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Syndecan-4 regulates subcellular localization of mTOR complex2 and Akt activation in a PKCα-dependent manner in endothelial cells

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SUMMARY

Mammalian target of rapamycin (mTOR) activity is regulated by assembly of two functionally distinct complexes, mTORC1 and mTORC2. In syndecan-4 (S4) null endothelial cells, mTORC2 activity is reduced, resulting in decreased Akt activation, while mTORC1 activity is increased. Levels of rictor, mLST8, and mSin-1 are unchanged in total cell lysates but decreased in the rafts of $S4^{-/-}$ endothelial cells, as is the level of PKCα. Expression of myristoylated-PKCα in $S4^{-/-}$ cells restores rictor, mLST8, and mSin-1 presence in the rafts and rescues Akt phosphorylation. PKCα knockdown mimics the effect of S4 deletion on mTORC2 localization and Akt activation. Reduced mTORC2 activity in $S4^{-/-}$ endothelial cells results in decreased FOXO1/3a and eNOS phosphorylation, decreased endothelial cell size and increased arterial blood pressure in $S4^{-/-}$ mice. Thus, S4-dependent targeting of PKCα to the plasma membrane is required for recruitment of mTORC2 components to the rafts and Akt activation.

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INTRODUCTION

Mammalian TOR (mTOR) resides in two protein complexes mTORC1 and mTORC2. Mammalian TORC1 is the rapamycin-sensitive complex consisting of mTOR, mLST8, and raptor while mTORC2 complex contains mTOR, mLST8, rictor, mSin1 and recently identified protor. mTORC2 is thought to activate Akt in response to various growth factors, while mTORC1 activation results in phosphorylation of the translational regulators p70S6 kinase 1 (p70S6K1) and 4E-BP1, augmenting protein synthesis (Yang and Guan, 2007).

Akt/protein kinase B plays a central role in numerous biological processes including cell growth, proliferation, migration and survival, eNOS activation, and angiogenesis among others (Kandel and Hay, 1999; Shiojima and Walsh, 2002; Somanath et al., 2006). It does so by phosphorylating an array of substrates such as GSK3, TSC2, BAD, the Forkhead family of transcription factors (FOXOs), and eNOS. Growth factors and cytokines activate Akt via PI 3-Kinase (PI 3-K) which phosphorylates phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to generate PI(3,4,5)P_3 that binds the PH domain of Akt and phosphoinositide-dependent protein kinase 1 (PDK1), recruiting them to the membrane. Once in the membrane, Akt is phosphorylated at two key residues - Thr^{308} and Ser^{473}. The Thr^{308} site is located in the catalytic loop and is phosphorylated by PDK1, while Ser^{473} is located in the C-terminal hydrophobic motif and is phosphorylated by “PDK2”. Recent studies have suggested that in most cell types and under most conditions, the predominant PDK2 is the mTOR complex 2 (mTORC2) (Hresko and Mueckler, 2005; Sarbassov et al., 2005). Syndecan-4, a single pass transmembrane protein, belongs to a 4-member syndecan family and is ubiquitously expressed and evolutionary conserved (Tkachenko et al., 2005). Cytoplasmic tails of syndecans consist of two conserved domains separated by a variable domain which is unique to each syndecan family member and a PDZ-binding motif that participates in formation of signaling complexes (Simons and Horowitz, 2001). Syndecan-4 is the only syndecan capable of binding phosphatidylinositols PI(4,5)P_2 with high affinity via a positively charged lysine-rich motif (Horowitz and Simons, 1998). Binding of syndecan-4, which under baseline conditions exists as a homodimer, to PIP_2 allows for further syndecan-4 oligomerization, and binding of PKCα (Keum et al., 2004; Tkachenko et al., 2005), translocation of PKCα to the membrane which in turn has been associated with increased PKCα activity (Horowitz and Simons, 1998; Lim et al., 2003). Therefore, while syndecan-4 does not possess a catalytic activity of its own, it participates in signal transduction by virtue of regulation of PKCα activity and by facilitating assembly of signaling complexes.

Recently, several unique syndecan-4 functions have been described, including modulation of cell adhesion, migration and morphology (Longley et al., 1999), regulation of eNOS activity, and angiogenesis (Echtermeyer et al., 2001; Li et al., 2002; Zhang et al., 2003). In this study, we report that homozygous disruption of syndecan-4 is associated with decreased mTORC2 and increased mTORC1 activities. In the absence of syndecan-4, there is a dysregulation of subcellular localization of mTORC2 components with the decreased presence of rictor, mLST8 and mSin-1 in the rafts and decreased Akt activation in endothelial cells, leading to reduced phosphorylation of Akt targets such as eNOS and FOXO subfamily of forkhead transcription factors. Furthermore, syndecan-4-dependent regulation of mTORC2 is mediated by PKCα since expression of myristoylated PKCα in $S4^{-/-}$ cells restores the presence of mTORC2 components in the rafts and rescues Akt phosphorylation. Functionally, syndecan-4-dependent disruption of mTORC2 assembly leads to systemic hypertension and decreased endothelial cell size in syndecan-$4^{-/-}$ mice.

RESULTS

Growth factor-induced Akt activation is decreased in absence of syndecan-4

To investigate the role of syndecan-4 in Akt activation, endothelial cells (EC) were isolated from heart or lungs of syndecan-4$^{-/-}$ (S4$^{-/-}$) and control littermates (S4$^{+/+}$) mice. EC were serum-starved and then stimulated with various growth factors at 50ng/ml for 5 minutes. FGF-2-induced-Akt phosphorylation at both Ser^{473} and Thr^{308} sites was decreased in S4$^{-/-}$ cells in comparison to the controls, while total Akt level was unchanged (Fig. 1A,B). At the same time, FGF-2-induced ERK phosphorylation was not different between S4$^{-/-}$ and S4$^{+/+}$ EC, suggesting a selective disruption of the PI 3-K-Akt signaling pathway (Fig. 1A). VEGF or IGF-1-induced Akt-Ser^{473} phosphorylation was also decreased in S4$^{-/-}$ EC, suggesting that reduced Akt activation is general and is not limited to the FGF signaling pathway (Fig. 1C). To confirm decreased Akt activation *in vivo*, S4$^{-/-}$ and $^{+/+}$ mice were injected intraperitoneally with FGF2 or vehicle followed by isolation of lung tissues 5 or 15 min later. FGF-2-induced Akt-Ser^{473} phosphorylation was dramatically decreased in S4$^{-/-}$ lungs compared to wild type controls (Fig. 1D). Akt kinase activity assay showed a decreased ability of Akt immunoprecipitated from S4$^{-/-}$ EC to phosphorylate its substrate GSK-3 *in vitro* compared to Akt immunoprecipitated from S4$^{+/+}$ EC (Fig. 1E). Adenoviral expression of a full-length syndecan-4 (Ad-S4), but not a control virus expressing green fluorescent protein (Ad-GFP), was able to fully restore FGF-2-induced-Akt phosphorylation, suggesting that this was indeed due to the absence of syndecan-4 (Fig. 1F).

Next, we explored the mechanism by which syndecan-4 regulates Akt activation. In order to compare FGF2-induced-PI3-K activation in S4$^{+/+}$ and S4$^{-/-}$ EC, we carried an immunoprecipitation with an anti-phosphotyrosine antibody from serum-starved S4$^{-/-}$ or S4$^{+/+}$ EC stimulated with PBS or FGF2 and then probed for the presence of p85 PI3-kinase regulatory subunit. While in S4$^{+/+}$ EC, stimulation with FGF2 was associated with an increase in the levels of p85PI3-K-bound phosphotyrosine, in S4$^{-/-}$ EC p85PI3-K phosphorylation was increased at baseline with little additional increase in phosphorylation following FGF2 treatment (Fig 1G). This suggests that the defect in Akt activation in S4$^{-/-}$ EC is not due to decreased tyrosine kinase receptor-induced PI3 kinase activation.

Next, we examined expression of different components of mTORC2. Levels of mTOR, rictor, mSin-1, protor (Fig 1H) and mLST8 (Fig 2B) measured by immunoblotting total cell lysates were similar between S4$^{+/+}$ and S4$^{-/-}$ EC. Furthermore, the interaction between the different components of mTORC2 was not disrupted, since immunoprecipitation of mSin-1 from total cell lysates brought down all the other members of the complex both in S4$^{-/-}$ and S4$^{+/+}$ EC (Fig 1H). Thus, syndecan-4 absence does not affect expression of mTORC2 components or their ability to interact with each other in the cytoplasm.

mTORC1 activity is increased in absence of syndecan-4

mTOR, when associated with raptor and mLST8, forms mTORC1 and regulates the activity of p70S6K1 and 4E-BP1. Thus, mTOR exists in the balance between mTORC1 and mTORC2. In S4$^{-/-}$ EC, there was an increase in the amount of raptor co-immunoprecipitated with mTOR in whole cell lysates (Fig 2A), suggesting the possibility of a shift of mTOR toward association with raptor versus rictor. At the same time, the total protein amounts of mTORC1 components raptor and mLST8 (Fig 2B) were similar in S4$^{-/-}$ and S4$^{+/+}$ EC.

To test the activity of mTORC1, we assessed phosphorylation of its downstream targets p70S6K1 and 4EBP1. The phosphorylation of p70S6K1 at Thr^{389} as well as phosphorylation of its downstream target S6 ribosomal protein (S6RP) at Ser$^{235/236}$ were increased in S4$^{-/-}$ EC (Fig 2B) while the total p70S6K level was unchanged (Fig 2B). Direct measurement of

p70S6K1 activity *in vitro*, carried out using the kinase immunoprecipitated from heart or lung tissues from $S4^{+/+}$ and $S4^{-/-}$ mice and a GST-ribosomal S6 peptide as substrate, confirmed a significant increase in p70S6K activity in $S4^{-/-}$ tissues in comparison to the wild type tissues (Fig 2C and 2D). FGF2-induced 4E-BP1 phosphorylation at $Thr^{37/46}$ was not significantly different between $S4^{-/-}$ and $S4^{+/+}$ EC (Fig 2B). There was, however, an increase in the basal level of phosphorylation of 4E-BP1 at the mTOR-dependent Thr^{70} site in $S4^{-/-}$ EC (Fig 2E).

Because of the significant increase in mTORC1 activity, one could hypothesize that the defective Akt phosphorylation in $S4^{-/-}$ EC is due to a negative feedback loop from p70S6K1 which has been previously reported(Shah et al., 2004). To investigate this possibility, $S4^{-/-}$ EC were exposed to rapamycin for 45 minutes prior to stimulation with FGF2. Pre-treatment with rapamycin inhibited FGF2-induced 4E-BP1 phosphorylation at Thr^{70}, as expected. While rapamycin treatment was associated with an increased Akt phosphorylation in $S4^{+/+}$ EC, it did not restore the level of Akt phosphorylation in $S4^{-/-}$ EC (Fig 2E), therefore ruling out a potential contribution of the negative feedback mechanism to reduced Akt activity in these cells.

Syndecan-4 regulates translocation of PKCα, rictor, mLST8, and mSin1 to the rafts

Whereas in unstimulated EC S4 is predominantly present in non-raft compartments, its clustering by ligands such as FGF2 or by an anti-syndecan-4 antibody induces a shift to detergent-insoluble membrane fractions (Tkachenko and Simons, 2002). It has also been reported that Akt-Ser^{473} kinase activity is enriched in buoyant, detergent-insoluble plasma membrane rafts (Hill et al., 2002). Therefore, we assessed the presence of Akt, mTOR, and different components of mTOR regulatory complexes in lipid raft fractions, which were isolated from $S4^{-/-}$ and $S4^{+/+}$ EC by sucrose gradient and ultracentrifugation as described in Methods. The raft fractions were detected using FITC-conjugated cholera toxin subunit that identifies the presence of the raft marker GM1 (Supl, Fig 1) and with flotillin-1 immunoblotting used as another raft marker (Fig 2F).

Akt phosphorylation in response to FGF2 was markedly decreased at both Ser^{473} and Thr^{308} sites in rafts of $S4^{-/-}$ compared to $S4^{+/+}$ EC, while total Akt level was unchanged (Fig. 2F). Levels of PDK1 and mTOR were increased in rafts of $S4^{-/-}$ EC (Fig. 2F). While the total mTOR level was increased, mTOR phosphorylation at Ser^{2448} was decreased in $S4^{-/-}$ rafts. When examined in total cell lysates, mTOR phosphorylation at Ser^{2448} was also decreased and was only partially reduced further by rapamycin treatment (Suppl. Fig 2).

Levels of the mTORC2 components, rictor, mLST8, and mSin1 were dramatically decreased in $S4^{-/-}$ rafts (Fig. 2F). On the other hand, the levels of p70S6K (both total and phosphorylated at Thr^{389}), and phospho-S6RP-$Ser^{235/236}$ were increased in $S4^{-/-}$ rafts. Finally, since syndecan-4 is known to bind PKCα and to regulate its recruitment to plasma membrane, we also examined PKCα levels in raft fractions. As expected, levels of both total PKCα and phospho-PKCα-Ser^{660} were markedly decreased in the raft domains of $S4^{-/-}$ compared to $S4^{+/+}$ EC (Fig. 2F).

Syndecan-4 regulates Akt activation via PKCα-dependent localization of mTORC2 components to the rafts

Since we had previously reported that alteration of PKCα expression or activity in endothelial cells interferes with Akt phosphorylation (Partovian and Simons, 2004), we hypothesized that the effect of syndecan-4 on mTORC2 and Akt maybe mediated via PKCα. To test this hypothesis, we first used pharmacological inhibitors. Human umbilical vein endothelial cells (HUVEC) were pre-incubated with Gö6976 (200 nM), an inhibitor of conventional PKCs, or PD98059 (40 μM), an inhibitor of MEK or H89 (20μM), a PKA inhibitor, and then stimulated

with FGF-2. FGF2-induced Akt phosphorylation was inhibited only in presence of Gö6976, while ERK activation was affected by PD98059, and eNOS phosphorylation was affected by both Gö6976 and H89 (Fig 3A). Next, we knocked down PKCα expression in AB1 EC using lentiviral-mediated shRNA stable transduction. PKCα knockdown resulted in a reduced phosphorylation of Akt Ser^{473} without affecting ERK phosphorylation (Fig 3B). The protein levels of rictor, mLST8, and mSin1 assessed by immunoblotting the total cell lysates were not altered in cells with decreased levels of PKCα (Fig 3C). However, the levels of rictor and mLST8 were decreased in the rafts of PKCα-knockdown EC (Fig 3D) while raptor levels were unchanged (Fig 3D), suggesting that PKCα regulates subcellular localization of mTORC2 components to the rafts.

Finally, $S4^{-/-}$ EC were transduced with adenoviral constructs encoding either a myristoylated PKCα (Ad-Myr-PKCα) or a green fluorescent protein (Ad-GFP), used as a control. The transduction of myristoylated PKCα results in its membrane targeting irrespective of any other factors. Expression of Myr-PKCα in $S4^{-/-}$ EC restored FGF2-induced Akt phosphorylation (Fig 3E) as well as mTOR Ser^{2448} phosphorylation (Fig 3E). There was also an increase in protein levels of Akt, rictor, mLST8, and mSin1 in the rafts of $S4^{-/-}$ EC expressing Myr-PKCα, while raptor levels remained unchanged (Fig. 3E). To understand whether PKCα functions as a kinase or a scaffold molecule, we repeated the same experiment with a kinase-dead-myristoylated-PKCα (Ad –Myr-KD-PKCα). Expression of Myr-KD-PKCα in $S4^{-/-}$ EC was not able to rescue Akt phosphorylation or increase localization of mTORC2 components to the rafts (Fig 3F). These results demonstrate that the effect of S4 on Akt activation and regulation of mTORC2 is mediated by PKCα and requires PKC*α* kinase activity.

Functional effects of reduced Akt activity in syndecan-4 mice

In order to determine whether S4 role in Akt activation is biologically relevant, we assessed key downstream Akt targets in $S4^{-/-}$ and $S4^{+/+}$ EC. The phosphorylation of eNOS at Ser^{1179}, a known direct substrate of Akt, was decreased in EC isolated from $S4^{-/-}$ compared to $S4^{+/+}$ mice following FGF2 stimulation, while total levels of eNOS were not different (Fig. 4A). The basal level of NO production measured as accumulation of nitrites over 18 hours was also significantly lower in $S4^{-/-}$ vs. $S4^{+/+}$ EC (Fig. 4B). Measurement of the cGMP content (a surrogate marker of eNOS activity) in lung and heart tissues isolated from $S4^{-/-}$ mice and wild type littermates also demonstrated a significant decrease in cGMP content in both tissues in $S4^{-/-}$ mice, which was most prominent in the heart (Fig. 4C). In agreement with this evidence of decreased eNOS activity in $S4^{-/-}$ EC and tissues, measurement of arterial blood pressure revealed significantly higher blood pressure in $S4^{-/-}$ mice compared to their wild type littermates (Fig. 4D), while there was no difference in their heart rates.

We then examined two other downstream targets of Akt, BAD and the forkhead family transcription factor members FoxO1 and 3a. While phosphorylation of BAD at Ser^{136} was not different, phosphorylation of FoxO1/3a at Thr^{24} and Thr^{32} was decreased in $S4^{-/-}$ EC (Fig 4E). Since Akt has been previously reported to control cell size via regulation of the forkhead family of transcription factors (Skurk et al., 2005), we compared the size of $S4^{+/+}$ vs. $S4^{-/-}$ EC by flow cytometry, using mean forward scatter height (FSC-H) as a measure of cell size (Fingar et al., 2002). Quiescent cells were analyzed for DNA content and CD31 (PECAM-1) expression, used as a marker of EC, and the mean FSC-H was determined for $CD31^{+}$ cells in the G1 phase of the cell cycle (Fig. 4F). As shown by the leftward shift of the mean FSC-H histogram, $S4^{-/-}$ EC were 9% smaller than the wild type cells (Fig. 4F, right panels).

DISCUSSION

This study provides several important new insights into the mTORC2/Akt signaling axis and new phenotypic observations for syndecan-4 null mice. Akt activation is decreased both in

endothelial cells *in vitro* and in mice tissues *in vivo*, in the absence of syndecan-4. This is associated with decreased phosphorylation of eNOS and FoxO1/3a, increased arterial blood pressure, and reduced endothelial cell size in $S4^{-/-}$ mice. Syndecan-4 regulates Akt activation via PKCα-dependent localization of mTORC2 components rictor, mLST8, and mSIN-1 to the rafts.

The impairment of Akt activation in syndecan-$4^{-/-}$ endothelial cells is not specific to any given growth factors and is not due to a decrease in activation of a cognate tyrosine kinase receptor, since other intracellular signaling pathways including ERK-1/2 and p70S6K1 phosphorylation are either maintained or increased. The decrease in growth factor-induced Akt phosphorylation at both Ser^{473} and Thr^{308} sites in $S4^{-/-}$ EC may suggest a possible defect in PI3-K activation. However, tyrosine kinase-induced PI 3-K activation is not only preserved but even increased in the absence of syndecan-4. In addition, treatment with rapamycin does not restore Akt phosphorylation in $S4^{-/-}$ cells, therefore ruling out the possibility of a negative feedback loop from mTORC1 being responsible for decreased Akt activation.

Regulation of Akt activation by mTOR has received much attention, as has, in particular, the role of mTORC2. Our data show, for the first time, that subcellular localization of mTORC2 components is critical to regulation of its function, assessed by Akt phosphorylation. There was a marked reduction in rictor, mLST8, and mSIN-1 levels in $S4^{-/-}$ membrane raft fractions, while the expression levels of mTORC2 components, as well as the stability of the complex, were unchanged in the total cell lysate of $S4^{-/-}$ compared to $S4^{+/+}$ endothelial cells. Since it has been shown that rictor, mLST8, and mSin-1 are all required for mTORC2 function (Guertin et al., 2006), their decreased presence in the rafts where Akt is normally phosphorylated (Hill et al., 2002) likely explains decreased Akt phosphorylation at Ser^{473} in $S4^{-/-}$ endothelial cells. However, the reason for decreased Akt phosphorylation at Thr^{308} is less clear since PDK1 expression was maintained in both total cell lysate and raft fractions of $S4^{-/-}$ EC. In addition, PDK1 activity seems normal since p70S6K activity, which is also PDK1-dependent, is maintained. The dependency of Akt-Thr^{308} phosphorylation on Ser^{473} phosphorylation is still a controversial issue in the field. In agreement with our data, others have shown that RNAi-mediated knockdown of rictor reduced Akt-Thr^{308} phosphorylation in tandem with that of Ser^{473} in multiple cell types ((Hresko and Mueckler, 2005; Sarbassov et al., 2005).

Interestingly, while mTORC2 activity was reduced in $S4^{-/-}$ endothelial cells, mTORC1 activity was increased, as shown by increased p70S6K activity *in vitro* and increased phosphorylation of p70S6K-Thr^{389} and S6RP-$Ser^{235/236}$. The levels of total mTOR and total p70S6K were similar in total cell lysates of $S4^{+/+}$ and $S4^{-/-}$ cells but were higher in $S4^{-/-}$ rafts. At the same time, raptor levels in the rafts and in total cell lysates were unchanged. Therefore, all of the known components required for mTORC1 function were abundantly present in $S4^{-/-}$ rafts, while there was a decreased availability of mTORC2 components. It should be noted that while mLST8 is also part of mTORC1, it is required for maintenance of the rictor-mTOR but not the raptor-mTOR interaction (Guertin et al., 2006). Another interesting observation was the increased amount of raptor co-immunoprecipitated with mTOR from total cell lysates of $S4^{-/-}$ compared to $S4^{+/+}$ endothelial cells, suggesting the possibility of a shift of mTOR towards association with raptor, thus leading to increased mTORC1 activity. However, because our attempts to immunoprecipitate mTOR complexes directly from the rafts fractions were not successful, we do not have a direct proof that this association is happening in the lipid rafts. Furthermore, one cannot exclude the possibility of the existence of a free mTOR or even of a third mTOR complex.

In the lipid rafts, S6RP phosphorylation at $Ser^{235/236}$ was high in $S4^{-/-}$ cells at baseline with no augmentation following FGF2 treatment, while phosphorylation of p70S6K at Thr^{389}, also high under basal conditions, was significantly reduced by FGF2 addition. Interestingly, levels

of a number of signaling molecules were decreasing in the lipid rafts following FGF2 stimulation in both $S4^{+/+}$ and $S4^{-/-}$ EC. It is possible that at this particular time point (3 to 5 minutes post FGF2 stimulation) these proteins were leaving the raft domains, since their presence in the raft is a very transient and dynamic process. The absence of correlation between FGF2-induced S6K and S6RP phosphorylation in the rafts could also be explained by the fact that other upstream kinases may regulate S6RP-$Ser^{235/236}$ phosphorylation independently of mTORC1 and p70S6K.

The other key finding in our study is that PKCα is required for syndecan-4-mediated recruitment of mTORC2 to the rafts and for Akt phosphorylation. There was a marked decrease in levels of both total PKCα as well as in phospho-PKCα-Ser^{657} in $S4^{-/-}$ rafts. This is not surprising, as we and others have previously shown that syndecan-4 plays a key role in localization of PKCα to the plasma membrane (Keum et al., 2004) and in regulation of its activity and stability (Horowitz and Simons, 1998). A knockdown of PKC*α* expression in wild type endothelial cells mimicked the results observed in syndecan-$4^{-/-}$ cells, including decreased Akt phosphorylation and decreased level of mTORC2 in the rafts. In addition, expression of a myristoylated-PKCα, but not its kinase-dead mutant, in syndecan-$4^{-/-}$ endothelial cells fully restored Akt activation as well as rafts levels of rictor, mLST8, and mSin1. Thus, these data show that syndecan-4 effects on regulation of mTORC2 sub-cellular localization and Akt activation are mediated by PKCα. These results are in agreement with previous studies in which overexpression of PKCα has been shown to increase Akt phosphorylation and activity (Li et al., 1999; Partovian and Simons, 2004).

The mechanism by which PKCα interacts with mTORC2 components and participates in Akt activation is not clear and is further complicated by the fact that PKCα phosphorylation and stability is mTORC2-dependent. Indeed, it has been reported that PKCα phosphorylation on the hydrophobic motif site as well as total protein levels are severely reduced in both rictor and mLST8 null mouse embryonic fibroblasts (Guertin et al., 2006). A recent study has demonstrated that mTORC2-dependent turn motif phosphorylation is essential for PKCα maturation, stability and signaling (Ikenoue et al, 2008). Furthermore, while PKCα is associated with rictor PKCα is not directly phosphorylated by mTORC2 (Ikenoue et al., 2008). Syndecan-4 null mice are viable suggesting that the residual Akt activity in these mice is sufficient for normal growth and survival. However, the difference between $S4^{-/-}$ and $^{+/+}$ mice may become more apparent in pathological conditions associated with increased Akt activation. Further studies will be required to evaluate the role of syndecan-4 in regulation of different Akt isoforms. That the syndecan-4-PKC*α*-dependent regulation of Akt is functionally significant is demonstrated by decreased eNOS activation in absence of syndecan-4 both *in vitro* and *in vivo*. Indeed, we have observed reduced eNOS Ser^{1179} phosphorylation, reduced tissues cGMP content, and increased mean arterial blood pressure in syndecan-$4^{-/-}$ mice, all consistent with reduced nitric oxide production from $S4^{-/-}$ endothelial cells. Furthermore, the extent of blood pressure increase in $S4^{-/-}$ mice was comparable to that observed in $eNOS^{-/-}$ mice (Beierwaltes et al., 2002). Interestingly, a prior study has demonstrated that endothelial overexpression of PKCα is associated with increased eNOS-Ser^{1179} phosphorylation, increased NO production in endothelial cells, and increased blood flow *in vivo* (Partovian et al., 2005).

The FoxO subfamily of forkhead transcription factors are another downstream target of Akt. FoxO3a is thought to be involved in regulation of cell size (Skurk et al., 2005). There was a significant decrease in FoxO1/3a phosphorylation in $S4^{-/-}$ endothelial cells which is consistent with a decrease in $S4^{-/-}$ EC sizeWhile Akt-$1^{-/-}$ (Peng et al., 2003) mice are significantly smaller in size than their wild type counterparts, $S4^{-/-}$ mice size was not different than the wild type mice and this may be related to increased mTORC1 activity in $S4^{-/-}$ mice.

Based on these results, we propose the following model of Akt activation by a growth factor (Fig 5). Growth factor stimulation of endothelial cells leads to activation of a cognate tyrosine kinase receptor as well as syndecan-4 clustering, perhaps via the latter's heparan sulfate chains or direct binding to the extracellular core protein domain (Tkachenko et al., 2005). The activated tyrosine kinase receptor signaling leads to PI 3-K activation and Akt translocation to the plasma membrane, while syndecan-4 oligomerization shifts it to the membrane rafts, allowing for recruitment and activation of PKC*α*. This in turn is associated with presence of rictor, mSin-1, and mLST8 in the rafts and Akt activation.

In the absence of syndecan-4, there is a marked reduction in PKC*α* recruitment to the membrane, leading to reduced presence of rictor, mSin-1, and mLST8 in rafts, and reduced Akt phosphorylation. The latter occurs despite normal presence of mTOR and PDK1 in the membrane rafts. Because raptor presence in the raft is not dependent on syndecan4/PKCα complex, there is an increased association of mTOR with raptor versus rictor, leading in turn to increased mTORC1 activation with increased p70S6K and S6RP phosphorylation. Decreased endothelial cell Akt activation is responsible for decreased eNOS activity and increased blood pressure and decreased endothelial cell size observed in syndecan-$4^{-/-}$ mice (Fig. 5).

In conclusion, our data show that syndecan-4 regulates Akt/eNOS activation via PKCα-dependent localization of rictor, mSin-1, and mLST8 to membrane raft domains.

Experimental Procedures

Endothelial cell culture—Primary mice EC were isolated from the heart as previously described (Allport et al., 2002). AB-1 endothelial cell line (Balconi et al., 2000) was obtained from Holmgren laboratory (Department of Oncology-Pathology, Cancer Center Karolinska, Stockholm, Sweden). AB-1 EC were grown in 10% CO2 in DMEM containing 10% FBS, supplemented with antiobiotics, 100 μg/ml heparin and 100 μg/ml EC growth supplement (ECGS; Biomedical Technologies, Stoughton, MA).

Adenoviral constructs and cell transduction—The myristoylated PKCα adenoviral construct was a gift from Dr. Robin R. Hodges (Schepens Eye Research Institute, Boston, MA) and was synthesized as previously described (Hodges et al., 2004; Rabinovitz et al., 1999). See Supplementary data for details of construct generation. Endothelial cells were incubated with adenoviruses in 0.5% FBS-containing medium. After 4 hrs, full-serum medium was added for overnight incubation. The day after, cells were washed with PBS and incubated in full-serum medium. The experimentations were performed between 48 and 72 hrs post-transduction following 18 hrs of serum starvation. In these conditions, the transduction efficiency was typically >80% as determined by GFP expression.

Lentivirus-mediated shRNA gene silencing—Lentiviral-based shRNA constructs (Sigma-Aldrich, Saint Louis, Missouri) were used to stably knockdown PKCα and a scrambled sequence was use as a control. See Supplementary data for target sequence. AB-1 EC were transduced with lentiviral particles at a MOI of 10, in the presence of hexadimethrine bromide or polybrene (final concentration of 8 μg/ml), in 10%-FBS-DMEM. After overnight incubation, the medium was replaced with fresh complete culture medium as described above. At day 4, puromycin (1 μg/ml) was added to the medium for selection of transduced cells. Several puromycine-resistant colonies were selected, assayed for PKCα knockdown, and then pooled and expanded.

Immunoprecipitation and Immunoblotting—For western blots, cells were washed with cold PBS and lysed in RIPA buffer (PBS 1X, Nonidet P-40 1%, Sodium Deoxycholate 0.5%

and SDS 0.1%) containing protease inhibitor cocktail (Complete™, Roche). Primary antibodies used include: phospho-Akt-Ser^{473} and Thr^{308}, total Akt, PDK1, mTOR, rictor, raptor, phospho-and total p70S6K1, phospho-4EBP1 and total 4EBP1, and phospho-ERK (Cell Signaling). Antibodies for flotillin-1, eNOS, and PKCα were from BD Transduction Laboratories. Antibodies for β tubulin and VE-Cadherin were from Santa Cruz Biotechnology and anti HA mAb from Roche diagnostics.

For mTOR complex immunoprecipitation, actively growing cells were washed with cold PBS, and lysed in ice-cold buffer A (40 mM Hepes [pH 7.5], 120 mM NaCl, 1mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na_3VO_4, 0.3% ChAPS, and EDTA-free protease inhibitor). 1 mg of protein was incubated with mSin-1 antibody (a gift from Dr. Su, Yale University) or mTOR antibody (sc-1549 from Santa Cruz Biotech) overnight at 4° C. 30 μl of a 50% slurry of protein G-sepharose was added the day after for 3 hrs. Immunoprecipitates were washed 2 times with the lysis buffer and 3 times with wash buffer (50 mM HEPES [pH 7.5] and 150 mM NaCl).

In vitro Kinase Assay—The non-radioactive Akt kinase assay kit from Cell Signaling Technology was used following the manufacturer's instructions. Primary $S4^{-/-}$ and $S4^{+/+}$ EC were serum-starved for 18 hrs, then stimulated with FGF-2 or vehicle for 3 minutes. Akt was immunoprecipitated from cell extracts using immobilized Akt (1G1) monoclonal antibody. Then, an *in vitro* kinase assay was performed using GSK-3 fusion protein as a substrate. For p70S6K1 kinase activity, p70S6K1 was immunoprecipitated from heart or lung tissues with a polyclonal antibody (Cell Signaling), and the *in vitro* assay was performed using an S6-ribosomal–GST fusion peptide as substrate.

Isolation of detergent-insoluble membrane domains or rafts—Isolation of rafts was performed as described previously (Lang et al., 2002). See Supplementary data for details.

Basal NO (nitrite) accumulation—Primary heart EC isolated from $S4^{-/-}$ and $S4^{+/+}$ mice were plated in 10 cm plates and allowed to reach confluency. On the day before the experiment, 3 ml of serum-free media was added to the cells. After 18 hrs, 500 μl of media was collected and centrifuged. Samples were assayed at the Macromolecular Core Equipment Facility, University of Wyoming, by chemiluminescence using a Sievers NO analyzer. The results were expressed as picomoles of nitrate per mg of protein.

Determination of cGMP content in tissues: cGMP levels were quantitated using the cGMP enzyme immunoassay kit (Cayman Chemicals) and results were normalized to tissue weight. See Supplementary material for details.

Determination of blood pressure in mice: All animal experiments were approved by the Institutional Animal Care and Use Committee. Blood pressure and heart rate were measured using a Millar transducer inserted into the right carotid artery. Four mice were measured in each group. See Supplementary material for details.

Statistical analysis: All p values were determined using a two-tailed Student's t-test. Results were considered significant at the $p<0.05$ level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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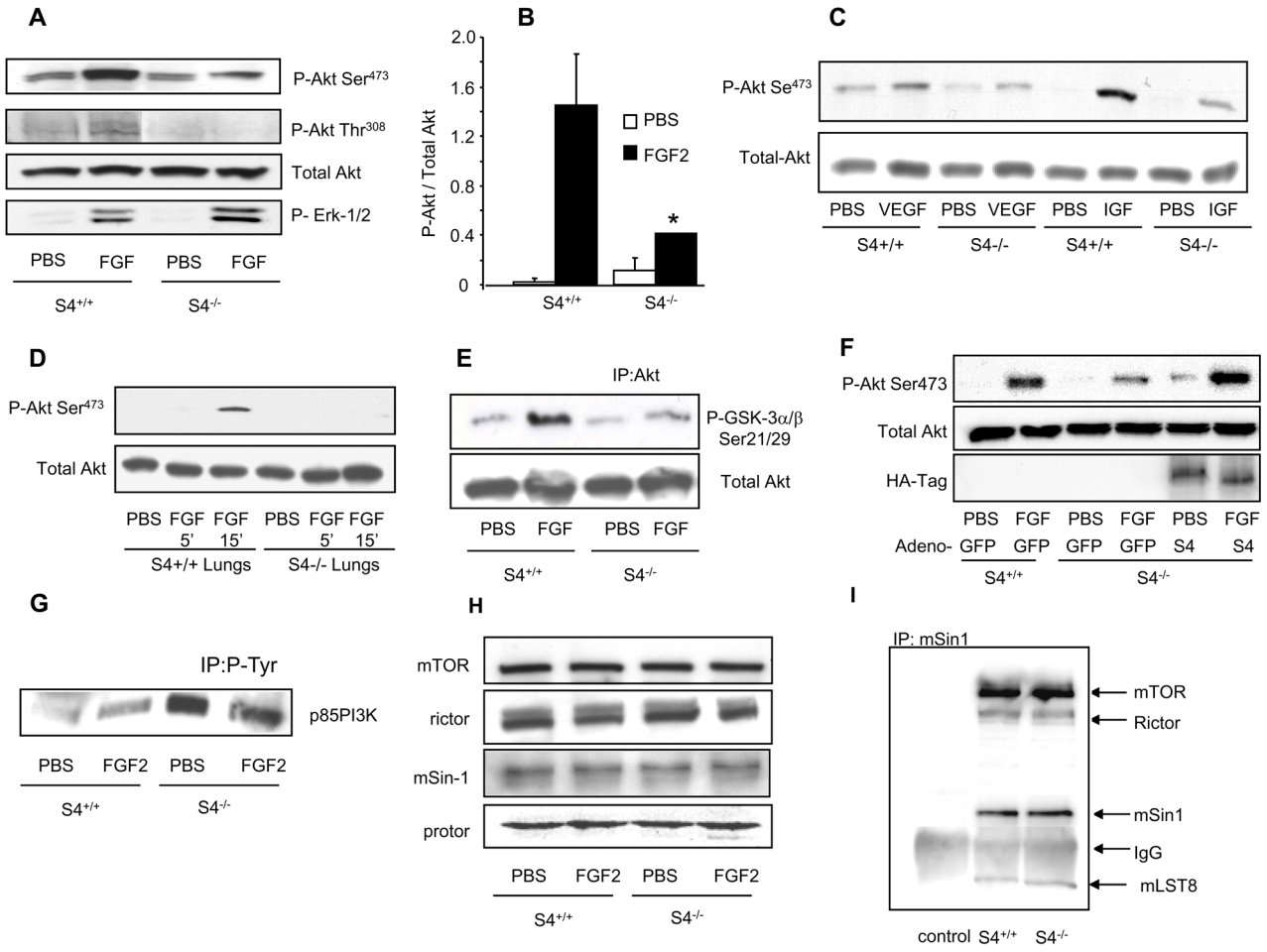

Figure 1. Growth factor-induced Akt activation is decreased in the absence of syndecan-4

(A) Akt phosphorylation in response to FGF2 is decreased in $S4^{-/-}$ EC while ERK phosphorylation is unchanged

(B) Quantitative analysis of P-Akt Ser^{473}/total Akt: Results represent the average of 3 different experiments performed in 3 different cell preparations. Results are means ± SEM. * $p<0.05$.

(C) Akt phosphorylation in response to VEGF or IGF1 stimulation. Note a decrease in $S4^{-/-}$ EC

(D) Akt phosphorylation in the lungs in response to FGF2 injected intraperitoneally in mice: Note a decrase in $S4^{-/-}$ mice

(E) *In vitro* Akt kinase assay performed using GSK-3 fusion protein as substrate: note decreased Akt activity in $S4^{-/-}$ EC

(F) Expression of syndecan-4 in $S4^{-/-}$ EC restores Akt phosphorylation: $S4^{+/+}$ and $S4^{-/-}$ EC were transduced with adenoviruses encoding full-length HA-tagged syndecan-4 (Adeno-S4) or GFP (Adeno-GFP) used as a control

(G) Tyrosine-kinase-induced PI3-K activation is increased in $S4^{-/-}$ EC: phosphotyrosine were immunoprecipitated from whole cell lysates and subjected to immunoblotting with an anti-p85 PI3-K antibody

(I) mTORC2 integrity is preserved in $S4^{-/-}$ EC: mSin-1 was immunoprecipitated from the total cell lysates of actively growing $S4^{-/-}$ and $S4^{+/+}$ cells using a CHAPS-containing buffer and immunoblotted for mTOR, rictor, and mLST8

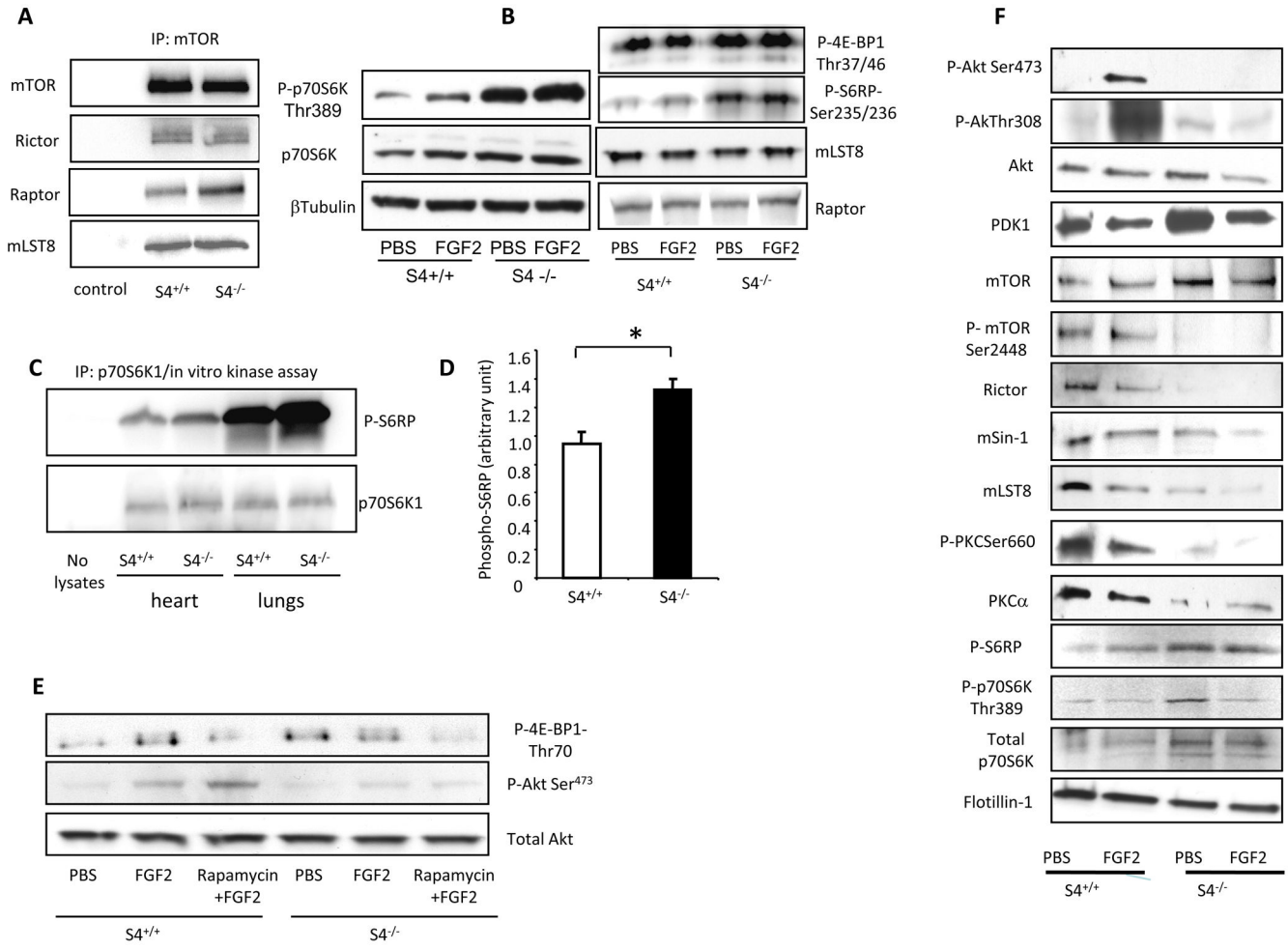

Figure 2. mTORC1 activity is increased in S4$^{-/-}$ EC and tissues

(A) mTOR was immunoprecipitated from total cell lysates and immunoblotted with raptor, rictor, and mLST8: Note the increased amount of raptor co-immunoprecipitated with mTOR in S4$^{-/-}$ EC

(B) Phosphorylation of p70S6K1 at Thr^{389} and S6RP at Ser$^{235/236}$ in response to FGF2 is increased in S4$^{-/-}$EC. Note similar levels of mTORC1 components mLST8 and raptor between S4$^{-/-}$ and S4$^{+/+}$ EC.

(C) *in vitro* p70S6K1 kinase activity, performed using S6 ribosomal fusion GST peptide as substrate: Note increased activity in S4$^{-/-}$ tissues

(D) Quantitative analysis of p70S6K1 activity assay in lung and heart tissues from 3 S4$^{+/+}$ and 3 S4$^{-/-}$ mice. The intensity of phospho-S6 band in S4$^{+/+}$ hearts was arbitrarily set to 1, and all other intensities were normalized to that threshold. Heart and lung ration in S4$^{+/+}$ vs. S4$^{-/-}$ mice are avereged together. The data are shown as a ratio of FGF2 to PBS stimulatrion. Results are means ± SEM. * $p<0.05$.

(E) Immunoanalysis of 4E-BP1 and Akt phosphorylation. Rapamycin treatment inhibited FGF2-induced 4E-BP1 phosphorylation at Thr^{70} but did not restore Akt phosphorylation in S4$^{-/-}$ EC.

(F) Immunoanalysis of plasma membrane raft domains isolated from S4$^{-/-}$ and S4$^{+/+}$ EC. Note the decreased phosphorylation levels of Akt, mTOR and PKCα as well as decreased levels of total PKCα, rictor, mLST8 and mSin-1 in S4$^{-/-}$ rafts. On the other hand the levels of mTOR, PDK1, p70S6K and P-S6RP are increased in S4$^{-/-}$ rafts

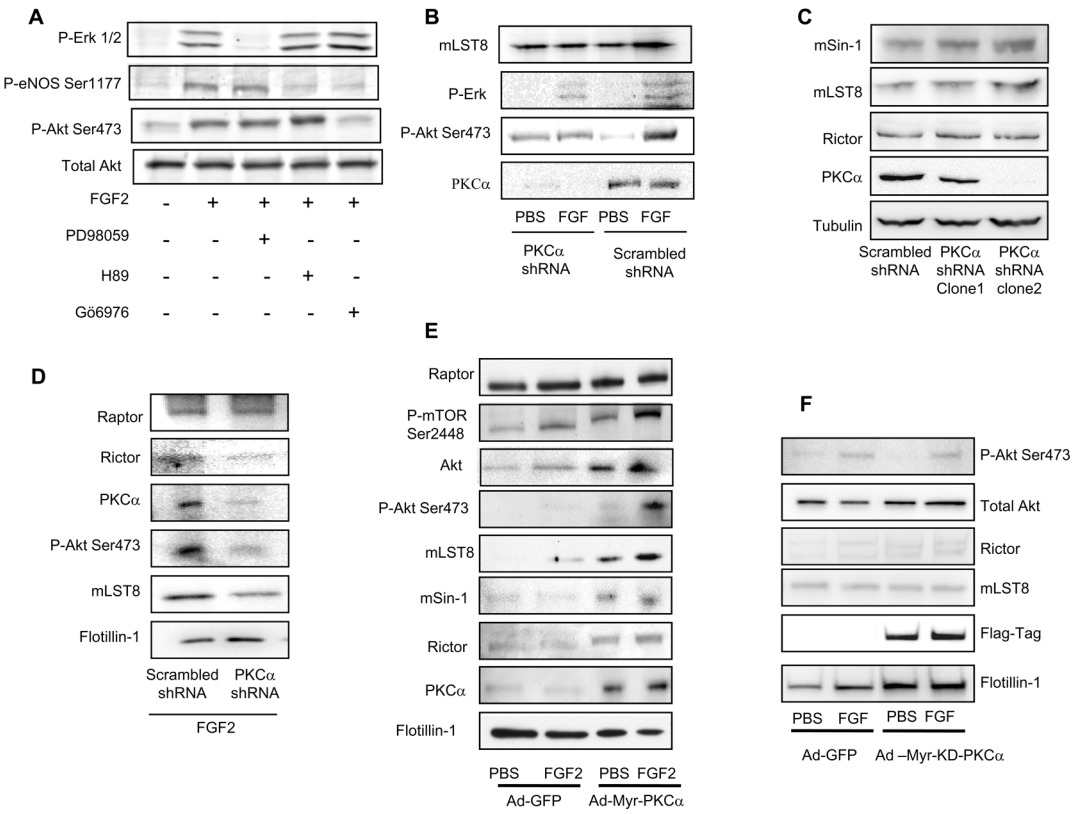

Figure 3. Syndecan-4 regulates Akt activation via PKCα-dependent translocation of rictor, mSin-1, and mLST8 to raft domains

(A) Immunoanalysis of FGF2 signaling in HUVEC. Note inhibition of Akt phosphorylation in presence of Gö6976, an inhibitor of conventional PKCs, while PD98059, an inhibitor of MEK or H89, a PKA inhibitor did not affect Akt phosphorylation

(B) PKCα knockdown is associated with decreased FGF2-induced Akt phosphorylation inAB-1 cells, while ERK phosphorylation is maintained

(C) Effect of PKCα knockdown on expression of rictor, mSin-1 and mLST8 in total cell lysates of AB-1 cells

(D) PKCα knockdown in AB-1 EC is associated with decreased FGF2-induced Akt-Ser^{473} phosphorylation and decreased expression of rictor and mLST8 in plasma membrane rafts. Note the unchanged raptor level.

(E) Immunoanalysis of raft domains isolated from S4$^{-/-}$ cells transduced with either myristoylated-PKCα (Ad-Myr-PKCα) or green fluorescent protein (Ad-GFP). Note the re-appearance of rictor, mLST8 and mSin-1 in rafts of Myr-PKCα transduced cells as well as increased Akt and mTOR phosphorylation

(F) Immunoanalysis of raft domains isolated from S4$^{-/-}$ cells transduced with either myristoylated-Kinase-Dead-PKCα (Ad-Myr-KD-PKCα) or green fluorescent protein (Ad-GFP): PKCα kinase activity is required for PKCα-mediated regulation of Akt and mTORC2

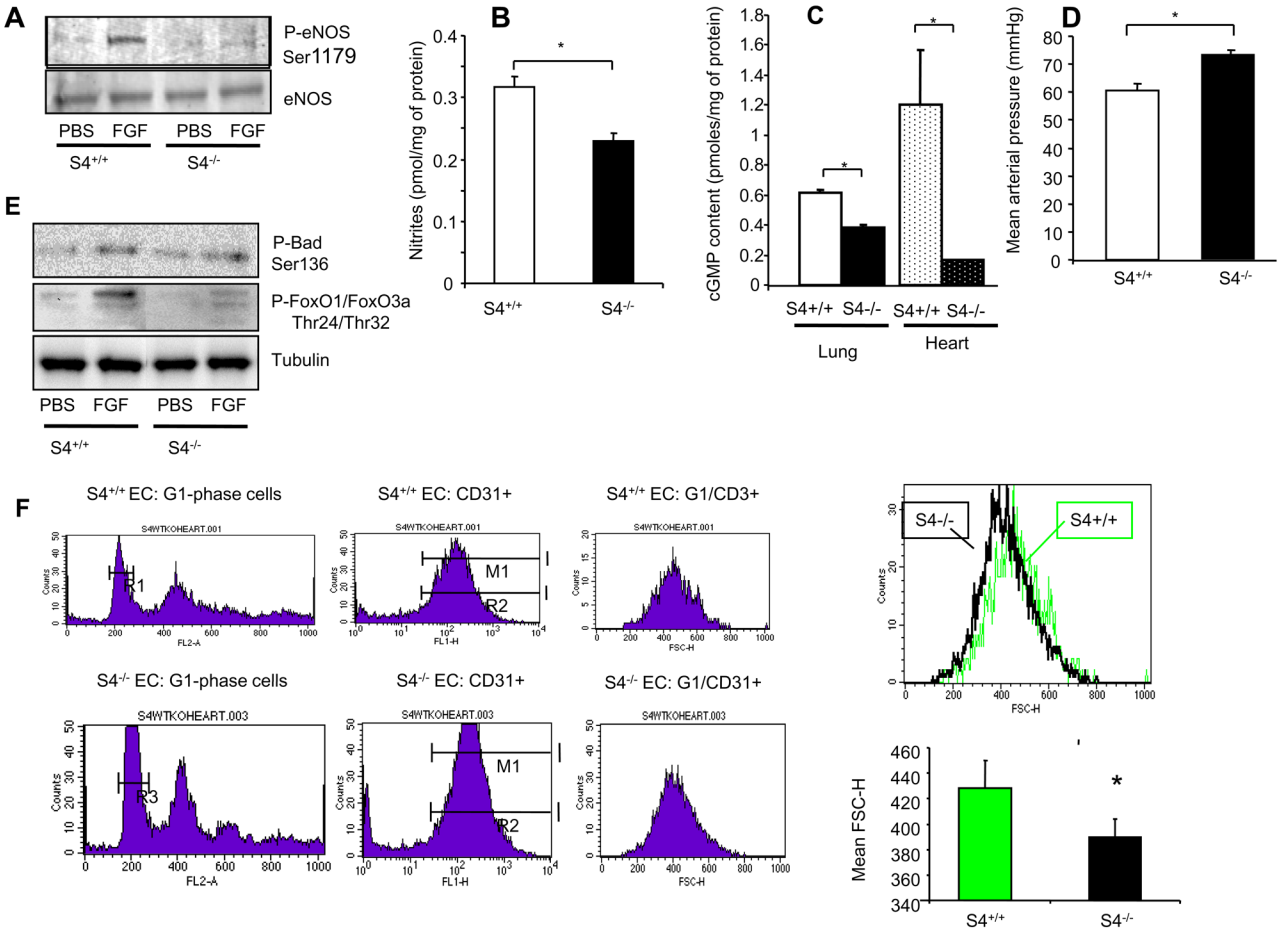

Figure 4. Decreased eNOS activation, increased blood pressure, and also smaller endothelial cell size in S4$^{-/-}$ mice

(A) FGF2-induced-eNOS phosphorylation at Ser^{1179} is decreased in S4$^{-/-}$ EC

(B) eNOS activity in S4$^{-/-}$ EC: Nitrite levels accumulated in the medium over 18 hrs and expressed as pmoles of nitrate per mg of protein. Data are means ± SEM of 6 plates from 2 different cell preparations.

(C) cGMP content of heart and lung tissues from S4$^{-/-}$ mice expressed as pmoles per mg of protein. Mean ± SEM, n=3 mice per group. * $p<0.05$

(D) Mean arterial blood pressure measured invasively in anesthetized animals is higher in S4$^{-/-}$ mice. Mean ± SEM, n=4 mice per group. * $p<0.05$

(E) FoxO1/3a and BAD phosphorylation in response to FGF2-stimulation in S4$^{-/-}$ EC. Tubulin was used as loading control

(F). Cell size of S4$^{-/-}$ versus S4$^{+/+}$ EC. Serum-starved cells were harvested for analysis by flow cytometry. The mean FSC-H of G1-phase and CD31-positive cell population was determined in both S4$^{+/+}$ (upper panels) and S4$^{-/-}$ (lower panels) EC. The mean FSC-H histogram curves for each cell type were shown separately and also overlapped (upper right panel). Note the shift to the left of the black curve which corresponds to S4$^{-/-}$ cells. The bar graphs (lower right panel) show the quantification from 2 different experiments.

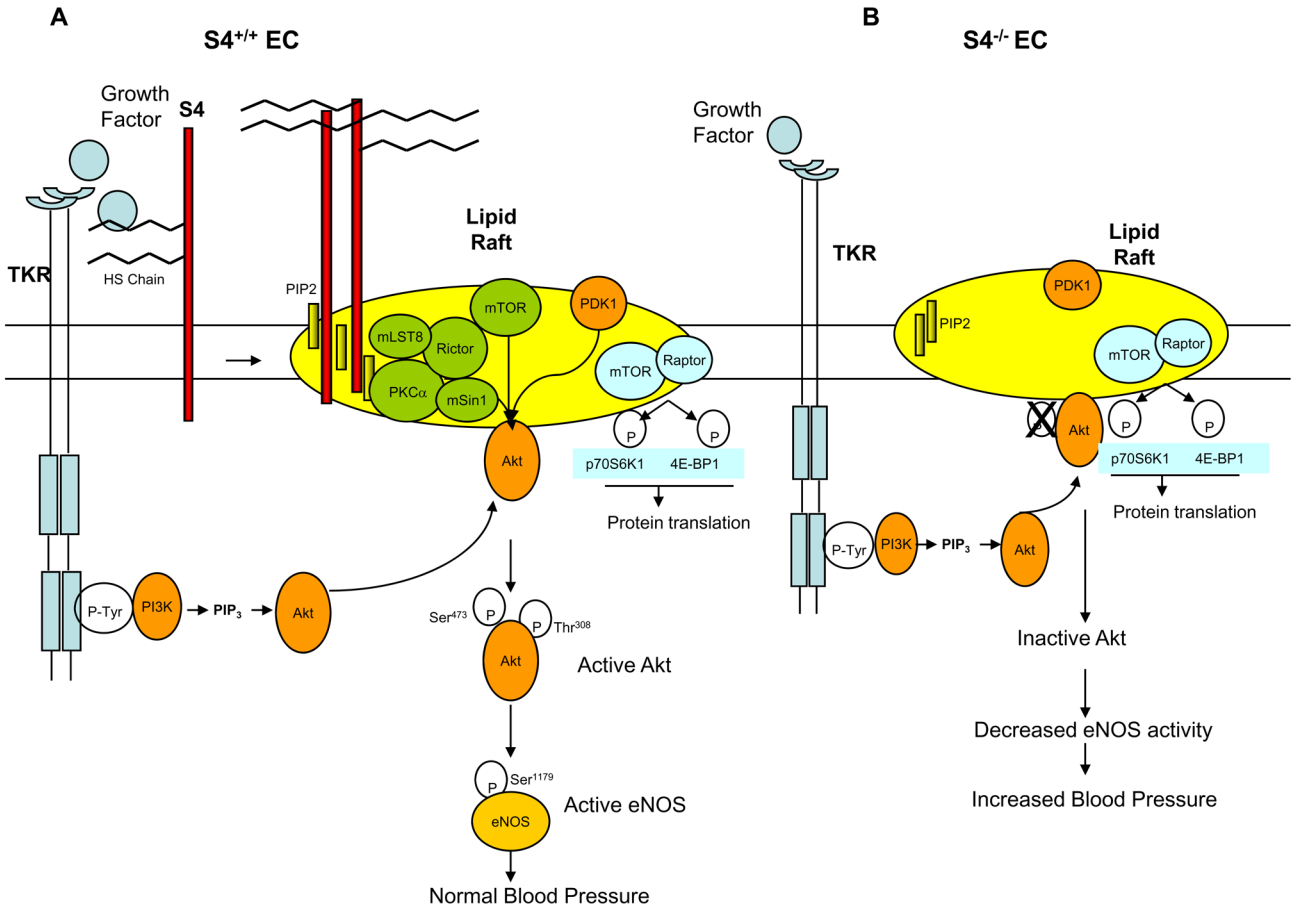

Figure 5. A proposed model of S4/PKCα-dependent regulation of mTORC2 localization in the membrane rafts and Akt activation

(A) Stimulation of $S4^{+/+}$ EC with a heparin-binding growth factor induces tyrosine kinase receptor activation and syndecan-4 clustering. The former leads to PI3-K activation, increased concentration of PI(3,4,5)P3 and translocation of Akt to the rafts. The latter is associated with S4 shift to the rafts, formation of the S4-PI(4,5)P2 complex and recruitment of PKCα. This in turn is associated with mSin-1, mLST8, and rictor translocation to the rafts and Akt phosphorylation leading to phosphorylation of downstream targets such as eNOS.

(B) In $S4^{-/-}$ EC, while Akt is still translocated to the membrane upon growth factor stimulation, its phosphorylation is impaired due to decreased level of PKCα, rictor, mLST8 and mSin-1 in the rafts which lead to decreased mTORC2 activity. mTOR shifts towards association with raptor whose presence in the rafts is independent of S4/PKCα complex. This leads to increased mTORC1 activity with increased p70S6K activation.