1-Grb2, indicating that the Sos1 PH-PI(4,5)P_2 interaction may be important for priming of mESCs. To investigate whether PI(4,5)P_2 engagement by the Sos PH domain is sufficient for mESC differentiation, we tethered the PI(4,5)P_2-specific PH domain of PLCδ (Kavran et al., 1998) to Sos1-Grb2 PH KR/EE (Figure 3C). Addition of an ectopic PLCδ PH domain rescued priming by the inactive Sos1-Grb2 PH KR/EE mutant, consistent with the notion that PI(4,5)P_2 is a physiological Sos1 PH domain ligand in mESCs.

The interaction between Ras-GTP and the Sos1 RasGEF module, via a pocket behind the catalytic site, allosterically stimulates RasGEF activity (Margarit et al., 2003). This interaction is abolished by a W729E mutation (henceforth "allo W/E") in the REM domain, either alone or in combination with L686E/R687A (henceforth "allo LRW/EAE") in the Cdc25 domain. Analysis of Dnmt3b levels upon expression of either allo W/E or allo LRW/EAE mutants of Sos1-Grb2 in Grb2$^{-/-}$ mESCs indicated that binding of allosteric Ras is essential for Sos1 to prime mESCs for differentiation (Figure 3B). We also confirmed that a truncated Sos1 protein lacking the SH2 domain of Sos1-Grb2 (Sos1ΔSH2), which could therefore not be recruited to pTyr sites, such as those in Frs2 and Ptpn11, was incapable of rescuing Dnmt3b induction in Grb2$^{-/-}$ mESCs (Figure 3B). Furthermore, restoration of priming by full-length Grb2 was abolished by inactivating either the Grb2 SH2 or N-terminal SH3 domain, supporting the idea that Grb2 induces priming by coupling Sos1 to pTyr sites (Figure S4D). Modulation of Dnmt3b expression by these Sos1-Grb2 or full-length Grb2 mutants was accompanied by reciprocal regulation of Nanog expression (Figures S4D and S4E).

To extend this analysis to the level of cellular morphology, we examined whether Sos1-Grb2 PH, REM/Cdc25, and SH2 domain-mediated interactions are similarly important for PrE lineage commitment. Sos1-Grb2 expression and prolonged clonal culture induced PrE differentiation in Grb2$^{-/-}$ mESCs, as measured by colony morphology (Figures 2G and 3D) and GATA4 induction (Figure 3E). The PH R/A mutation in the Sos1 PH domain had a minor effect on PrE differentiation (Figures 3D and 3E), whereas PH KR/EE, which more strongly interfered with phospholipid binding, abolished Sos1-Grb2-mediated PrE differentiation. Similarly, blocking allosteric Ras binding to the Sos1 RasGEF domain (allo W/E) prevented rescue of GATA4 induction and colony differentiation, as did removal of the Grb2 SH2 domain (Sos1ΔSH2). Similar results were obtained with WT and PH KR/EE Sos1-Grb2 expressed at subendogenous levels (Figure S4F). In summary, direct coupling of Sos1 to activated receptor complexes through Grb2, recognition of membrane lipids such as PI(4,5)P_2 by the PH domain, and binding of Ras to the allosteric site are all indispensable for Sos1 to not only prime mESCs for differentiation but also direct PrE lineage commitment.

Autocrine FGF4 is proposed to specify PrE by activating the downstream Ras-Erk pathway, which we confirmed by stimulating Fgf4$^{-/-}$ mESCs with FGF4 (Figure 3F). We therefore investigated the effects of Sos1-Grb2 mutations on Erk activation in FGF-stimulated Grb2$^{-/-}$ mESCs. Control Grb2$^{-/-}$ cells had low levels of phosphorylated Erk, which was increased by expression of WT Sos1-Grb2 (Figure 3G). Reduction of the Sos1 PH domain-PI(4,5)P_2 interaction with the PH R/A mutation decreased, whereas the more severe PH KR/EE mutation abolished, Erk phosphorylation. Similarly, inhibiting allosteric Ras binding (allo W/E) or uncoupling Sos1-Grb2 recruitment to pTyr sites (Sos1ΔSH2 mutant) prevented FGF-dependent Erk activation (Figure 3G). This fully correlates with the relative capability of these Sos1-Grb2 mutants to regenerate PrE lineage specification.

In order to more fully investigate the relationship between Erk activation and mESC lineage commitment, we examined Dnmt3b induction kinetics upon addition of FGF4 to Fgf4$^{-/-}$ mESCs. Strong Erk activation within 15 min was seen in response to 10 ng/ml FGF4, and this efficiently induced Dnmt3b from 8 hr onward. By contrast, 1 ng/ml FGF4 poorly induced Erk activation and Dnmt3b expression (Figure S4G). Therefore, the amplitude of acute Erk activation by FGF4 correlates with lineage priming, consistent with our results using Sos1-Grb2 mutants. We also examined autocrine FGF4-mediated Erk activation induced by Sos1-Grb2 expression in actively differentiating mESCs. As anticipated, Erk activation was undetectable in Grb2$^{-/-}$ mESCs. However, expression of Sos1-Grb2 rescued Erk activation in either primed or fully differentiated Grb2$^{-/-}$ mESCs, whereas expression of Sos1-Grb2 PH KR/EE failed to restore Erk activation (Figure S4H), just as it failed to restore either mESC priming or PrE specification. Collectively, our data demonstrate that Sos1 activation in mESCs is tightly controlled by combinatorial interactions with pTyr sites, PI(4,5)P_2, and allosteric Ras, and that each interaction is essential for FGF-dependent Erk activation, mESC priming, and PrE specification.

A Charge Neutralization Mechanism in the Sos1 HF Domain Is Conserved to Prevent Inappropriate Sos1 Activation and Lineage Commitment

As well as its autoinhibitory function, the N-terminal HF domain has been implicated in Sos1 activation via a putative membrane phospholipid-interaction surface, containing a patch of basic residues punctuated by a central acidic residue, E108 (Figure 4A; Gureasko et al., 2010). E108 is mutated to lysine in NS, whereupon Sos1 is activated by an increase in the affinity of the HF domain for membranes (Yadav and Bar-Sagi, 2010). Therefore, we examined the role of Sos1 HF domain-mediated membrane interaction during mESC differentiation. Mutating a key basic residue in the membrane-interaction surface (K121E) diminished Dnmt3b induction by Sos1-Grb2 (Figure 4B) and suppressed PrE specification (Figure S5A), suggesting that membrane recognition by the HF domain plays a functional role in PrE specification.

We also addressed the influence of the activating E108K NS mutation on Sos1-Grb2 activity and mESC fate determination. When expressed in Grb2$^{-/-}$ mESCs, Sos1-Grb2 E108K induced elevated Erk activation compared to WT Sos1-Grb2 (Figure 4C). Incorporation of the K121E substitution into E108K Sos1-Grb2 partially reversed the effect of the NS mutation on Erk signaling, consistent with the view that hyperactivation of the E108K Sos1-Grb2 mutant occurs as a result of increased electrostatic attraction between the HF domain and the membrane.

We speculated that increased membrane binding induced by the E108K HF mutation may subvert the requirement for coincident interaction with multiple membrane-based ligands to induce biological Sos1 activation. Indeed, incorporation of E108K into the Sos1ΔSH2 variant, which is defective for Erk activation because it cannot couple to pTyr sites, fully rescued Erk activation in Grb2$^{-/-}$ mESCs (Figure 4C), indicating that increased membrane recruitment by the Sos1 HF domain uncouples Sos1 from pTyr regulation. The E108K mutation also increased Dnmt3b induction by the Sos1ΔSH2 mutant

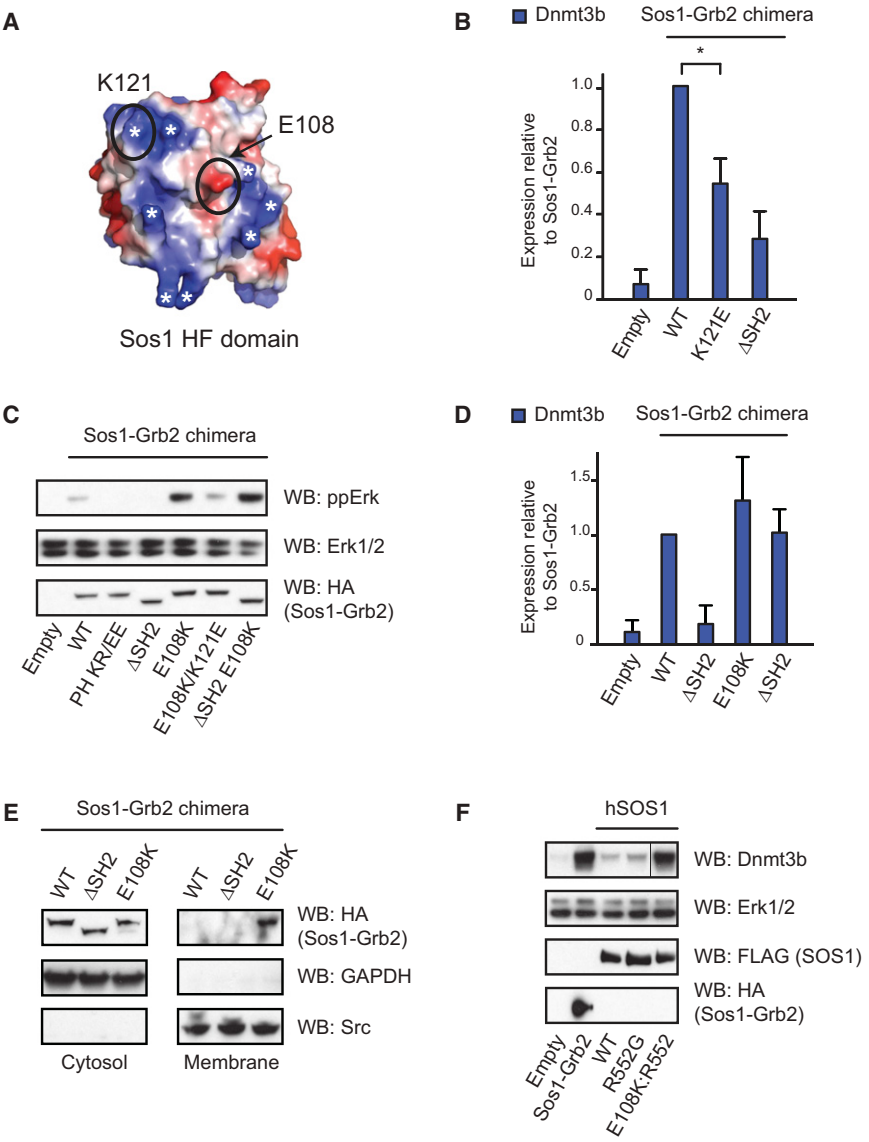


Figure 4. Charge Neutralization in the HF Domain Ensures Receptor-Dependent Sos1 Activation and Cell-Fate Determination

(A) Electrostatic potential of the membrane-interacting surface of the Sos1 HF domain. Putative membrane-interacting residues are labeled by an asterisk (*).

(B) Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2. Data shown are average ± SD (n = 3); *p ≤ 0.02.

(C) ppErk levels in Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2.

(D) Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2. Data shown are average ± SD (n = 3).

(E) Cytosolic (left) and membrane (right) compartments of Grb2$^{-/-}$ mESCs expressing WT or mutant Sos1-Grb2. GAPDH and Src mark cytosol and membrane, respectively.

(F) Dnmt3b levels in Grb2$^{-/-}$ mESCs expressing full-length WT or mutant SOS1 proteins. Intervening lanes were removed.

See also Figure S5.

from baseline to a level similar to that of WT Sos1-Grb2 (Figure 4D), and this was accompanied by Nanog suppression (Figure S5B). Following prolonged culture, GATA4 expression was induced to levels similar to those in WT Sos1-Grb2, and differentiated mESC colonies appeared (Figures S5C and S5D). We conclude that the E108K HF variant activates Sos1-dependent signaling, thereby aberrantly priming mESCs and driving PrE differentiation in the absence of a pTyr-Grb2 input.

The WT Sos1 HF domain appears to have evolved limited, but biologically optimized affinity toward membrane phospholipid ligands so as to maintain responsiveness of Sos1 to FGFR2 activation. The importance of finely tuned membrane binding by the Sos1 HF domain is supported by the conservation of basic residues from *C. elegans* to human and of aspartate or glutamate at the position corresponding to E108 in human SOS1 (Figure S5E) (Yadav and Bar-Sagi, 2010). We therefore propose that one biological consequence of the invariant HF acidic residue is to limit HF domain membrane affinity, preventing Sos1 activation and initiation of PrE differentiation in the absence of an appropriate FGFR2-pTyr signal.

In light of these findings, we directly tested whether E108 restricts Sos1 membrane affinity in mESCs. Fractionating extracts of Grb2$^{-/-}$ mESCs expressing Sos1-Grb2 revealed that neither WT Sos1-Grb2 nor Sos1ΔSH2 were stably membrane associated (Figure 4E). However, the Sos1-Grb2 E108K mutant yielded a stable membrane-associated pool, supporting the hypothesis that charge neutralization by E108 attenuates Sos1 HF domain membrane affinity, thus maintaining the requirement for a pTyr signal in PrE development.

We also pursued the physiological relevance of charge neutralization on the HF domain by examining the ability of full-length SOS1 to prime Grb2$^{-/-}$ mESCs for differentiation. SOS1 expressed very poorly in Grb2$^{-/-}$ cells (data not shown). Thus, we sensitized our assay using another NS mutation, R552G, which destabilizes autoinhibitory interactions and hyperactivates SOS1 only when it is on membranes (Gureasko et al., 2008). As anticipated, expression of WT SOS1 or SOS1 R552G failed to prime Grb2$^{-/-}$ mESCs for differentiation (Figure 4F), presumably because SOS1 is inefficiently recruited to membranes in the absence of Grb2. By contrast, expressing SOS1 containing both R552G and E108K NS mutations induced Dnmt3b expression in Grb2$^{-/-}$ mESCs (Figure 4F), indicating that these cells are primed to differentiate in the absence of a pTyr-Grb2 signal. These data show that charge neutralization on the HF domain is required to properly regulate full-length SOS1.

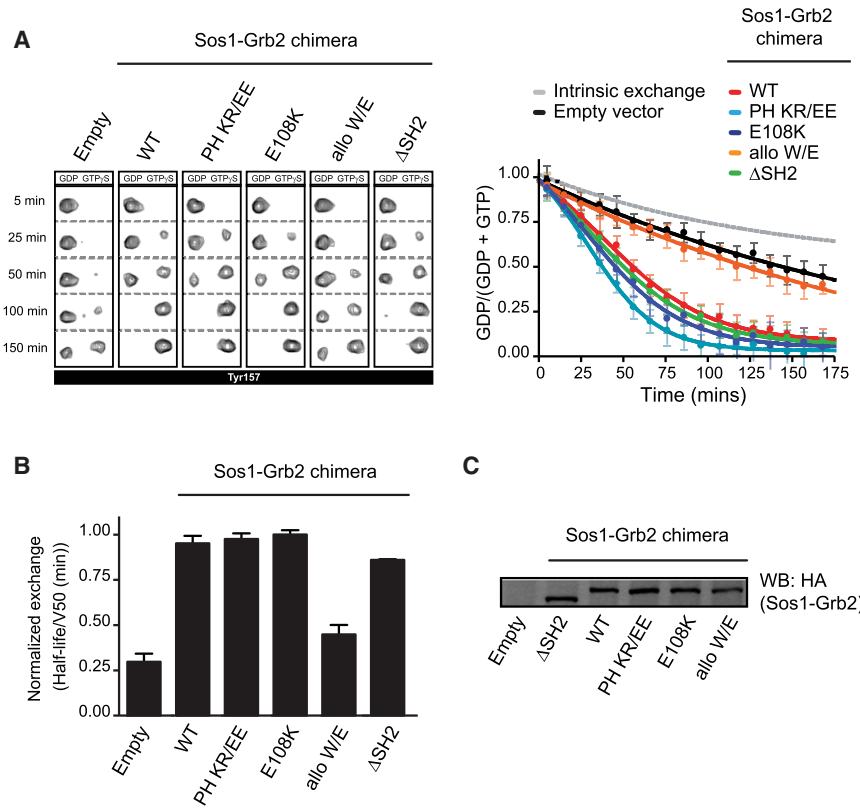


Figure 5. The Modular Interaction Domains of Sos1 Control Cell Fate by Directly and Indirectly Influencing Sos1 RasGEF Activity

(A) Measurement of Sos1 RasGEF activity by NMR. (Left) Representative HSQC spectra showing chemical shift changes between Ras Tyr157 in the GDP- and GTPγS-bound conformations. (Right) Chemical shifts were measured over time, quantified, and plotted to determine relative RasGEF activity of Sos1-Grb2 expressed in Grb2$^{-/-}$ mESCs. Data shown are average ± SD (n = 2).

(B) Normalized rate of the GDP-GTP exchange reaction on Ras. Data shown are average ± SD (n = 2).

(C) Sos1-Grb2 expression from Grb2$^{-/-}$ mESC lysates used for NMR assays.

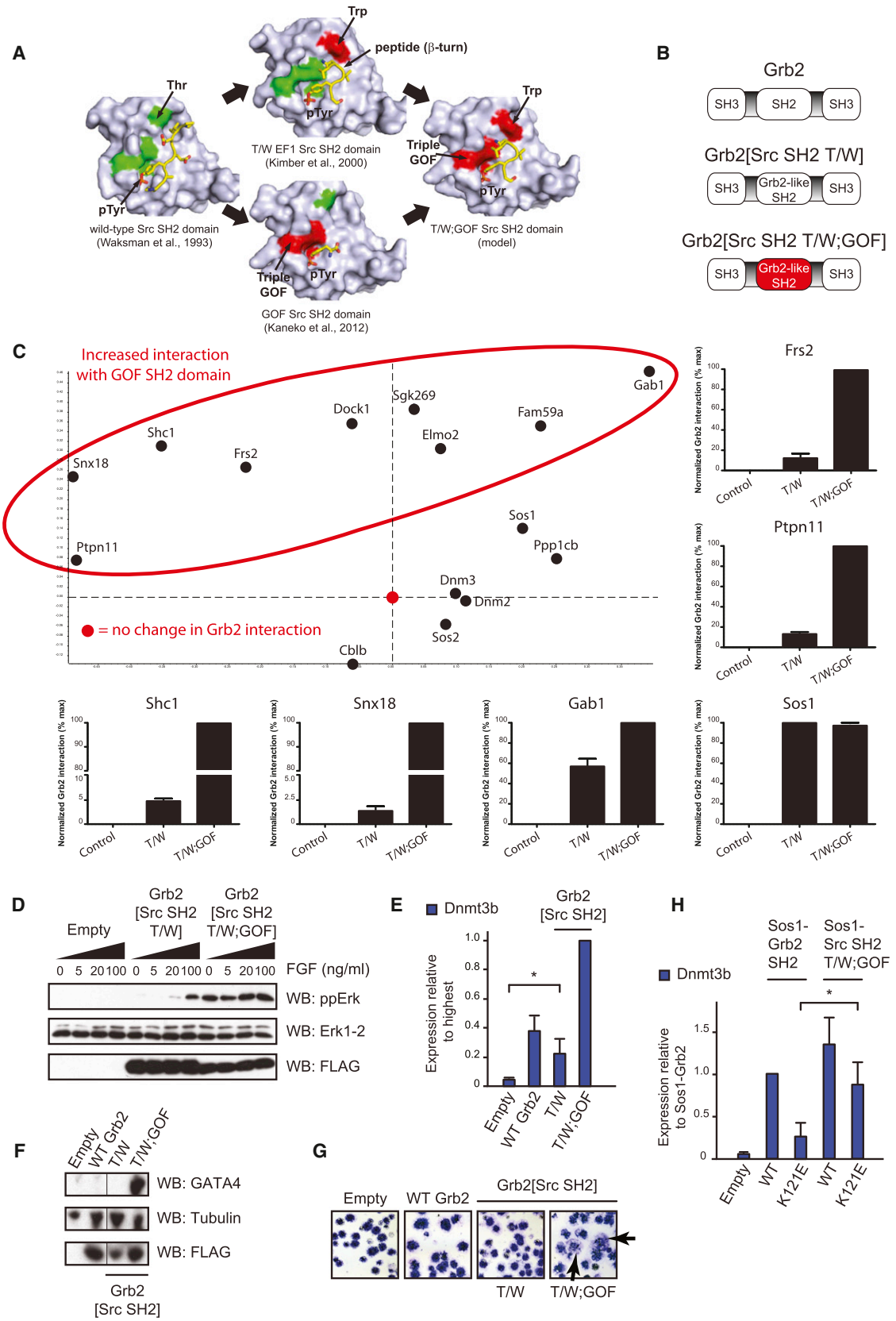
Protein and Lipid Interactions Modulate Sos1-Signaling Output in Embryonic Cells through Both Direct and Indirect Effects on GEF Activity

The functionally important Sos1 interactions identified in mESCs could potentially have a direct effect on Sos1 RasGEF activity or influence Sos1 activity indirectly through membrane-based interactions that colocalize Sos1 with its Ras GTPase substrate. To distinguish these possibilities, we used an NMR-based assay that monitors GEF-catalyzed exchange of GDP for GTP on Ras. This assay is based on the principle that conversion of ^{15}N-labeled Ras-GDP to Ras-GTP induces specific chemical-shift perturbations in the Ras ^{1}H-^{15}N *h*eteronuclear *s*ingle *q*uantum *c*oherence (HSQC) spectrum, as previously employed to monitor regulation of the Ras superfamily GTPases RhoA and Rheb (Gasmi-Seabrook et al., 2010; Marshall et al., 2009). Identification and quantification of peak intensities at these unique chemical shifts, exemplified by Ras Tyr157 (Figure 5A), can be used to determine the rate of conversion of Ras-GDP to Ras-GTP, enabling direct real-time measurement of RasGEF activity. Uniquely, this assay provides sufficient sensitivity to measure the GEF activity of Sos1 from cell lysates.

In the absence of an added GEF and in the presence of an excess of nonhydrolyzable GTPγS, Ras intrinsically exchanges GDP for GTP with a half-time (T1/2) of 126.2 ± 19.0 min (Figure 5A). Addition of Grb2$^{-/-}$ mESC lysate expressing WT Sos1-Grb2 increased this rate of conversion (T1/2 = 38.6 ± 1.7 min; Figure 5A). However, neither the E108K HF domain mutation (T1/2 = 36.7 ± 0.9 min), the PH KR/EE mutation (T1/2 = 37.6 ± 1.2 min), nor SH2 domain removal (SosΔSH2; T1/2 = 42. ± 0.2 min) had a significant effect on Sos1-Grb2 RasGEF activity, consistent with the notion that these domains function by juxtaposing Sos1 and Ras at the membrane. In contrast, the allo W/E mutation, which inhibits the interaction of Ras-GTP with the allosteric site on the RasGEF domain, suppressed the ability of Sos1-Grb2 to catalyze GDP/GTP exchange on Ras in Grb2$^{-/-}$ mESCs (T1/2 = 82.9 ± 9.7 min). The normalized exchange rate and expression level of each Sos1-Grb2 variant were confirmed (Figures 5B and 5C). Together, these data argue that the various interactions mediated by different Sos1 domains can regulate Sos1 function either by directly modulating RasGEF activity, as in the case of allosteric Ras binding, or by localizing the GEF to its Ras substrate via membrane targeting without modifying enzymatic activity per se, as in the case of the Sos1 HF and PH domains and the Grb2 SH2 domain.

Physiological Grb2 SH2 Domain Ligand Affinity Is Optimized for Appropriate PrE Lineage Commitment

Our demonstration that the HF domain of Sos1 has evolved a mechanism to prevent inappropriate PrE differentiation in the absence of physiological growth factor signals suggests that other modular interaction domains required for Sos1 activation, such as the Grb2 SH2 domain, might also be finely balanced with respect to their ligand affinities. We tested this notion with an SH2 domain engineered for potently increased pTyr affinity (Figure 6A) (Kaneko et al., 2012). This mutant is based on the Src SH2 domain and contains substitutions in the pTyr-binding pocket (threonine βC3 to valine, cysteine βC3 to alanine, and lysine βD6 to leucine, collectively termed "gain of function" [GOF]; Figure S6A). Analogous mutants in the Grb2 SH2 domain were unstable in cells (data not shown). Therefore, in order to apply this GOF Src SH2 domain to Grb2-dependent mESC differentiation, we exploited our previous observation that substituting tryptophan for threonine at the EF1 position (Figures



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6A and S6A; termed "T/W") converts the Src SH2 domain-binding properties to those of a Grb2 SH2 domain (Marengere et al., 1994). By combining T/W and GOF mutations in the Src SH2 domain, we obtained a synthetic Grb2-like SH2 domain with increased pTyr-binding activity (Figure 6A).

We initially confirmed that these mutations alter SH2 ligand binding in the predicted manner. The WT Src SH2 domain bound modestly (K_D 11.4 ± 2.4 μM; Figure S6B) to a pY392 Frs2 peptide, a putative ligand for Grb2 in mESCs (Figure 2A). The T/W mutation reduced this K_D to 1.4 ± 0.23 μM, consistent with an increased selectivity for Grb2 SH2 ligands. Introduction of the GOF mutations into the Src SH2 T/W mutant reduced the K_D of the SH2 domain for the Frs2 peptide by 35-fold to 41 ± 8 nM. The combined Grb2-like T/W;GOF Src SH2 domain therefore shows a remarkable increase in binding to the Frs2 peptide. Surface plasmon resonance kinetic analysis indicated an increased on-rate (>10×) and decreased off-rate (0.14×) for binding of the Frs2 pY392 peptide ligand to Src SH2 T/W;GOF compared to Src SH2 T/W (Figure S6C), explaining the striking increase in affinity.

In order to test whether the GOF substitutions in the Grb2-like Src SH2 domain increased coupling to endogenous pTyr sites in mESCs, we examined the interaction of synthetic Grb2 proteins consisting of a Grb2-like SH2 domain flanked by Grb2 SH3 domains (termed Grb2[Src SH2]; Figure 6B) with the Grb2 SH2 domain ligands identified in Figure 2A. With SWATH-MS analysis, we found that Frs2, Ptpn11, Shc1, and Snx18 coprecipitated with Grb2[Src SH2 T/W] and bound much more strongly to the Grb2[Src SH2 T/W;GOF] variant with increased pTyr-binding activity (Figures 6C and S6D). In contrast, binding of these Grb2 variants to Sos1 was independent of SH2 domain ligand affinity. The enhanced in vitro pTyr-binding properties of the Src SH2 T/W;GOF mutant domain are therefore replicated in the context of a full-length synthetic Grb2 protein expressed in mESCs.

We also investigated whether SH2 domain ligand affinity influences activation of the Erk pathway. $Grb2^{-/-}$ mESCs did not significantly activate Erk even in the presence of FGF, but Grb2[Src SH2 T/W] expression rescued Erk activation by FGF in a dose-dependent fashion (Figure 6D). Grb2[Src SH2 T/W;GOF] induced Erk activation in the absence of exogenous FGF, which was not further increased upon FGF stimulation. Grb2[Src SH2 T/W;GOF] also augmented the kinetic profile of Erk activation (Figure S6E). Importantly, Erk activation by Grb2 [Src SH2 T/W;GOF] was blocked by the FGFR inhibitor SU5402 (Figure S6E), consistent with the notion that pTyr-binding affinity of the Grb2 SH2 domain is tuned to allow an appropriate response to FGF stimulation. Intriguingly, phosphorylation of Frs2 on Tyr196, a pYxN Grb2 SH2-binding site, was increased in Grb2[Src SH2 T/W;GOF]-expressing cells (Figure S6E), indicating that the increased on-rate and decreased off-rate of Src SH2 T/W;GOF toward SH2 ligands (Figure S6C) may protect them from dephosphorylation. Taken together, these results confirm that the Src SH2 T/W;GOF domain markedly potentiates pTyr signaling and Erk activation.

Based on these observations, we asked whether increased SH2 pTyr binding influences priming of $Grb2^{-/-}$ mESCs expressing synthetic Grb2 proteins. Grb2[Src SH2 T/W;GOF] significantly enhanced Dnmt3b induction compared with WT Grb2 or Grb2[Src SH2 T/W] (Figures 6E and S6F), and this was accompanied by Nanog suppression (Figure S6G). Similar results were obtained with a Sos1 chimera incorporating the GOF Grb2-like Src SH2 domain (Sos1-Src SH2; Figure S6H).

Finally, we measured whether increased Grb2 SH2 phosphopeptide binding drives inappropriate PrE lineage commitment. $Grb2^{-/-}$ mESCs re-expressing WT Grb2 displayed a differentiated colony morphology, accompanied by expression of the PrE marker GATA4 by around day 13 (Figures 2G and 3E). However, by day 9, no detectable GATA4 was expressed by $Grb2^{-/-}$ mESCs, or cells rescued with WT Grb2 or Grb2[Src SH2 T/W] (Figure 6F), indicating a normal delay in PrE differentiation. By contrast, cells expressing Grb2[Src SH2 T/W;GOF] displayed high levels of GATA4 by day 9 (Figure 6F). $Grb2^{-/-}$ mESC colonies expressing Grb2 or Grb2[Src SH2 T/W] proteins also continued to display a packed, undifferentiated morphology similar to that of control cells at day 9 (Figure 6G). However, $Grb2^{-/-}$ mESCs expressing Grb2[Src SH2 T/W;GOF] developed a differentiated morphology by this stage (Figure 6G). Our data show that increased SH2 domain pTyr binding induces precocious PrE differentiation, arguing that the WT Grb2 SH2 domain is biochemically restrained with respect to ligand affinity and has been selected to control appropriate timing of biological events such as embryonic PrE differentiation.

Our results suggest that the Sos1/Grb2 complex functions as a finely tuned coincidence detector to ensure appropriate initiation of cell-fate change. Consistent with this, the E108K mutation, which increased the affinity of the Sos1 HF domain for membranes, caused aberrant mESC priming in the absence of Grb2 SH2 domain-mediated pTyr engagement (Figure 4). One important prediction of our model is that reciprocally

Figure 6. Grb2 SH2 Domain Ligand Affinity Is Tuned to Prevent Precocious Differentiation

(A) Generation of a Grb2-like SH2 superbinder. WT Src SH2 domain complexed with a pYEEI phosphopeptide (left; Waksman et al., 1993). Mutation of the EF1 Thr to Trp introduces a preference for Grb2 ligands (top; Kimber et al., 2000), whereas mutations in the pTyr-binding pocket increase SH2 domain pTyr affinity (bottom; Kaneko et al., 2012). The combination mutant is a high-affinity SH2 domain with preference for Grb2 ligands (right). Green = WT residues; red = mutant.

(B) Synthetic Grb2 in which the SH2 domain is replaced by the Src SH2 T/W (Grb2-like SH2, white) or T/W;GOF (high-affinity Grb2-like SH2, red).

(C) PCA plot of Grb2[Src SH2 T/W] and Grb2[Src SH2 T/W;GOF] interacting proteins quantified by SWATH-MS. Circles represent the relative behavior of individual proteins (see Table S1). Graphical data are average ± SEM (n = 3).

(D) ppErk levels following 10 min aFGF stimulation of $Grb2^{-/-}$ mESCs expressing Grb2[Src SH2] proteins.

(E) Dnmt3b levels in $Grb2^{-/-}$ mESCs expressing WT Grb2 or Grb2[Src SH2] proteins. Data shown are average ± SD (n = 3); *$p \leq 0.04$.

(F) GATA4 expression in day 9 $Grb2^{-/-}$ mESCs expressing WT Grb2 or Grb2[Src SH2] proteins. Intervening lanes were removed.

(G) Colony morphology of $Grb2^{-/-}$ mESCs expressing WT Grb2 or Grb2[Src SH2] proteins.

(H) Dnmt3b levels in $Grb2^{-/-}$ mESCs expressing Sos1-Grb2 and Sos1-Src chimeras. Data shown are average ± SD (n = 3); *$p \leq 0.05$.

See also Figure S6 and Table S1.

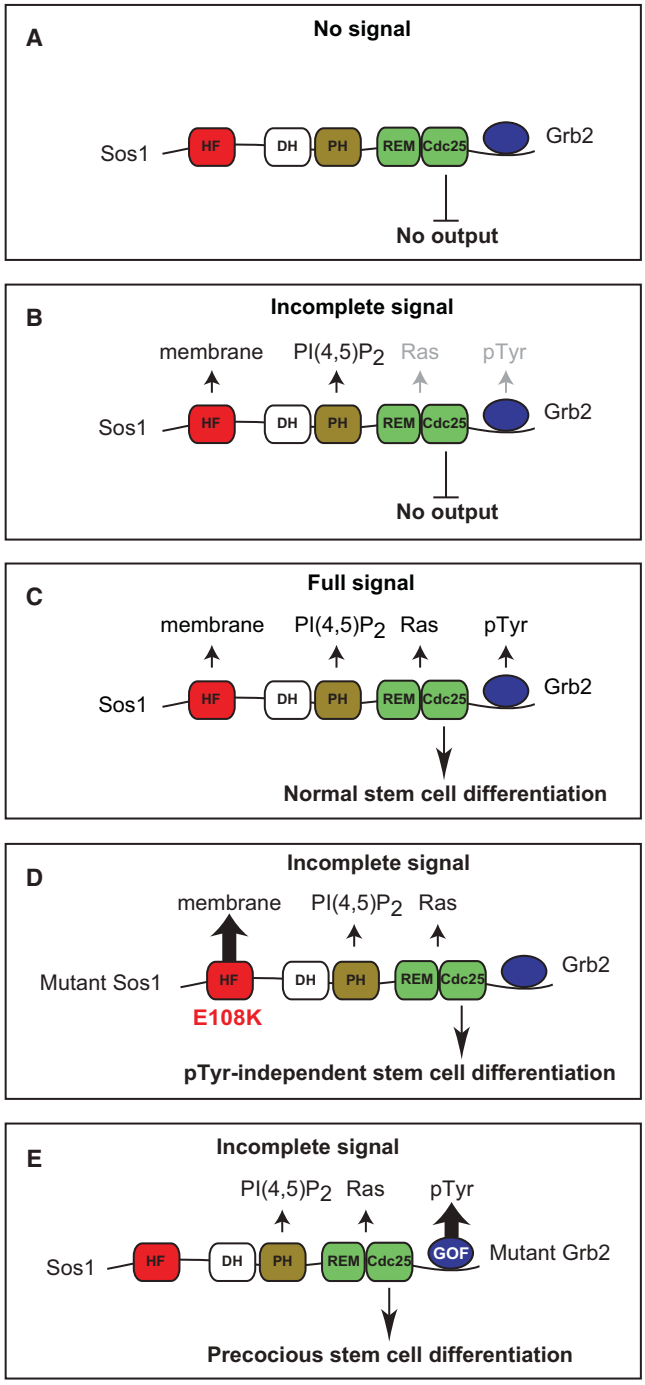

Figure 7. Multiple Limited-Affinity Ligand Interactions Allow Sos1/Grb2 to Appropriately Specify Biological Output

(A) Under resting conditions, the Sos1/Grb2 complex remains inactive in the cytosol and does not induce stem cell differentiation.

(B and C) Incomplete cellular stimulation may result in some interaction of Sos1/Grb2 with membrane ligands (B), but this is insufficient to induce stem cell differentiation until a full signal is received (C), and all ligand binding domains are occupied.

(D) E108K NS mutation in the HF domain increases the affinity of Sos1 for membrane ligands and induces aberrant stem cell differentiation in the absence of a pTyr signal.

(E) Similarly, a Grb2 SH2 domain with increased pTyr affinity drives precocious stem cell differentiation. Therefore, in order for multiple, coincident ligand interactions to appropriately specify stem cell differentiation, individual ligand affinities must be selected to remain below a certain threshold.

altering the ligand affinities of this domain pair, i.e., increasing Grb2 SH2 pTyr affinity while reducing HF membrane engagement, will result in similarly inappropriate mESC differentiation. As previously demonstrated, the Sos1-Grb2 K121E reduced HF membrane affinity and inhibited mESC priming (Figure 6G). However, increasing Grb2 SH2 ligand affinity (Sos1-Src SH2 T/W;GOF) initiated mESC differentiation even in the face of reduced HF membrane engagement. Therefore, reciprocally tuning ligand affinities of the Grb2 SH2 and Sos1 HF domains elicits mESC fate change, despite incomplete ligand engagement. We propose that the relatively modest binding properties of the Sos1 HF and Grb2 SH2 domains form part of a larger system of limited-affinity interactions to tightly control Sos1 activation, ensuring that PrE specification is initiated only upon formation of multiple, coincident biological interactions (Figure 7).

DISCUSSION

Evolution of Multiple Sos1 Protein- and Lipid-Interaction Domains Forms an Integrated System to Ensure Appropriate Induction of Cell-Fate Change

Comparison of eukaryotic protein sequences suggests that metazoans have evolved proteins with increased domain complexity. To understand the importance of multidomain organization for metazoan development, we investigated the extent to which the Sos1 RasGEF integrates multiple protein-protein and protein-lipid interactions to control an essential mammalian morphogenetic process, the formation of PrE. Our data suggest that Sos1 does not simply act as a RasGEF but has also evolved as a coincidence detector, in which the affinity of each module for its cognate ligand has been fine-tuned to monitor the strength and duration of upstream FGF signals. By reciprocally adjusting ligand affinities of individual domains to induce inappropriate cell-fate change, we demonstrate that multiple, balanced ligand interactions within a single protein confer signaling fidelity. This ensures appropriate timing of PrE specification by preventing aberrant activation of the Ras-Erk pathway in pluripotent cells and precocious differentiation of epiblast and PrE lineages (Figure 7). Moreover, the metazoan ability to differentiate distinct cell types, such as epiblast and PrE, may have been facilitated by the evolution of such sophisticated multidomain proteins.

Limited-Affinity Biomolecular Interactions Can Maintain Signaling Specificity

This cooperative system of domain-based interactions depends on the recruitment of Sos1 to pTyr ligands via the Grb2 adaptor protein. The affinity of Grb2 for pTyr-containing sites has been selected to safeguard against inappropriate lineage commitment (Figure 6). Therefore, the Grb2-mediated recruitment of Sos1 to activated receptors defines the timing of events in embryonic development by tightly coupling PrE cell-fate determination to

exposure of pluripotent cells to an appropriately strong and sustained FGF stimulus. The affinity of the Grb2 SH2 domain for pTyr sites therefore explains how a signaling pathway conveys temporal regulation of cellular development.

As a corollary, Sos1 must otherwise have limited membrane affinity to prevent spurious pTyr-independent activation. Indeed, our results indicate that the HF membrane-interaction surface is required for Sos1 activation and PrE specification (Figure 4) but is not in itself sufficient to drive Sos1 activation in the absence of Grb2 (Figure 3). However, a NS mutation in the Sos1 HF domain (E108K) increases HF domain membrane affinity (Figure 4) (Yadav and Bar-Sagi, 2010), uncoupling Sos1 activation and PrE differentiation from Grb2-dependent receptor recruitment. Therefore, the precise affinity of the HF domain for phospholipids is critical in balancing the membrane association that supports Sos1 activation, with a requirement for FGF stimulation to switch on Sos1 RasGEF signaling (Figure 7).

In conclusion, we propose that signaling complexes such as Sos1/Grb2, which control key cell-fate decisions requiring precise temporal and spatial control, employ multiple biologically optimized interaction surfaces that together form a cooperative signaling device. This may be a general regulatory principle. Indeed, an example from yeast suggests that a membrane-binding domain in the Ste5 scaffold protein has imperfections that ensure that activation of the yeast mitogen-activated protein kinase (MAPK) pathway is robustly controlled by mating pheromone (Winters et al., 2005). In the case of Sos1/Grb2, the requirement for concomitant ligand binding by the HF, PH, and RasGEF domains of Sos1 and SH2 domain of Grb2 to trigger pathway activation provides a mechanism by which noise, such as stochastic exposure to growth factors, can be buffered and the timing of differentiation events in the sophisticated process of embryogenesis enforced. Our findings illustrate how the interplay between distinct interaction domains in a protein such as Sos1 not only supports the transmission of a biochemical signal but also ensures a robust response to developmental cues at precisely the right time and with sufficient specificity to safeguard against precocious and hence disastrous induction of cell-fate change.

EXPERIMENTAL PROCEDURES

ESC Culture

mESCs were cultured on gelatin under standard conditions. WT and Sos1$^{-/-}$ mESCs were infected with lentivirus for 24 hr and selected with puromycin after 48 hr. Grb2$^{-/-}$ mESCs were transfected using Lipofectamine LTX (Invitrogen) and selected with puromycin. Unless otherwise stated, Nanog and Dnmt3b levels were determined by immunoblotting.

Immunostaining and Cellomics Analysis

Nanog/Dnmt3b immunostaining of WT, Grb2$^{-/-}$, and Fgf4$^{-/-}$ mESCs was carried out by confocal microscopy, and that of Sos1$^{-/-}$ mESCs was carried out by the Cellomics automated platform (Thermo Scientific).

PrE Differentiation Assays

WT, Grb2$^{-/-}$, Fgf4$^{-/-}$, or Sos1$^{-/-}$ mESCs were either cultured at clonal density, electroporated or infected with lentiviral shRNAs, and selected with puromycin after 24 hr. After 4 days, leukemia inhibitory factor (LIF) was withdrawn from the media, and colonies were cultured further to induce PrE differentiation.

Immunoprecipitation

Grb2$^{-/-}$ mESCs were lysed in FLAG IP-MS lysis buffer (Bisson et al., 2011), and triple-FLAG-tagged proteins immunoprecipitated with FLAG-M2 Agarose (Sigma-Aldrich). Immunoprecipitates were washed thoroughly prior to immunoblot analysis or trypsin digestion followed by mass spectrometry.

Fluorescence Polarization

GST Sos PH domains were incubated with 200 nM BODIPY-labeled soluble PI(4,5)P_2 derivative (GloPIPs BODIPY TMR-PI(4,5)P_2; Echelon), and His-Src SH2 domains were incubated with 20 nM FITC-labeled Frs2 pTyr392 peptide (MHNpYVNTE) prior to fluorescence polarization measurements.

SH2 Domain NMR-Based RasGEF Assay

Lysates from Grb2$^{-/-}$ mESCs transfected with Sos1-Grb2 were incubated with isotopically labeled, GDP-bound ^{15}N-Ras and 10-fold molar excess GTPγS. Peak intensities were extracted from individual spectra to calculate the GDP/GTPγS ratio and plotted against time.

Surface Plasmon Resonance Kinetic Analysis

Comparative evaluation of Src T/W and Src T/W;GOF SH2 domain binding to a FRS2 pY392 peptide was performed with a BIAcore 3000 instrument (Biacore Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.056.

ACKNOWLEDGMENTS

The authors thank Ivica Letunic, Derek Ceccarelli, Bernard Liu, and Geraldine Mbamalu for technical input. We also thank Edvard Smith, Cheryl Seguin, Michael Kofler, Oliver Rocks, Nicolas Bisson, John Kuriyan, and Jody Gureasko for helpful discussions. G.M.F. and T.P. conceived the study, interpreted the data, and wrote the paper. Experiments were performed primarily by G.M.F. and by M.J.S., F.L., M.S.H., G.D.G., and M.T. T. Kaneko, H.H., R.D.B., T. Ketela, J.M., M.I., S.S.C.L., S.S.S., and J.R. provided conceptual insights and reagents. Mass spectrometry was supported by AB SCIEX. This work was supported by grants to T.P. from the Terry Fox Foundation/CIHR (#TFF-105268), the CCSRI, and the ORF; to T.P., J.R., and S.S.S. from the CIHR (#MOP-6849, #FRN-77803, and #MOP-93684); and to M.I. from the CRS. A 600 MHz NMR spectrometer was funded by the CFI. Support for the lentiviral shRNA platform was from the CFI, ORF, OICR, and the Terry Fox Research Institute. M.J.S. is supported by a CBCF fellowship.

Received: June 5, 2012
Revised: October 18, 2012
Accepted: January 31, 2013
Published: February 28, 2013

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