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ERK5 induces ankrd1 for catecholamine biosynthesis and homeostasis in adrenal medullary cells.

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Abstract

Extracellular signal-regulated kinases (ERKs) play important roles in proliferation, differentiation and gene expression. In our previous study, we demonstrated that both ERK5 and ERK1/2 were responsible for neurite outgrowth and tyrosine hydroxylase (TH) expression in rat pheochromocytoma cells (PC12) (*J Biol Chem* 284, 23564–23573, 2009). However, the functional differences between ERK5 and ERK1/2 signaling in neural differentiation remain unclear. In the present study, we show that ERK5, but not ERK1/2 regulates TH levels in rat sympathetic neurons. Furthermore, microarray analysis performed in PC12 cells using ERK5 and ERK1/2-specific inhibitors, identified ankyrin repeat domain 1 (ankrd1) as an ERK5-dependent and ERK1/2-independent gene. Here, we report a novel role of the ERK5/ankrd1 signaling in regulating TH levels and catecholamine biosynthesis. Ankrd1 mRNA was induced by nerve growth factor in time- and concentration-dependent manners. TH levels were reduced by ankrd1 knockdown with no changes in the mRNA levels, suggesting that ankrd1 was involved in stabilization of TH protein. Interestingly, ubiquitination of TH was enhanced and catecholamine biosynthesis was reduced by ankrd1 knockdown. Finally, we examined the relationship of ERK5 to TH levels in human adrenal pheochromocytomas. Whereas TH levels were correlated with ERK5 levels in normal adrenal medullas, ERK5 was down-regulated and TH was up-regulated in pheochromocytomas, indicating that TH levels are regulated by alternative mechanisms in tumors.

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Taken together, ERK5 signaling is required for catecholamine biosynthesis during neural differentiation, in part to induce ankrd1, and to maintain appropriate TH levels. This pathway is disrupted in pathological conditions.

Keywords

nerve growth factor (NGF); extracellular-signal regulated kinase (ERK); ankyrin repeat domain 1 (ankrd1); tyrosine hydroxylase (TH); sympathetic neurons; pheochromocytoma

1. Introduction

The mitogen-activated protein kinase family includes extracellular signal-regulated kinases (ERKs), ERKs are involved in proliferation, differentiation and gene expression. ERK1/2 are activated by variety of stimuli, and the signaling pathway leading to ERK1/2 activation has been well characterized [1–3]. The ERK5 kinase domain shares approximately 50% of homology with ERK1/2, but its unique long carboxy-terminus encodes two proline-rich regions, a nuclear export domain and a nuclear localization domain [4, 5] and it plays a critical role in activating transcription [6]. The threonine and tyrosine residues on ERK5 are specifically phosphorylated by the upstream kinase, MEK5. ERK5 is also activated by growth factors, neurotrophic factors, cytokines and stressors, but the precise signaling pathways leading to ERK5 activation remain unclear. For example, involvement of small G proteins such as Ras and Rap1 in ERK5 activation remains controversial [7]. Ras-mediated ERK5 activation in rat pheochromocytoma cells (PC12 cells) and Rap1-mediated ERK5 activation in cortical neurons have been reported [8, 9]. In contrast, we have shown neither Ras nor Rap1 was required for ERK5 phosphorylation by nerve growth factor (NGF) or epidermal growth factor (EGF) in PC12 cells [10], whereas these small G-proteins are involved in the NGF-induced sustained ERK1/2 phosphorylation [11, 12].

The ERK5 gene knockout is lethal at E9.5–10.5 due to cardiovascular defects [13]. These defects resulted from abnormal vasculogenesis and angiogenesis in ERK5-lacking endothelial cells [14] rather than development of myocytes. Pathophysiological roles for ERK5 have been proposed for tumor development and cardiac hypertrophy [4, 15]. There were no specific ERK5 inhibitors prior to the development of BIX02189 [16], and transfection efficiency in neuronal cells is low, so the approaches available to clarify the roles of ERK5 in neuronal cells have been limited. Genetic deletion studies showed that ERK5 is necessary and sufficient for neural differentiation of progenitor cells [17], and is critical for adult hippocampal neurogenesis regulating several forms of hippocampus-dependent memory formation [18, 19]. We have shown that ERK5, along with ERK1/2, is essential for neurite outgrowth and expression of the neurotransmitter synthesizing enzyme, tyrosine hydroxylase (TH) in PC12 cells [10]. In rat C6 glioma cells, ERK5 and ERK1/2 were critical factors for gene expression of glial cell-derived neurotrophic factor [20]. Nevertheless, functional differences between ERK5 and ERK1/2, especially during the neural differentiation process remain unclear and requires investigation.

In the present study, we report novel findings that ankyrin repeat domain 1 (ankrd1) is induced by ERK5, independent of ERK1/2, during neural differentiation and this ERK5-

specific signaling molecule regulates TH levels and catecholamine biosynthesis. In addition, ERK5 regulation was observed in normal human adrenal medullas, but was disrupted in adrenal pheochromocytomas.

2. Materials and Methods

2.1. Materials

NGF, basic fibroblast growth factor (bFGF), Hoechst-33258 and antibodies against neurofilament light chain, β-tubulin and β-actin were purchased from Sigma-Aldrich (St. Louis, MO). U0126, antibodies against phospho-ERK1/2, ERK1/2, phospho-ERK5, ERK5, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), flag (DYKDDDDK) and TH and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling (Beverly, MA). This phospho-specific ERK5 antibody also recognizes phospho-ERK1/2. Antibodies against ERK2, ankrd1 and ubiquitin were purchased from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence (ECL) assay kit, protein sepharose G and HRP-conjugated anti-mouse IgG were purchased from GE Healthcare (Buckinghamshire, England). Another ECL kit was purchased from PerkinElmer (Waltham, MA). Lipofectamine 2000, Neon electroporation kit, Alexa488-conjugated anti-rabbit IgG antibody and G418 were purchased from Invitrogen (Grand Island, NY). Non-immune rabbit immunoglobulin was purchased from Dako Japan (Tokyo, Japan). Growth factor-reduced Matrigel matrix was purchased from BD Biosciences (Franklin Lakes, NJ). MG132 was purchased from Peptide Institute (Osaka, Japan). TriPure isolation reagent (for total RNA extraction) was purchased from Roche (Indianapolis, IN). RT-PCR kit was purchased from Toyobo (Osaka, Japan). SYBR Premix Ex Taq (a kit for real-time PCR) was purchased from Takara (Otsu, Japan) or Roche. siRNA for rat ankrd1 was synthesized by B-Bridge (Mountain View, CA), and the cocktail of three duplexes was used, i.e. 1) sense 5'-GUU CAG AAA UGG AGA GUA UTT- 3' and antisense 5'-AUA CUC UCC AUU UCU GAA CTT-3'; 2) sense 5'-GAG CAU GCU UAG AAG GAC ATT-3' and antisense 5'-UGU CCU UCU AAG CAU GCU CTT-3'; and 3) sense 5'- GAA UGG AAC CAA AGC GAU ATT-3' and antisense 5'-UAU CGC UUU GGU UCC AUU CTT-3'. Rat ERK5 shRNA in pBAsi-mU6 vector (target Sequence 5'-CCA GCA ACT GTC CAA GTC T-3') was created by Takara (Otsu, Japan). siRNA for rat ERK5 was synthesized by Invitrogen (sense 5'-CCG CGA UCU UAA ACC CUC UAA CCU U-3' and antisense 5'-AAG GUU AGA GGG UUU AAG AUC GCG G-3'). DNA plasmid encoding flag-ankrd1 was kindly provided by Dr. Francesco Acquati (University of Insubria, Italy). BIX02189 was kindly provided by Boehringer Ingelheim (Ridgefield, CT).

2.2. Cell culture

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Cell Culture Laboratory, Cleveland, OH), 5% horse serum (Invitrogen), penicillin (50 units/ml), and streptomycin (50 μg/ml) in an incubator containing 5% CO_2 at 37°C. PC12 cells that stably overexpress ERK5 shRNA or flag-ankrd1 were cultured in the presence of G418. Sympathetic neurons were dissociated from the superior cervical ganglia (SCG) of newborn rat pups as described [21, 22]. Both sexes of rat pups were used without regard to sex. Neurons were plated onto plates coated

with poly-L-lysine and mouse type IV collagen. Cultures were maintained at 37 °C in 5% CO_2. Neurons were cultured in C2 media (DMEM/F12 1:1, BSA 0.5 mg/ml, l-glutamate 1.4 mM, selenium 30 nM, transferrin 10 μg/ml, insulin 10 μg/ml) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 50 ng/ml NGF, and 3% fetal bovine serum. Neurons were cultured with the anti-mitotic agent AraC (1 μM) for two days to deplete non-neuronal cells. Half the media was changed every two days, including new NGF. For transfection of sympathetic neurons, SCG were dissected from P3 Sprague Dawley rat pups. We used the Neon™ Transfection system and followed the manufacturer's instructions. Briefly, neurons were electroporated with specific parameters regarding pulse voltage (1500 V), pulse width (20 ms), and pulse number (1). Neurons were plated onto poly-L-lysine/collagen treated plates and grown in C2 media supplemented with NGF (50 ng/ml) and 3% fetal bovine serum. Neurons were grown in culture for 4 days before lysates were isolated and Western blotting was performed.

2.3. Assay for neurite outgrowth

The neurite extension from PC12 cells was regarded as an index of neuronal differentiation. The cells were fixed with 4% paraformaldehyde and the nuclei were stained with Hoechst-33258. The photographs were taken with CELAVIEW-RS100 (Olympus, Tokyo, Japan). The number of nuclei and total length of neurites were calculated with the CELAVIEW software (Olympus, Tokyo, Japan), then the value of total neurite length divided with nucleus number was expressed as a neurite length per cell (μm/cell). Data are expressed as means ± S.E.M. of the values of three wells [10]. To test the role of ERK5 in axon outgrowth, we used explants of SCG, which contain abundant sympathetic neurons. Ganglia were desheathed to facilitate axon outgrowth, embedded in reduced growth factor Matrigel matrix, and covered with serum-free DMEM/F12 with penicillin-streptomycin and 2 ng/ml NGF. Explants were maintained at 37°C with 5% CO_2. A day after being plated, explants were photographed and incubated with or without U0126 and BIX02189, and then photographed again 6 h later. Axon length was measured using Image-J (Version 1.36b, National Institute of Health), and the rate of axon growth per hour was calculated as reported [23].

2.4. Microarray analysis

Total RNA from PC12 cells stimulated with NGF (100 ng/ml, 4 h) in the presence or absence of U0126 (30 μM) or BIX02189 (30 μM) was extracted using TriPure isolation reagent. Microarray slides (whole rat genome oligo microarray 4 × 44 k, Agilent Technology) were used for the transcript analysis. RNA labeling (Quick RNA amplification and labeling kit, Agilent Technology) and microarray hybridization (17 h at 65°C) were carried out according to the supplied manual. The microarrays were washed after hybridization according to the Agilent manual and air-dried arrays were scanned with a 5 μm resolution in a high resolution Agilent microarray scanner. The scanned images were analyzed using GeneSpring GX7.3 (Agilent Technologies). Signal intensities for each probe were normalized to the 75th percentile. Genes that were up-regulated by more than three times by NGF were defined as NGF-stimulated genes. Among them, genes whose expression was inhibited by more than 50% by U0126 and BIX02189 were defined as ERK1/2-dependent genes and ERK5-dependent genes, respectively.

2.5. SDS-polyacrylamide gel electrophoresis and Western blotting

The electrophoresis was performed on 8–11% acrylamide gels. Proteins were transferred electrically from the gel onto polyvinylidene difluoride membrane (GE Healthcare) by the semi-dry blotting method. The blots were blocked for 0.5–1 h with 5% low fat milk in Tris-buffered saline containing 0.1% tween-20 (TBST) at room temperature, and incubated with primary antibodies overnight at 4°C. The blots were washed several times and incubated with HRP-conjugated anti-rabbit or anti–mouse IgG antibody as a secondary antibody in TBST containing 5% low fat milk at room temperature for 2 h. After rinsing with TBST, blots were developed using a chemiluminescence assay kit, and visualized by exposing the chemiluminescence from the membrane to the Hyper-film ECL or with ChemiDocXRS (BioRad, Hercules, CA, USA). The densities of the bands corresponding to TH and GAPDH as an internal control were analyzed by densitometry using Image-J software.

2.6. Semi-quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from PC12 cells or sympathetic neurons was extracted using TriPure isolation reagent. Samples were reverse transcribed using an RT-PCR kit and real-time PCR was carried out using SYBR Premix Ex Taq and a thermal cycler (Opticon; MJ Research, Waltham, MA or Light cycler nano; Roche). The PCR primers used for experiments in PC12 cells were as follows: ankrd1 (5’-CCC TAT GGA TCT GGT GTT GC −3’ and 5’-CTA ACC CAG GGT CCT TCC AT-3’), GAPDH (5’-ACC ACA GTC CAT GCC ATC AC −3’ and 5’-TCC ACC ACC CTG TTG CTG TA-3’), TH (5’-GCT ACC GAG AGG ACA GCA TC-3’ and 5’-GCA CCA TAA GCC TTC AGC TC −3’), β-actin (5’-AGG GAA ATC GTG CGT GAC AT-3’ and 5’-TCC TGC TTG CTG ATC CAC AT-3’), neurofilament light chain (5’-TCA CCA GCG TGG GTA GCA TAA C-3’ and 5’-TGG GGC TCA ATC TTT CTT CTT AGC-3’). The PCR primers used for human tissue experiments were as follows: TH (5’-ACT GGT TCA CGG TGG AGT TC-3’ and 5’-AGC TCC TGA GCT TGT CCT TG-3’), ERK5 (5’-TTT GCC TTA CTT CCC ACC TG-3’ and 5’-CCC ATG TCG AAA GAC TGG TT-3’), ankrd1 (5’-GAT CGA ATT CCG TGA TAT GCT-3’ and 5’-AAA CAT CCA GGT TTC CTC CA-3’). For human ankrd1 analysis, Taqman Probe method was carried out using the probe (5’-CTG GGC AA-3’) conjugated with FAM and the quencher (UPL#61, Roche). The amount of each PCR product was normalized to that of GAPDH, and expressed as a fold change relative to the control.

2.7. Immunofluorescence

PC12 cells transfected with flag-ankrd1 were fixed with 4% paraformaldehyde and stained with anti-flag primary antibody (1:500 dilution) and Alexa 488-conjugated anti-rat IgG secondary antibody (1:200 dilution). The nuclei were also stained with Hoechst-33258 (1 μg/ml). Then, cells were observed with a fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan) [24].

2.8. Subcellular fractionation

After cells were incubated with or without NGF for a day, cells were homogenized in buffer A (10 mM Hepes, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1.5 mM Na_3VO_4, pH 7.3). A portion of these homogenates was

dissolved in Laemmli buffer (final concentration, 75 mM Tris-HCl, 2% SDS, 15% glycerol, 3% 2-mercaptoethanol, pH 6.8) and regarded as total cell lysate and the remainder was centrifuged at 800 × g at 4°C for 10 min. The resulting pellets (P1) were dissolved in Laemmli buffer and regarded as a nuclear fraction. The supernatant (S1) was centrifuged at 108,800 × g at 4°C for 30 min. The supernatant (S2) was dissolved in Laemmli buffer and regarded as the cytosolic fraction. The pellet (P2) was dissolved in buffer B (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% 2-mercaptoethanol, 10 mM glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 50 mM NaF, 5 mM Na_2HPO_4, pH 7.5), and was centrifuged at 108,800 × g at 4°C for 30 min, again. The resulting supernatant (S3) was dissolved in Laemmli buffer and regarded as crude membrane fraction. Fractions dissolved in Laemmli buffer were boiled at 95°C for 5 min.

2.9. Immunoprecipitation and detection of ubiquitinated TH

Briefly, PC12 cells were transfected with control siRNA or ankrd1 siRNA and cultured in the presence or absence of NGF. After the cells were incubated with a proteasome inhibitor, MG132 (1 μM) for 6 h, the cells were lysed in RIPA buffer with protease inhibitors (1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 125 mM NaCl, 50 mM Tris, 10% glycerol, 1 mM EDTA, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml antipain, 10 μg/ml aprotinin, 2 μg/ml Na_3VO_4, pH 8.0). Then, after sonicating the lysates, total cell lysate (750 μg) was mixed with anti-TH antibody (1:100 dilution) and protein sepharose beads G. After immunoprecipitation at 4°C for overnight, the immunoprecipitates were washed and eluted by the addition of Laemmli buffer.

2.10. Spectrophotometric measurement of total catecholamine content

Total catecholamines contained in the cells in 60 mm plates were extracted with 0.4 M perchloric acid and adsorbed on aluminum hydroxide. Their amounts were estimated by the ethylenediamine condensation method [25, 26], using a fluorescence spectrophotometer (FP-6500, JASCO, Tokyo, Japan) at an excitation wavelength of 425 nm and an emission wavelength of 540 nm. Dopamine was used as a standard.

2.11. Catecholamine determinations by LC-MS/MS

Sample vials, sodium hydroxide and silanized inserts were obtained from Sun Sri (a subdivision of Fisher Scientific, Rockwood, TN). Boric acid, 3,4-dihydroxybenzylamine hydrobromide (DHBA) dopamine (DA), norepinephrine (NE), and ammonium acetate were from Sigma-Aldrich (St. Louis, MO). Dansyl chloride (DNS) was from Fluka (Buchs, Switzerland). Acetonitrile (ACN) and water were purchased from Burdick and Jackson (Muskegon MI). Formic acid was from J.T. Baker (Phillipsburg, NJ). Siliconized (pre-lubricated) tubes were purchased from Corning Costar (Corning, NY). Gemini-NX 100×2.00 mm C18 column was purchased from Phenomenex (Torrance, CA). 20 mm Javelin βetabasic C18 pre-columns were purchased from ThermoFisher Scientific (Waltham, MA)

The analysis of neurotransmitters was determined by adapting the methods described by Cai et al [27, 28]. Concentrated stocks of neurotransmitters and internal standards were prepared at 1 mg/ml in 0.1 M perchloric acid in siliconized tubes. DHBA internal standard was

prepared in 0.1M perchloric acid at 10 ng/5μl. Dansyl chloride derivatization reagent was prepared at 3.5 mg/ml in acetonitrile on the day of the analysis. Samples containing 3×10^6 cells were prepared and resuspended in 350 μl as described [29]. The derivatized samples were analyzed by LC-MS/MS.

Neurotransmitters were analyzed using a 4000 QTRAP hybrid/triple quadrupole linear ion trap mass spectrometer (ABSciex) with electrospray ionization (ESI) in positive mode. The mass spectrometer was interfaced to a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler followed by 2 LC-20AD XR LC pumps and analysis on an Applied Biosystems/SCIEX Q4000 instrument (Foster City, CA). Data were acquired using Analyst 1.6.2 software and analyzed using Multiquant 3.0.1 software. The LOQ for the compounds was 1 ng/ml for NE, and 5 ng/ml for DA where the relative standard deviation was less than 20%.

2.12. Human tissue samples of adrenal pheochromocytomas and normal adrenal medullas

Fresh frozen tumor specimens resected surgically from eleven patients with pathologically confirmed adrenal pheochromocytoma in Yamagata University Hospital from 2000 to 2014 were included in the present study. Fresh normal adrenal tissues were also obtained from seven patients who received radical nephrectomy for treatment of renal cell carcinoma in the institution during the period [30]. We confirmed pathologically that these adrenal glands were without any permeation or metastasis. Adrenal medullas were carefully separated from the cortex by the surgeon. All fresh samples were used for semi-quantitative RT-PCR and Western blotting analysis. Paraffin-embedded formalin-fixed specimens used for routine pathological examination were collected and analyzed by immunohistochemistry. Tissue samples of normal adrenal glands were compared to those of pheochromocytomas as controls. Clinical demographics of the patients for the study were collected from medical archives, and were presented in Table 1. For RT-PCR analysis, tissue samples in TriPure Isolation Reagent were sonicated and total RNA was isolated as described above. For Western blotting, tissue samples in RIPA buffer were sonicated. The insoluble pellets were discarded and the protein concentration was measured using DC protein assay reagents (BioRad), followed by Western blotting. The present study was carried out in accordance with a protocol approved by the Ethical Committee of Yamagata University School of Medicine (approval No. H25–98). Informed consent was obtained in written forms with patient's signatures.

2.13. Immunohistochemical staining

Antibodies against ERK5 (1:200 dilution), ankrd1 (1:100 dilution) and TH (1:500 dilution) were used for immunohistochemistry. The staining was carried out with standard procedure as described elsewhere [31, 32]. Briefly, tissue sections (3 μm) were deparaffinized in xylene, rehydrated, and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol in for 15 min. After heat-induced antigen retrieval with an autoclave (120°C, 10 min), anti-ERK5, ankrd1 and TH antibodies were incubated overnight with sections. Detection of primary antibodies was performed by peroxidase method using Histofine simple stain MAX-PO MULTY (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine in the presence of H_2O_2. Following immunohistochemical staining, the sections were

counterstained with hematoxylin for 30 sec to enhance nuclear detection. Slides were dehydrated and mounted in permanent histological specimens. The primary antibodies were substituted with non-immune rabbit immunoglobulin for negative controls of ERK5 and ankrd1 staining. The negative control reactions demonstrated a complete absence of specific reactions in all cases (data not shown). Paraffin-embedded formalin-fixed sections of human kidney and cardiac muscle were used as positive controls for ERK5 and ankrd1, respectively. The kidney and heart tissues were positively stained in cytoplasm, but not in the nuclei, of renal mesangial cells and ventricular myocytes, respectively (data not shown). Cellular accumulation of ERK5 and ankrd1 was defined as positive cytoplasmic staining of more than 50% of tumor and adrenal medullary cells despite their staining intensity.

2.14. Statistics

Data were expressed as the mean values ± S.E.M, and significant differences were analyzed using Student's t-test or Tukey's and Dunnett's methods for multiple comparison.

3. Results

3.1. ERK5 regulates TH protein in PC12 cells and rat sympathetic neurons

Previously, we have shown that NGF promotes both ERK5 and ERK1/2 phosphorylation in PC12 cells and ERK5 is responsible for neurite outgrowth and TH expression along with ERK1/2 [10]. In that study, the role of ERK5 was examined using dominant-negative mutants of ERK5 or MEK5, or novel pharmacological inhibitors, BIX02188 or BIX02189. In the present study, we generated PC12 cells which stably expressed ERK5 shRNA. In response to NGF (100 ng/ml, 5 min), both ERK5 and ERK1/2 were phosphorylated in PC12 cells transfected with empty vector. However, only ERK1/2 phosphorylation was induced by NGF in PC12 cells where ERK5 expression was knocked-down by ERK5 shRNA (Fig. 1A). When these cells were cultured in the presence or absence of NGF (100 ng/ml) for a day, TH levels were selectively abolished by ERK5 knockdown in both basal and NGF-stimulated states, whereas other neuronal marker/cytoskeletal protein levels including neurofilament light chain, β-tubulin, β-actin and GAPDH were not affected at all by ERK5 knockdown (Fig. 1B). We could observe the same effect in another ERK5-shRNA cell clone (data not shown). In addition, TH levels were down-regulated by another ERK5 siRNA (Fig. 1C), minimizing the possibility of off-target effects accounting for these results. The loss of TH by ERK5 shRNA was greater than that by the biochemical interfering mutants and pharmacological inhibitors as observed in a previous study [10], suggesting that constitutive ERK5 knockdown can cause a more dramatic effect. Taken these results together, ERK5 is required for TH levels in PC12 cells.

PC12 cells are a model of immature neurons [33], but it remains unclear whether the role of ERK5 observed in PC12 cells is similar in sympathetic neurons. Therefore, we next examined the role of ERK5 in sympathetic neurons, which express TrkA and p75 NGF receptors. First, SCG explants were incubated with NGF in the presence or absence of U0126 (20 μM) or BIX02189 (3, 30 μM). NGF alone (2 ng/ml) promoted axon elongation at approximately 30 μm/h, and BIX02189 completely blocked axon elongation whereas U0126 did not in our condition (Figs. 2A and 2B). Next, we examined the involvement of ERK5 in

regulating TH levels in dissociated sympathetic neurons. Four days after ERK5 shRNA was transfected into the sympathetic neurons, TH levels were reduced significantly (Fig. 2C). There was no change in TH levels in neurons incubated with U0126 (20 μM) for 1 or 3 days, although phosphorylation of ERK1/2 was inhibited (Fig. 2D), which is consistent with our previous data. These results suggest that ERK5 plays a critical role in axon elongation and regulating TH levels in cultured sympathetic neurons.

3.2. Ankrd1 gene expression was induced by ERK5, but not ERK1/2 in PC12 cells

Next, we attempted to investigate the functional difference between ERK5 and ERK1/2 during neural differentiation by microarray analysis. PC12 cells were stimulated with NGF (100 ng/ml) for 4 h in the presence or absence of U0126 (30 μM) or BIX02189 (30 μM) at the time point when growing neurites become visible in response to NGF. 374 genes were induced 3-fold by NGF (i.e. NGF-stimulated genes). Expression of 232 NGF-stimulated genes (62.0%) was attenuated by both U0126 and BIX02189 (i.e. ERK1/2 and ERK5-dependent genes), expression of 49 genes (13.1%) genes was inhibited by U0126 only (i.e. ERK1/2-dependent genes), and induction of 46 genes (12.3%) was blocked by BIX02189 only (i.e. ERK5-dependent genes) (Fig. 3A). We also checked the selectivity of BIX02189 and U0126 at the same time when the samples for microarray were prepared. NGF (100 ng/ml, 5 min) promoted phosphorylation of both ERK5 and ERK1/2, which was selectively blocked by BIX02189 (30 μM) and U0126 (30 μM), respectively (Fig. 3B). Examples of the ERK5-dependent and ERK1/2-independent (BIX02189-sensitive and U0126-insensitive) genes were shown in Fig. 3C. Gene expression patterns of ankrd1 and hairy and enhancer of split-1 (hes1) were ERK5-dependent and ERK1/2-independent (BIX02189-sensitive and U0126-insensitive) genes. Neurofilament light chain was an example of ERK1/2- and ERK5-dependent gene. GAPDH was an NGF-independent gene and constant regardless of NGF stimulation or inhibition by U0126 or BIX02189.

Although ankrd1 is abundantly expressed in heart and its role as a transcription modulator, stretch sensor and apoptosis suppressor has been suggested [34], its role in neural cells is poorly understood. Thus, we focused on ankrd1 as an ERK5-selective signaling molecule in the present study. The structure of ankrd1 is shown in Fig. 4A. Four ankyrin repeats are encoded by its carboxy-terminal half and a coiled-coil domain is encoded by its amino-terminal half, suggesting ankrd1 can interact with other proteins through these domains. In addition, a nuclear localizing signal (NLS) and a nuclear export signal (NES) exist in ankrd1, which enable ankrd1 to translocate between the cytosol and the nucleus [34]. Because the microarray suggested that NGF-stimulated ankrd1 gene expression is ERK5-dependent, we attempted to confirm that by RT-PCR. PC12 cells were stimulated with NGF (100 ng/ml) for 0.5–6 h or NGF (1–100 ng/ml) for 2 h, then ankrd1 gene expression was examined. NGF induced ankrd1 gene expression in a time- and concentration-dependent manner (Fig. 4B). Ankrd1 mRNA was rapidly induced in response to NGF (0.5–2 h) and then gradually returned to control levels after 2 h. Endogenous ankrd1 protein levels were elevated 4 h after NGF stimulation (Fig. 4C), and remained high for 12 hr. PC12 cells were stimulated with NGF (100 ng/ml) for 2 h in the presence or absence of U0126 (30 μM) and BIX02189 (30 μM). Consistent with the microarray results, the increase in ankrd1 mRNA was blocked by inhibiting ERK5, but not ERK1/2 (Fig. 4D). β-Actin mRNA was also

ERK5-dependent and ERK1/2-independent. Neurofilament light chain mRNA was induced in an ERK5 and ERK1/2-dependent manner. NGF induction of TH mRNA was blocked significantly by BIX02189, but U0126 also showed tendency to inhibit the expression although it was not significant in our condition. NGF (100 ng/ml, 5 min) stimulated the phosphorylation of ERK5 and ERK1/2, which was blocked by BIX02189 (30 μM) and U0126 (30 μM), respectively (Fig. 4E), confirming the selectivity of these inhibitors. bFGF stimulates GDNF gene expression via ERK5 activation in C6 glioma cells [20], so we also treated cells with bFGF. bFGF (100 ng/ml, 5 min) stimulated phosphorylation of ERK1/2 but not ERK5 in PC12 cells (Fig. 4E), consistent with the observation that bFGF did not stimulate expression of ankrd1 mRNA (Fig. 4D). Ankrd1 intracellular localization was examined by immunostaining and fractionating into nuclear, membrane, and cytosolic fractions. Flag-ankrd1 localized in nucleus abundantly, but was also seen in cytosol and membrane fractions in undifferentiated and differentiated PC12 cells (data not shown).

3.3. Ankrd1 regulation of TH levels and catecholamine biosynthesis in PC12 cells

Previously, we demonstrated ERK5 is involved in stabilization of TH protein [10]. In the present study, we show that ERK5 is also required for NGF induction of TH mRNA (Fig. 4D), indicating ERK5 regulates TH levels by stimulating TH gene expression as well as stabilizing the protein. To investigate whether ankrd1 is involved in regulation of TH, ankrd1 was knocked-down by siRNA, and then TH levels were examined in PC12 cells (Fig. 5). Flag-ankrd1 expression was abolished by cocktail of three siRNA duplexes (Fig. 5A). In this condition, ankrd1 siRNA decreased basal TH levels (by 30.3%) and NGF (100 ng/ml, a day)-stimulated TH (decreased by 61.7%). In addition, two individual siRNAs for ankrd1 also reduced TH levels (Fig. 5B). However, TH protein levels in PC12 cells stably overexpressing flag-ankrd1 were comparable to that in control cells (Fig. 5C). These results suggest ankrd1 is necessary but not limiting for TH levels in PC12 cells. Transfection of PC12 cells with ankrd1 siRNA blocked NGF induction of ankrd1 mRNA, but had no effect on basal or NGF (100 ng/ml, 2 and 4 h)-stimulated TH mRNA (Fig. 5D). This suggests that ankrd1 alters TH protein stabilization rather than gene expression. Ankrd1 is induced by nerve injury, and involved in neurite outgrowth of dorsal root ganglion sensory neurons [35]. However, NGF (100 ng/ml)-induced neurite outgrowth was not significantly altered by ankrd1 siRNA or overexpression of flag-anlkrd1 in PC12 cells (supplemental Fig. 1), suggesting ankrd1 is not involved in neurite outgrowth in PC12 cells.

Because ERK5 regulates TH stability and ankrd1 regulates TH protein but not mRNA levels (Figs. 5A, 5B and 5D), we hypothesized that ERK5 stabilizes TH via ankrd1. TH is degraded through the ubiquitin-proteasome pathway [21]. PC12 cells were transfected with ankrd1 siRNA, then incubated with NGF (100 ng/ml) for 24 h. Six hours before harvesting the cells, proteasome inhibitor, MG132 (1 μM) was added to cultures. After immunoprecipitation of TH, ubiquitinated TH protein was examined by Western blotting. Ubiquitination of TH was promoted in ankrd1-siRNA treated cells (Fig. 5E), suggesting that ankrd1 blocks ubiquitination of TH protein. Furthermore, ankrd1 knockdown did not decrease TH protein levels when proteasome activity was blocked by MG132 (Fig. 5E).

Our data show that ERK5 and ankrd1 regulate TH levels in PC12 cells, suggesting they also alter catecholamine biosynthesis. Total catecholamine content including dopamine, norepinephrine and epinephrine was measured by the ethylenediamine condensation method in untreated and NGF (100 ng/ml)-treated PC12 cells. NGF stimulated catecholamine biosynthesis in PC12 cells, and ERK5 knockdown decreased catecholamine biosynthesis (Fig. 6A), which is consistent with the low TH protein levels (Fig. 1B). Furthermore, ankrd1 knockdown with siRNA prevented the NGF (100 ng/ml)-induced increase in catecholamine levels (Fig. 6B). We also measured dopamine and norepinephrine levels in PC12 cells by LC-MS/MS method. NGF stimulated dopamine biosynthesis compared with drug-free control, and ankrd1 knockdown prevented the NGF-induced increase in dopamine compared to control siRNA with NGF. For norepinephrine, ankrd1 knockdown prevented the norepinephrine levels compared to control siRNA with or without NGF (Fig. 6C). These results suggest both ERK5 and ankrd1 regulate TH levels and catecholamine biosynthesis.

3.4. Correlation between ERK5 signaling and TH levels is disrupted in human pheochromocytoma cells

We demonstrated that ERK5 signaling was required for TH levels in PC12 cells and sympathetic neurons. Adrenal pheochromocytoma is a cancer of adrenal medulla where TH is expressed abundantly and catecholamines are produced at high levels [30, 36]. Hence, the pheochromocytoma cells secrete extraordinary amount of catecholamines, which causes serious symptoms including hypertension, hypermetabolism, hyperglycemia, headache, and hyperhidrosis. Because it has been demonstrated that TH is increased in adrenal pheochromocytomas, we sought to examine whether ERK5 was overexpressed and might contribute to the up-regulation of TH in this disease.

Microscopically on conventional haematoxylin and eosin-stained sections, the normal adrenal glands and pheochromocytomas had typical histopathological features in all of the patients (n=7 and 11, respectively; upper left panels in Figs. 7A and 7B). Both normal medullas and tumors were immunostained strongly with antibody against TH (upper right panels in Figs 7A and 7B). The normal adrenal medulla chromaffin cells showed weak and uniform cytoplasmic immunohistochemical positivity for ERK5 and ankrd1 in most instances with normal adrenals (n = 6). The normal adrenal cortex stained a little more strongly with ERK5 and ankrd1 antibodies than normal medulla (lower panels in Fig. 7A). Positive nuclear expression of ERK5 was seen in some of the medulla (n = 4), but ankrd1 immunoreactivity was rarely observed in the nuclei of the cells (Fig. 7A, insets). No medullary cells exhibited moderate or strong immunointensity for ERK5 and ankrd1 in these studies. Distinct staining patterns were seen in adrenal pheochromocytomas. ERK5 (n = 9) and ankrd1 (n = 11) were positively stained, the tumor cells generally showed weak immunopositivity in cytoplasm with the nuclei stained variably for ERK5 and rarely for ankrd1 (lower panels in Figs. 7B, and 7C). Some nuclei in the tumor cells were positive for ERK5 immunostaining (n = 5), but others were negative (n = 6), as observed in normal adrenal medullas. In three of the cases with pheochromocytomas, there were some tumor cells that were located sparsely in tumor masses and had strong immunostaining for ERK5 and ankrd1 proteins in cytosol (Fig. 7B, insets). Positive but weak immunostaining to both proteins was also seen in the other eight instances (Fig. 7C, insets).

Next, we examined whether ERK5 and ankrd1 mRNA levels were enhanced in pheochromocytomas, using RT-PCR. Although TH mRNA was significantly elevated in pheochromocytomas as reported previously [30, 36], ERK5 mRNA levels were unexpectedly decreased in pheochromocytomas. Ankrd1 mRNA levels were similar between normal adrenal medullas and pheochromocytomas (Fig. 8A). We also investigated the protein levels of TH and ERK5 by Western blotting. Surprisingly, the protein levels of TH and ERK5 varied widely within individual samples. Densitometric analysis revealed that TH was significantly up-regulated and ERK5 was significantly down-regulated in pheochromocytomas (Fig. 8B), which is consistent with their mRNA levels (Fig. 8A). Furthermore, we noticed that TH levels were relatively high in the samples that contained high levels of ERK5 in normal adrenal medullas and low levels of ERK5 is associated with low TH levels, we therefore examined the relationship between TH and ERK5 levels in normal adrenal medullas and pheochromocytomas. As expected, ERK5 protein levels correlated with TH levels in normal adrenal medullas (R=0.792, $p < 0.05$), but no significant correlation was observed in pheochromocytomas (R=0.422, p=0.196) (Fig. 8B). This suggests that ERK5 plays a role in maintaining appropriate TH levels in normal adrenal medulla, but that ERK5 regulation of TH is disrupted in pheochromocytomas. Thus, other factors must contribute to the high levels of TH and catecholamines in pheochromocytoma.

4. Discussion

In the present study, we have demonstrated that ERK5 regulates TH levels in both PC12 cells and primary cultured sympathetic neurons. Furthermore, we first showed that ankrd1 induced by ERK5 but not ERK1/2 is involved in TH protein expression and catecholamine biosynthesis (Fig. 9). This ERK5-specific ankrd1 signaling is one of the functional differences from ERK1/2. Furthermore, the correlation between ERK5 and TH levels was observed in normal adrenal medullas, whereas this correlation was absent in adrenal pheochromocytomas.

We have previously showed that TH protein expression is reduced by dominant-negative mutants of ERK5 and MEK5 or a pharmacological inhibitor, BIX02189 [10]. In the present study, we used PC12 cells clones that stably express ERK5 shRNA to constitutively lower ERK5 protein levels. Using these cells, we could observe a more efficient reduction of TH levels and catecholamine biosynthesis in these cells (Figs. 1B and 6A). It has been shown that the carboxy-terminus of ERK5 has an independent role in activation of transcription factors [6], hence we presume that complete loss of ERK5 by ERK5 knockdown more efficiently blocks ERK5 function than does either the interfering mutant or the pharmacological inhibitor. Furthermore, we speculated that down-regulation of TH by ERK5 inhibition results from the reduced gene expression (Fig. 4D) and destabilization of TH [10]. It has been shown that ERK5 is activated by NGF in primary cultured sympathetic neurons [37] and is required for the survival response to NGF [38]. In the present study, we also examined TH levels in sympathetic neurons in addition to PC12 cells, We showed significant loss of TH protein by ERK5 shRNA in these neurons (Fig. 2C), suggesting the role of ERK5 in TH expression is not limited to PC12 cells, but rather is universal to TH-expressing cells/tissues. The rat TH promoter contains binding sites of transcription factors such as activator protein-1, cAMP response element binding protein, Sp1 and early growth

response 1 [39], and it has been shown that these transcription factors are activated by ERK5 directly or indirectly [6, 20, 40, 41]. Thus, these transcription factors may mediate TH gene expression downstream of ERK5.

In our previous study, we observed dominant negative mutants of ERK5 and MEK5 and its pharmacological inhibitor attenuated the NGF-induced neurite outgrowth in PC12 cells [10]. In addition to PC12 cells, axon elongation from sympathetic ganglia was largely blocked by ERK5 inhibition, whereas the MEK inhibitor U0126 had no effect (Figs. 2A and 2B). This was surprising because U0126 caused significant inhibitory effect on neurite outgrowth in PC12 cells [10] and dissociated sympathetic neurons [42]. One of the differences in experimental conditions is that we examined axon elongation where there were already short axons from the beginning of drug incubation, reflecting axon elongation rather than axon initiation. Therefore, we suggest that ERK1/2 activity is necessary for axon initiation and not necessary for axon elongation in sympathetic neurons.

To clarify the functional differences in ERK5 and ERK1/2 in the process of neural differentiation, microarray screening was performed using ERK5 and ERK1/2-selective inhibitors in PC12 cells. The selectivity of these inhibitors was confirmed in the previous and the present studies (Figs. 3B and 4E) [10]. Although other groups have also reported that U0126 does not block ERK5 efficiently at low doses as observed in our study [43–45], there are conflicting reports showing U0126 inhibits ERK5 in addition to ERK1/2 [8, 46]. The reason for these inconsistent results remains unclear, but a cross-talk between ERK1/2 and ERK5 may exist. In fact, we observed lower band shift of phosho-ERK5 in U0126-treated cells although phosphorylation status of ERK5 at the TEY activation site was not blocked by U0126 (Figs. 3B and 4E).

We indentified ankrd1 as an ERK5-dependent and ERK1/2-independent gene. Ankrd1 contains protein-protein interacting domains including four tandem ankyrin repeats and a coiled-coil domain with no apparent enzymatic activity. The coiled-coil domain can contribute to self dimerization, and some ankrd1 binding partners have been identified including YB1, myopalladin, cardiac calsequestrin, muscle-specific RiNG finger 1 and 2 ubiquitin ligases (MURF1/2), titin and desmin [34]. In addition to the protein-interacting domains described above, ankrd1 encodes a nuclear localization motif, potential PEST protein degradation sequence, and putative phosphorylation and glycosylation sites [34, 47]. Ankrd1 plays critical roles in transcriptional regulation, myofibrillar assembly, stretch sensing and communication between the sarcoplasmic reticulum and the nucleus. As a transcriptional modulator, ankrd1 binds to p53 tumor suppressor protein and enhances its transcriptional activity [48], whereas ankrd1 represses some cardiac gene expression [34, 47, 49, 50]. With regard to signaling leading to ankrd1 expression, it is induced by α_1- or β-adrenergic receptor stimulation [51, 52] or transforming growth factor-β/Smads signaling although other growth factors such as bFGF and vascular endothelial growth factor caused no effect [53]. Serum also induces ankrd1 gene expression [52] and ankrd1 is presently a hypothetical serum-response factor target [54]. Other transcription factors such as Nkx2.5, GATA-4 and Sp3 are also required for induction of ankrd1 expression [34]. It has been shown that ERK5 and ERK1/2 can activate the serum response element by phosphorylating ternary complex factors (TCF) such as Sap1 and Elk1, respectively [8]. Sp3 is also activated

by phosphorylation by ERKs [55], suggesting these factors are involved in ERK5-mediated ankrd1 gene expression. One unresolved findings is that ankrd1 expression is induced by only ERK5, and not ERK1/2.

Although ankrd1 has seen mainly studied in cardiac myocytes, it has been reported that ankrd1 is expressed after nerve injury and involved in neurite outgrowth in dorsal ganglion neurons and cells derived from dorsal ganglion neurons [35]. However, neurite outgrowth was neither affected by ankrd1 knockdown nor overexpression in PC12 cells (supplemental Fig. 1), suggesting the effect of ankrd1 on neurite outgrowth is cell type-specific. Ankrd1 may regulate gene expression or modify cytoskeletal protein functions that are necessary and sufficient for neurite outgrowth in dorsal root ganglion neurons, and that are absent in PC12 cells.

In the present study, ankrd1 knockdown reduced TH protein levels and catecholamine biosynthesis without changing the mRNA levels (Fig. 5). However, overexpression of ankrd1 did not cause increased TH protein levels, suggesting additional signals are required for up-regulation of TH protein. ERK5 contributes to both gene expression of TH and stabilization of TH protein (Figs. 1 and 4)[10], but ankrd1 protein potentially stabilizes TH protein by blocking ubiquitination without promoting gene expression (Fig. 5), therefore blockade of catecholamine biosynthesis by ankrd1 knockdown was partial whereas it was completely inhibited by ERK5 knockdown (Fig. 6). In previous studies, NGF induced rapid phosphorylation of TH on Ser31 which was important for regulating the stability of TH protein [10, 56]. However, we revealed that this phosphorylation was ERK1/2-dependent but ERK5-independent, suggesting that ERK5 stabilizes TH protein using a distinct mechanism. The ability of ankrd1 to block ubiquitination of TH may be regulated by an ERK5-specific mechanism. How ankrd1 contributes to stabilization of TH protein will be examined in further studies. Ankrd1 may block the ubiquitination and degradation of TH protein by binding to TH protein directly or indirectly. It has been shown that ankrd1 interacts with E3-ubiquitin ligase, MuRF1 and MuRF2 through the coiled-coil domain [57]. Thus, although ubiquitin ligase for TH has not been reported previously, ankrd1 may interact with the unidentified ubiquitin ligase for TH to protect it from ubiquitination and degradation. In addition, ERK5 knockdown caused no effect on other neuron-specific cytoskeletal protein levels such as neurofilament light chain or β-tubulin in ERK5-deficient cells (Fig. 1). One of the reasons why ERK5 regulates TH levels specifically may result from the fact ankrd1 inactivates the unidentified TH-specific ubiquitin ligase.

In human adrenal pheochromocytomas, TH was overexpressed at both mRNA and protein levels as reported previously (Fig. 8), and excess catecholamine biosynthesis caused hypertension in nine of eleven patients (Table 1). At first, we hypothesized that ERK5 and ankrd1 were also overexpressed in adrenal pheochromocytomas because ERK5 signaling increased TH levels in PC12 cells and sympathetic neurons. However, ERK5 expression levels were significantly reduced in pathological conditions (Fig. 8). ERK5 expression levels correlated with TH levels in normal adrenal medullas, but did not in adrenal pheochromocytomas. Therefore, ERK5 signaling is required for maintenance of appropriate catecholamine levels in normal medulla. In addition, ERK5 expression was slightly higher in normal adrenal cortex than adrenal medulla, suggesting that ERK5 also plays important

roles in expression of mineralcorticoid or glucocorticoid synthesizing enzymes in normal cortex (Fig. 7A). As mentioned above, loss of ERK5 causes down-regulation of TH in PC12 cells and sympathetic neurons, but this observation is not applied to the pathological conditions such as adrenal pheochromocytomas. Hence, unidentified factor (s) other than ERK5 that function in adrenal pheochromocytomas may cause enhancement of TH expression. In these tumors we could not observe ankrd1 protein levels, but ankrd1 mRNA levels did not significantly change between normal adrenal medullas and adrenal pheochromocytomas. For this reasons, although ankrd1 mainly localized in cytoplasm where TH exists, it is unlikely that ankrd1 promotes TH expression in pheochromocytomas.

5. Conclusion

In summary, we demonstrated that ERK5 regulated TH levels, catecholamine biosynthesis and neurite/axon outgrowth in PC12 cells and primary cultured sympathetic neurons. In addition, we found for the first time that ankrd1 gene expression was induced by ERK5 signaling, but not ERK1/2 signaling and ankrd1 was required for stabilization of TH protein and promotion of catecholamine biosynthesis. Furthermore, we showed that the relationship between ERK5 and TH is important for normal catecholamine homeostasis and this correlation is lost in human adrenal pheochromocytoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ERK	extracellular signal-regulated kinase
PC12	pheochromocytoma cells
TH	tyrosine hydroxylase
ankrd1	ankyrin repeat domain 1
NGF	nerve growth factor
EGF	epidermal growth factor

bFGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HRP	horseradish peroxidase
ECL	enhanced chemiluminescence
DMEM	Dulbecco's modified Eagle's medium
SCG	superior cervical ganglia
TBST	Tris-buffered saline containing 0.1% tween-20
RT-PCR	reverse transcription-polymerase chain reaction
NLS	nuclear localizing signal
NES	nuclear export signal
TCF	ternary complex factors

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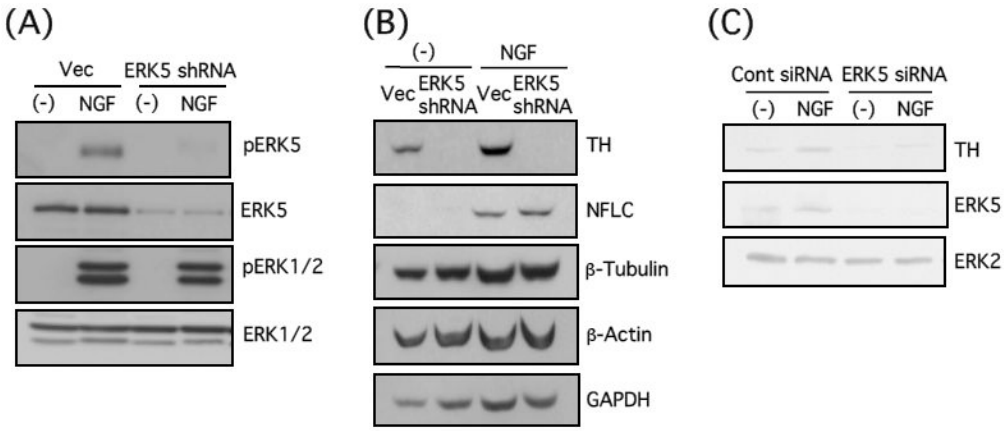

Figure 1.

ERK5 is required for TH expression in PC12 cells. (A) PC12 cells stably expressing ERK5 shRNA were stimulated with or without NGF (100 ng/ml) for 5 min, then phosphorylation of ERK5 and ERK1/2 was examined by Western blotting. (B) PC12 cells stably expressing ERK5 shRNA were stimulated with or without NGF (100 ng/ml) for 24 hours then protein levels of TH, neurofilament light chain (NFLC), β-tubulin, β-actin and GAPDH were examined by Western blotting. (C) PC12 cells transfected with control siRNA or ERK5 siRNA were incubated with or without NGF (100 ng/ml) for a day, then TH, ERK5 and ERK2 were examined by Western blotting.

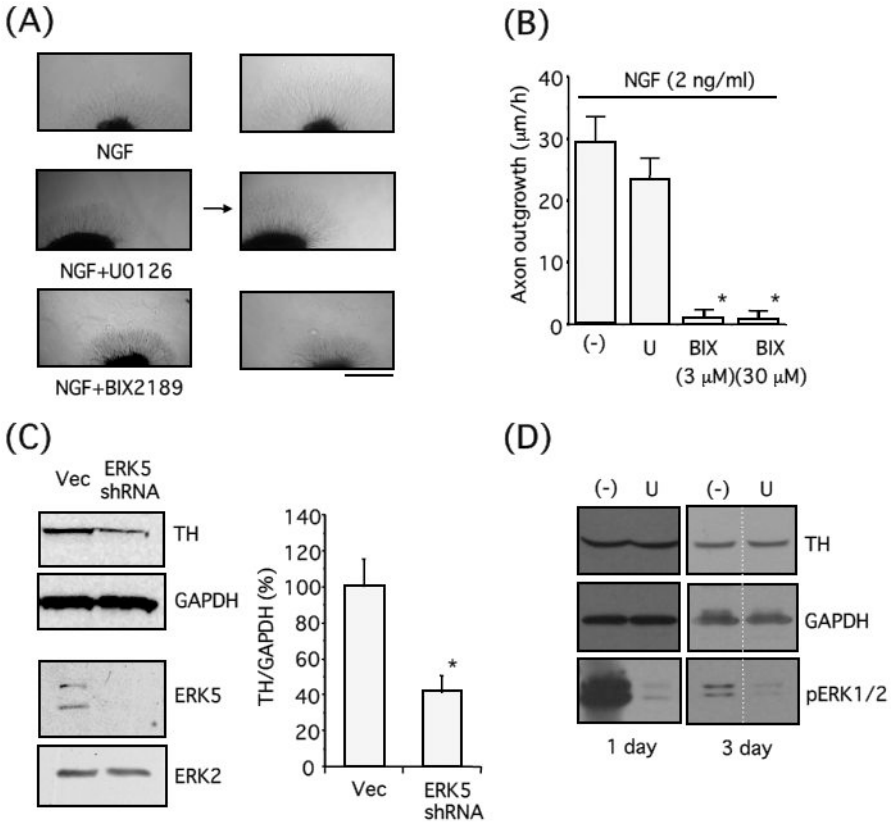

Figure 2.

ERK5 is required for axon elongation and TH expression in sympathetic neurons. (A) Sympathetic ganglia were incubated with NGF (2 ng/ml) in the presence or absence of U0126 (20 μM) and BIX02189 (30 μM) for 6 h, then axon elongation was observed using phase-contrast microscopy. Scale bar=500 μm. (B) Axon outgrowth length in sympathetic neurons was measured as described in Materials and Methods. Axon outgrowth was significantly blocked by BIX02189 (BIX) (* = $p < 0.05$) (n=3). (C) Sympathetic neurons transfected with empty vector (Vec) or ERK5 shRNA were incubated in the presence of NGF (4 days), then levels of TH, GAPDH, ERK5 and ERK2 were examined by Western blotting. Density of TH was normalized by that of GAPDH and, the ratio was expressed as a percentage of Vec alone (n=8). TH protein levels were significantly inhibited by ERK5 knockdown (* = $p < 0.05$). (D) Sympathetic neurons were incubated with or without U0126 (U, 20 μM) in the presence of NGF (1 or 3 days), then levels of TH, GAPDH and phopho-ERK1/2 (pERK1/2) were examined by Western blotting.

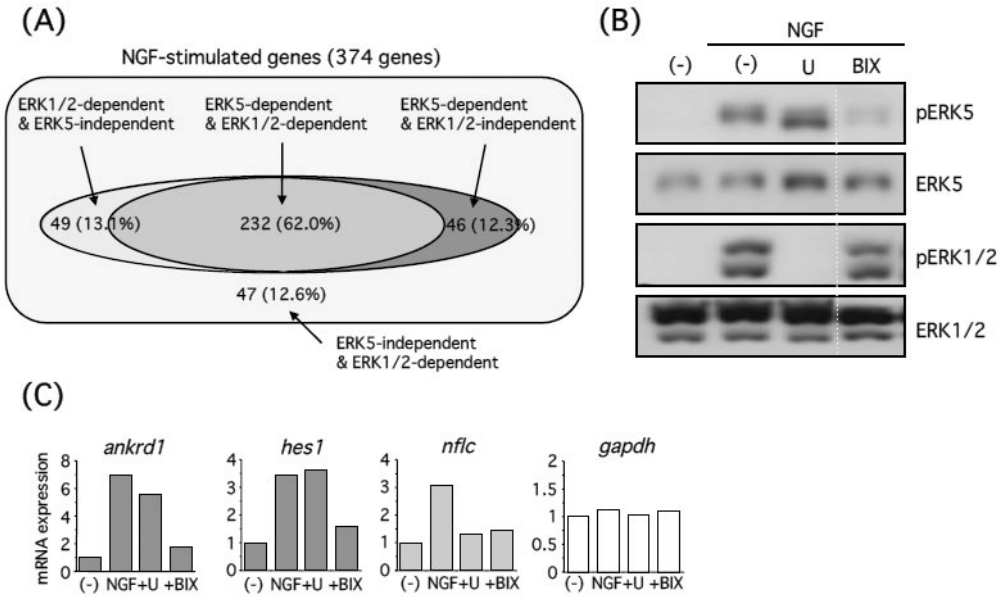

Figure 3.

Identification of ERK5-dependent genes. (A) PC12 cells were stimulated with or without NGF (100 ng/ml) for 4 h in the presence or absence of U0126 (30 μM) and BIX02189 (30 μM), then gene expression was examined by microarray analysis. Genes that were up-regulated more than three fold by NGF were defined as NGF-stimulated genes. Among them, genes whose expression was inhibited by more than 50% by U0126 and BIX02189 were defined as ERK1/2-dependent genes and ERK5-dependent genes, respectively. (B) PC12 cells were incubated with or without NGF (100 ng/ml) for 5 min in the presence or absence of U0126 (U, 30 μM) and BIX02189 (BIX, 30 μM), then phosphorylations of ERK5 and ERK1/2 were examined. These samples were prepared at the same time when samples for microarray analysis were prepared. (C) Examples of ERK5-specific genes (dark gray), ERK5 and ERK1/2-dependent genes (light gray) and NGF-independent genes (white).

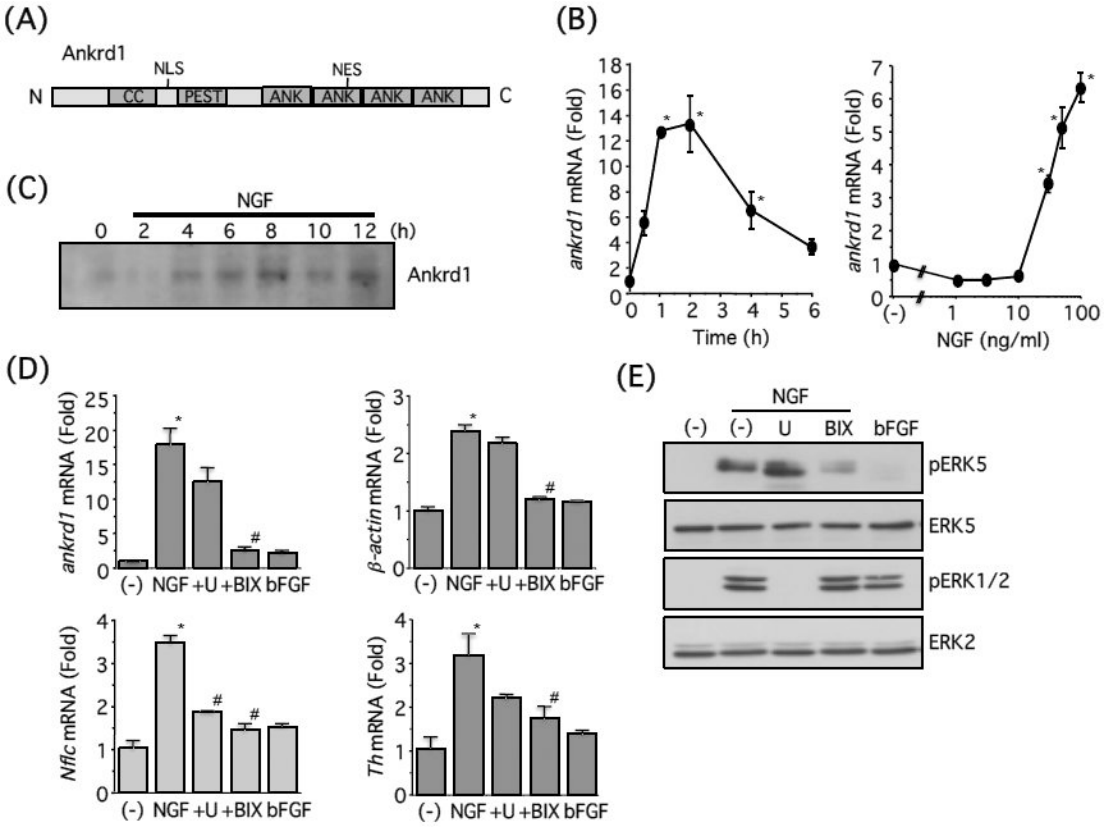

Figure 4.

Ankrd1 is expressed via ERK5 in PC12 cells. (A) Structure of Ankrd1. (B) PC12 cells were stimulated with NGF (100 ng/ml) for 0.5-6 h (left) or NGF (1-100 ng/ml) for 2 h (right), then ankrd1 gene expression was examined by real-time RT-PCR (n=3). (C) PC12 cells were stimulated with NGF (100 ng/ml) for 0 to 12 h as indicated, and ankrd1 protein levels were examined by Western blotting. (D) PC12 cells were stimulated with or without NGF (100 ng/ml) or bFGF (100 ng/ml) in the presence or absence of U0126 (30 μM) and BIX02189 (30 μM), then mRNA encoding ankrd1, β-actin, neurofilament light chain (NFLC), and TH was examined by real-time RT-PCR. NGF significantly induced expression of all four genes compared with drug-free control (* = $p < 0.05$). The induction of ankrd1, TH and β-actin was blocked by BIX02189 (BIX, 30 μM) compared with NGF (# = $p < 0.05$) and the induction of neurofilament light chain was blocked by both U0126 (U, 30 μM) and BIX02189 (30 μM) (# = $p < 0.05$) (n=3). (E) PC12 cells were stimulated with or without NGF (100 ng/ml) or bFGF (100 ng/ml) for 5 min in the presence or absence of U0126 (U, 30 μM) and BIX02189 (BIX, 30 μM), then phosphorylation of ERK5 and ERK1/2 was examined by Western blotting.

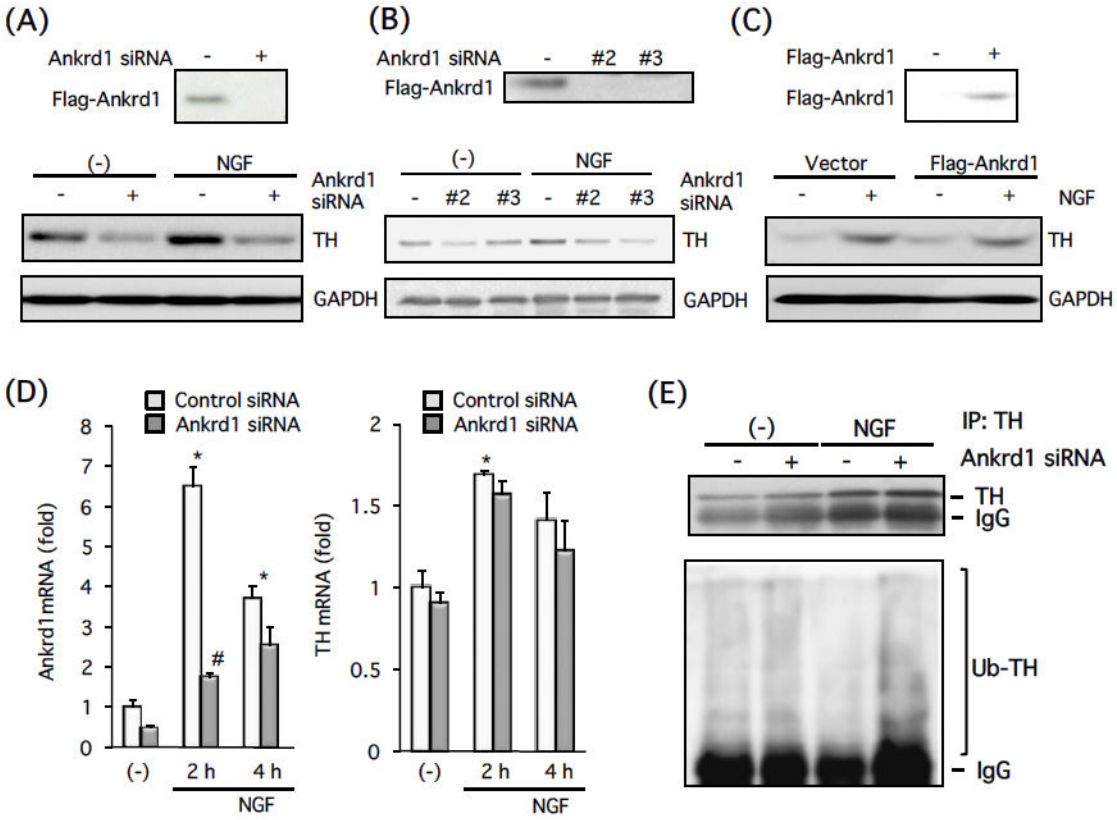

Figure 5.

Ankrd1 is necessary for TH expression and blocks ubiquitination of TH in PC12 cells. (A) PC12 cells were transfected with Flag-ankrd1 and control siRNA or ankrd1 siRNA, then Flag-ankrd1 was examined by Western blotting (upper panel). PC12 cells transfected with control siRNA or ankrd1 siRNA were incubated with or without NGF (100 ng/ml) for a day, then TH and GAPDH were examined by Western blotting. These cells were not transfected with Flag-ankrd1 (lower panels). (B) PC12 cells were transfected with Flag-ankrd1 and control siRNA or individual siRNAs (#2 and #3) for ankrd1, then Flag-ankrd1 was examined by Western blotting (upper panel). PC12 cells transfected with control siRNA or individual siRNAs (#2 and #3) for ankrd1 were incubated with or without NGF (100 ng/ml) for a day, then TH and GAPDH were examined by Western blotting. These cells were not transfected with Flag-ankrd1 (lower panels). (C) PC12 cells stably overexpressing flag-ankrd1 or transfected with empty vector were incubated with or without NGF (100 ng/ml) for 24 hours, then levels of TH and GAPDH were examined by Western blotting. (D) PC12 cells transfected with control siRNA or ankrd1 siRNA were incubated with NGF (100 ng/ml) for 2 or 4 h as indicated, then gene expression of ankrd1 and TH was examined by real-time RT-PCR. NGF significantly induced ankrd1 and TH expression (* = $p < 0.05$), and ankrd1 expression was inhibited by ankrd1 siRNA (# = $p < 0.05$) (n=3). (E) PC12 cells transfected with ankrd1 siRNA or control siRNA were incubated in the presence or absence of NGF (100 ng/ml) for 18 h, and the cells were further incubated in the presence of the proteasome inhibitor, MG132 (1 μM) for 6 h. After TH immunoprecipitation, ubiquitinated TH was

examined by Western blotting. Anti-TH antibody (upper) and anti-ubiquitin antibody (lower).

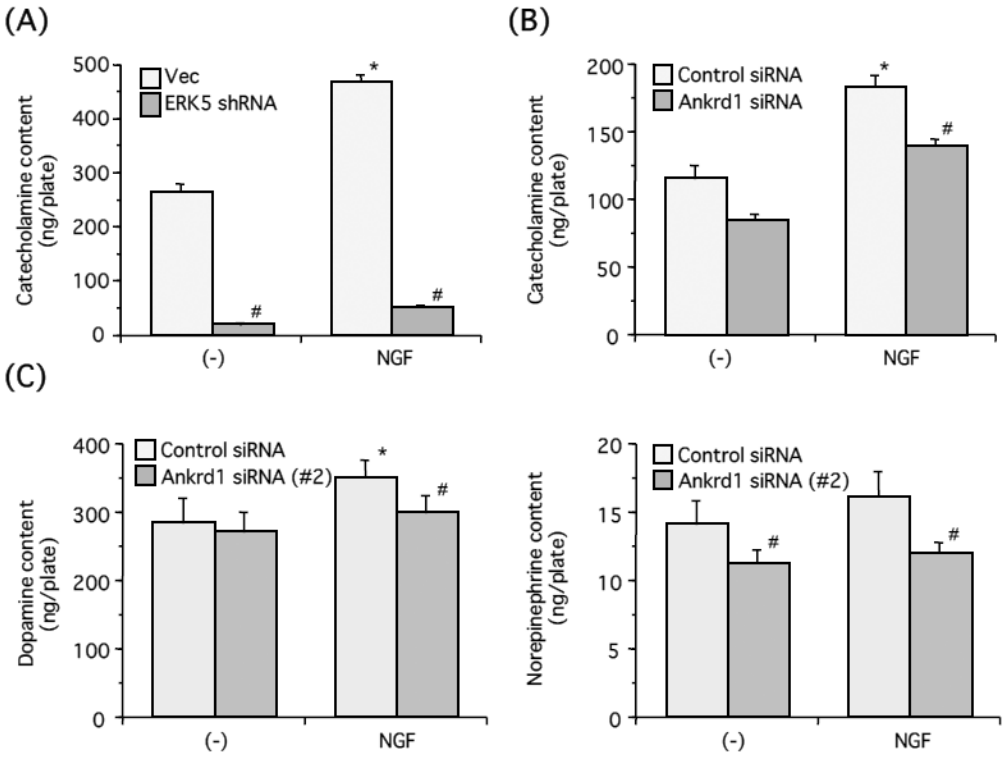

Figure 6.

ERK5 and ankrd1 regulate catecholamine biosynthesis in PC12 cells. (A) PC12 cells stably expressing ERK5 shRNA or transfected with empty vector (Vec) were incubated in the presence or absence of NGF (24 hours), and total catecholamine content was measured. NGF significantly increased catecholamine content (* = $p < 0.05$), while ERK5 knockdown decreased basal and NGF-stimulated catecholamines (# = $p < 0.05$). (n=3) (B) PC12 cells transfected with control siRNA or ankrd1 siRNA were incubated in the presence or absence of NGF (24 hours), and total catecholamine content was measured. NGF significantly stimulated catecholamine biosynthesis compared with drug-free control (* = $p < 0.05$), and ankrd1 knockdown prevented the NGF-induced increase in catecholamines compared to control siRNA with NGF (# = $p < 0.05$) (n=3). (C) PC12 cells transfected with control siRNA or ankrd1 siRNA (#2) were incubated in the presence or absence of NGF. Then dopamine and norepinephrine levels were measured by LC-MS/MS as described in Materials and Methods. NGF significantly stimulated dopamine biosynthesis compared with drug-free control (* = $p < 0.05$), and ankrd1 knockdown prevented the NGF-induced increase in dopamine compared to control siRNA with NGF (# = $p < 0.05$) (n=10). For norepinephrine, ankrd1 knockdown prevented the norepinephrine levels compared to control siRNA with or without NGF (# = $p < 0.05$) (n=10).

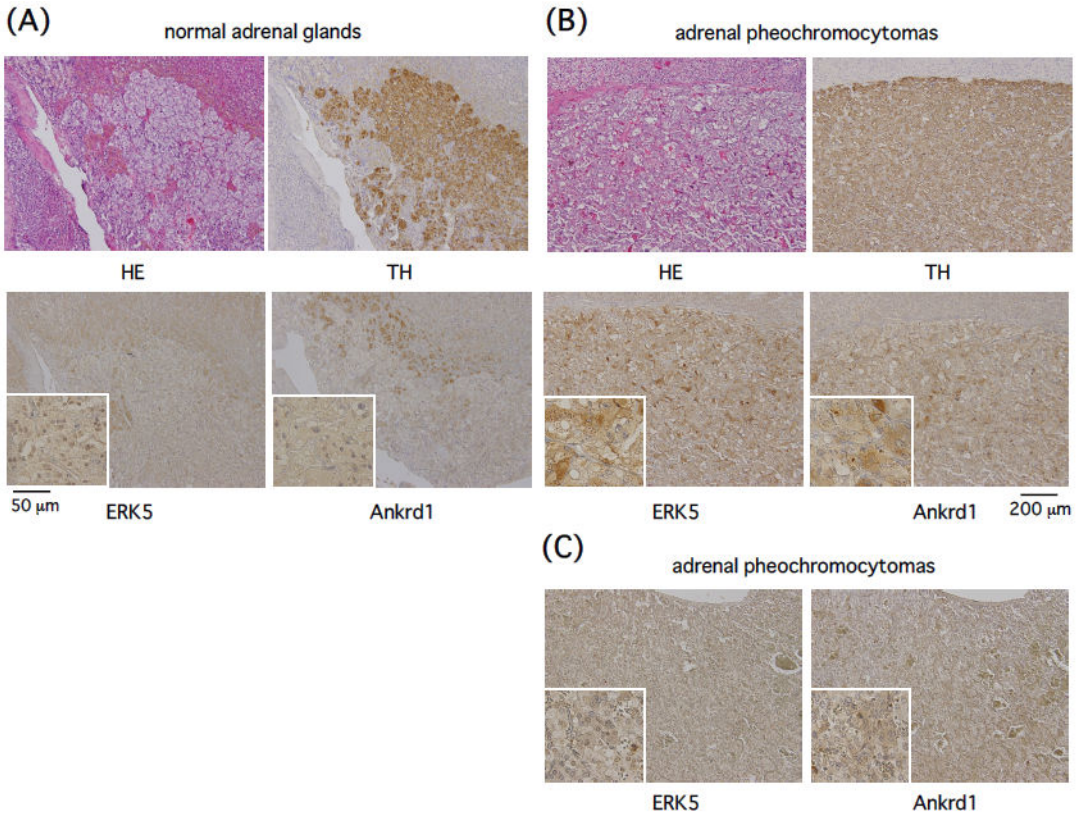

Figure 7.

Expression of ERK5 and ankrd1 in normal human adrenal glands and adrenal pheochromocytomas. Representative microscopic photos of a normal adrenal glands (A) and adrenal pheochromocytomas (B,C), stained for TH, ERK5 and ankrd1 as described in Materials and Methods. TH was strongly expressed in cytoplasm of normal adrenal medulla cells and adrenal pheochromocytomas, but not at all in the adrenal cortex (Figs. 7A and 7B, upper). ERK5 and ankrd1 proteins were detected diffusely in normal medullas and the tumors (Figs. 7A and 7B, lower). Normal medullary cells showed weak cytoplasmic staining with anti-ERK5 and ankrd1 antibodies, but showed variable nuclear staining for ERK5, and little nuclear staining for ankrd1. (Fig 7A, lower insets). Some medullary cells situated near the cortex had intracellular yellow pigmentation (Fig. 7A, HE, ERK5 and ankrd1) that did not represent specific staining. Pheochromocytomas had different profiles of staining compared to normal medulla (Figs. 7B and 7C). ERK5 and ankrd1 staining were considered positive in almost all tumor cells. The intensity of immunoreactions to ERK5 and ankrd1 varied among tumor cells and patient samples (Figs. 7B and 7C, insets). Fig. 7B shows a representative case in which parts of tumor cells within a whole tumor mass expressed strong positive immunostaining to ERK5 and ankrd1 proteins (Fig. 7B, lower and insets). Figure 7C shows a representative case in which tumor cells showed weak and homogeneous cytoplasmic staining for ERK5 and ankrd1. Original magnification was set at ×100 and ×400 for each panel and inset, respectively. Scale bars were indicated as in the figure.

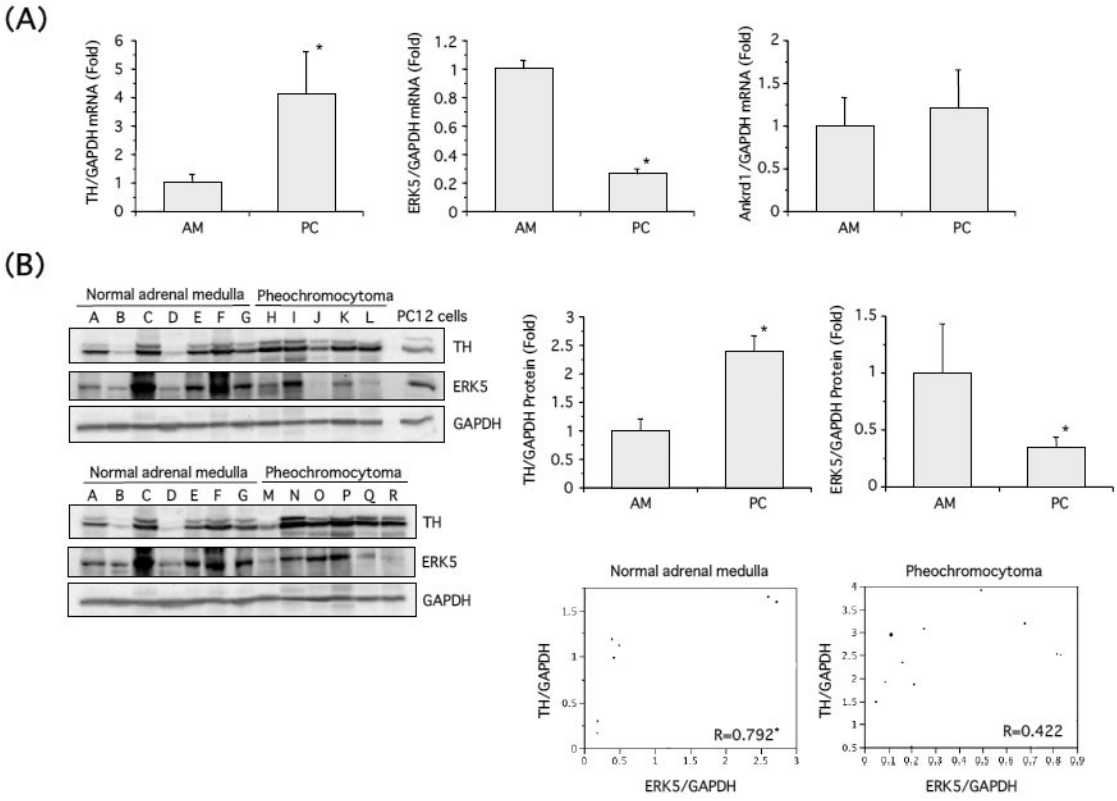

Figure 8.

ERK5 levels are correlated with TH levels in normal human adrenal glands, but not in adrenal pheochromocytomas. (A) Total RNA was extracted from normal human adrenal glands (AM) and pheochromocytomas (PC), then expression of TH, ERK5 and ankrd1 mRNA was examined by real-time RT-PCR. TH mRNA was up-regulated in pheochromocytomas (* = $p < 0.05$) and ERK5 mRNA was down-regulated (* = $p < 0.05$). (B) Tissues of normal human adrenal glands (AM) and pheochromocytomas (PC) were lysed, and protein levels of TH, ERK5 and GAPDH were examined by Western blotting. A-R indicates individual sample ID. Densitometric analysis revealed that levels of TH in pheochromocytomas were significantly higher (* = $p < 0.05$) and levels of ERK5 were significantly lower than those in normal adrenal glands (* = $p < 0.05$). Furthermore, TH and ERK5 levels were correlated in normal adrenal medulla (R=0.792, * = $p < 0.05$) although less correlation was observed in case of pheochromocytomas (R=0.422, p=0.196).

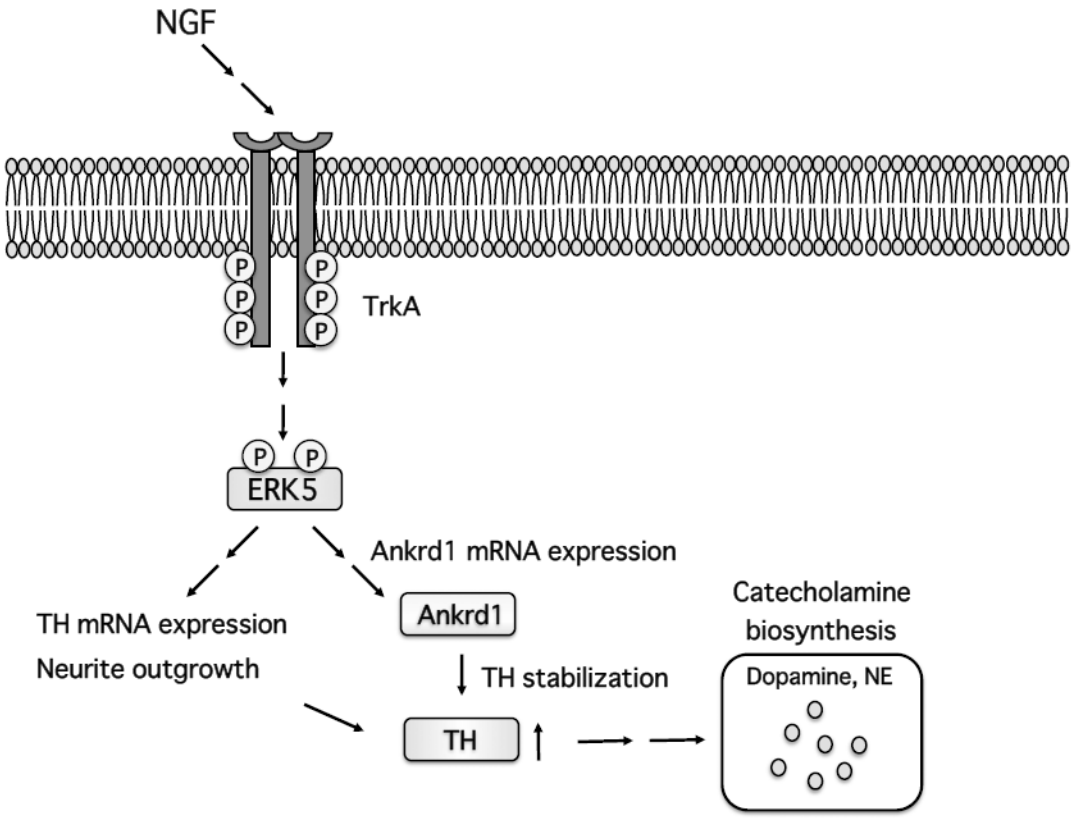

Figure 9.

A putative signaling pathway of ERK5/ankrd1 for promoting catecholamine biosynthesis. ERK5 is activated by NGF through TrkA. Whereas ERK5 signaling is required for TH gene expression and neurite outgrowth, ERK5 induces ankrd1 that promotes stabilization of TH protein and catecholamine biosynthesis.

Table 1.

Patients and tumor characteristics. Tumor size was estimated by examining the tumor images and expressed as length × width × depth (the depth was not recorded in some cases). SP means “sporadic”, there was no familial case in this study.

Patients and tumor characteristics									Urine Catecholamines (μg/day)			Urine Metabolites (mg/day)		Urine Metanephrines (mg/day)	
ID	**Age**	**Gender**	**Pathological diagnosis**	**Laterality**	**Malignancy**	**Tumor Size**	**Genetic**	**Hypertension**	**Adrenaline**	**Noradrenaline**	**Dopamine**	**HVA**	**VMA**	**MN**	**NMN**
AM-A	69	M	Renal cell carcinoma	R	−										
AM-B	53	F	Renal cell carcinoma	L	−										
AM-C	79	M	Renal cell carcinoma	L	−										
AM-D	83	F	Renal cell carcinoma	L	−										
AM-E	43	M	Renal cell carcinoma	L	−										
AM-F	71	F	Renal cell carcinoma	L	−										
AM-G	57	F	Renal cell carcinoma	R	−										
PC-H	67	M	aderenal pheochromocytoma	R	−	45*35*30 mm	SP	+	34.5	243	666	NA	12.9	1.5	2.6
PC-I	74	M	aderenal pheochromocytoma	L	−	60*50 mm	SP	+	235	264	751	14.5	NA	NA	NA
PC-J	78	F	aderenal pheochromocytoma	L	+	not accessed (metastatic lesion)	SP	+	NA	NA	NA	NA	NA	NA	NA
PC-K	55	F	aderenal pheochromocytoma	L	−	135*110*100 mm	SP	+	NA	NA	NA	NA	NA	NA	NA
PC-L	34	M	aderenal pheochromocytoma	L	−	45*27*25 mm	SP	+	106.6	2890.8	1686.8	NA	NA	0.38	3.73
PC-M	14	F	aderenal pheochromocytoma	L	−	45*3 7mm	SP	+	2	74	548	2.88	2.06	0.06	0.27
PC-N	43	M	aderenal pheochromocytoma	R	−	35*30*30 mm	SP	+	139	333	675	3.9	8.58	3.64	1.73
PC-O	33	F	aderenal pheochromocytoma	L	−	56*44 mm	SP	+	NA	NA	NA	NA	NA	NA	NA
PC-P	56	M	aderenal pheochromocytoma	L	−	45.7*45.0*40.5 mm	SP	+	28	267	828	5.28	13.9	0.33	7.09
PC-Q	73	M	aderenal pheochromocytoma	L	−	40*37*31 mm	SP	−	39	155	431	NA	NA	1.42	0.4
PC-R	66	F	aderenal pheochromocytoma	L	−	156*140*11.2 mm	SP	−	19.34	193.35	536.31	6.1	56.3	2.83	17