Biochemical and Biophysical Research Communications 400 (2010) 500–506

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Wnt5a/Ror2-induced upregulation of xPAPC requires xShcA

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ARTICLE INFO

Article history:
Received 12 August 2010
Available online 21 August 2010

Keywords:
ShcA
Ror2
Wnt-5a
Xenopus

ABSTRACT

Ror receptor-tyrosine kinases act as Wnt-5a receptors in beta-catenin independent Wnt-signaling pathways. In *Xenopus*, expression of xPAPC is regulated by a Wnt-5a/Ror2 pathway, which resembles typical signaling cascades downstream of receptor-tyrosine kinases. Here, we have identified the phospho-tyrosine binding protein ShcA as an intracellular binding partner of Ror2. ShcA binds to a conserved motif in Ror2 via its SH2-domain. Wnt-5a induces clustering of Ror2 in the cell membrane and recruitment of ShcA to the Ror2 receptor complex. We further show that ShcA is co-expressed with Ror2 in developing *Xenopus* embryos and ShcA is required for Wnt-5a/Ror2 mediated upregulation of xPAPC, demonstrating the functional relevance of this interaction.

1. Introduction

Ror receptor-tyrosine kinases (RTK) are evolutionary conserved single-pass transmembrane receptors with characteristic domain architecture [1] including a Wnt-binding Frizzled-like cysteine-rich domain. Ror receptors have been shown to activate non-canonical Wnt-pathways [2–5] and to mediate inhibition of canonical Wnt/β-catenin signaling [6,7]. Ror2 also mediates Wnt5a-induced filopodia formation via its interaction with filamin A (FLNa) in murine fibroblasts [8,9]. In addition, Ror2 has been shown to interact with casein-kinase 1ε (CK1 ε, [10]), Glycogen-synthase kinase 3 (GSK3, [11]), TGF-β-activated kinase 1 (TAK1 [12]), Dishevelled (Dvl [13]) and 14-3-3β [14].

Tyrosine-kinase activity seems to be dispensable for a subset of Ror2 functions including inhibition of canonical Wnt-signaling in certain contexts [7,12], Wnt-5a-induced cell migration [8] and AP1-activation [15]. On the other hand, tyrosine-autophosphorylation of Ror2 upon ligand binding [16,17] is required for the antagonism of canonical Wnt-signaling by Wnt-5a [17]. We have reported previously that Ror2 controls transcription of the xpapc gene in early *Xenopus* embryos. Regulation of xPAPC transcription requires xRor2 tyrosine-kinase activity, PI3K, cdc42 and JNK [3].

Typically, RTK signaling is initiated by ligand-induced dimerization and autophosphorylation of the receptor. Intracellularly, binding of adaptor proteins with phospho-tyrosine binding domains such as SH2-, SH3- or PTB-domains, is required for the subsequent activation of downstream effectors including PI3K and the small GTPases Ras, Rac1 or cdc42 [18].

Here, we show that the SH2- and PTB-domain protein ShcA binds to Ror2 *in vitro* and *in vivo*. ShcA interaction with Ror2 occurs via its SH2-domain, which binds to Shc-SH2 motif located in the Ror2 tyrosine-kinase domain. In *Xenopus* embryos, xShcA is co-expressed with xRor2 and required for the xRor2-dependent upregulation of the xWnt-5a target gene xPAPC.

2. Materials and methods

2.1. Frog handling and microinjections

RNA for microinjections was prepared using the mMessage mMachine Kit (Ambion, Austin/TX, USA). If not indicated otherwise, injection amounts were 100 pg pCS2 + LacZ DNA, 30 pg xRor2-EGFP and xRor2-mCherry RNA, 100 pg xWnt-5a RNA, xShcA p52, xShcA p52 ΔN and xShcA p52 ΔC RNA, 1.6 pmol xWnt-5a MO, 0.8 pmol xRor2 MO [3], 2.4 pmol xShcA MO p52 (5′-taacaaaccccctgtcttctacagc-3′). A control MO was provided by GeneTools and used in the same concentration as experimental MOs.

Embryos were obtained by in vitro fertilization and cultured as described previously [19]. Embryos were injected in both blastomeres at the two-cell stage for Animal Cap experiments or in one dorsal blastomere at the four-cell stage for in situ hybridization and cultured till they reached the desired stage according to the normal table of Nieuwkoop and Faber (NF) [20].

2.2. Animal caps, immunostaining and in situ hybridization

Xenopus embryos were injected as indicated and cultured till NF Stage 9. The Animal Cap was dissected and cultured in xWnt-5a conditioned or control Medium from stable transfected 3T3 cells,

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doi:10.1016/j.bbrc.2010.08.074

brought to *Xenopus* physiological ion concentration (approx. 100 mM NaCl) with distilled water. For RT-PCR Animal Caps were stimulated, cultured and processed as described in (Schambony and Wedlich 2007).

For immunostaining, Animal Caps were fixed after xWnt-5a stimulation for 1 h (4% formaldehyde, 100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$, pH 7.4), blocked in 10% horse serum/PBT (PBS, 0.1% Tween 20), incubated with the primary antibody overnight at 4 °C, washed three times with PBT, incubated with the secondary antibody for 4 h at RT, washed and nuclei stained with DAPI, and mounted in Mowiol (Roth). Images of optical sections were taken using a fluorescence microscope equipped with Apotome optics (Zeiss).

In situ hybridization was carried out as described in [21].

2.3. Antibodies, cells and transfections

The following antibodies were used: mouse anti-β-Actin (Sigma–Aldrich); goat anti-Ror2 (R&D Systems); rabbit anti-ShcA, rabbit anti-Grb2, rabbit anti-Shp2, rabbit anti-GFP, rabbit anti-pAKT, rabbit anti-EEA1 (Abcam); rabbit anti-AKT, rabbit anti-JNK (Santa Cruz); rabbit anti pJNK (Promega); rabbit anti-Flag, rabbit anti-myc (Cell Signaling); mouse anti-myc 9E10, mouse anti-β-tubulin (DSHB); mouse anti-mCherry was obtained from the antibody facility at MPI CBG; anti-mouse-AP, anti-rabbit-AP (Cell Signaling), anti-goat AP, anti-mouse-Cy3 (Dianova), anti-rabbit-Alexa Fluor 488 (Invitrogen). HEK 293 cells and 3T3 cells stably expressing xWnt-5a were generated using the pMSCV system (Clontech) and cultured in DMEM supplemented with 10% FCS and 100 μg/ml G418. Conditioned medium from xWnt-5a expressing cells (xWnt-5a CM) and control cells (control CM) was collected after 4 days. Cos-1 and HEK 293 cells were cultured in DMEM supplemented with 5% FCS and transfected with TransPass D2 (New England Biolabs) according to manufacturer's instructions.

2.4. Co-immunoprecipitation

Two days after transfection, cells were washed with cold PBS. Proteins were solubilized in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40) supplemented with complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail (Roche). Proteins were precipitated with anti-Flag affinity agarose gel (Sigma–Aldrich) or purified 9E10 anti-myc and Dynabeads protein G (Invitrogen), and further analyzed by standard Western blotting procedure.

3. Results

3.1. Wnt-5a induces clustering of xRor2

The xWnt-5a triggered xRor2-dependent signaling cascade in early *Xenopus* embryos and *Xenopus* Animal Cap explants includes PI3K, cdc42, MKK7 and JNK [3] and thus strongly resembles other RTK pathways. In addition, dimerization and autophosphorylation of mammalian Ror2 in response to Wnt-5a stimulation has been shown [16,17]. We hypothesized that *Xenopus* xRor2 acts similarly and indeed observed enhanced co-localization and clustering of xRor2-EGFP and xRor2-mCherry in Animal Cap explants after stimulation with xWnt-5a conditioned medium (xWnt-5a CM) in comparison to control CM (Fig. 1A and B). Intracellular and submembraneous clusters and vesicle-like structures do not represent endosomes as they failed to co-localize with the early endosomal marker EEA1 (Supplementary Fig. 1A–C).

After transfection of xRor2-EGFP into Cos-1 cells we observed partial co-localization of xRor2-EGFP with endogenous Ror2 that increased significantly after stimulation with Wnt-5a protein for 15 min (Supplementary Fig. 1D–E). Consistently, the amount of xRor2-EGFP that co-immunoprecipitated with xRor2-Flag significantly increased in Wnt-5a stimulated Cos-1 cells (Fig. 1C). Notably, Wnt-5a stimulation also resulted in increased AKT phosphorylation in these cells, indicating that PI3K was activated and thus Wnt-5a/Ror2 signaling recapitulated the PI3K, cdc42, JNK cascade [3].

3.2. xRor2 binds SH2-domain proteins

Based on these observations, we assumed that PI3K activation downstream of xRor2 could be mediated by the same adaptor proteins that are well-characterized effectors in other RTK signaling cascades. We have cloned the *Xenopus* orthologs of the SH2-domain proteins Grb2, Shp2 and ShcA (Fig. 2A) and confirmed endogenous expression of these genes in gastrula stage embryos by RT-PCR (Supplementary Fig. 2A).

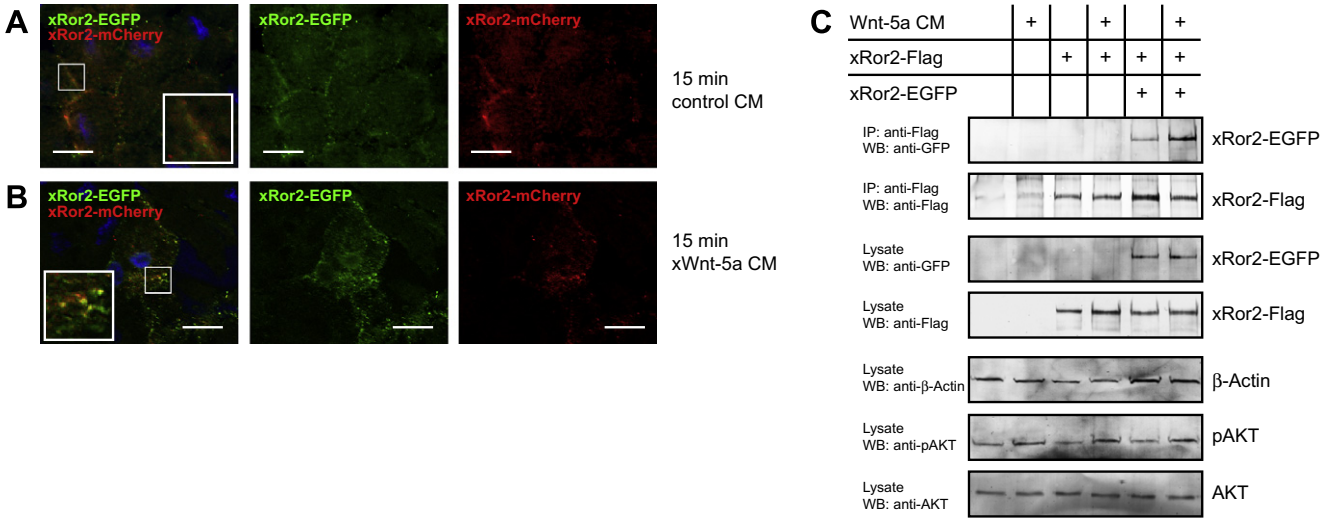

Fig. 1. xRor2 clusters in response to Wnt-5a. (A) EGFP- and mCherry-tagged xRor2 was expressed in *Xenopus* embryos (NF stage 10) after injection of 30 pg RNA each. In Animal Caps incubated in control CM for 15 min, both proteins partially co-localized at the cell membranes. (B) Stimulation with xWnt-5a CM induced a more pronounced co-localization at the membrane and intracellularly. (C) Co-immunoprecipitation of xRor2-Flag and xRor2-EGFP overexpressed in Cos-1 cells was strongly increased after stimulation with Wnt-5a conditioned medium for 15 min. Wnt-5a stimulation also induced AKT phosphorylation, indicating activation of PI3K.

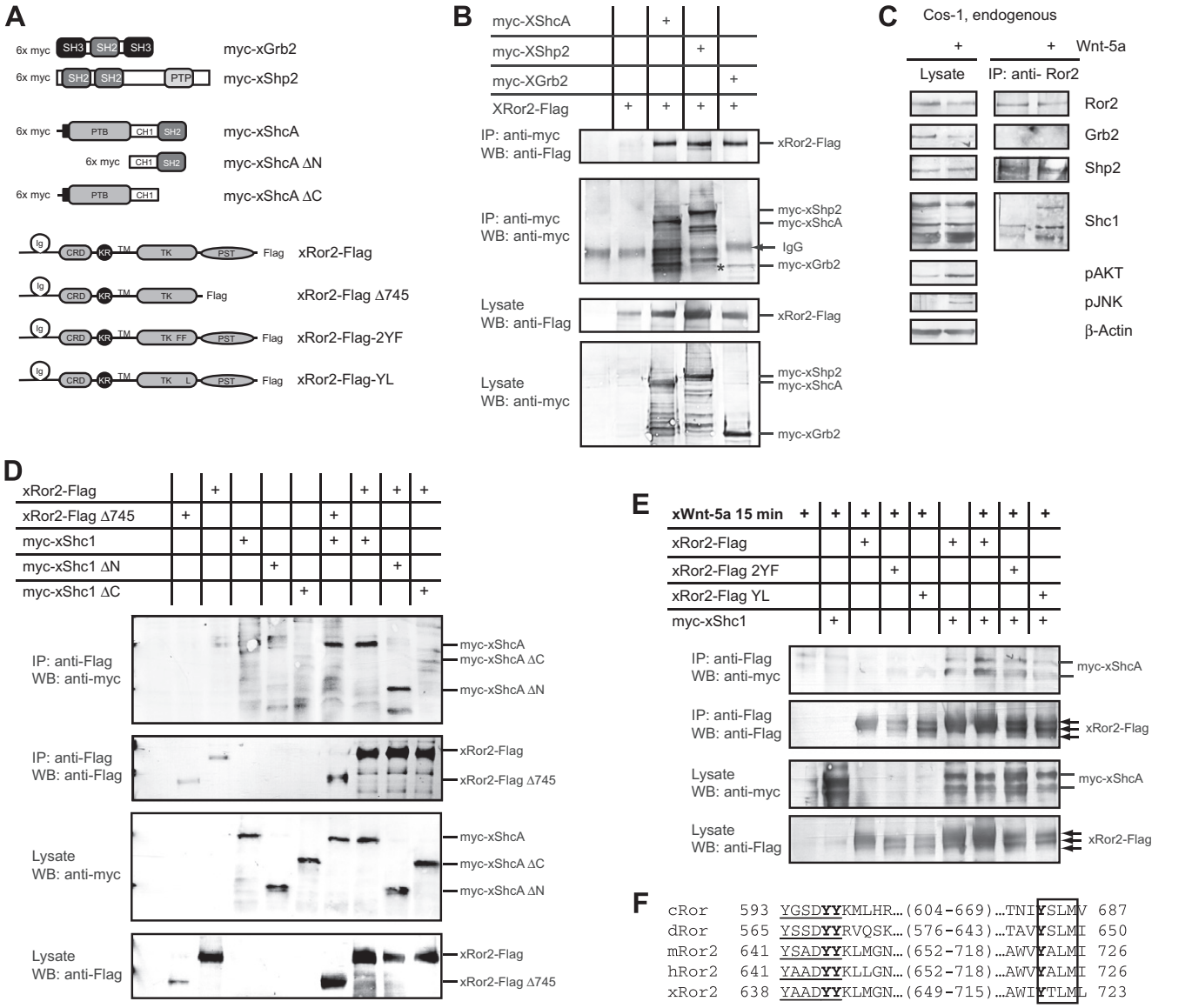

Fig. 2. SH2-domain proteins bind to xRor2. (A) Schematic illustration of xGrb2, xGab1, xShp2, xShcA and xRor2 constructs used for co-immunoprecipitation. (B) myc-tagged xShcA (NCBI NM_001090463), xShp2 (NCBI BC073687), and xGrb2 (NCBI AJ223061) were co-expressed with xRor2-Flag in Cos-1 cells. All proteins co-immunoprecipitated with xRor2-Flag without Wnt-5a stimulation. (C) Endogenous Ror2 was immunoprecipitated from Cos-1 cells stimulated with control CM and Wnt-5a CM for 15 min and co-immunoprecipitation of endogenous proteins was detected by immunoblotting. Activation of PI3K and JNK was monitored by AKT and JNK phosphorylation. (D) Co-immunoprecipitation of xShcA and xRor2 in Cos-1 cells: xShcA bound equally well to FL xRor2 and to xRor2Δ745. xShcAΔN, lacking the PTB-domain, was still able to bind to xRor2, but xShcAΔC, lacking the SH2-domain, failed to do so. (E) Co-immunoprecipitation of xShcA and xRor2 in HEK 293 cells was induced by xWnt-5a stimulation. Mutation of Y642 and Y643 (xRor2-Flag 2YF) moderately, mutation of Y719 (xRor2-Flag-YL) strongly reduced binding of xShcA to xRor2. Arrows indicate xRor2-Flag bands. (F) Conserved tyrosine residues mutated in xRor2-Flag 2YF and xRor2-Flag-YL are indicated in bold type, the conserved Shc-SH2 binding motif is boxed, the motif described as required for activation of the related RTK TrkA [23] is underlined. The respective regions of *C. elegans* Ror (Cam-1, NCBI NP_001021907.1), *Drosophila melanogaster* Ror (AAF52885), human (NCBI NP_004551.2) and mouse Ror2 (NCBI NP_038874.3) are aligned with the xRor2 sequence (NCBI NP_001082312); numbers indicate the respective amino acid.

Co-immunoprecipitation showed that all, xGrb2, xShp2 and xShcA, bind to xRor2 when overexpressed in Cos-1 cells (Fig. 2B). In this model system experimental artifacts due to overexpression of the proteins could not be ruled out as overexpressed xRor2 was not only observed at the cell membrane but also to a large extend intracellularly in Cos-1 cells (see Supplementary Fig. 1D and E). However, we have shown previously that Cos-1 cells endogenously recapitulate the Ror2 signaling cascade we have characterized in *Xenopus* embryos [3,13] and were therefore suitable to investigate ligand-dependent SH-2 domain protein recruitment to endogenous Ror2.

Endogenous Ror2 was immunoprecipitated from Cos-1 cells and we found that Wnt-5a stimulation induced recruitment of ShcA (Fig. 2C). In mammalian cells, ShcA is expressed in three isoforms named p66, p52 and p46 according to their molecular weight. In unstimulated cells the ShcA p46 and ShcA p52 co-immunoprecipitated with Ror2. Wnt-5a stimulation resulted in enhanced binding of ShcA p46 and p52 and in addition ShcA p66 was co-immunoprecipitated with Ror2 (Fig. 2C). Shp2 was constitutively associated with Ror2 while Grb2, although present in the lysate, was not bound to Ror2 in Cos-1 cells neither with nor without Wnt-5a stimulation (Fig. 2C). Activation of downstream signaling was confirmed by immunoblotting for pAKT and pJNK, which were increased in the lysates of Wnt-5a stimulated cells (Fig. 2C). The levels of phospho-p38 MAPK and phospho-ERK1/2 remained unchanged (not shown). This indicated a specific activation of the

JNK-mediated branch of MAPK signaling. Of the SH2-domain proteins investigated only ShcA showed recruitment to Ror2 upon Wnt-5a stimulation in Cos-1 cells, therefore we focused on the role of ShcA as an intracellular effector of Ror2 signaling.

3.3. xShcA binds to the tyrosine-kinase domain of xRor2 via its SH2-domain

We generated truncated versions of xShcA lacking either the PTB or the SH2-domain and an xRor2 construct that lacks the region C-terminal of the tyrosine-kinase domain (xRor2Δ745, see also Fig. 2A). Co-immunoprecipitation showed that full length xShcA binds to full length xRor2 as well as to xRor2Δ745, indicating that xShcA binds to a motif located within the tyrosine-kinase domain of xRor2 (Fig. 2D). Binding of xShcA to xRor2 required the SH2, but not the PTB-domain of xShcA, as xShcAΔN was still able to bind xRor2, but xShcAΔC was not (Fig. 2D). Indeed, xRor2 contains a conserved binding motif for Shc-SH2-domains within its tyrosine-kinase domain [1,22] but no PTB-domain binding motif. In addition, a motif described as required for activation of the related RTK TrkA [23] in vertebrate Ror2 and *Caenorhabditis elegans* Ror also matches the consensus sequence for Shc-SH2-domains pYΦX(I/L/M) with Φ representing a neutral, hydrophobic and X any amino acid [22]. We have mutated both motifs (Fig. 2E) and performed co-immunoprecipitation in HEK293 cells. In these cells, strong binding of xShcA to xRor2 was only observed after stimulation with xWnt-5a CM for 15 min, demonstrating again that this interaction was ligand-induced (Fig. 2E). Mutation of Y642 and Y643, located in the motif required for activation of the related TrkA resulted in weaker binding of xShcA probably due to reduced activation of xRor2. Mutation of Y719 in the conserved Shc-SH2 binding motif however almost completely abolished binding of xShcA (Fig. 2E). Notably, both mutations resulted in a reduction of the dominant band of lower electrophoretic mobility observed for wt xRor2 and the appearance of an additional band of higher mobility, indicating that the resulting proteins carry less post-translational modifications. It can be concluded that xShcA interacts with Ror2 in a ligand-dependent manner and binds to a phospho-tyrosine containing SH2-domain binding motif in the Ror2 tyrosine-kinase domain, which is conserved in all Ror orthologs (Fig. 2F).

3.4. xShcA is co-expressed and co-localizes with xRor2 in Xenopus embryos

We investigated next, if xShcA is co-expressed with xRor2 *in vivo* and whether it is required for xRor2 signaling in early *Xenopus* embryos. In mammalian cells, ShcA is expressed in the three isoforms ShcA p46, p52 and p66. All isoforms contain an N-terminal phospho-tyrosine-binding (PTB) domain, a central CH1 domain and a C-terminal SH2-domain (Fig. 3A). The p66 isoform shows an extended N-terminus due to alternative splicing, while p52 and p46 isoforms are generated by usage of alternative translation starts from the same transcript (for review see [24]).

In *Xenopus* embryos the p52 isoform is present maternally and dominates through gastrula and neurula stages. Only from tadpole

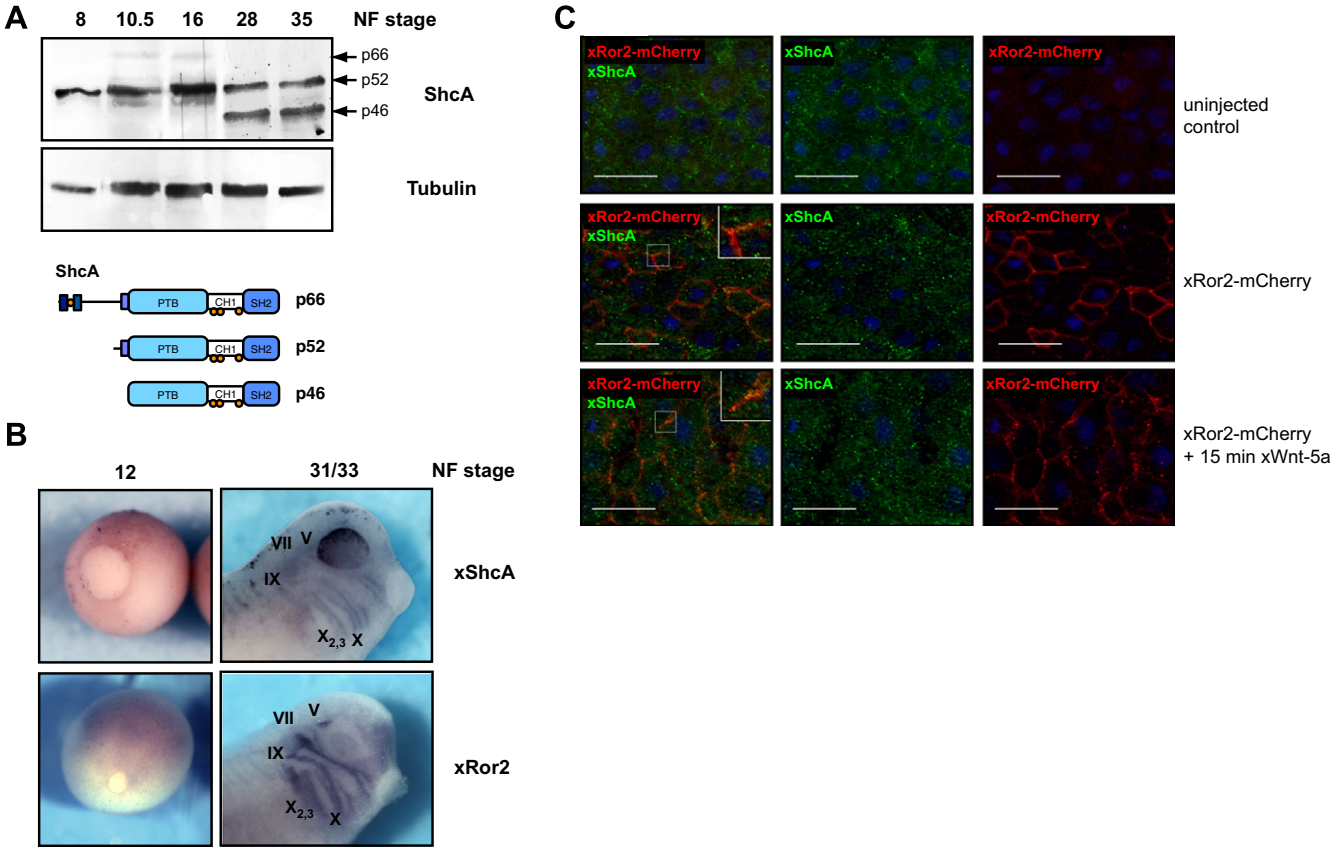

Fig. 3. xShcA is co-expressed and co-localizes with xRor2. (A) Western blotting of *Xenopus* embryo lysates showed expression of only p52 xShcA until neurula stages; in tadpole stages (NF stage 28 and 35) also p46 xShcA was detected. Schematic illustration of the three ShcA isoforms p66, p52 and p46. (B) Whole mount in situ hybridization for xShcA and xRor2 at NF stage 12 and 31/33. Cranial ganglia and nerves are labeled as follows: V = profundal-trigeminal, VII = facial, IX = glossopharyngeal, X = first vagal, $X_{2,3}$ = second and third vagal. (C) Immunostaining for endogenous ShcA showed largely membrane proximal localization of the protein. In Animal Caps injected with 60 pg xRor2-mCherry RNA, xRor2-mCherry protein weakly co-localized with xShcA at the cell membrane without exogenous ligand. xWnt-5a stimulation induced clustering of xRor2-mCherry and strongly enhanced co-localization with endogenous xShcA.

stages onwards we have observed expression of the p46 isoform (Fig. 3A). The p66 isoform was not detected in significant amounts in *Xenopus* embryo lysates. Whole mount in situ hybridization of *Xenopus* embryos showed that xShcA expression overlapped with xRor2 expression on the dorsal side of gastrula stage embryos (Fig. 3B). At later stages overlapping expression was detected in the nervous system, cranial ganglia and cranial nerves (Fig. 3B and Supplementary Fig. 2B–J).

3.5. xShcA is required for xPAPC expression in Xenopus embryos

Immunostaining for endogenous ShcA on *Xenopus* Animal Caps showed a low level expression and mostly membrane proximal localization of the protein (Fig. 3C). In Animal Caps expressing mCherry-tagged xRor2 we observed partial co-localization of the two proteins at cell membranes (Fig. 3C). Co-localization could be enhanced by treating the explants with xWnt-5a CM for 15 min (Fig. 3C), indicating that xWnt-5a stimulates binding of xShcA to xRor2 *in vivo*.

To further investigate the role of xShcA in early *Xenopus* development we generated antisense Morpholino oligonucleotides directed against xShcA p52 and xShc p46 (Supplementary Fig. 3). Interestingly, already at gastrula stages which express only xShcA p52 the translation of the p52 isoform was inhibited stronger with a combination of both MOs than with xShcA p52 MO alone. Therefore, we used both, the p52 MO alone and a combination of both MOs for the subsequent experiments.

xPAPC is a target gene of the Wnt-5a/Ror2 pathway in gastrulating *Xenopus* embryos. Therefore, we investigated the effect of xShcA gain-of-function and loss-of-function on xPAPC gene expression at early gastrula stages (NF stage 10.5). RT-PCR revealed that knock-down of xShcA resulted in downregulation of *xPAPC* mRNA levels (Fig. 4A). Overexpression of xShcA only slightly upregulated *xPAPC*, but overexpression of either xShcAΔN or xShcAΔC almost completely suppressed *xPAPC* transcription, indicating that both constructs behaved as dominant-negatives with respect to *xPAPC* expression in gastrula stage *Xenopus* embryos (Fig. 4A). When we performed in situ hybridizations for *xPAPC* mRNA at late gastrula stages (NF stage 12–12.5), we found that knock-down of xShcA was sufficient to downregulate *xPAPC* transcription (Fig. 4E) in 53% and 62% of the embryos, respectively (Fig. 4B). xRor2 MO injection downregulated *xPAPC* in 61% of the embryos (Fig. 4B and F) and thus had a slightly stronger effect than xShcA p52 MO alone, but was comparable to xShcA p52 + p46 MOs. Injection of the lineage tracer LacZ or a control MO had no significant effect on *xPAPC* expression with 92% and 87% of the embryos showing a normal expression pattern (Fig. 4B, C and D). Overexpression of xWnt-5a resulted in an upregulation of *xPAPC* in 60% of the embryos (Fig. 4B and G). Consistent with a role of xShcA in the Wnt-5a/Ror2 mediated transcriptional regulation of the xpapc gene, xShcA p52 MO was sufficient to suppress xWnt-5a induced upregulation of xPAPC (Fig. 4B and H) to 5% of the injected embryos. Moreover, the percentage of embryos showing a downregulation of *xPAPC* increased to 16%, which, although not statistically

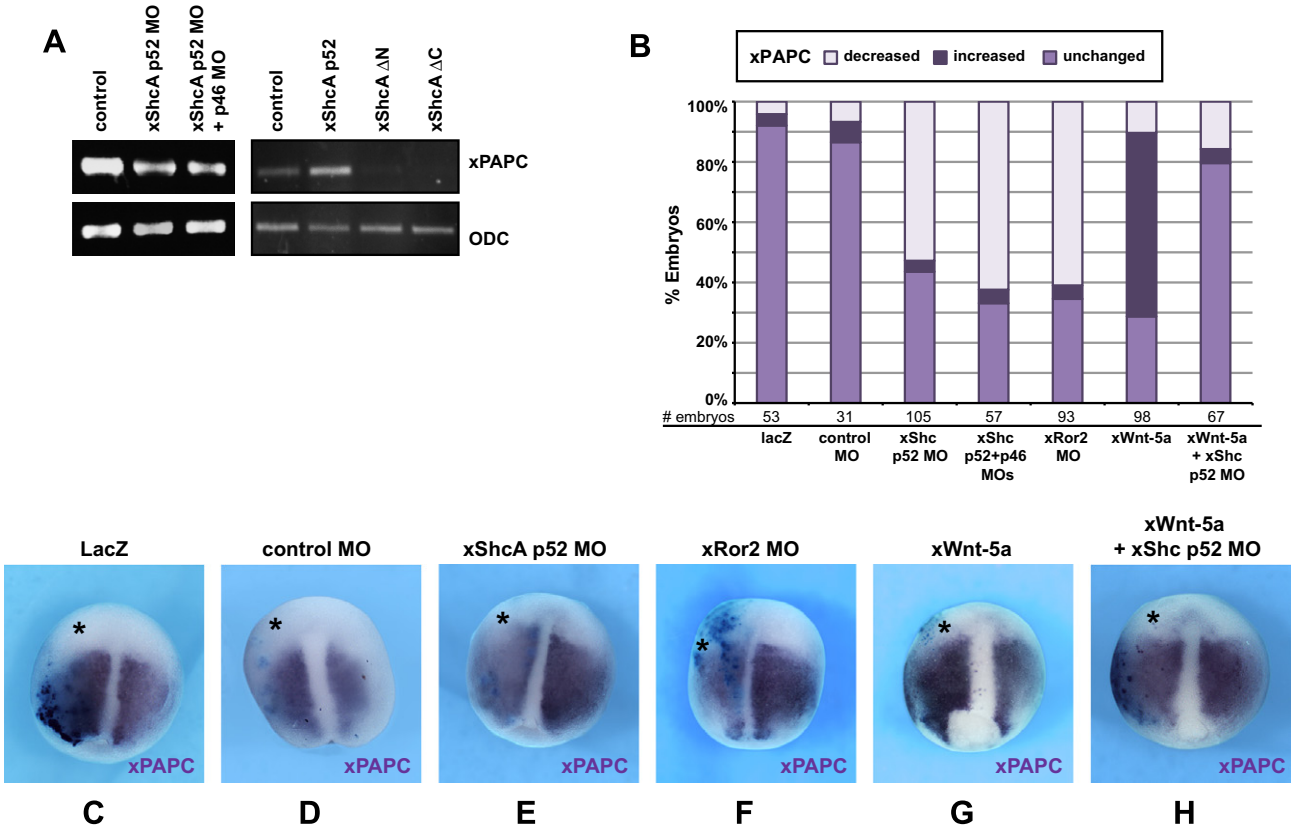

Fig. 4. xShcA is required for xRor2-dependent transcriptional regulation of xPAPC. (A) Injection of 2 pmol xShcA p52 MO or 2 pmol of each xShcA p52 and p46 MOs significantly downregulated xPAPC mRNA levels at NF stage 11. Overexpression of xShcA p52 (injection of 200 pg RNA) slightly upregulated xPAPC while overexpression of either xShcA p52 ΔN or xShcA p52 ΔC completely blocked xPAPC expression. (B) Statistical evaluation of xPAPC in situ hybridization. Embryos were injected in one dorsal blastomere and xPAPC staining on the injected (labeled with LacZ) side was scored relative to un injected side of the same embryo and the percentage of embryos showing enhanced, equal and reduced staining was calculated; the graph represents average values of at least three independent experiments (** significantly different from LacZ and control MO, *t*-test, $p > 0.99$). (C–H) Representative images of embryos; injected side is to the left and labeled with an asterisk: LacZ (100 pg, C), control MO (2.7 pmol, D), xShcA p52 MO (2.7 pmol, E), xRor2 MO (0.8 pmol F), xWnt-5a RNA (100 pg, G) and xWnt-5a RNA + xShcA p52 MO (100 pg + 2.7 pmol, H).

significant, further supported the conclusion that xShcA acts downstream of xWnt-5a in the regulation of xPAPC gene expression.

4. Discussion

Dimerization and autophosphorylation are common functional mechanisms for receptor-tyrosine kinases. For mammalian Ror2 autophosphorylation induced by forced dimerization via a cross-linking antibody [16] or the Wnt-5a ligand [17] has been reported. Consistent with these reports, we have shown Wnt-5a-induced dimerization of xRor2-Flag and xRor2-EGFP in a cell culture model and clustering of xRor2 *in vivo* in *Xenopus* Animal Cap explants. In the same cellular systems we show that ShcA is recruited to the xRor2 RTK in response to xWnt-5a stimulation.

Our results demonstrate ligand-induced interaction of Ror2 with a phospho-tyrosine binding protein. The sequence motif YxLM represents a ShcA SH2 binding site and is conserved in all Ror family proteins [1,22]. Consistently, we have mapped ShcA interaction with Ror2 to the ShcA SH2-domain and confirmed that mutation of the tyrosine residue in this YTLM motif (Fig. 2F) abrogates the ability of xRor2 to recruit xShcA.

A second interaction motif, YxxDYY, is found in all Ror family RTKs (Fig. 2F, [1]) and is required for activation of the related family of Trk receptors [23]. Mutation of the last two tyrosine residues in this motif rendered xRor2 less capable to recruit xShcA than wt xRor2, but stronger than mutation of the YTLM motif (Fig. 2E). These results indicate that the YTLM motif is the Shc binding motif of Ror2 and the YxxDYY motif may be an activation motif of Ror family RTKs similar to Trk receptors.

The tyrosine-kinase domain and the Shc-SH2 binding motif are evolutionary conserved in all Ror family receptors. In contrast, the proline-/serine-/threonine (PST)-rich C-terminus is variable in invertebrates and missing in the *Drosophila melanogaster* Ror ortholog. So far, all known cytoplasmic interaction partners of Ror2 bind to this PST-rich region [10–13]. ShcA is the first interacting protein that binds to a motif present in all Ror family members, suggesting that signaling through ShcA might represent an evolutionary conserved function.

In *Xenopus* embryos, we demonstrated that xShcA and xRor2 are co-expressed in dorsal tissues including cranial ganglia and cranial nerves at tadpole stages. XRor2 expression appeared generally broader than xShcA expression suggesting that xShcA could mediate a subset of xRor2 functions in *Xenopus* embryos. Ror2 has multiple signaling functions likely achieved by interaction of Ror2 with context-specific partners, but not necessarily requiring tyrosine-kinase activity [2–9]. Therefore, signaling through ShcA is probably one of several signaling option downstream of Ror2 receptors. Whether xShcA function in the nervous system involves interaction with xRor2 remains to be investigated.

However, we have confirmed that xShcA is required downstream of xWnt-5a in the regulation of xPAPC expression during gastrulation. Thus, xShcA mediates xRor2 signaling in a physiological context, likely by transducing the signal from xRor2 to PI3K.

ShcA has been shown to activate PI3K through recruitment of Grb2 and Grb2-associated binder 2 (Gab2, [25]). In addition, Gab1 mediates PI3K activation downstream of the Ror2 related TrkA receptor [26]. On the other hand, the regulatory p85 subunit of PI3K can bind directly to some RTKs. PI3K p85 contains one SH3 and two SH2-domains with the SH2 consensus binding motif YXXM (reviewed in [27]). The YTLM motif found in xRor2 represents a reasonable match and direct binding of PI3K to Ror2 cannot be ruled out at this point. Further studies are required to elucidate the molecular mechanism of ShcA function downstream of Ror2 in more detail and the potential role of other adapter proteins.

In conclusion, we have identified ShcA as a novel interacting protein of Ror2 RTKs. Binding was ligand-induced and mapped to the conserved YXLM motif in the Ror2 tyrosine-kinase domain. The functional requirement of ShcA downstream of Ror2 has been shown in *Xenopus* embryos where it is required for Wnt-5a/Ror2 mediated regulation of xPAPC expression. These results confirm that in early *Xenopus* embryos Ror2 signals through a typical RTK pathway and demonstrate to our knowledge for the first time the relevance of RTK activation downstream of a Wnt-signal.

Acknowledgments

The authors thank Robert Slany for his support in generating a stable xWnt-5a expressing 3T3 line, Patrick Keller for the mCherry antibody, and Iris Babion for technical support. The 9E10 and E7 hybridoma developed by J.M. Bishop and M. Klymkowski were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. This work was financially supported by a DFG grant to A.S. (SCHA 965/2-3).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.074.

References

[1] W.C. Forrester, The Ror receptor tyrosine kinase family, Cell. Mol. Life Sci. (2002) 83–96.
[2] I. Oishi, H. Suzuki, N. Onishi, R. Takada, S. Kani, B. Ohkawara, I. Koshida, K. Suzuki, G. Yamada, G.C. Schwabe, S. Mundlos, H. Shibuya, S. Takada, Y. Minami, The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway, Genes Cells (2003) 645–654.
[3] A. Schambony, D. Wedlich, Wnt-5A/Ror2 regulate expression of xPAPC through an alternative noncanonical signaling pathway, Dev. Cell (2007) 779–792.
[4] S. Yamamoto, O. Nishimura, K. Misaki, M. Nishita, Y. Minami, S. Yonemura, H. Tarui, H. Sasaki, Cthrc1 selectively activates the planar cell polarity pathway of Wnt signaling by stabilizing the Wnt-receptor complex, Dev. Cell (2008) 23–36.
[5] J.L. Green, T. Inoue, P.W. Sternberg, Opposing Wnt pathways orient cell polarity during organogenesis, Cell (2008) 646–656.
[6] A.J. Mikels, R. Nusse, Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context, PLoS Biol. (2006) e115.
[7] J.L. Green, T. Inoue, P.W. Sternberg, The *C. elegans* ROR receptor tyrosine kinase, CAM-1, non-autonomously inhibits the Wnt pathway, Development (2007) 4053–4062.
[8] M. Nishita, S.K. Yoo, A. Nomachi, S. Kani, N. Sougawa, Y. Ohta, S. Takada, A. Kikuchi, Y. Minami, Filopodia formation mediated by receptor tyrosine kinase Ror2 is required for Wnt5a-induced cell migration, J. Cell. Biol. (2006) 555–562.
[9] A. Nomachi, M. Nishita, D. Inaba, M. Enomoto, M. Hamasaki, Y. Minami, Receptor tyrosine kinase Ror2 mediates Wnt5a-induced polarized cell migration by activating c-Jun N-terminal kinase via actin-binding protein filamin A, J. Biol. Chem. (2008) 27973–27981.
[10] S. Kani, I. Oishi, H. Yamamoto, A. Yoda, H. Suzuki, A. Nomachi, K. Iozumi, M. Nishita, A. Kikuchi, T. Takumi, Y. Minami, The receptor tyrosine kinase Ror2 associates with and is activated by casein kinase Iepsilon, J. Biol. Chem. (2004) 50102–50109.
[11] H. Yamamoto, S.K. Yoo, M. Nishita, A. Kikuchi, Y. Minami, Wnt5a modulates glycogen synthase kinase 3 to induce phosphorylation of receptor tyrosine kinase Ror2, Genes Cells (2007) 1215–1223.
[12] A. Winkel, S. Stricker, P. Tylzanowski, V. Seiffart, S. Mundlos, G. Gross, A. Hoffmann, Wnt-ligand-dependent interaction of TAK1 (TGF-beta-activated kinase-1) with the receptor tyrosine kinase Ror2 modulates canonical Wnt-signalling, Cell. Signal. (2008) 2134–2144.
[13] F. Witte, O. Bernatik, K. Kirchner, J. Masek, A. Mahl, P. Krejci, S. Mundlos, A. Schambony, V. Bryja, S. Stricker, Negative regulation of Wnt signaling mediated by CK1-phosphorylated dishevelled via Ror2, The FASEB journal: official publication of the Federation of American Societies for Experimental Biology (2010).
[14] Y. Liu, J.F. Ross, P.V.N. Bodine, J. Billiard, Homodimerization of Ror2 tyrosine kinase receptor induces 14-3-3(beta) phosphorylation and promotes osteoblast differentiation and bone formation, Mol. Endocrinol. (2007) 3050–3061.
[15] M. Nishita, S. Itsukushima, A. Nomachi, M. Endo, Z. Wang, D. Inaba, S. Qiao, S. Takada, A. Kikuchi, Y. Minami, Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating dishevelled polymerization, Mol. Cell Biol. (2010) 3610–3619.

[16] Y. Liu, B. Rubin, P.V.N. Bodine, J. Billiard, Wnt5a induces homodimerization and activation of Ror2 receptor tyrosine kinase, J. Cell. Biochem. (2008) 497–502.
[17] A. Mikels, Y. Minami, R. Nusse, The Ror2 receptor requires tyrosine kinase activity to mediate Wnt5a signaling, J. Biol. Chem. 284 (2009) 30167–30176.
[18] J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell (2000) 211–225.
[19] F. Unterseher, J.A. Hefele, K. Giehl, E.M. De Robertis, D. Wedlich, A. Schambony, Paraxial protocadherin coordinates cell polarity during convergent extension via Rho A and JNK, EMBO J 23 (2004) 3259–3269.
[20] P.D. Nieuwkoop, J. Faber, Normal Table of *Xenopus laevis* (Daudin), Garland Publishing, 1994.
[21] T. Hollemann, F. Panitz, T. Pieler, In situ hybridization techniques with *Xenopus* embryos, in: J.D. Richter (Ed.), A Comparative Methods Approach to the Study of Oocytes and Embryos, Oxford University Press, 1999, pp. 279–290.
[22] Z. Songyang, L.C. Cantley, Recognition and specificity in protein tyrosine kinase-mediated signalling, Trends Biochem. Sci. (1995) 470–475.
[23] M.E. Cunningham, R.M. Stephens, D.R. Kaplan, L.A. Greene, Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor, J. Biol. Chem. (1997) 10957–10967.
[24] K.S. Ravichandran, Signaling via Shc family adapter proteins, Oncogene (2001) 6322–6330.
[25] H. Gu, H. Maeda, J.J. Moon, J.D. Lord, M. Yoakim, B.H. Nelson, B.G. Neel, New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway, Mol. Cell. Biol. (2000) 7109–7120.
[26] M. Holgado-Madruga, D.K. Moscatello, D.R. Emlet, R. Dieterich, A.J. Wong, Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor, Proc. Natl. Acad. Sci. USA (1997) 12419–12424.
[27] M.P. Wymann, L. Pirola, Structure and function of phosphoinositide 3-kinases, Biochim. Biophys. Acta (1998) 127–150.