ERBIN is a new SARA-interacting protein: competition between SARA and SMAD2 and SMAD3 for binding to ERBIN

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Summary
SARA, an early endosomal protein, plays a key role in TGFβ signalling, as it presents SMAD2 and SMAD3 for phosphorylation by the activated TGFβ receptors. Here, we show that ERBIN is a new SARA-interacting protein that can be recruited by SARA to early endosomes. ERBIN was recently shown to bind and segregate phosphorylated SMAD2 and SMAD3 (SMAD2/3) in the cytoplasm, thereby inhibiting SMAD2/3-dependent transcription. SARA binds to ERBIN using a new domain, which we have called the ERBID (ERBIN-binding domain), whereas ERBIN binds to SARA using a domain (amino acids 1208–1265) that also interacts with SMAD2 and SMAD3, which we have called the SSID (SARA- and SMAD-interacting domain). We additionally show that SARA competes with SMAD2/3 for binding to ERBIN. In agreement, overexpression of SARA or the ERBID peptide reverses the inhibitory effect of ERBIN on SMAD2/3-dependent transcription. Taken together, these data suggest that the response of cells to TGFβ and activin A can be influenced by the relative concentrations of SARA, ERBIN and SMAD2/3.

Key words: ERBIN, SARA, TGFβ, Activin A, SMAD

Introduction
The transforming growth factor β (TGFβ) family of ligands consists of evolutionarily conserved pleiotropic secreted cytokines, which include TGFβ1, activins and bone morphogenetic proteins (BMPs). Individual members of this family play crucial roles in multiple processes throughout development and in the maintenance of tissue homeostasis in adult life (Massague and Gomis, 2006; Feng and Derynck, 2005). As a consequence, deranged signalling by TGFβ family members has been implicated in many human diseases, including cancer, fibrosis, and autoimmune and vascular diseases (ten Dijke and Arthur, 2007; Gordon and Blobe, 2008). TGFβ ligands trigger heteromeric complex formation between specific transmembrane type I and type II Ser/Thr kinase receptors, in which the type II receptor transphosphorylates and activates the type I receptor. Receptor SMADs (R-SMADs) are phosphorylated by type I receptors, and in turn can form heteromeric complexes with SMAD4. These activated SMAD complexes accumulate in the nucleus, where they directly or indirectly bind to specific promoter regions on target genes together with transcription factor and/or co-activators and co-repressors (Massague and Gomis, 2006).

SMAD anchor for receptor activation (SARA) was originally identified as a protein that functions to recruit non-phosphorylated SMAD2 and/or SMAD3 (SMAD2/3) to the activated receptors for phosphorylation by controlling their localisation. Indeed, SARA binds SMAD2/3 proteins through a specific domain called the SMAD-binding domain (SBD). Receptor-induced phosphorylation of SMADs causes their dissociation from SARA and formation of SMAD2/3–SMAD4 complexes, which subsequently translocate to the nucleus to initiate signalling (Tsukazaki et al., 1998). Although SARA is present at the plasma membrane, where it has been found to interact with TGFβ receptors (Di Guglielmo et al., 2003), it is mainly enriched on the early endosomal membrane through interactions mediated by its FYVE domain (Panopoulou et al., 2002). The predominant localisation of SARA on early endosomes raises the question of whether phosphorylation of SMAD2/3 occurs at the plasma membrane or whether endocytosis to early endosomes is a prerequisite. Several reports, using a number of approaches, have clearly shown that trafficking of the TGFβ receptor into the endocytic compartment enables TGFβ signalling (Hayes et al., 2002; Penheiter et al., 2002). Indeed, the current view is that TGFβ receptors localise to both raft and non-raft membrane domains, and that the internalisation route dictates whether signalling or degradation will ensue.Internalisation of TGFβ receptors, through the clathrin-coated pathway into EEA1- and SARA-positive endosomes, promotes signalling. However, internalisation through the raft-caveolar pathway, where SMAD7 and SMURF2 are localised, promotes ubiquitin-dependent receptor degradation. Indeed, inhibition of this pathway leads to receptor stabilisation, suggesting that trafficking of receptors to the SARA-positive early endosomes functions to sequester receptors from the rafts and caveolae, thereby stabilising the receptors (Di Guglielmo et al., 2003; Felberbaum-Corti et al., 2003). Thus, partitioning between these two internalisation pathways appears to be a dynamic and balanced process influencing the signalling outcome of the activated TGFβ family receptors (Di Guglielmo et al., 2003).
SARA not only presents SMADs to the ligand–receptor complex for phosphorylation, but also recruits protein phosphatase 1 (PP1) to a specific domain downstream (i.e. further towards the C-terminal) of the SBD, thereby mediating TGFβ receptor dephosphorylation (Bennett and Alphey, 2002). Because SARA appears to play multiple roles in TGFβ and activin A signalling, and particularly because of its endosomal localisation, we have undertaken the task of identifying SARA-interacting proteins with the aim of identifying additional endosomal proteins that participate in TGFβ signalling. Among several interacting proteins, we have focused on the protein ERBIN, a protein that interacts with ERBB2 (also known as HER2) and is involved in the localisation and signalling of ERBB2 in epithelia (Borg et al., 2000). Importantly, ERBIN has previously been shown to interact with SMAD proteins (Warner et al., 2003; Dai et al., 2007). Here, we dissect the domains of SARA and ERBIN responsible for their interaction, we define ERBIN as a protein that can be recruited to the early endocytic compartment by SARA and we address the functional consequences of the ERBIN–SARA interaction in TGFβ and activin A signalling.

Results
SARA interacts with ERBIN and recruits it to the early endocytic compartment

To identify SARA-interacting proteins, we carried out a yeast two-hybrid (Y2H) screen using the C-terminal region of SARA (SARAΔ1–664) as bait. Interestingly, besides known SARA-interacting proteins, such as the R-SMADs SMAD2 and SMAD3 (Tsukazaki et al., 1998) and PP1 (Bennett and Alphey, 2002), the screen identified ERBIN as a putative new SARA-interacting protein. Four yeast two-hybrid clones were identified, mapping to a region of ERBIN between amino acids 1081 and 1265, which we designated as the Y2H interaction domain (Y2HID).

To validate the results of the Y2H screen, we carried out GST-pulldown experiments using GST–Y2HID and cell lysates from HEK-293 cells infected with adenoviruses expressing FLAG–SARA or FLAG–SARAΔ1–664. Indeed, the ERBIN Y2HID strongly interacted with both SARA and SARAΔ1–664 and not with control GST (Fig. 1A). Likewise, Myc–ERBIN was co-immunoprecipitated with FLAG–SARAD1–664 in HEK-293 cell lysates, both in the presence and in the absence of TGFβ1 (Fig. 1B). Moreover, we generated a polyclonal antibody to ERBIN and could co-immunoprecipitate endogenous ERBIN and SARA (Fig. 1C). In addition, immunoprecipitation of SARA pulled down endogenous ERBIN (Fig. 1C). Therefore, we conclude that ERBIN is an interaction partner of SARA.

SARA interacts strongly with the membrane lipid PtdIns(3)P, through its FYVE domain, being thus targeted to early endosomes (Panopoulou et al., 2002; Itoh et al., 2002). Indeed, when placental membranes were floated over a sucrose gradient (Papanikolaou et al., 2005), a small fraction of ERBIN was present in the same membrane fractions as SARA (Fig. 1D).

Fig. 1. SARA interacts and colocalises with ERBIN. (A) SARA and ERBIN interact in vitro. HEK-293 cell lysates expressing either FLAG–SARA or FLAG–SARAΔ1–664 were incubated with GST–ERBIN Y2HID or GST alone. The complexes were washed, analysed by SDS-PAGE and immunoblotted (IB) with an anti-FLAG antibody. (B) SARA and ERBIN interact as judged by co-immunoprecipitation of overexpressed proteins. HEK-293 cells were transiently transfected with Myc–ERBIN and infected with FLAG–SARAΔ1–664. Cell lysates were subjected to anti-FLAG immunoprecipitation (IP) and immunoblotted with an anti-Myc antibody. The membranes were re-incubated with an anti-FLAG antibody to confirm the immunoprecipitation of FLAG–SARAD1–664. (C) Endogenous SARA and ERBIN interact as judged by co-immunoprecipitation. Upper panel: HEK-293 cell lysates were incubated with an anti-ERBIN antibody (α-ERBIN) or pre-immune serum, complexes were washed and analysed by immunoblotting using an anti-SARA antibody. The membranes were re-incubated with an anti-ERBIN antibody to confirm the immunoprecipitation of ERBIN. Lower panel: lysates from HCC-78 cells were incubated with an anti-SARA or anti-rabbit-IgG antibody. 10% of the complexes were probed with an anti-SARA antibody, whereas 90% were probed with an anti-ERBIN antibody. (D) Placental homogenate was fractionated using sucrose-gradient flotation followed by western blot analysis. Membranes were probed as indicated. EE indicates early-endosome-enriched fractions. Fractions are labelled 1–10 where 10 represents the bottom of the tube. I, input.
However, despite its association with SARA, as shown above, ERBIN has not been previously demonstrated to exhibit early endosomal localisation. Thus, we tested the colocalisation of overexpressed untagged ERBIN with the early endosomal marker EEA1 (Mu et al., 1995; Simonsen et al., 1998). In agreement with previous reports (Borg et al., 2000), ERBIN was found both at the plasma membrane and in the cytoplasm, exhibiting no colocalisation with EEA1 (Fig. 2A–C). However, upon coexpression of GFP–SARA, ERBIN exhibited considerable colocalisation (in ~50% of the cells) with GFP–SARA on early endosomes (Fig. 2D–F). Overexpression of SARA caused enlargement of early endosomes, as shown previously (Fig. 2D–G) (Hu et al., 2002). Apparently, the quantity of ERBIN recruited to early endosomes by endogenous levels of SARA is insufficient to be detected by the antibody used, although the flotation experiment showed that some ERBIN was present in the same membrane fractions as SARA (Fig. 1D).

Evidently, overexpression of GFP–SARA enriches this protein on early endosomes, thereby allowing detection of its interacting partner ERBIN.

A new domain of SARA (amino acids 730–926) interacts with ERBIN

To characterise the domain of SARA responsible for the interaction with ERBIN, we investigated, in lysates of HEK-293 cells, the interaction of ERBIN Y2HID fused to GST with several HA-tagged constructs spanning the bait sequence of SARA (amino acids 664–1323) used in the Y2H screen (Fig. 3A). GST–Y2HID interacted with SARA-1 (amino acids 667–926), but not with SARA-2 (amino acids 906–1204) or SARA-3 (amino acids 1170–1323) constructs (Fig. 3B). As SARA-1 contains the SBD and PP1-phosphatase-binding motif (PP1BM), we investigated the involvement of these known domains of SARA in the interaction with ERBIN. We did not detect any binding of Myc–ERBIN to the SARA SBD, whereas, as a control, SMAD2 bound strongly to this GST-fusion protein (Fig. 3C).

To characterise the domain of SARA responsible for the interaction with ERBIN, we tested the interaction of GST–Y2HID of ERBIN (Fig. 3D), we conclude that neither the SBD nor the PP1BM of SARA participate in the interaction of SARA with ERBIN. Therefore, it appears that SARA binds to ERBIN through a region between amino acids 730 and 926. We named this domain the ERBID (ERBIN-binding domain). To narrow down further the SARA area that interacts with ERBIN, we utilised a SARA N-terminal construct extending from amino acids 1 to 753 (Fig. 3E). Indeed, this construct interacted with GST–Y2HID, suggesting that the region of SARA that interacts with ERBIN extends from amino acids 730 to 753. However, amino acids 730 to 760 of SARA interacted with ERBIN in a considerably weaker manner compared with that of amino acids 730–926 (Fig. 3F), suggesting that additional amino acids are required for strong SARA–ERBIN interaction.

A new domain of ERBIN (amino acids 1208–1265) interacts with SARA, SMAD2 and SMAD3

To define more accurately the region of ERBIN that mediates binding to SARA, we tested the interaction of GST–Y2HID deletion mutants (Fig. 4A) with FLAG–SARAΔ1–664 using GST-pulldown assays in lysates of HEK-293 cells (Fig. 4B). Initially, we used three overlapping regions of the ERBIN Y2HID, which we named E1 (amino acids 1081–1188), E2 (amino acids 1124–1215) and E3 (amino acids 1159–1265), and a fourth construct E4 (amino acids 1240–1371) containing the last 25 amino acids of the Y2HID followed by the PDZ domain (Fig. 4A). As the Y2HID domain (amino acids 1081–1265) of ERBIN includes the SMAD-interacting domain (SID, amino acids 1172–1282) that has been previously shown to interact with SMAD2 and SMAD3 proteins (Dai et al., 2007), we extended the GST-pulldown assays by also using FLAG–SMAD2, FLAG–SMAD3, Myc–SMAD1 and Myc–SMAD4 constructs (Fig. 4C–F). The ERBIN E3 and E4 GST-fusion constructs interacted strongly with SARAΔ1–664 (Fig. 4B) and SMAD2 (Fig. 4C), whereas SMAD3 interacted predominantly with the E3 construct (Fig. 4D). SMAD1 interacted mainly with the E3 construct, albeit in a much weaker manner (Fig. 4F). SMAD4 interacted weakly and exclusively with the E1 construct (Fig. 4E). Overall, these data demonstrate that SARA, SMAD2 and SMAD3 interact with the same area of ERBIN, extending from amino acids 1208 to 1265.

To define further the minimal region crucial for the interactions of SARA, SMAD2 and SMAD3 with ERBIN, we generated three additional ERBIN constructs, E3C (amino acids 1208–1265, E3C.1 (amino acids 1208–1240) and E3C.2 (amino acids 1241–1265) (Fig. 4A). Whereas SARA and SMAD2 interacted only with E3C.2 (Fig. 4G,H), SMAD3 interacted with both E3C.1 and E3C.2 (Fig. 4I). As expected SARA, SMAD2 and SMAD3 interacted with the larger E3C construct (Fig. 4G–I). By confirmation, SARA, SMAD2 and SMAD3 interacted with the E4 construct (Fig. 4J), whereas none of the constructs interacted with E5 (Fig. 4J), which is derived from E4 by deletion of amino acids 1240–1262 (Fig. 4A). Thus, SARA and SMAD2 interact with ERBIN between amino acids 1241 and 1265, whereas SMAD3 binds ERBIN between amino acids 1208 and 1265. Collectively, these data establish a new binding
Fig. 3. Mapping of the ERBIN interaction domain of SARA. (A) Domain organisation of SARA and the constructs generated to test the interaction with ERBIN (shown on the right-hand side). (B) ERBIN interacts with HA-SARA-1. 293 cell lysates expressing either HA–SARA-1, HA–SARA-2 or HA–SARA-3 were incubated with GST–ERBIN Y2HID or GST alone. The complexes were washed, analysed by SDS-PAGE and immunoblotted (IB) with an anti-HA antibody. (C) ERBIN does not interact with the SARA SBD. HEK-293 cell lysates expressing either Myc–ERBIN or FLAG–SMAD2 were incubated with GST–SBD or GST alone. The complexes were washed, analysed by SDS-PAGE and immunoblotted with either an anti-Myc or anti-FLAG antibody to detect tagged ERBIN or SMAD2, respectively. (D) The ERBIN–SARA interaction does not require the SARA SBD or the PP1BM. HEK-293 cell lysates expressing either HA–SARA-1ΔSBD or HA–SARA-1ΔSBD-PP1BM (HA-SARA-1ΔSBD-PPI) were incubated with GST–ERBIN Y2HID or GST alone. The complexes were visualised using an anti-HA antibody. (E) ERBIN interacts with SARA 1–753. HEK-293 cell lysate expressing FLAG–SARA1-753 was incubated with GST–ERBIN Y2HID or GST alone. The complexes were visualised using an anti-FLAG antibody. (F) ERBIN interacts weakly with SARA 730–760 and SARA 730–773. HEK-293 cell lysate expressing HA–ERBIN was incubated with GST–SARA-1, GST–SARA1ΔSBDΔPP1, GST–SARA 730–773, GST–SARA 730–760 or GST alone. The complexes were visualised as in B. I, input.
domain in ERBIN (amino acids 1208–1265), which is responsible for the interaction with the SARA, SMAD2 and SMAD3 proteins. We have named this domain SSID (SARA- and SMAD-interacting domain).

Given that the same short region of ERBIN is responsible for its interaction with SARA, SMAD2 and SMAD3, we analysed in more detail the SSID and the Y2HID sequences. The SSID sequence is unusually rich in disorder-promoting residues, especially Pro and Gln, and poor in order-promoting amino acid residues (Trp, Ile, Cys and Val) (Fig. 4K). Indeed, several relevant programs (see Materials and Methods) gave a convincing prediction of disorder for SSID, with the short segment comprising amino acids 1246–1257, expected to be α-helical, representing a ‘dip’ in the disorder prediction (Fig. 4K).

Moreover, the 1246–1257 segment could be classified as an α-MoRF (for molecular recognition feature, also known as a molecular recognition element, MoRE). MoRFs are short, loosely structured protein regions embedded within longer largely disordered sequences, which, upon binding to their partner(s), undergo disorder-to-order transitions (Cheng et al., 2007).

**SMAD2/3 and SARA interact with ERBIN independently of each other, thereby competing with each other for binding to the SSID of ERBIN**

As SARA binds to ERBIN using a distinct domain from the SBD (i.e. the ERBID, SARA 730–926, Fig. 3) and the interaction was detected using the Y2H system, SARA and ERBIN must bind to each other directly. However, indirect interaction of ERBIN with SMAD2/3, through mutual binding to SARA, is also possible. To address this, we investigated whether ERBIN could interact with the phosphorylated SMAD2/3 proteins, which do not bind SARA (Tsukazaki et al., 1998). To this end, we tested the interaction by examining co-immunoprecipitation of HA–ERBIN with Myc-tagged versions of either full-length SMAD2/3 or the constitutively active (ca) SMAD2 and SMAD3, in which the SSMS motif is mutated to EDME and EDVE, respectively, thereby mimicking the phosphorylated molecules (Ofr et al., 2002). Both the non-phosphorylated and the constitutively active forms of SMAD2 and SMAD3 bound ERBIN to a similar extent (Fig. 5A) suggesting that binding of SMAD2/3 to ERBIN is independent of their binding to SARA. Consequently, given the fact that, first, SMAD2/3 and SARA can bind independently of each other to ERBIN and, second, SMAD2/3 and SARA bind to the SSID of ERBIN, we hypothesised that the interaction of SMAD2/3 and SARA to ERBIN could be competitive and mutually exclusive. Indeed, expression of increasing amounts of FLAG–SARA dramatically decreased the amount of Myc–SMAD2ca (Fig. 5C) or Myc–SMAD3ca (Fig. 5B) pulled down by the EC3 construct of ERBIN (the SSID, amino acids 1208–1265). Thus, SMAD2/3 and SARA interact with ERBIN independently of each other, thereby competing with each other for binding to the SSID of ERBIN.

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**SARA or its ERBID peptide attenuates the inhibitory effect of ERBIN on TGFβ and activin A transcriptional responses**

Because ERBIN binds both SARA and SMAD2/3, we investigated its role in TGFβ and activin A signalling using a loss-of-function approach. To this end, we made two A431 stable cell lines in which the CAGA-luc SBE (SMAD-binding element) (responsive to TGFβ and activin A) and the Renilla luciferase gene (constitutively expressed to correct the CAGA-luc values) were stably integrated. Using two different small interfering RNAs (siRNAs) for ERBIN, we were able to decrease the levels of endogenous ERBIN protein in stably transfected A431 cell lines compared with that in control cells as judged by western blot analysis (Fig. 6A). Using both siRNAs in transcriptional response assays, we observed a significant increase of both activin-A- and TGFβ-induced CAGA-luc levels (Fig. 6A). Collectively, these results show that ERBIN plays an inhibitory role in TGFβ and activin A transcriptional responses.

To verify the effect of ERBIN on TGFβ and activin A signalling, we used a gain-of-function approach. For this purpose, we overexpressed ERBIN in HEK-293 cells co-transfected with each of the following three reporter constructs: (1) the CAGA-luc reporter, responsive to TGFβ and activin A through SMAD3, (2) the ARE-luc (luciferase driven by the activin response element), responsive to activin A signalling through SMAD2 and cooperation with the transcription factor FAST, and (3) the BRE-luc (luciferase driven by the BMP response element) activated by BMPs through SMAD1 and/or SMAD5. As can be seen in Fig. 6B, overexpression of ERBIN strongly inhibited transcription of the CAGA-luc and ARE-luc reporters and also, to a lesser extent, BRE-luc transcription, whereas it exerted no effect on a SMAD-independent promoter (E-selectin) (Fig. 6B). The effect of ERBIN on the SBE-luc reporter was dose dependent (data not shown). Thus, ERBIN inhibits TGFβ and activin A transcriptional activation and additionally has a negative effect on BMP-induced transcription, probably through its weak interaction with SMAD1 (Fig. 4F).

Importantly, overexpression of SARA and ERBIN (SARA amino acids 730–926) or even the minimal interacting area (SARA amino acids 730–773) reverses the negative effect of ERBIN overexpression on the TGFβ-induced transcriptional activation of CAGA-luc in a statistically significant manner (Fig. 6C, D). Moreover, overexpression of the ERBIN or SARA amino acids 730–773 enhances the transcriptional activity of TGFβ, even in the absence of ERBIN overexpression (Fig. 6D).

As SARA binds to ERBIN using a distinct domain from the SBD (i.e. the ERBID, SARA 730–926, Fig. 3) and the interaction was detected using the Y2H system, SARA and ERBIN must bind to each other directly. However, indirect interaction of ERBIN with SMAD2/3, through mutual binding to SARA, is also possible. To address this, we investigated whether ERBIN could interact with the phosphorylated SMAD2/3 proteins, which do not bind SARA (Tsukazaki et al., 1998). To this end, we tested the interaction by examining co-immunoprecipitation of HA–ERBIN with Myc-tagged versions of either full-length SMAD2/3 or the constitutively active (ca) SMAD2 and SMAD3, in which the SSMS motif is mutated to EDME and EDVE, respectively, thereby mimicking the phosphorylated molecules (Ofr et al., 2002). Both the non-phosphorylated and the constitutively active forms of SMAD2 and SMAD3 bound ERBIN to a similar extent (Fig. 5A) suggesting that binding of SMAD2/3 to ERBIN is independent of their binding to SARA. Consequently, given the fact that, first, SMAD2/3 and SARA can bind independently of each other to ERBIN and, second, SMAD2/3 and SARA bind to the SSID of ERBIN, we hypothesised that the interaction of SMAD2/3 and SARA to ERBIN could be competitive and mutually exclusive. Indeed, expression of increasing amounts of FLAG–SARA dramatically decreased the amount of Myc–SMAD2ca (Fig. 5C) or Myc–SMAD3ca (Fig. 5B) pulled down by the EC3 construct of ERBIN (the SSID, amino acids 1208–1265). Thus, SMAD2/3 and SARA interact with ERBIN independently of each other, thereby competing with each other for binding to the SSID of ERBIN.
Fig. 4. See next page for legend.
**Fig. 5. SARA and ERBIN compete for binding to unphosphorylated and phosphorylated SMAD2 and SMAD3.** (A) ERBIN interacts with phosphorylated and nonphosphorylated SMAD2/3. HEK-293 cells were transiently co-transfected with Myc-SMAD3 and HA-ERBIN, Myc-SMAD2 and HA-ERBIN, Myc-SMAD3ca and HA-ERBIN, or Myc-SMAD2ca and HA-ERBIN. Anti-HA antibody or control rat IgG immunoprecipitations (IP) were performed and immunoblotted (IB) with anti-Myc antibody. The membranes were re-incubated with anti-HA antibody to confirm the immunoprecipitation of HA-ERBIN. (B) SARA competes with SMAD2 and SMAD3 for binding to ERBIN. HEK-293 cell lysate expressing Myc-SMAD3ca was incubated with GST-E3C, complexes were washed and increasing amounts of FLAG-SARA were added for competition. The complexes were washed, analysed by SDS-PAGE and immunoblotted with an anti-FLAG antibody to detect FLAG-SARA, and an anti-Myc antibody to detect Myc-SMAD3ca. The experiment was repeated, as in B, using Myc-SMAD2ca.

3-P has important consequences on the regulation of the TGFβ and activin A functions in cells. Indeed, both silencing of the ERBIN gene and overexpression of the ERBID (SARA 730–926) exerted a positive effect on TGFβ-dependent induction of plasminogen activator inhibitor 1 (PAI-1) expression (Fig. 7E), whereas overexpression of the ERBID enhanced the inhibitory effect of TGFβ on HaCaT cell proliferation (Fig. 7F).

**Discussion**

In a Y2H screen for SARA-interacting proteins, among other proteins, we identified ERBIN, a member of the LAP family [containing LRR (leucine-rich repeat) and PDZ domains] (Borg et al., 2000). LAP family members contain 16 LRRs at their N-termini and up to four PDZ domains at their C-termini. The LAP family is composed of LET-413 in *C. elegans*, Scribble in *Drosophila melanogaster*, and ERBIN, densin-180 (also known as LRRC7), scribble and Lano (also known as LRRC1) in mammals, proteins that are considered to be adaptor proteins involved in polarity and/or receptor targeting (reviewed by Bryant and Huwe, 2000). Indeed, ERBIN is a basolaterally localised LAP protein and has been shown to interact, through its PDZ domain, with ERBB2 of the epidermal growth factor receptor (EGFR) family, thereby restricting ERBB2 to the basolateral membrane of polarised epithelial cells (Borg et al., 2000). Moreover, ERBIN interacts, through its LRR domain, with the scaffold protein Sur-8 disrupting the Sur-8–Ras–Raf complex, thereby inhibiting activation of extracellular signal-regulated kinase 1 or 2 (ERK1/2) (Dai et al., 2006; Huang et al., 2003). ERBIN also plays a crucial role in myelination, given that it binds to and stabilises ERBB2 receptor, which is necessary for NRG1 signalling (Tao et al., 2009).
Fig. 6. See next page for legend.
ERBIN has been implicated in TGFβ signalling through its interaction with both non-phosphorylated and phosphorylated SMAD2/3 (Warner et al., 2003; Dai et al., 2007). ERBIN overexpression inhibited TGFβ-dependent transcription, whereas siRNA against ERBIN stimulated ligand-induced responses. ERBIN overexpression had no effect on ligand-induced phosphorylation of SMAD2/3; however, accumulation of SMAD2 in the nucleus was decreased. Therefore, a sequestering role for ERBIN was proposed, according to which ERBIN binds to phosphorylated SMAD2/3 preventing their association with SMAD4 and subsequent accumulation in the nucleus, thereby inhibiting TGFβ signalling (Dai et al., 2007). In agreement, we report that overexpression of ERBIN inhibits, whereas siRNA silencing of ERBIN enhances, TGFβ- and activin-A-dependent transcription. However, we additionally demonstrate that ERBIN interacts also with SARA, another regulator of the TGFβ and activin A pathway. Indeed, the initial Y2H interaction was validated using pull-down and immunoprecipitation assays. Moreover, ERBIN floated, in sucrose gradients, in the same membrane fractions as SARA and EEA1, known early endocytic markers, and overexpressed SARA recruited ERBIN to early endosomes. We cannot rule out that there is also a minor colocalisation at the plasma membrane, as SARA has been shown to interact with TGFβ receptors at the plasma membrane (Di Guglielmo et al., 2003) and ERBIN is also detected at the plasma membrane (Borg et al., 2000). Although the localisation of ERBIN on early endosomes has not been reported previously, individual ERBIN-positive cytotoxic vesicles have been described, by immunohistochemistry, in basal cell carcinoma (BCC) (Lebeau et al., 2005), and punctate ERBIN staining has been observed, by immunofluorescence, in proximal dendrites (Calin-Jageman et al., 2007). Taken together, we define ERBIN as a newly identified SARA-interacting protein, which can colocalise with the latter on early endosomes.

We mapped the ERBIN interaction domain of SARA (Fig. 3) and concluded that it is contained within the region comprising amino acids 730–926, downstream of the SBK (Tsukazaki et al., 1998) and PP1BM (Bennett and Alphey, 2002), which we have called the ERBID. We additionally mapped the SARA interaction domain of ERBIN (Fig. 4) and found that a 25-amino-acid domain (amino acids 1241–1265) of ERBIN is responsible for the interaction with SARA. Because these 25 amino acids were included in a longer 110-amino-acid stretch (1172–1282) that has been shown to interact with SMAD2/3 (Warner et al., 2003; Dai et al., 2007), we further defined the interaction domain of SMAD2 and SMAD3 on ERBIN. Much to our surprise, the SMAD2 and SARA-interacting domains of ERBIN were identical, being contained between amino acids 1241 and 1265. The interaction domain of ERBIN with SMAD3 was contained within a slightly larger area between amino acids 1208 and 1265. Additional electrostatic contacts between Arg279 of the MH2 domain of SMAD3 and Glu1321 of the PDZ domain of ERBIN might also participate in the SMAD3–ERBIN interaction, as suggested by recent molecular dynamics simulations (Deliot et al., 2009). Because the area between amino acids 1208 and 1265 binds SARA, SMAD2 and SMAD3 we have called it the SSID.

The fact that the same short unstructured region of ERBIN (i.e. the SSID) can bind both SARA and SMAD2/3 is not so surprising in light of many recent studies that have revealed that the true functional state for many proteins and protein domains is intrinsically unstructured (Wright and Dyson, 1999; Uversky et al., 2005; Fink, 2005; Dyson and Wright, 2005; Tompa, 2002; Dunker and OBradovic, 2001; Dyson and Wright, 2002). Protein regions or full-length proteins that are intrinsically disordered (IDPs) lack a single stable three-dimensional structure under physiological conditions, but adopt a well-defined conformation upon interacting with a target molecule in a manner different from that of the induced fit. Similar suggestions have been made in the past for the interactions of the SMAD2/3 MH2 domain, which is known to bind a diverse group of ligands that are completely unrelated in sequence and structure, ranging from transcription factors to receptors and membrane-anchoring proteins. X-ray, nuclear magnetic resonance (NMR), biophysical and bioinformatics studies have recently suggested that at least some of these interactions, including the one with SARA, are mediated by a number of hydrophobic patches distributed over the surface of MH2 that are able to recognise disordered regions of proteins in an extended conformation (Wu et al., 2000; Chong et al., 2004). SARA, in particular, interacts with SMAD2/3 through its natively unstructured SBD, which makes extensive hydrophobic contacts with MH2 and folds upon binding. Thus, the data presented in this work render ERBIN as a new potential hub protein with its intrinsically disordered regions, such as the SSID, contributing to several of its multiple interactions, including those with SARA and SMAD2/3.

The present work adds an unexpected twist to the sequestration model in which ERBIN physically sequesters SMAD2/3-P and prevents it binding to SMAD4, thereby inhibiting their accumulation in the nucleus and blocking TGFβ-dependent transcriptional activation (Dai et al., 2007). Indeed, this work provides evidence that SARA plays a regulatory role in the SMAD2/3–ERBIN interaction. We have shown that SARA and SMAD2/3 bind to ERBIN independently of each other, thereby competing for binding to the SSID of ERBIN. This result suggests that the response of a cell to TGFβ and activin A can be

**Fig. 6.** ERBIN is a negative regulator of activin A and TGFβ signalling. (A) Two A431 clones (C1 and C2) stably harbouring the CAGA-luc reporter gene were transected with two siRNAs targeting ERBIN, and were then serum starved and stimulated with either activin A (ActA) or TGFβ. Firefly and Renilla luciferase levels (RLU) were measured after 16 hours. Immunoblot (IB) analysis following siRNA transfection was performed using an anti-ERBIN antibody. Tubulin is shown as a loading control. (B) Overexpression of Myc–ERBIN inhibits activin A, TGFβ and BMP2 signalling. HEK-293 cells were co-transfected with Myc–ERBIN or vector control, and CAGA-luc, ARE-luc, BRE-luc or E-selectin-luc. Luciferase assays were performed as outlined in the Materials and Methods. Lysates were immunoblotted with an anti-Myc antibody to ensure expression of Myc–ERBIN. (C) Overexpression of SARA reverses the inhibitory effect of ERBIN on TGFβ signalling. HEK-293 cells were co-transfected with either CAGA-luc and ERBIN, or transfected with ERBIN and infected with SARA adenovirus. Cells were serum starved and TGFβ added for 16 hours. Luciferase assays were performed as outlined in the Materials and Methods. The lysates were immunoblotted with anti-Mye and anti-FLAG antibodies to ensure expression of Myc–ERBIN and FLAG–SARA. (D) Overexpression of SARA 730–926 and SARA 730–773 reverses the inhibitory effect of ERBIN on TGFβ signalling. HEK-293 cells were co-transfected with CAGA-luc and either Myc–ERBIN or vector control, with or without GFP–SARA 730–926 or GFP–SARA 730–773 as indicated. Luciferase assays were performed as outlined in the Materials and Methods. The lysates were immunoblotted with anti-ERBIN and anti-GFP antibodies to ensure expression of Myc–ERBIN, GFP, GFP–SARA 730–926 and GFP–SARA 730–773. The data are means ± s.d. of triplicate samples from three independent experiments (a representative blot is shown). *P<0.05.
Fig. 7. See next page for legend.
regulated by the relative concentrations of SMAD2/3, SARA and ERBIN, as well as the binding affinities, of these proteins. Indeed, a cell with a high ratio of ERBIN/SARA concentration is expected to sequester ligand-induced SMAD2/3-P, thereby inhibiting their nuclear accumulation and transcriptional effects, as shown previously (Dai et al., 2007). By contrast, a cell with high ratio of SARA/ERBIN concentration is expected to compete the sequestering capacity of ERBIN; following SMAD2/3 phosphorylation, higher levels of SARA should be able to maximize the nuclear accumulation of SMAD2/3-P as SARA

Fig. 7. The competition between SARA and ERBIN for binding to SMAD2/3 has functional consequences for TGFβ signalling. (A) Overexpression of GST–SSID inhibits, whereas ERBIN (isofrom 7) has no effect on, TGFβ signalling. HEK-293 cells were transfected with CAGA-luc and GST–SSID, ERBIN (isofrom 7) or ERBIN, and vector controls. Luciferase assays were performed as outlined in the Materials and Methods. The lysates were immunoblotted with antibodies against GST, ERBIN or Myc. (B) SARA 730–926 overexpression and ERBIN knockdown do not affect SMAD2/3 phosphorylation kinetics. HEK-293 cells were either transfected with GFP–SARA 730–926, GFP alone or siRNA targeting ERBIN, and were then serum starved and stimulated with TGFβ. Phosphorylation of SMAD2/3 was detected by immunoblotting (IB) with antibodies specific for SMAD2-P (PS2) or SMAD3-P (PS3). (C) SARA 730–926 overexpression affects complex formation between SMAD3 and SMAD4. HEK-293 cells were transfected with the indicated plasmids and cell lysates subjected to antiFLAG immunoprecipitation (IP). Immunoprecipitates were immunoblotted with anti-Myc antibody to detect Myc–SMAD3 co-immunoprecipitated with FLAG–SMAD4, and anti-FLAG antibody to ensure equal immunoprecipitation of FLAG–SMAD4. Total lysates were immunoblotted with anti-GFP antibody to detect GFP and GFP–ERBIN, anti-HA antibody to detect HA-ALK5ca, anti-FLAG antibody to detect FLAG–SMAD4 and anti-Myc antibody to detect Myc–SMAD3 and Myc–ERBIN. SMAD3 and SMAD4 bands were quantified by densitometry and the SMAD3 co-immunoprecipitated by SMAD4 is shown in the graph, after normalisation against the intensity of the immunoprecipitated SMAD4 band. The ratio of SARA/SMAD3 band intensities in the control experiment was given the arbitrary value 1. The data are means ± s.e.m for four independent experiments (a representative blot is shown). *P<0.05. (D) SARA 730–926 enhances and ERBIN overexpression inhibits nuclear accumulation of GFP–SMAD2 upon TGFβ stimulation. HaCaT cells were transfected with GST–SARA 730–926 or HA–ERBIN, and were then serum starved and stimulated with TGFβ for 1 hour. Statistical analysis of cells transfected with GST–SARA 730–926 or GST alone with a ratio of fluorescence intensity (nucleus:cytoplasm) higher than 2.8. In the case of HA–ERBIN overexpression, GFP–SMAD2 accumulation in the nucleus or retention in the cytoplasm was estimated in >150 cells per experiment in three independent experiments. The graph represents the results from the three experiments. (E) siRNA against ERBIN or overexpression of SARA 730–926 enhances TGFβ-induced expression of plasminogen activator inhibitor 1 (PAI-1). HEK-293 cells were transfected either with an siRNA against ERBIN or infected with adenoviruses expressing GFP alone, GFP–SARA 730–926 or GFP–SARA 730–773. Western blot analysis following siRNA transfection was performed using an anti-ERBIN polyclonal antibody. β-Actin is shown as a loading control. Anti-GFP antibody was used to detect viral infection. siRNA against ERBIN enhances TGFβ induced expression of PAI by ~1.83-fold (*P<0.05 compared with control; n=4) and overexpression of ERBIN and SARA 730–773 enhances TGFβ-induced expression of PAI-1 by ~2.6- and by ~2.4-fold, respectively (*P<0.05 compared with control; n=3). (F) Overexpression of SARA 730–926 or SARA 730–773 enhances TGFβ-induced growth inhibition. HaCaT cells were transfected with GFP–SARA 730–926, GFP–SARA 730–773 or GFP alone, and were then serum starved and stimulated with TGFβ. BrdU incorporation was estimated in >300 cells in each experiment and results are expressed as a percentage of the proliferating cells (*P<0.05 compared with control; n=4).

Fig. 8. A model illustrating the interactions between SARA, ERBIN and SMAD2/3. ERBIN interacts with SARA using a domain that interacts also with SMAD2 and SMAD3 (amino acids 1208–1265, SSD). As a consequence, SARA competes with SMAD2/3 for binding to ERBIN. As ERBIN binds and segregates phosphorylated SMAD2/3 in the cytoplasm, SARA not only ensures proper presentation of SMAD2/3 for phosphorylation by TGFβ and activin A receptors but also facilitates the nuclear transfer of phosphorylated SMAD2/3 by competing for their cytoplasmic segregation by ERBIN. Thus, the cellular response to TGFβ/activin A might be regulated by a complex interplay between the relative concentrations of SARA, ERBIN and SMAD2/3, as well as their binding affinities. See the Discussion for details.
algorithm (Iakoucheva et al., 2004); these have been also experimentally detected in a large-scale phosphoproteomic survey of tyrosine kinase activity using a large number of non-small cell lung cancer (NSCLC) cell lines and NSCLC tumours (Rikova et al., 2007). Questions remaining include: does phosphorylation within the SSID in some way regulate the affinity of SARA and/or SMAD3 binding? How is the effect of ERBIN on non-SMAD and SMAD-dependent responses integrated in the overall response of a cell to TGFβ? Are the effects of ERBIN on ERBB2 localisation and inhibition of ERK1/2 phosphorylation functionally linked with those of TGFβ signalling in the cells? Definitely, more work is required to elucidate these issues.

In conclusion, we have identified ERBIN as a novel SARA-interacting protein that can be recruited by the latter to early endosomes, where SARA predominantly resides. SARA binds to ERBIN using a new domain downstream of the SBD and PP1BM, which we have called the ERBID. ERBIN interacts with SARA using a domain that interacts also with SMAD2 and SMAD3 which we called the SSID (amino acids 1208–1265). As a consequence, SARA competes with SMAD2/3 for binding to ERBIN. Because ERBIN binds and segregates phosphorylated SMAD2/3 in the cytoplasm, thereby inhibiting SMAD2/3-dependent transcription, overexpression of SARA or the ERBID peptide reverses the inhibitory effect of ERBIN on SMAD2/3-dependent transcription. Thus, SARA not only ensures proper presentation of SMAD2/3 for phosphorylation by TGFβ and activin A receptors but also facilitates the nuclear transfer of phosphorylated SMAD2/3 by competing for their cytoplasmic segregation by ERBIN. Our data suggest that the response of cells to TGFβ and activin A might be regulated by a complex interplay between the relative concentrations of SARA, ERBIN and SMAD2/3, as well as their binding affinities.

Materials and Methods
Identification of SARA interacting proteins by Y2H screening
Yeast two-hybrid screening and data analysis were performed by Hybrigenics (Paris, France). SARA (amino acids 665–1323) was cloned into a Y2H vector optimized by Hybrigenics. A random primed human placental cDNA library was used for the screen.

Expression plasmids and siRNAs
GST–ERBIN 1240–1371 (E4) and Myc–ERBIN 1–1371 (isoform 2) were kindly provided by Jean-Paul Borg (Institut de Cancérologie de Marseille, Marseille, France). ERBIN (isoform 7) was kindly provided by Borradori Luca (Geneva University Hospital, Geneva, Switzerland) (Favre et al., 2001). pGEX5X-3–ERBIN 1081–1265 (Y2HID), pGEX6P-1–ERBIN 1081–1188 (E1), pGEX6P-1–ERBIN 1124–1215 (E2), pGEX6P-1–ERBIN 1159–1265 (E3), pGEX6P-1–ERBIN 1208–1265 (E3C), pGEX6P-1–ERBIN 1208–1240 (E3C1), pGEX6P-1–ERBIN 1241–1265 (E3C2), pcDNA3-HA–SARA 667–926 (SARA-1), pcDNA3-HA–SARA 906–1204 (SARA-2), pcDNA3-HA–SARA 1170–1323 (SARA-3), pcDNA3-HA–SARA 722–926 (SARA-1ASBD) and pGEX6P-1–SBD 667–721 (SBD), pGEX-6P-3–SARA 730–773, pGEX-6P-3–SARA 730–760, pCMV-Shuttle-FLAG–SARA 735 (SARA N-terminus) were generated by PCR using the primers in supplementary material Table S1. GFP–SARA 722–926, GFP–SARA 730–773 or GFP alone using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were starved of serum (0.2% FCS) and then added with 0.5 ng/ml TGFβ1 for 24 hours. At 48 hours after transfection, cells were starved of serum (0.2% FCS) overnight and then infected with 5 ng/ml TGFβ1 for 10 hours. Cell lysates were subjected to SDS-PAGE analysis, followed by western blot analysis using an antibody against PAI-1.

Proliferation assay
HaCaT cells were transiently transfected with GFP–SARA 730–926, GFP–SARA 730–773 or GFP alone using Lipofectamine 2000 (Invitrogen). After 72 hours, cells were subjected to SDS-PAGE analysis, followed by western blot analysis using an antibody against PAI-1.

BMP-2 was from Immunotools. An anti-EEA1 rabbit polyclonal antibody was kindly provided by Mariano Zerial (MPI-CBG, Dresden, Germany). A mouse monoclonal anti-EEA1 antibody was purchased from Sigma-Aldrich. Rat monoclonal anti-HA antibody was purchased from Sigma-Aldrich. Rat monoclonal-anti-HA antibody was kindly provided by Syuzuru Etoh (Ajinomoto, Japan) and Marko Hyvonen (Department of Biochemistry, University of Cambridge, Cambridge, UK) (Harrington et al., 2006). Indirect immunofluorescence
NIH 3T3 cells were transfected with Effectene (Qiagen). Indirect immunofluorescence, confocal microscopy and image acquisition were carried out as described previously (Panopoulou et al., 2002).

GST pull-down assays
GST fusion proteins or GST alone (~15–30 µg, equimolar amounts) were incubated for 1 hour at 4°C with 20 µl of glutathione–agarose beads (Amersham Biosciences), in assay buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40 and protease inhibitors from Roche). After washing three times with assay buffer, the beads were combined with HEK-293 cell extracts transiently transfected or infected with the protein of interest and incubated for a further 4
hours at 4°C with rotation. The beads were washed three times before eluting the bound proteins with SDS sample buffer.

**Immunoprecipitation and immunoblotting**

HEK-293 cells were transfected for 36 hours using FuGENE 6 (Roche) and lysed in buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100 and protease inhibitors from Roche). The lysates were pre-absorbed with protein G beads and incubated with the appropriate antibodies for 12–18 hours at 4°C with rotation. Protein G beads were added for 4 hours at 4°C, with rotation, followed by five washes with lysis buffer. Immunoprecipitates were separated by SDS-PAGE and immunoblotted. The lysis buffer for complex formation contained 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM sodium vanadate, 25 mM sodium fluoride, 50 mM o-phenylacetic acid and 10 mM 2-glycerophosphate and protease inhibitors.

**Reporter assays for SMAD-dependent transcription**

HEK-293 cells were transfected with FuGENE 6 in medium, 20 hours later the cells were placed into reduced serum medium (0.2% FBS) for 8 hours. Then, cells were treated or not with 50 ng/ml activin A, 2.5 ng/ml TGFβ1 or 100 ng/ml BMP2 and incubated for an additional 16 hours. Cells were processed for luciferase as described in the Promega E4030 luciferase kit, and β-galactosidase (β-gal) activity was measured using a standard protocol. Relative light units were measured using a Berthold luminometer and standardized for transfection efficiency using the β-gal values. Cells were plated in triplicate wells and assays were repeated three times.

**siRNAs assays in stable cell lines**

For the generation of stable transfecants, A431 cells were co-transfected with CAGA-luc-pri and pSRV55-puro unstructured plasmids using FuGENE 6. After 24 hours, cells were subcultured into medium containing 0.4 μg/ml puromycin. Two clones highly responsive to TGFβ1 and activin A were selected for re-transfection with Renilla pRL-TK vector plus a neomycin resistance cassette, and isolated clones were selected in the presence of 0.75 mg/ml G418 and 0.4 μg/ml puromycin and tested for CAGA-luc-pri activation by activin A and TGFβ1 and constitutive expression of Renilla luciferase. A total of two clones were selected and were used for the studies. The Dual luciferase kit was used according to the manufacturer’s instructions (Promega).

**Preparation of ERBIN polyclonal antiserum**

A rabbit polyclonal antibody was generated using as antigen GST-ERBIN 1240–1371 protein. Crude serum was first depleted of anti-GST antibodies, then a rabbit polyclonal antibody was generated using as antigen GST–ERBIN 1240–1371 protein. Crude serum was first depleted of anti-GST antibodies, then a rabbit polyclonal antibody was generated using as antigen GST–ERBIN 1240–1371 protein. Crude serum was first depleted of anti-GST antibodies, then a rabbit polyclonal antibody was generated using as antigen GST–ERBIN 1240–1371 protein. Crude serum was first depleted of anti-GST antibodies, then a rabbit polyclonal antibody was generated using as antigen GST–ERBIN 1240–1371 protein. Crude serum was first depleted of anti-GST antibodies.

**Sequence analysis**

The internet server PredicProtein was used for secondary structure predictions (Yang et al., 2005), DisEMBL (Linding et al., 2003), IUPred (Dosztanyi et al., 2004). Disorder predictions were carried out using the network ENDOTRACK (EU FP6, LSH-2004-1.1.5-2). G.S. was supported by the PENED 03EA562 program, co-financed by E.U. European Social Fund (75%) and the Greek Ministry of Development GRST (25%).

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**References**


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Table S1. Oligonucleotides used for PCR amplification of all constructs generated

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