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Coactosin accelerates cell dynamism by promoting actin polymerization

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ABSTRACT

During development, cells dynamically move or extend their processes, which are achieved by actin dynamics. In the present study, we paid attention to Coactosin, an actin binding protein, and studied its role in actin dynamics. Coactosin was associated with actin and Capping protein in neural crest cells and N1E-115 neuroblastoma cells. Accumulation of Coactosin to cellular processes and its association with actin filaments prompted us to reveal the effect of Coactosin on cell migration. Coactosin overexpression induced cellular processes in cultured neural crest cells. In contrast, knock-down of Coactosin resulted in disruption of actin polymerization and of neural crest cell migration. Importantly, Coactosin was recruited to lamellipodia and filopodia in response to Rac signaling, and mutated Coactosin that cannot bind to F-actin did not react to Rac signaling, nor support neural crest cell migration. It was also shown that deprivation of Rac signaling from neural crest cells by dominant negative Rac1 (DN-Rac1) interfered with neural crest cell migration, and that co-transfection of DN-Rac1 and Coactosin restored neural crest cell migration. From these results we have concluded that Coactosin functions downstream of Rac signaling and that it is involved in neurite extension and neural crest cell migration by actively participating in actin polymerization.

Introduction

Particular types of cells such as neural crest cells migrate long way from their origin to differentiate into many kinds of cell types (Le Douarin, 2008). Extension of the leading processes of the migrating cells relies on actin polymerization/depolymerization for its dynamism. ADP-actin is lost from the pointed edge of filamentous actin (F-actin), and ATP-actin is added to the barbed end of F-actin at the front of the leading process (Gungabissoon and Bamburg, 2003). This actin treadmilling allows the leading process to extend so that it can drive cell movement. Similar mechanism is also served as developing neurons, which extend axons and dendrites to make neuronal circuits.

For actin treadmilling, actin depolymerizing factor homology (ADF-H) family plays a central role (Lappalainen et al., 1998; Yang et al., 1998). Among ADF-H family proteins, Cofilin accelerates treadmilling of actin filaments by removing actin monomer from the pointed end (Yang et al., 1998; Bamburg et al., 1999). Twinfilin forms a 1:1 complex with ADP-actin monomers to inhibit nucleotide exchange on actin monomers, further prevents assembly of the monomer into filaments (Palmgren et al., 2002). Conversely, Drebrin binds only to F-actin but not to G-actin (Lappalainen et al., 1998). In the developing axon, Drebrin is localized in filopodia of growth cones (Sasaki et al., 1996), and attenuates actin dynamics by inhibiting activity of the binding of Cofilin to F-actin. Coactosin is also a member of ADF-H family, which binds to F-actin (Goroncy et al., 2009). It was suggested that Coactosin inhibits actin depolymerization by counteracting activity of Capping proteins, which cap the barbed end to prevent actin polymerization (Röhrig et al., 1995). Recently, we have cloned chick Coactosin, and reported that Coactosin is expressed in cells that migrate or extend cellular processes, such as neural crest cells and young neurons in chick embryos (Hou et al., 2009).

We raised a question if Coactosin plays an active role in morphogenesis by accelerating actin dynamics. In order to reveal the role of Coactosin, we focused on neural crest cells, which arise at the dorsal part of the neural tube and migrate long way to differentiate into pigment cells, neurons and supporting cells of the dorsal root ganglion, sympathetic and parasympathetic cells etc. (Le Douarin, 2008). Function of Coactosin in young neurons was also analyzed with N1E-115 neroblastoma cells, which differentiates into neurons in serum deprived medium.

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Here, we show that Coactosin binds with actin and Capping protein. Knock-down of Coactosin resulted in disruption of actin polymerization, and of neural crest cell migration, which suggests that Coactosin is actively involved in cellular dynamism by promoting actin polymerization. We further show that Coactosin functions downstream of Rac signaling to mediate lamellipodia and filopodia formation. Mutated Coactosin that cannot bind to F-actin could not mediate Rac signaling to make lamellipodia, nor support neural crest cell migration. These results suggest that Coactosin is actively involved in actin polymerization, and that plays a crucial role in actin dynamism downstream of the Rac signaling.

Materials and methods

Chick embryos and fixation

Fertile chicken eggs from a local supplier (Yamagishi, Japan) were incubated at 38 °C in a humid atmosphere until embryos reach desired stages.

In situ hybridization and immunohistochemistry

Embryos and cultured cells for histological processes were fixed in 4% paraformaldehyde in PBS at 4 °C for overnight. For visualization of Rac1 by immunohistochemistry, explants were fixed in 10% trichloroacetic acid as previously described by (Yonemura et al., 2004).

Whole-mount *in situ* hybridization was performed as described by (Bally-Cuif et al., 1995) or by (Stern, 1998). *In situ* hybridization for sections was carried out as described by (Ishii et al., 1997).

Primary antibodies used for Immunohistochemistry were: polyclonal anti-Coactosin antibody, which was raised in rabbits using bacterially expressed peptide, NH2-DHKELDEDYIKNELK-COOH, by Sawady Technology (Tokyo) as described previously (Hou et al., 2009), mouse monoclonal antibodies, HNK-1 (anti-CD57, which specifically stains neural crest cells, Zymed), 3F2.3 (anti-β2 subunit of non-muscle capping protein, Developmental Studies Hybridoma Bank (DSHB)), 0.T.127 (Anti-Rac1, Abcam), and M2F6, (Anti-Drebrin E. a kind gift of Prof. Tomoaki Shirao), rat monoclonal antibody 3F10 (Anti-HA (Hemoagglutinin), Roche Applied Science), rabbit monoclonal anti-GFP (Invitrogen) was used.

Secondary antibodies used were anti-mouse Alexa-594 (Invitrogen), anti-mouse Alexa-488, anti-rat Alexa-488 (Invitrogen), and anti-rabbit Alexa-594 (Invitrogen) antibodies.

Visualization of F-actin

Actin filaments were visualized by Rhodamine or Oregon Green 488 conjugated Phalloidin (Invitrogen).

In ovo electroporation

In ovo electroporation was performed at stage 10 as previously described (Funahashi et al., 1999; Odani et al., 2008) using expression vectors; HA tagged Coactosin in pMiwII (Suemori et al., 1990; Wakamatsu et al., 1997; Araki and Nakamura, 1999) (pMiwII-Coactosin-HA), HA tagged mutant Coactosin, in which lysine75 (actin binding site) was changed to alanine by Mutagenesis Kit (Stratagene), in pMiwII (pMiwII-CoactosinK75A-HA), a dominant negative form of Rac1 (N17-Rac1) and constitutive active Rac1 (V12-Rac1) in pEF-BOS-HAx3 vector. GFP fusion was made for the following expression vectors; Coactosin in pEGFP-C1 (Clontech) (pEGFP-Coactosin), mutant Coactosin in pEGFP-C1 (pEGFP-CoactosinK75A) and β-actin expression vector (Clontech) (pAcGFP1-Actin).

In some cases stock solution of DiI (C-7000, Molecular Probe) (0.5% in 100% ethanol) was diluted 10 times in 0.3 M sucrose solutions, and was added to the same volume of plasmid solution at electroporation to label neural crest cells (Omi et al., 2002).

siRNA construction

siRNA was designed referring to Katahira and Nakamura (2003), and the sequences were 5′-AGCTAATTACGATGCACAGAC-3′ for *Coactosin* siRNA and 5′-AGCAAATTACCATCCAGAGAC-3′ for control siRNA. pSuper-GFP (Oligoengine) that contains H1 promoter and a nine-base hairpin loop sequence (5′-TTCAAGAGA-3′) was used to make GFP-coupled siRNA. We also prepared pSuper-siRNA expression vector by deleting GFP construct. We confirmed that the *Coactosin* siRNA does not cross react with other chick ADF-H family members by BLAST search.

Cell culture

HEK 293 cells and neural crest cells were cultured at 38 °C in 10% FBS (Fetal Bovine Serum) containing DMEM (Dlubecco's Modified Eagle's Medium) in an atmosphere of 5% CO_2. N1E-115 cells were maintained in DMEM (NISSUI) containing 10% fetal bovine serum and 1% penicillin, 1% streptomycin, in a humidified incubator with 5% CO_2 at 37 °C as undifferentiated, and were induced to differentiate to neuronal cells by moving to differentiation medium, DMEM with 2% FBS and 1.25% Dimethyl sulfoxide (DMSO).

Transfection of pMiwII-Coactosin-HA and pMiwII-Capping protein β2-Myc to HEK 293 cells, and transfection of pEGFP–Coactosin, pMiwII-Capping protein β2-Myc, pEGFP–CoactosinK75A and/or V12-Rac1 to N1E-115 cells was carried out by Lipofectamine™ 2000 (Invitrogen).

Neural crest cells were cultured as described by Newgreen and Thiery (1980). Briefly, chick embryos were electroporated at stage 10. At 12 h after electroporation, neural tubes were taken out, digested in 1.5 mg/ml Dispase (GODO SHUSEI) for 30 min, washed in Leibovitz-15 media (GIBCO), and cut into small pieces. The tissue pieces were then cultured on 35 mm plastic dish or coverslips coated with fibronectin (BD Biosciences).

After neural crest cells were pre-cultured for 6 h, time lapse images were collected for 12 h by scanning laser microscope (FV300 on IX-81, OLYMPUS). Single-track z-sections (10–20 μm in thickness) at 10 min intervals were collected to produce a 3D time-lapse movie.

Quantification

For statistical analysis of number of filopodia, software of Image-Pro (Nippon Roper) was used. We set the field for the image capture in consideration of cell migration for recording period. Images were captured every 10 min, and all the cells in the field were counted. Image-Pro automatically counts cell number according to the diameter and contours of the object. If 2 or 3 cells were counted as one, modification was made by the author for the final number of cells. Filopodia were abstracted according to the manufacture's protocol, and those that have longer than 4-pixel-length were counted. Then mean number of filopodia per cell and standard error were calculated.

For statistical analysis for migration distance of cultured neural crest cells, lines were made every 20 μm in parallel with the edge of the neural tube (start point) to the cell of farthest migration, and the number of cells between 2 lines was counted. Statistical analysis was performed with Prism 5.0 (MDF) and determined the mean and standard error of percentage.

Sagittal explant culture and live imaging

Saggital explant culture elaborated by Kasemeier-Kulesa et al. (2005) was modified to show neural crest cell migration. Embryos were electroporated at stage 10, and taken out 12 h later. Shallow cut was made along the midline of the spinal cord with a sharpened tungsten needle. It allows the dorsal side of the embryo to flat and smooth on the culture filter (Millicell Culture Insert, Millipore) which was coated with 20 μg/ml fibronectin. Culture was carried out on the Microscope Stage Heat plate Chamber (MI-IBC; Olympus) in serum free neural basal medium (GIBCO, 17504-044) supplemented with B27 (Invitrogen) in the atmosphere of 5% CO_2, 40% O_2. Images were collected by scanning laser microscope (FV300 on IX-81, OLYMPUS). Single-track z-sections (10–20 μm in thickness) at 5 min intervals were collected to produce a 3D time-lapse movie.

Immunoprecipitation and immunoblotting

For immunoprecipitation and immunoblotting, cultured cells were homogenized in the lysis buffer (10 mM Tris HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 0.1% polyoxyethylene octylphenyl ether) supplemented with cocktail of protease inhibitors (Roche), and were lysed by sonication with a Misonix 2000 sonicator (Qsonica) (1 min total sonication time, 12 s pulses, power level 2, 4 °C throughout). Immunoprecipitation with anti-actin antibody was performed as described by Erickson et al. (1997). Lysates were cleared by centrifugation at 15,000 g for 10 min at 4 °C, and boiled in sample buffer. Total protein concentration was determined by performing a Bradford assay (Bio-Rad). For immunoprecipitation, Protein Sepharose 4 Fast Flow (GE Healthcare) or Dynabeads protein A/G beads (Invitrogen), which had been soaked with 2 μg of each antibody, was incubated with the lysate including 200 μg proteins for an hour at 4 °C. The beads were washed four times with 1 ml of the lysis buffer and eluted by boiling for 5 min in the sample buffer. After centrifugation, equal amount of eluate was loaded on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) using 12.5 or 20% gels (ATTO), with 250 V, 20 mA for 60 min. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) with 50 V, 2 mA/cm^2. After blocking with 2% dry milk in TBS-Tween (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% Tween) for 1 h, membranes were probed with primary antibodies as indicated. After 3 washes of 30 min each in TBS-Tween, blots were incubated with HRP (Horse radish peroxidase)-conjugated anti-rabbit IgG or anti-mouse IgG light chain specific secondary antibodies (Jackson ImmunoResearch; 1/5000). SuperSignal West Pico system (Thermo) was used for detection of immunoreactivity. Antibodies for immunoprecipitation and immunoblotting were: anti-Coactosin antibody, rabbit anti-Coactosin-like protein antibody (Millipore), anti-HA tag antibody (12CA5, Roche Applied Science), anti-Myc monoclonal antibody (9E10, DSHB), anti-actin monoclonal antibody (Genscript), anti-capping protein β2 subunit antibody (3F2.3, DSHB), anti-Tubulin 4α (GeneTex). For the control, normal rabbit IgG was used (Santa Cruz Biotechnology).

Results

Association of Coactosin with actin

To reveal the role of Coactosin in actin dynamics, we first checked if Coactosin is really associated with actin in neural crest cells. Homogenate of cultured neural crest cells was immunoprecipitated with anti-Coactosin or anti-actin antibody, and the precipitates were analyzed by Western blotting. Immunoblotting with each antibody demonstrated a band of Coactosin at 16 kDa (Fig. 1A, arrow) and that of actin at 42 kDa (Fig. 1B, arrow), respectively. Immunoprecipitation and immunoblotting with these antibodies showed that Coactosin was immunoprecipitated with anti-actin antibody (Fig. 1A, arrowhead), and vice versa

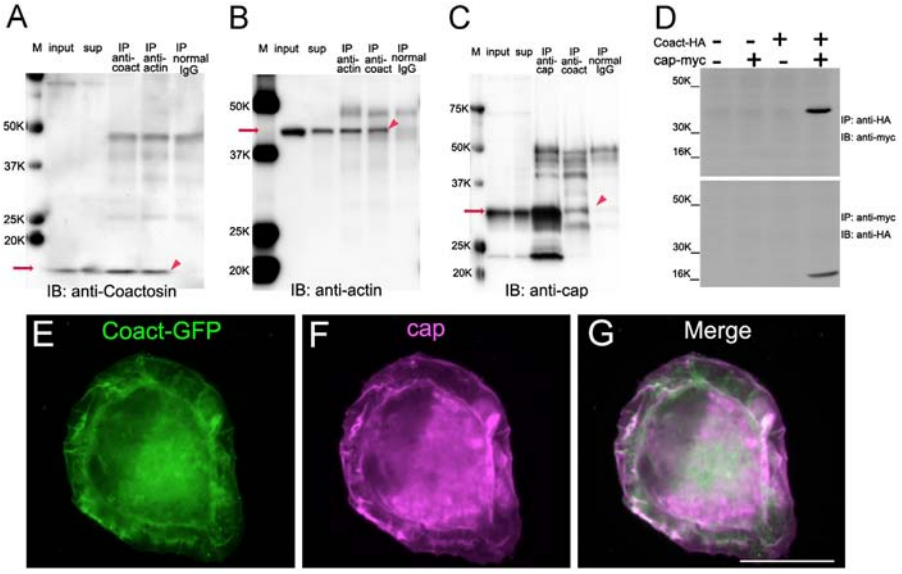


Fig. 1. Association of Coactosin with actin and Capping protein β2. (A) Immunoblotting (IB) with anti-Coactosin antibody after immunoprecipitation (IP) with anti-actin or anti-Coactosin antibody. Lanes from the left, molecular marker (M), input (1/40), supernatant (sup), IP with anti-Coactosin antibody (IP anti-coact), IP with anti-actin antibody (IP anti-actin) and IP with normal IgG. Band of Coactosin at 16 kDa is indicated by a red arrow. Band of Coactosin (arrowhead) was detected after IP with anti-actin antibody. (B) Immunoblotting (IB) with anti-actin antibody after immunoprecipitation with anti-actin or anti-Coactosin antibody. Lanes from the left, molecular marker (M), input (1/40), supernatant (sup), IP with anti-actin antibody (IP anti-actin), IP with anti-Coactosin antibody (IP anti-coact) and IP with normal IgG. Band of actin at 42 kDa is indicated by a red arrow. Band of actin (arrowhead) was detected after IP with anti-Coactosin antibody. (C) Immunoblotting (IB) with anti-Capping protein β2 after immunoprecipitation with anti-Capping protein β2 or anti-Coactosin antibody. Lanes from the left, molecular marker (M), input (1/40), supernatant (sup), IP with anti-Capping protein antibody (IP anti-cap, input), IP with anti-Coactosin antibody (IP anti-coact) and IP with normal IgG. Band of Capping protein β2 at 31 kDa (indicated by the red arrow) was also detected after IP with anti-Coactosin antibody (arrowhead). (D) Immunoprecipitation with anti-HA antibody and immunoblotting with anti-Myc antibody (upper panel), and vice versa (lower panel) from lysate of the HEK 293 cells that were transfected with HA tagged Coactosin (Coact-HA) and Myc tagged Capping protein β2 (cap-myc) expression vectors. (E–G) Association of Coactosin and Capping protein β2 in N1E-115 cells. Exogenous Coactosin-GFP (E, green) and Capping protein β2 (F, magenta) are co-localized in N1E-115 cells (G). Scale bar is 10 μm.

(Fig. 1B, arrowhead). The results indicate that Coactosin and actin are associated in neural crest cells. Furthermore, immunoprecipitation with anti-Coactosin antibody and immunoblotting with anti-capping protein β2 showed the band at 31 kDa (Fig. 1C, arrowhead), which indicates that Coactosin is associated with Capping protein β2 (Fig. 1C, arrow). Association of Coactosin and Capping protein was confirmed by exogenous transfection *in vitro*. HA tagged Coactosin and Myc tagged Capping proteins β2 were transfected to HEK 293 cells, and the lysate of the cultured cells was immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Myc antibody. Reciprocal experiment was also carried out. Since molecular weight of HA tag and of Myc tag is about 2.5 kDa and 3.6 kDa, tagged Coactosin and Capping protein β2 are recognized as a band at 18.5 kDa and 34.5 kDa, respectively (Fig. 1D). The result clearly shows that Coactosin and Capping protein β2 are associated in HEK 293 cells.

Association of Coactosin and Capping protein β2 in N1E-115 neuroblastoma cells was examined by immunohistochemistry. Coactosin–GFP and Capping protein β2 expression vectors were transfected to N1E-115 cells, and these proteins were visualized by anti-GFP and anti-Capping protein β2 antibody (Fig. 1E and G). The merged figure reveals that Coactosin and Capping protein β2 co-localize at lamellipodia in N1E-115 cells.

Coactosin is localized in lamellipodia and filopodia

We then examined subcellular localization of Coactosin in N1E-115 neuroblastoma cells. N1E-115 cells are round and proliferate in the presence of 10% serum, but exhibit neurite outgrowth upon serum deprivation (Amano et al., 1972; Hirose et al., 1998). In proliferation phase, Coactosin was widely distributed in the cytoplasm (Fig. 2A–C). Some cells extended a few lamellipodia, where Coactosin was localized in association with actin fibers in $89.33 \pm 4.8\%$ cells (6 times of experiments, and about 30 cells observed in each experiment) (Fig. 2A–C). In differentiation phase, cells extended lamellipodia, filopodia and neurites, where Coactosin was accumulated in all the cells examined (4 times of experiments, and about 20 cells observed in each experiment) (Fig. 2D–F). Distribution of Coactosin and Drebrin (Shirao, 1995) was also examined immunohistochemically. Interestingly, although Coactosin and Drebrin were co-localized at the proximal part of the filopodia, Coactosin was accumulated more distally in the filopodia than Drebrin in $73.65 \pm 4.38\%$ cells (3 times of experiments, about 20 cells observed in each experiment) (Fig. 2G–I).

Overexpression of Coactosin augments number of filopodia on neural crest cell

The subcellular accumulation of Coactosin to lamellipodia and filopodia, and its association with actin filaments suggest that Coactosin is involved in formation of cellular processes through actin dynamism. We then evaluated effects of overexpression in cultured neural crest cells. At 12 h after electroporation of Coactosin–GFP expression vector, neural tube of the transfected area was taken out, and cultured as described by Newgreen and Thiery (1980). In the culture, neural crest cells that were transfected with GFP migrated away from the dorsal neural tube (Fig. 3A and B, see Fig. 5A). Neural crest cells that had been transfected with Coactosin extended and withdrew lamellipodia/filopodia more frequently (Fig. 3C and D) than those that were transfected with GFP (Fig. 3A and B). But time lapse image demonstrates that movement of Coactosin-transfected neural crest cells was disordered compared to directed migration of GFP-transfected cells (supplementary movie 1A, 1B). We quantified number of filopodia on 3 movies per each group (Fig. 3E). On the time lapse image, filopodia were abstracted by Image-Pro, and the number was counted at every 10 min. Quantification shows that the

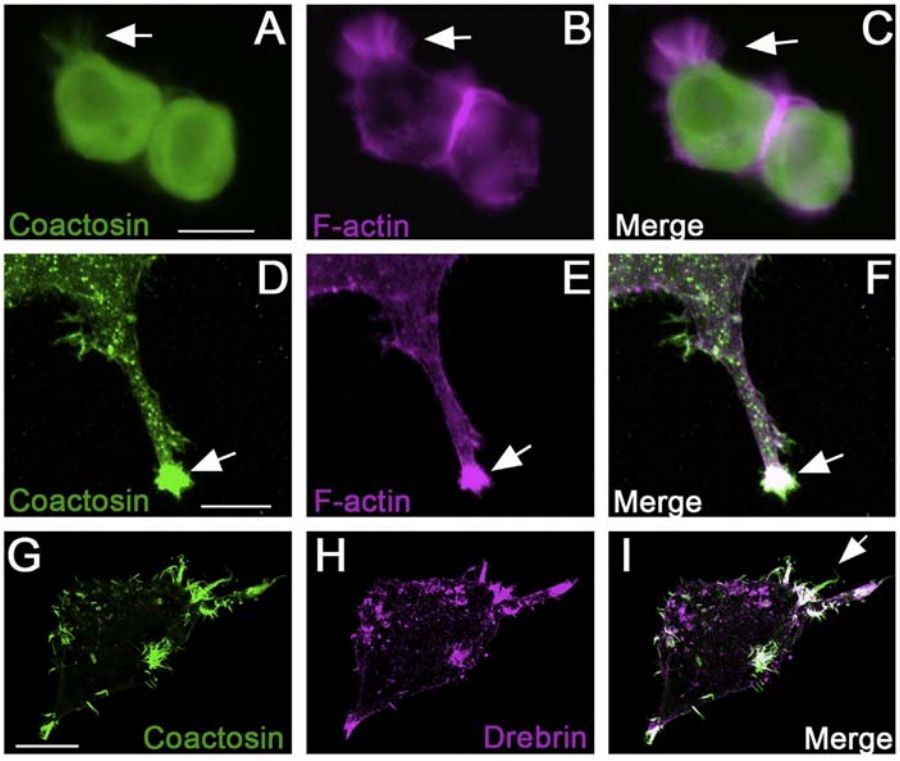


Fig. 2. Subcellular localization of Coactosin in N1E115 cells. Localization of transfected Coactosin–GFP in N1E-115 cells during growth phase (A–C) or differentiation phase (D–I). Coactosin, actin filaments and Drebrin were visualized with anti-Coactosin antibody (A, D, G, green), Rhodamine-Phalloidin (B, E, magenta), anti-Drebrin antibody (H, magenta), respectively. Coactosin is detected in the lamellipodia (arrows, A–C) and growth cone (arrows, D–F) in association with actin filaments. Both Coactosin and Drebrin are localized in filopodia (G–I). At the proximal part, Coactosin and Drebrin are co-localized but Coactosin is accumulated into more distal part of the filopodia (arrow on I). Scale bar: 10 μm.

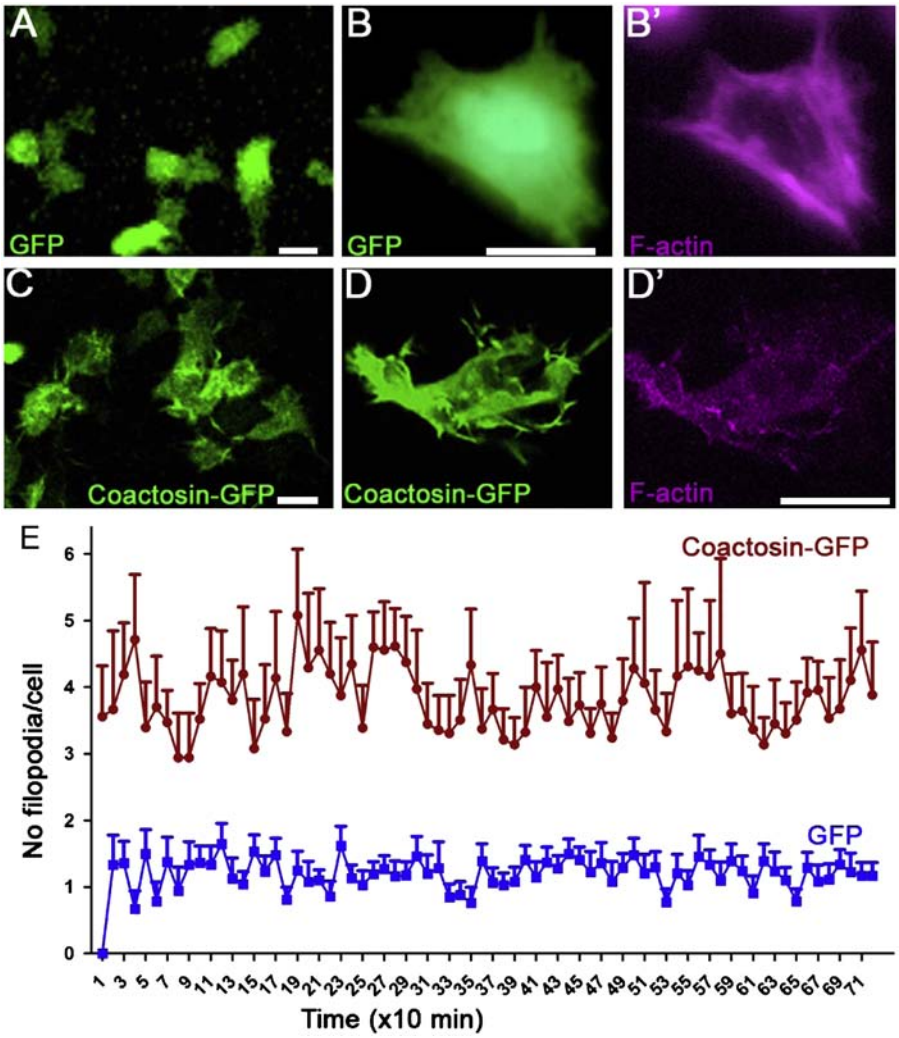


Fig. 3. Augmentation of filopodia by overexpression of Coactosin. The images from the time lapse movies for GFP (A and B) or Coactosin-GFP transfected neural crest cells (C and D). (A–D) GFP fluorescence. (B′ and D′) visualization of F-actin by Rhodamine–Phalloidin. Cells that were transfected with Coactosin extend more cellular processes than that transfected with GFP. (E) Statistical analysis of the number of filopodia. On the time lapse images captured every 10 min, cell number in the whole field was counted. Filopodia were abstracted by Image-Pro, and the mean number of filopodia per cell and the standard error were calculated and shown on the ordinate and abscissa. Because cells in the whole field were the objects of statistic, the number of cells at each time point is different, but about 20–30 cells in the control and 30–40 cells in the experimental field existed. Scale bar: 10 μm.

control cell has about 1.5 filopodia while the Coactosin transfected cell has about 4 filopodia, more than twice that of the control throughout the recording period (Fig. 3E). Thus, Coactosin was implicated in formation of cellular processes, corresponding to its preferential accumulation in association with actin filaments (Fig. 2).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2013.04.006.

Knockdown of Coactosin disrupts neural crest cell migration

It was shown that Coactosin transcripts are detected in neural crest cells, axons and growth cones of the oculomotor nerve and in cells that are forming mesonephric duct (Hou et al., 2009). Furthermore, induction of cellular processes by Coactosin and its association with actin filaments suggest that Coactosin is involved in cell movement. To examine the role of Coactosin in neural crest cell migration, we knocked down Coactosin by electroporating GFP-coupled siRNA expression vector (Hou et al., 2011) (Fig. 4A). Neural crest cells that were transfected with control siRNA-GFP are migrating through sclerotome, and have arrived near the level of the ventral limit of the neural tube at 24 h after electroporation (Fig. 4B–D). On the other hand, most neural crest cells that were transfected with *Coactosin* siRNA–GFP stayed near the dorsal neural tube (Fig. 4E–G). Staining of neural crest cells with HNK1 antibody confirmed that migration of cells was disrupted by Coactosin knockdown (Compare Fig. 4D and G). For quantification, a line to represent migration route was drawn, and vertical lines to the migration route were made at every 20 μm on Image-Pro software (Fig. 4H). Relative cell numbers between two vertical lines were counted (Fig. 4I). In control, neural crest cells reached 180 μm away from the dorsal midline and distributed evenly on the route (Fig. 4I, mean migration distance; 59.1 ± 2.54 μm (mean ± standard error), 8 sections from 5 times of experiments). Neural crest cells that had Coactosin knocked down reached 120 μm away, but most cells stayed within 80 μm from the dorsal midline (Fig. 3I, mean migration distance; 30.5 ± 1.14 μm, 11 sections from 5 times of experiments)

Then we examined the effects of *Coactosin* knockdown on neural crest cell migration in culture. At 12 h after electroporation of siRNA expression vector, neural tube of the transfected area was taken out, and cultured for 16 h. In the culture, neural crest cells transfected with control siRNA-GFP migrated away from the dorsal neural tube, as well as non-transfected cells (Fig. 5A and B). The cells transfected with *Coactosin* siRNA-GFP showed impairment of

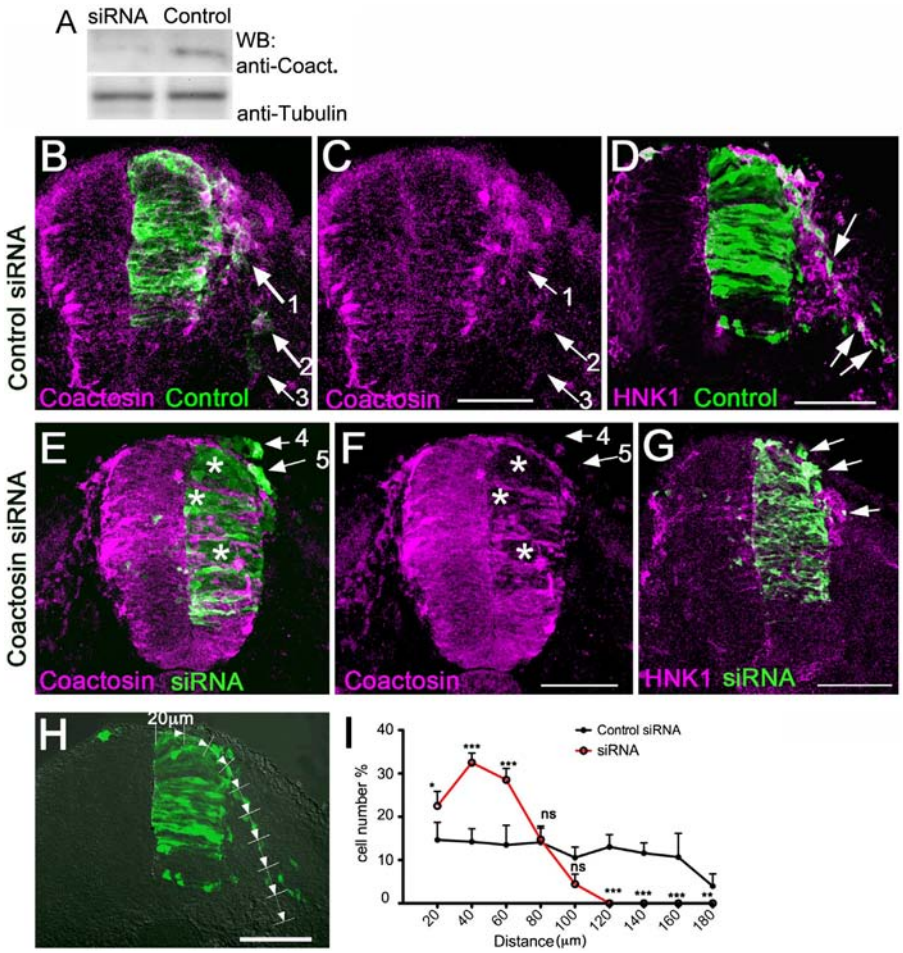


Fig. 4. Impairment of neural crest cell migration by Coactosin knockdown *in vivo*. (A) Immunoblotting with anti-Coactosin antibody of neural crest cells transfected with siRNA expression vectors. *Coactosin* siRNA effectively knocked down Coactosin in neural crest cells. Immunoblotting with anti-tubulin antibody was served as the control. (B–G) Immunohistochemical staining on a transverse section after electroporation of control siRNA-GFP (B–D) or *Coactosin* siRNA-GFP (E–G). (B, C, E, F) staining with anti-Coactosin antibody (magenta) and with anti-GFP antibody (green). (D, G) staining with HNK-1 antibody (magenta) and with anti-GFP antibody (green). The arrows indicated by 1, 2, 3 on B and C and by 4, 5 on E and F point the same transfected cells, respectively. Coactosin expression was effectively decreased in *Coactosin* siRNA transfected cells compared with non-transfected side (E, F, asterisks). Coactosin-knockdown impaired neural crest cell migration, that is, cells stayed inside or near the neural tube compared with control cells (arrows). Scale bar: 50 μm. (H, I) For quantification of neural crest cell distribution, migration route was drawn, and perpendicular lines to the route were drawn at every 20 μm (H), and relative cell numbers between the lines are shown on I (control versus *Coactosin* siRNA, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, total 266 cells for control, 238 cells for *Coactosin* siRNA, $n=5$, *t*-test).

migration. They stayed within or near the neural tube (Fig. 5C and D). We then examined distribution of neural crest cells by drawing parallel lines to the edge of the neural tube at every 20 μm and by counting cell number between the lines. Non-transfected cells and control siRNA transfected cells distributed rather evenly from the starting point to 100 μm and gradually diminished up to 200 μm. In contrast, neural crest cells transfected with *Coactosin* siRNA remained near the tube, and most of the cells stayed within 100 μm (Fig. 5E). The mean migration distance was 89.6 ± 2.86 μm for non-transfected cells and 90.4 ± 3.96 μm for control siRNA-GFP transfected cells in the control culture (4 times of experiments). Conversely, the mean distance was 82.3 ± 2.91 μm for non-transfected cells and 37.1 ± 1.64 μm for *Coactosin* siRNA-GFP transfected cells in the culture for knockdown (4 times of experiments). The former 3 groups can be regarded as the control and the mean migration distance of the control was about 2.5 times that of Coactosin knockdown.

Then, behavior of neural crest cells was visualized in sagittal explant culture (Kasemeier-Kulesa et al., 2005), which well recapitulates *in vivo* state. In this system, neural crest cells migrate through sclerotome and underneath the surface ectoderm as *in vivo* (Fig. 5F). Neural crest cells that were transfected with control siRNA migrated similarly to cells that were labeled only by DiI in the anterior part of the sclerotome (Fig. 5F). The cells transfected with *Coactosin* siRNA moved very slowly, and stayed near the neural tube, while neural crest cells that were labeled only by DiI migrated through the anterior half of the sclerotome (Fig. 5G). The mean migration distance was 55.7 ± 1.82 μm (4 times of experiments) for the neural crest cells transfected with control siRNA and 25.2 ± 0.79 μm (4 times of experiments) for those transfected with *Coactosin* siRNA. Neural crest cells transfected with control siRNA distributed evenly up to 90 μm and diminished gradually, but most cells transfected with *Coactosin* siRNA distributed up to 60 μm from the starting point (Fig. 5H).

Coactosin participates in actin polymerization

Then, we attempted to show if impairment of cell migration by Coactosin knockdown results from disruption of actin polymerization. First, we examined effects on actin polymerization *in vivo* by electroporating actin–GFP expression vector with *Coactosin* siRNA or with control siRNA at stage 10, and fixing embryos at 24 h later. In this series of experiments, we used siRNA expression vector that was deleted of GFP expression construct. Since co-electroporation of 2 types of expression vector transfects identical cells (Momose et al., 1999), actin–GFP expressing cells could be considered as

those transfected with siRNA. In control embryos, actin–GFP expressing neural crest cells migrate through sclerotome (Fig. 6A). In a merged figure of actin–GFP and Rhodamine-Phalloidin, we can see white color in the cytoplasm of neural crest cells (Fig. 6A and C), which indicates that exogenous actin–GFP molecules are integrated into F-actin. *Coactosin* siRNA transfected cells once again showed impairment of migration (Fig. 6D). In these cells, GFP and Rhodamine are segregated (Fig. 6D–F), which indicates that actin–GFP molecules were not integrated into F-actin in the absence of Coactosin. Involvement of Coactosin in actin polymerization was further examined at cellular level *in vitro*. Twelve hours after electroporation of actin–GFP with *Coactosin* siRNA or with control siRNA expression vectors at stage 10, the neural tube was taken out and cultured. Neural crest cells that had migrated out from the tube were dissociated and passed to secondary culture. In the control culture, exogenous actin–GFP spread throughout the cytoplasm, and was incorporated into F-actin in $91.23 \pm 2.47\%$ cells (3 times of experiments, about 30 cells observed in each experiment) (Fig. 6G–I). In the Coactosin-knockdown cells, F-actin arrangements were disrupted, and exogenous actin–GFP was not incorporated into F-actin but it was rather accumulated in the cytoplasm in $70.65 \pm 3.7\%$ cells (4 times of experiments, about 25 cells observed in each experiment) (Fig. 6J–L). In about 30% of cells ($29.35 \pm 3.6\%$), actin–GFP was incorporated into F-actin. The results indicate that Coactosin is actively involved in actin polymerization in living cells. Thus, Coactosin may play a crucial role in neural crest cell migration by mediating actin polymerization.

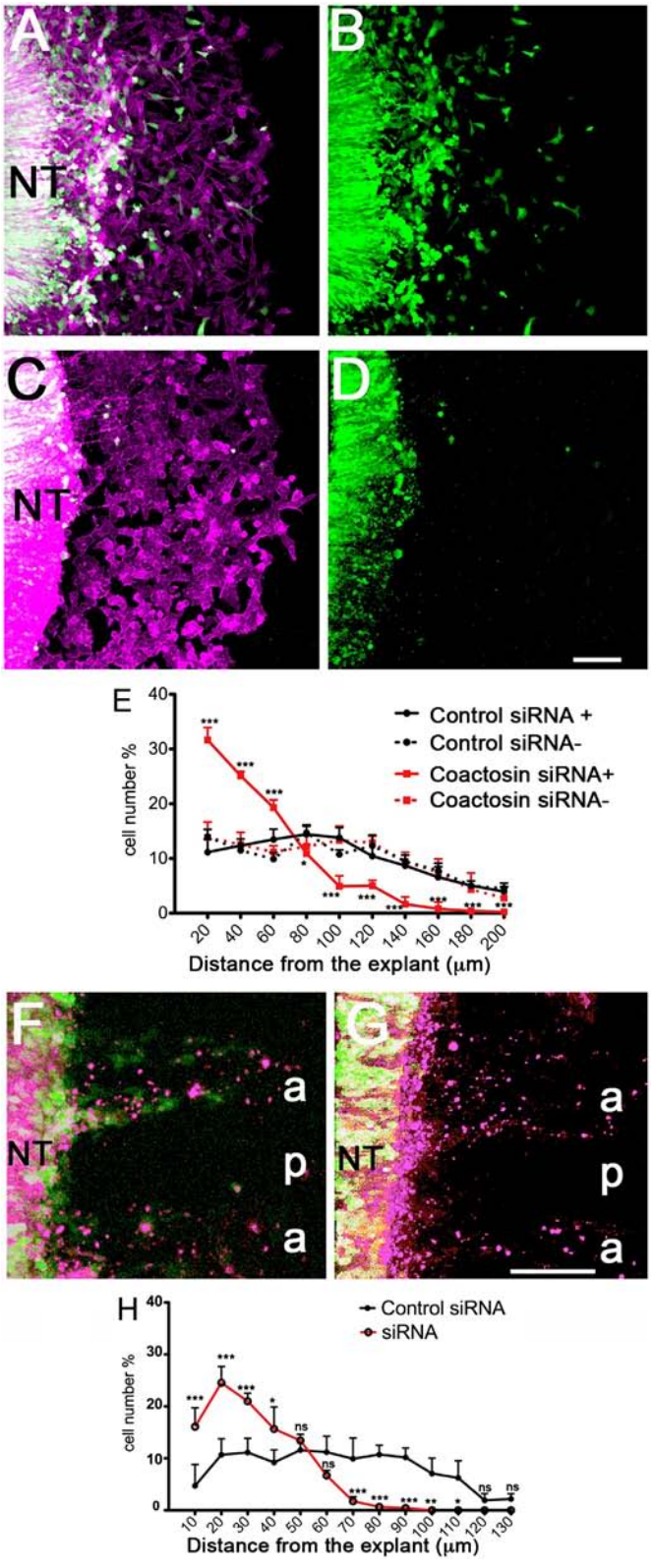


Fig. 5. Impairment of neural crest cell migration by Coactosin knockdown *in vitro*. (A–D) Neural crest cell migration in culture. Electroporation of control siRNA-GFP (A, B), or of *Coactosin* siRNA-GFP expression vector (C, D) Cells are visualized with anti-GFP (green) and HNK1 antibodies (magenta). (E) Quantification of migration in culture. Lines parallel to the edge of the neural tube were drawn on the photos of neural crest cell culture at every 20 μm, and the relative cell numbers between the lines were counted. Mean of cell number and standard error (vertical bars) are shown (versus control+, $^{*}p < 0.05$, $^{***}p < 0.001$, 330 cells for control+, 293 cells for control-, 225 cells for *Coactosin*+, 257 cells for *Coactosin*-, $n=4$, two-way ANOVA). (F, G) Neural crest cell migration in saggital explant culture. Electroporation of control siRNA-GFP (F), or *Coactosin* siRNA-GFP expression vector (H). Neural crest cells were also labeled with DiI (magenta). In saggital explant culture, which well recapitulates *in vivo* system, control siRNA-transfected (green in F) and DiI labeled neural crest cells (magenta), migrate through anterior part of the somite (a). Coactosin-knockdown cells (green in G) stayed within or near the neural tube (NT), while DiI labeled neural crest cells (magenta) migrate through anterior part of the somite (a). p: posterior part of the somite. Scale bar: 40 μm. (H) Distribution of neural crest cells. Neural crest cell migration was impaired by Coactosin knockdown (versus control siRNA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, 269 cells for control, 183 cells for *Coactosin*, $n=4$, *t*-test).

Coactosin functions downstream of Rac signaling

It was reported that Rac activation leads to assembly of a meshwork of actin filaments to protrude lamellipodia (Watanabe, 2010; Lundquist, 2003). We examined if Coactosin is involved in the event under Rac signaling. N1E-115 cells actively protruded lamellipodia and filopodia by transfection of constitutive active Rac1 as shown by Kozma et al. (1997). When constitutive active Rac1 and Coactosin-GFP were transfected to NIE-115 cells, Rac and Coactosin were co-localized to the lamellipodia in $84.3 \pm 4.8\%$ cells (3 indepentent experiments, and about 20 cells were counted in each experiment) (Fig. 7A–C).

It was suggested that SKYSK motif of Coactosin is essential in actin binding, where K75 in the motif is a key molecule (Le Clainche and Carlier, 2008). In order to elucidate if actin–Coactosin association is essential for effectuation of Rac signaling, we prepared Coactosin mutant where K75 was replaced to alanine (Coactosin K75A), which does not bind to actin (Provost et al., 2001). After transfection with Coactosin K75A-GFP to N1E-115 cells, Coactosin K75A was detected by anti-GFP (Fig. 7D–F). Fig. 7D shows that transfected Coactosin K75A stayed in the whole cytoplasm in $82.06 \pm 4.05\%$ cells (4 times of experiments and about 30 cells observed in each experiment), which is contrasted by the localization of wild type Coactosin into the tip of the lamellipodia and filopodia (Fig. 7A–C, Fig. 2). The result indicates that Coactosin accumulation to lemellipodia and filopodia requires binding to actin. Then we analyzed if Coactosin K75A affects neural

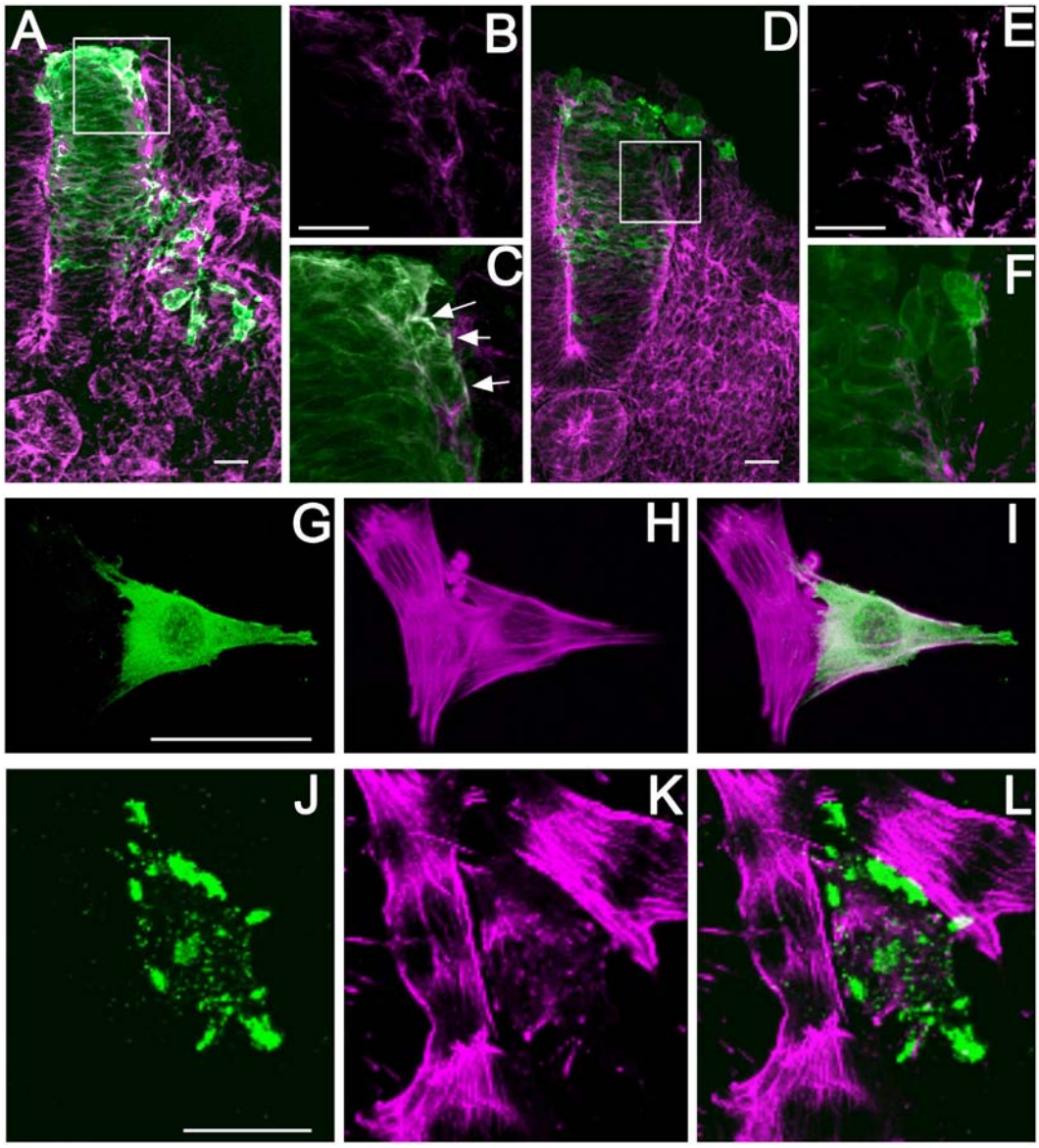
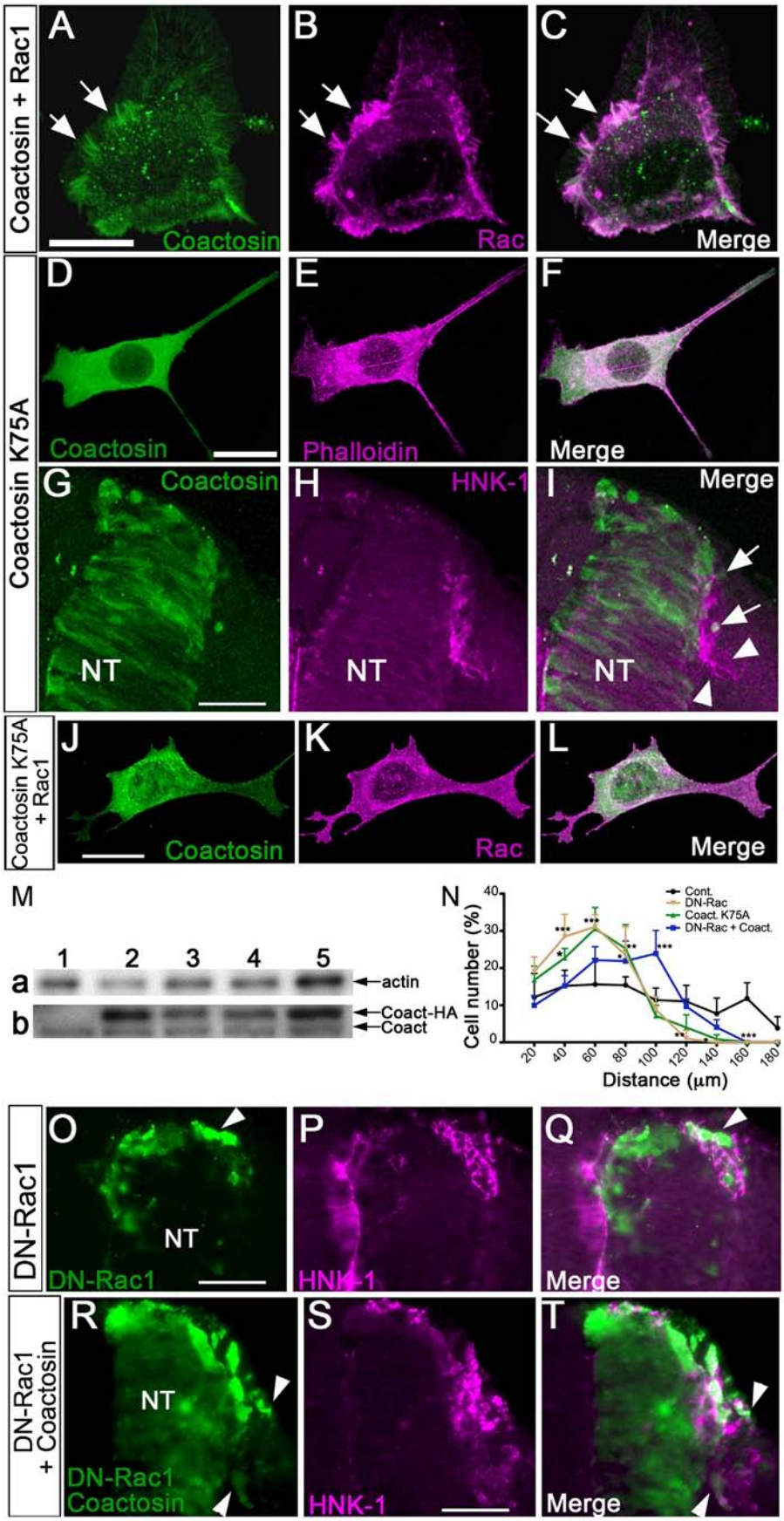


Fig. 6. Coactosin is involved in actin polymerization in the neural crest cells. Distribution of newly synthesized actin molecules with control (A–C, G–I) or *Coactosin* siRNA (D–F, J–L). Green fluorescence indicates actin molecules expressed from the transgene. F-actin is visualized by Rhodamine-Phalloidin (magenta). Incorporation of actin–GFP molecules into F-actin was detected *in vivo* with control siRNA (A–C, arrows) but not with *Coactosin* siRNA (D–F). *In vitro* analysis (G–L) shows that exogenous actin–GFP molecules were integrated into F-actin in the control cells (G–I), but not in Coactosin knockdown cells (J–L), where actin–GFP accumulation was segregated from F-actin, and structure of F-actin was disrupted. Scale bar: 20 μm.

crest cell migration *in vivo*. Transfection of Coactosin K75A interfered with neural crest cell migration as transfection of *Coactosin* siRNA (Fig. 7G–I). Quantification shows that Coactosin K75A transfected cells migrated to 140 μm at 24 h after electroporation (Fig. 7N), and mean migration distance was 38.5 ± 1.75 μm (12 sections from 5 times of experiments).

Fig. 7. Coactosin functions downstream of Rac signaling. (A–C) Recruitment of constitutive active Rac and Coactosin–GFP to lamellipodia. Co-localization of Rac and Coactosin in lamellipodia (arrows) was visualized by anti-Rac (magenta) and anti-Coactosin (green) antibody in N1E-115 cells. Scale bar: 10 μm. (D–F) Distribution of Coactosin K75A-GFP, which cannot bind to actin, in N1E-115 cells. Staining with anti-Coactosin antibody (green) and Rhodamine-conjugated Phalloidin (magenta) shows that mutated Coactosin is seen throughout cytoplasm. Scale bar: 10 μm. (G–I) Disruption of neural crest cell migration by HA tagged Coactosin K75A. Staining with HNK-1 (magenta) and anti-HA (green) antibody shows that migration of Coactosin K75A transfected cells (green) was disrupted (arrows), while non-transfected cells (magenta) reached middle part of the neural tube (NT, arrowheads). Scale bar: 50 μm. (J–L) Distribution of Coactosin K75A-GFP and constitutive active Rac in N1E-115 cells. Staining with anti-Rac (magenta) and anti-Coactosin (green) antibody shows that lamellipodia are few and the cell looks smooth. Scale bar: 10 μm. (M) Immunoprecipitation with anti-Coactosin antibody and immunoblotting with anti-actin (a) or anti-Coactosin (b) antibody. Interference of Coactosin K75A with actin–Coactosin association was examined by electroporation with different doses of Coactosin K75A expression vectors. Lysates from non-transfected neural crest cells (lane 1), from neural crest cells transfected with 5 μg/μl (lane 2), 2 μg/μl (lane 3), 1 μg/μl (lane 4) of Coactosin K75A expression vector, and from neural crest cells transfected with 5 μg/μl of Coactosin expression vector (lane 5) were used for immunoprecipitation. Since transgenes are tagged with HA, they could be distinguished by immunoblotting with anti-Coactosin (b), which shows that endogenous Coactosin is almost the same in all the lanes. (N) Distribution of neural crest cells was quantified. Migration patterns after DN-Rac1 transfection and Coactosin K75A transfection were very similar. Quantification shows that Coactosin partially restored neural crest cell migration in cells that had Rac signaling disrupted (versus control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, 201 cells for DN-Rac1, 188 cells for K75A, 194 cells for Coactosin+DN-Rac1, 260 cells for control GFP, $n = 5$, two-way ANOVA). O-Q: Effect of dominant negative Rac1 (DN-Rac1) on neural crest cell migration. Staning with anti-GFP (green) and HNK-1 antibody (magenta) shows that disruption of Rac signaling interfered with neural crest cell migration (arrowheads). Scale bar: 50 μm. (R–T) Rescue of neural crest cell migration by Coactosin. Staining with anti-HA (green) and HNK-1 (magenta) antibodies reveal that co-eletroporation of HA-tagged Coactosin with DN-Rac1 partially restored neural crest cell migration (arrowheads). Scale bar: 50 μm.


Coactosin + Rac1
A
Coactosin
B
Rac
C
Merge
Coactosin K75A
D
Coactosin
E
Phalloidin
F
Merge
G
Coactosin
NT
H
HNK-1
NT
I
Merge
NT
Coactosin K75A + Rac1
J
Coactosin
K
Rac
L
Merge
M
1 2 3 4 5
a
actin
b
Coact-HA
Coact
N
Cont.
DN-Rac
Coact. K75A
DN-Rac + Coact.
Cell number (%)
Distance (μm)
DN-Rac1
O
NT
DN-Rac1
P
HNK-1
Q
Merge
DN-Rac1 + Coactosin
R
NT
DN-Rac1
Coactosin
S
HNK-1
T
Merge

Then co-transfection of Coactosin K75A and constitutive active Rac1 to N1E-115 cells was carried out. Transfected cells had smooth contour and extended fewer lamellipodia/filopodia in 75.11 ± 8.7% cells (4 times of experiments, and about 20 cells observed in each experiment) (Fig. 7J–L), compared with cells that were transfected with wild type Coactosin and constitutive active Rac1 (Fig. 7A–C). Both mutated Coactosin and activated Rac1 were distributed throughout the cytoplasm. The results indicate that Coactosin K75A worked as dominant negative to the Rac signal, and that Rac signal to extend lamellipodia/filopodia needs actin–Coactosin binding. Thus, binding of Coactosin to F-actin may be prerequisite to mediate actin dynamism.

We further examined interference effect of Coactosin K75A on association of Coactosin and actin *in vivo*. Homogenates of neural crest cells that had been electroporated with different doses of Coactosin K75A expression vectors were immunoprecipitated with anti-Coactosin antibody. Lower panel of Fig. 7M shows that immunoblotting with anti-Coactosin antibody was able to distinguish endogenous Coactosin (all lanes) and exogenous HA-tagged Coactosin (lane 2–4 for K75A or lane 5 for wild type). The amount of endogenous Coactosin was constant, which indicates equal loading of different samples. The amount of exogenous Coactosin well reflected the dose of transfected expression vector. Immunoblotting with anti-actin antibody revealed that Coactosin K75A interfered with Coactosin–actin association in a dose-dependent manner (2–4), while transfected wild type Coactosin enhanced the association (lane 5) (Fig. 7M, upper panel).

Next, we examined behavior of neural crest cells by depriving Rac signaling by co-transfection with dominant negative Rac1 (DN-Rac1) and GFP. Staining with anti-GFP antibody, which represents DN-Rac1 transfection, and HNK-1 antibody indicated that neural crest cells delaminated from the tube, but that they stayed near the tube in all 5 embryos examined (Fig. 7O–Q) as shown by Shoval and Kalcheim (2012). Mean migration distance for DN-Rac1 transfected cells was 34.9 ± 1.29 μm (10 sections from 5 times of experiments) and that for control GFP transfected cells was 61.7 ± 2.46 μm (8 sections, 5 times of experiments) at 24 h after electroporation. The distribution of neural crest cells along the migration route (Fig. 7N) further shows that disruption of Rac signaling impaired neural crest cell migration. Then we carried out co-electroporation of DN-Rac1 and Coactosin expression vectors. The results showed that co-electroporation of DN-Rac1 and Coactosin partially restored neural crest cell migration (Fig. 7R–T, 7N). The mean migration distance was slightly but significantly recovered in co-electroporation with DN-Rac1 and Coactosin (49.5 ± 1.78 μm, 10 sections from 5 times of experiments, versus DN-Rac1, $p = 0.0002$, Mann Whitney test).

Discussion

In the present study, we obtained the following results: (1) Coactosin was associated with Capping protein and with actin filaments, involved in cellular process formation, (2) knock-down of Coactosin resulted in disruption of actin polymerization, and of neural crest cell migration, (3) mutated Coactosin that cannot bind to F-actin interfered with neural crest cell migration, (4) Coactosin was recruited to lamellipodia and filopodia in response to Rac signaling, and (5) Coactosin partially rescued migration of neural crest cells even in disruption of Rac signaling.

In treadmiling, G-actin is added at the barbed end, and dissociated from the pointed end of F-actin, and then the cellular leading process is extended. Capping protein caps barbed ends of actin filaments, and interferes with actin elongation (Le Clainche and Carlier 2008; Cooper and Sept, 2008). It was indicated that Coactosin inhibits actin depolymerization by counteracting capping activity of Capping proteins in cell free system (Rörig et al., 1995). Indeed, we have shown that Coactosin and Capping protein are associated in cultured neural crest cells and in the transfected HEK 293 and N1E-115 cells. Coactosin was also associated with F-actin, and localized in lamellipodia and filopodia, where Coactosin was localized more distally than Drebrin. Accumulation of Coactosin in lamellipodia and filopodia required binding of Coactosin to actin since mutated Coactosin, Coactosin K75A, which does not bind to actin, stayed throughout cytoplasm. These observations suggest that Coactosin plays a crucial role in formation of cellular processes during morphogenesis. Along this line, we carried out gain- and loss-of function experiments of Coactosin in neural crest cells. Overexpression of Coactosin augmented cellular processes and transfected cells extended and retracted filopodia more than the control cells. In contrast, knock-down of Coactosin slowed down cell movement, and almost completely impaired neural crest cell migration. Thus, Coactosin may be involved in actin dynamism and play an important role in cellular process formation, which is indispensable for neural crest cell migration.

Then we assumed if Coactosin actively participates in actin polymerization. In neural crest cells where Coactosin exists, G-actin was incorporated into F-actin both *in vitro* and *in vivo*, but in neural crest cells where Coactosin had been knocked down by siRNA, G-actin was not incorporated into F-actin. Thus, it became clear that Coactosin actively participates in actin polymerization. In cell free system with a condition of low salt condensation, actin polymerization proceeded in the absence of Coactosin (Doucet et al., 2002). Our data demonstrated that Coactosin is rather required for actin polymerization in living cells, where some other molecules are most likely involved in actin–Coactosin association.

For cellular dynamic processes, Rac and Rho signals play crucial roles. Rho induces stress fiber formation and focal adhesion of cells. On the other hand Rac signaling increases membrane ruffling, and induces lamellipodia (Burridge and Wennerberg, 2004). In N1E-115 cells, Rac signal increased lamellipodia and filopodia formation, and augmented neurite outgrowth (Kozma et al., 1997). In the present study, Rac also increased lamellipodia and filopodia, and recruited Coactosin to these processes in N1E-115 cells. Then as shown by Shoval and Kalcheim (2012), Rac signal is necessary for neural crest cell migration. Neural crest cells were stacked and stayed near the neural tube when Rac signaling had been disrupted by DN-Rac1. Coactosin could partially restore neural crest cell migration even in disruption of Rac signaling. It was indicated that intrinsic polarization of Rac signaling in response to environmental signals is further important to determine the direction of cellular process formation, and to guide migrating cells (Kuriyama and Mayor, 2008). Coactosin overexpression augmented cellular processes in neural crest cells, and then drove frequent yet disordered movement in cultured neural crest cells. The results indicate that Coactosin restores process formation but not guidance machinery to direct migration. Thus, neural crest cells, where Rac signaling is disrupted and Coactosin is overexpressed, may migrate randomly. Consequently, the result looks as if neural crest cell migration had been partially restored. Upon Rac signaling, Coactosin may interact with molecule(s) that is/are activated by the signaling, and directed migration of neural crest cells would be executed.

Then we wanted to know if actin–Coactosin association is necessary for transducing Rac signaling. Coactosin binds to actin through K75 in SKYSK motif so that Coactosin K75A, where lysine 75 is replaced with alanine, cannot bind to actin. Abundance of Coactosin K75A interfered with actin–Coactosin association, indicating that it interacts to a molecule that executes actin association at another domain. Importantly, this mutated Coactosin disrupted neural crest cell migration as well as dominant negative Rac1, and further prevented constitutive active Rac1 inducing lamellipodia and filopodia formation in N1E-115 cells. The results indicate that actin–Coactosin association is necessary for execution of Rac signaling. Thus Coactosin K75A may behave as dominant negative against Rac signaling.

The present study revealed that Coactosin functions in relation to effectors of Rac signaling. Upon Rac activation, Coactosin is

recruited to lamellipodia, where Coactosin binds to F-actin and decap its barbed end. Then G-actin may be incorporated into F-actin and actin filaments elongate. On the other hand depolymerization occurs at the pointed end (Le Clainche and Carlier, 2008; Lappalainen et al., 1998). Thus cell movement and process extension can be achieved. Actin–Coactosin association may be critical for this cell dynamism, so that effects of interference with the association looked like that of deprivation of Rac singaling. Taken together, the binding of Coactosin to F-actin may be prerequisite to Rac signal effectuation in cell dynamism.

In conclusion, Coactosin is a very important effecter of Rac signaling to promote actin polymerization, and plays a crucial role in morphogenetic movement such as neurite extension and neural crest cell migration, which is regulated by Rho and Rac signaling (Kashef et al., 2009). Coactosin may actively contribute to neural crest cell migration by executing Rac signaling and by assisting actin polymerization.

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