Detection and Organ-Specific Ablation of Neuroendocrine Cells by Synaptophysin Locus-Based BAC Cassette in Transgenic Mice

Chieh-Yang Cheng, Zongxiang Zhou, Alexander Yu. Nikitin*

Department of Biomedical Sciences, Cornell University, Ithaca, New York, United States of America

Abstract

The role of cells of the diffuse neuroendocrine system in development and maintenance of individual organs and tissues remains poorly understood. Here we identify a regulatory region sufficient for accurate in vivo expression of synaptophysin (SYP), a common marker of neuroendocrine differentiation, and report generation of Tg(SypEGFPloxP-DTA)147yl يوم (SypELDTA) mice suitable for flexible organ-specific ablation of neuroendocrine cells. These mice express EGFP and diphtheria toxin fragment A (DTA) in SYP positive cells before and after Cre-loxP mediated recombination, respectively. As a proof of principle, we have crossed SypELDTA mice with Elia-Cre and PB-Cre4 mice. Elia-Cre mice express Cre recombinase in a broad range of tissues, while PB-Cre4 mice specifically express Cre recombinase in the prostate epithelium. Double transgenic Elia-Cre; SypELDTA embryos exhibited massive cell death in SYP positive cells. At the same time, PB-Cre4; SypELDTA mice showed a substantial decrease in the number of neuroendocrine cells and associated prostate hypertrophy. As no increase in cell death and/or Cre-loxP mediated recombination was observed in non-neuroendocrine epithelium cells, these results suggest that neuroendocrine cells play an important role in prostate development. High cell type specificity of Syp locus-based cassette and versatility of generated mouse model should assure applicability of these resources to studies of neuroendocrine cell functions in various tissues and organs.

Introduction

Neuroendocrine (NE) cells have both neuronal and endocrine phenotypes [1]. The diffuse neuroendocrine system (DNES) is composed of NE cells scattered throughout the entire body either as single cells or clusters, such as solitary pulmonary NE cells (PNECs) and neuroepithelial bodies (NEBs) [2], the islets of Langerhans in the pancreas [3,4], gastrointestinal NE cells [5,6], dermal NE cells (so-called Merkel cells) [7], adrenal medullary NE cell [8–10], and prostate NE cells [11]. PNECs are implicated in regulation of lung maturation and growth, function as oxygen-sensing chemoreceptors and are likely important for lung stem cell niches [2]. Gastrointestinal NE cells are known to control gastrointestinal secretion, motility, growth, immune cell function and food intake [5]. Though there has been progress in understanding the function of NE cells, the physiological role of NE cells in most other organs is not well understood.

Cells with NE differentiation are also present in many cancer types, with their representation ranging from being the major component in small cell carcinomas of the lung [2] and prostate [12], as well as NE tumors of gastrointestinal tract [13], to more limited quantity in other cancers, such as adenocarcinomas of the lung [2] and prostate [12]. Unfortunately, the cell of origin of neoplastic NE cells and their contribution to cancer progression remain insufficiently elucidated [1,2,12,14]. NE cells are detected by a number of markers, such as chromogranin A (CgA) [15], neuron-specific enolase (NSE) [16], neural cell adhesion molecules (NCAMs, so-called CD56) [17], calcitonin gene-related peptide (CGRP) [18] and SYP [19]. However, the use of NSE [20–22] or CD56 [23,24] is limited because of their poor specificity and/or sensitivity. CgA reactivity is strongly dependent on the number of neurosecretory vesicles per cell and is frequently lost in neoplastic NE cells [25], while only subset of NE cells express CGRP [26]. In contrast, SYP is expressed in a broad-spectrum of normal and neoplastic NE and neural cells [19,27].

SYP is a major integral membrane protein of small synaptic vesicles and belongs to a family of proteins that includes synaptogyrin (SYG) and synaptoporin [28]. It has been reported that in cell culture transfection experiments the 1.2 kb upstream region of rat Syn promoter is insufficient to confer cell type specific expression [29]. It has also been suggested that NE cell specific silencer elements lay within the 2.6 kb upstream fragment of Syp [29]. At the same time, other cell culture studies have reported that neuron-restrictive silencer element (NRSE), a binding site for RE-1 silencing transcription factor (REST), a.k.a. neuron-restrictive silencer factor (NRSF), is located within the first intron of Syp gene [30]. However, the regulatory region sufficient for accurate in vivo expression of SYP remains unknown, thereby preventing
development of genetic constructs allowing Syp-specific gene expression.

Since SYP is among the most reliable markers for NE cells, we generated mice with Syp locus-based Bacterial Artificial Chromosome (BAC) cassettes. We show that in combination with the preserved NRSE in the first intron, only the 121 kb upstream and 36 kb downstream regions, but not the 3 kb upstream region, allow for accurate expression of reporter gene in SYP expressing cells in the mouse. We also show that SYP positive cells can be accurately ablated in either the embryo or in the postnatal adult prostate after induction of DTA expression [31] by Cre-loxP mediated recombination in crosses of SypELDTA mice with EIIA-Cre [32] or PB-Cre[33] mice, respectively. The Syp containing BAC cassette and generated mice should provide useful tools for studies of NE cell biological roles in development and maintenance of various tissues and organs.

Materials and Methods

Bioinformatics Analyses

Analysis of sequence and species comparisons were performed by using the University of California Santa Cruz Genome Browser (UCSC, http://genome.ucsc.edu/).

Generation of SypELDTA Mice

A BAC clone containing approximately 121 kb and 36 kb of 5' and 3' DNA flanking the Syp locus was modified by insertion of a loxP-EGFP-Neo cassette-Stop-loxP-DTA-bpA cassette to replace the sequence spanning intron 1 downstream of NRSE to exon 7 of Syp locus by homologous recombination. The BAC constructs were microinjected into male pronuclei of fertilized oocytes from FVB/N mice to generate the SypELDTA mice. EIIA-Cre (FVB/N-Tg(EIIa-cre)C5379Lmgd/J) transgenic mice (The Jackson Laboratory, Bar Harbor, ME, stock number #003314) [32], Rosa26Stop<sup>fl</sup>lacZ<sup>2</sup>(B6;129S4-Gt(ROSA)26Sor<sup>tm1Sor</sup>/J) reporter mice (The Jackson Laboratory, stock number #003309) [34], and ARR-PB-Cre transgenic male mice on FVB/N (PB-Cre[33]) mice were described previously. Details about generation of the targeting construct and BAC recombineering are described in the Materials and Methods S1.

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Laboratory Animal Use and Care Committee at Cornell University ( Permit Number: 2000-0116). All efforts were made to minimize animal suffering.

Histotechnology

Mice euthanized according to schedule were subjected to cardiac perfusion by phosphate-buffered 4% paraformaldehyde. After digital camera photography during necropsy, collected tissues were processed for embedding in paraffin. Histological evaluations were done on 4 μm-thick sections stained with hematoxylin (Mayer’s haemalum) and eosin. Transverse sections of the whole prostate were scanned by ScanScope (Aperio Technologies, Vista, CA) with 40x objective followed by lossless compression and assessment of all alterations in identical anatomic regions.

Immunohistochemical Analyses

Immunoperoxidase staining of paraffin sections of paraformaldehyde-fixed tissue was performed by a modified avidin-biotin-peroxidase (ABC) technique [35]. Antigen retrieval was done by boiling the slides in 10 mM citric buffer (pH 6.0) for 10 minutes. The primary antibodies to cyto-keratin-8 (CK8), cytokeratin-5 (CK5), SYP and cleaved Caspase-3 were incubated with deparaffinized sections at 4°C overnight. After incubation with methanol (Fisher Scientific, Boemia, NY, #A545-4) containing 0.3% hydrogen peroxide (Sigma, St. Louis, MO, #H1009), sections were subsequently incubated with biotinylated secondary antibody for 30 minutes at room temperature and subsequently detected with the ABC Elite kit (Vector Laboratories, Burlingame, CA, #PK-6100) and 3,3-diaminobenzidine (DAB; Sigma, #D4418) as substrate. Hematoxylin was used as the counterstain in immunoperoxidase stainings. Double immunofluorescence staining was performed by incubation of SYP and EGFP, or β-galactosidase primary antibody at 4°C overnight, followed by Alexa-Fluor 594-conjugated and Alexa-Fluor 488-conjugated secondary antibodies. To stain cell nuclei, sections were incubated with a 10 μg/ml solution of 4',6-diamidino-2-phenylindole (DAPI; Sigma, #D9542) for 3 min. Antibody sources and dilutions are listed in the Materials and Methods S1.

Morphometric quantitative analyses

Five digital images of serial sections scanned by ScanScope with 40x objective were captured for each slide and transferred to Image J for manual counting of all epithelial cells (at least 1,000 cells) and SYP positive cells among them. The sizes of prostate lobes were determined by measuring the distance from the edge of each lobe to the urethra. The size of each prostatic duct was determined by measuring its diameter.

Western Blot Analyses

PCN1, PCN2, and PCN3 cell lysates were prepared using RIPA buffer (Tris-HCl 50 mM, pH 7.4; Nonidet P-40 1%; Na-deoxicholrate 0.25%; NaCl 150 mM; EDTA 1 mM; PMSF 1 mM; Aprotinin, leupeptin, pepstatin: 1 μg/ml each; Na3VO4 1 mM; NaF 1 mM), separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore). The membrane was incubated overnight at 4°C with antibodies to detect SYP (Dako, CA, #A0010, 1:100), followed by incubation for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, CA, #sc-2301, 1:2000) and developed using chemiluminescent substrate (Thermo scientific, Rockford, IL, #34077).

Statistical Analyses

Statistical analyses were performed with InStat 3.10 and Prism 5.01 software. (GraphPad, Inc., San Diego, CA). Two-tailed unpaired t-test was used in all calculations.

Results

Genomic Structure of Syp Locus and BAC Engineering for the Generation of SypELDTA Constructs

To identify the region containing all transcriptional cis-elements sufficient for accurate SYP expression in vivo, we have analyzed the genomic Syp locus by using the UCSC Genome Browser. The Syp locus is located on mouse chromosome X and contains 7 exons and 6 introns. Locations of Syp locus and other surrounding genes are preserved among different species, such as rat and human (Figure 1A). Notably, NRSE within the first intron of Syp is highly conserved among closely related mammalian species (Figure 1B). Furthermore, comparison of the Syp upstream region also displays high conservation in proximal (approximate 0 to−600 bp) and distal (approximate−2000 to−3000 bp) regions relative to transcription start site (Figure 2). It may imply that not only NRSE but
also the sequences of those conserved regions are involved in conferring the cell type specific expression of SYP. Therefore, for molecular engineering we have decided to use mouse BAC clone RP23-267C15 because it encompasses the entire Syp locus and, thus, is likely to contain all transcriptional cis-elements required for recapitulation of the endogenous cell specific gene expression.

First, loxP-EGFP-Neo cassette-Stop-loxP-DTA-bpA sequence was constructed to replace the sequence between 3’ NRSE and exon 7 of Syp locus in BAC (Figure S1A). As a result, the Syp promoter specifically drives EGFP expression to label the SYP expressing cells. Following Cre-loxP mediated recombination, DTA can be expressed to ablate the SYP expressing cells. Second, the loxP site in the backbone of RP23-267C15 was also replaced with β-lactamase sequence to avoid unintended Cre-mediated recombination due to multiple loxP sites (Figure S1B). The modified BAC construct is named SypELDTA (173 kb) (Figure 3A). It contains 121 kb and 36 kb upstream and downstream DNA sequences flanking the Syp gene, respectively. To evaluate applicability of shorter upstream sequence of Syp for cell type specific expression, the sequence Syp-loxP-EGFP-Neo cassette-Stop-loxP-DTA-bpA from SypELDTA was retrieved into pGEM-T vector. The retrieving BAC construct is named sSypELDTA (12 kb; Figure 3B). It contains a 3 kb upstream fragment of the Syp promoter region.

### BAC Transgene Function in Mammalian and Bacterial Cell Culture

Functionality of BAC construct was tested in cultured prostate cells and bacteria. sSypELDTA was transfected into the mouse p53 and Rb deficient prostate adenocarcinoma cells line PCN1 and PCN3, which were established from prostate carcinomas of PB-Cre4; p53<sup>−/−</sup>/Rb<sup>−/−</sup> mouse [36]. Consistent with our observation that PCN3 cells but not PCN1 cells express SYP (Figure S2A), EGFP positive cells have been observed only in PCN3 cells after transfection of sSypELDTA. Thus, 3 kb upstream fragment of Syp promoter region was sufficient to drive the transgene expression specifically in NE cells. To test for Cre-loxP mediated recombination, sSypELDTA was electroporated into the *Escherichia coli* EL350 system, which contains a tightly controlled arabinose-inducible Cre gene. As shown by PCR, successful Cre-loxP mediated recombination of sSypELDTA in bacterial system was observed after arabinose induction (Figure S2H). The construct resulting from Cre-loxP mediated recombination was named SypOTA. To confirm that DTA expression driven by Syp promoter could be detected after Cre-loxP mediated recombination in mouse cells, reverse transcriptase PCR (RT-PCR) was performed with different constructs transfected into PCN1 and PCN3 after Cre-4; p53loxP/loxP; RbloxP/loxP adenovirus infection. DTA expression could be detected in SypDTA-infected PCN3 cells and sSypELDTA-infected PCN3 cells followed by Ad-Cre infection. Conversely, no DTA expression could be detected in PCN1 cells. Thus, DTA was expressed specifically in NE cells after Cre-loxP mediated recombination (Figure S2I).

#### BAC Transgene Expression in SypELDTA Transgenic Mice

To test transgene expression in vivo, SypELDTA and SypELDTA DNA were used to generate transgenic lines 141–143 and 144–148, respectively (Table 1). Based on Southern blotting and quantitative PCR (qPCR), all mice of lines 144–148 carried a single copy of transgene, while copy number of SypELDTA transgene was variable among lines 141–143 (Figure S3, Table 1).

To confirm the specificity of transgene expression in transgenic mice, co-localization of transgene-derived EGFP and endogenous SYP has been determined by double immunofluorescence staining in prostate NE cells (Figure 4A). SypELDTA transgenic mice had expression of transgene in non-NE epithelium cells of the prostate (Figure S4, Table 1). In contrast, no EGFP expression was detected in SYP negative cells (Figure S5, Table 2) of SypELDTA mice. Among 5 tested transgenic lines, the line 147 had the highest

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**Figure 1. Genomic structure of the Syp gene.** (A) Location of the Syp locus on mouse, rat, and human chromosome X. Mouse Syp contains 7 exons (black boxes). The translation initiation codon, ATG, is located in the first exon. The NRSE is located within the first intron of Syp. (B) Sequence comparison of the NRSE derived from Syp across species.

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frequency (90%) of SYP positive NE cells co-expressing EGFP (Table 1). Furthermore, co-expression of EGFP and SYP in line 147 has also been observed in the lung NE cells (Figure 4B), medulla of adrenal gland (Figure 4C), islets of Langerhans in pancreas (Figure 4D), and brain (Figure 4E).

**DTA Expression in EIIA-Cre; SypELDTA Embryo**

To confirm that DTA expression driven by Syp promoter is able to ablate SYP positive cell lineage, DTA expression was examined by crossing the male *SypELDTA* mice of line 147 with female *EIIA-Cre* transgenic mice, following collection of embryos on gestational day (GD) 10.5 (Figure 5A). Adenoviral *EIIA* promoter targets expression of Cre recombinase to the early mouse embryo and Cre-mediated recombination occurs in a wide range of tissues, thereby allowing assessing effects of transgene expression in multiple organs and tissues [32]. *EIIA-Cre; SypELDTA* embryos had rare SYP positive cells in the brain and dorsal root ganglia (Figure 5B, D, F). Consistent with induction of apoptosis pathway by DTA [37,38], a significant number of cleaved Caspase-3 positive cells were detected in the same structures (Figure 5C, E, G). The brain and dorsal root ganglia of *EIIA-Cre* littermates had abundant SYP positive cells (Figure 5H, J, L), but almost no cleaved Caspase-3 positive cells (Figure 5I, K, M). Thus, our construct was effective in conditional ablation of SYP expressing cells by DTA *in vivo*.

**Effect of Prostate Epithelium-Specific NE Cell Ablation**

Modified *probasin* promoter drives expression of Cre recombinase in the prostate epithelium of postnatal mouse prostate of *PB-Cre4* transgenic mice [33,39]. By using *PB-Cre4* mice we have previously determined that deletion of tumor suppressor genes *p53* and *Rb* results in prostate carcinomas with NE differentiation [36,40]. We have also reported that expansion of NE cells is observed in prostate adenocarcinomas in *PB-Cre4; Pten*<sup>loxP/loxP</sup> mice, particularly after castration [41]. To verify that Cre recombinase under the control of *probasin* promoter is expressed in NE cells, which are concentrated in the prostate proximal region (Figure S6), Cre-mediated recombination in prostate NE cells was examined by crossing *PB-Cre4* transgenic mice [33] with *R26R* reporter mice [34]. The expression of β-galactosidase is possible only after Cre-mediated deletion of a stop codon flanked by *loxP* sites. Double immunofluorescence staining showed colocalized expression of β-galactosidase and SYP in the proximal...
Figure 3. BAC transgenic constructs. (A) SypELDTA construct. The purple bar represents the EGFP probe. Restriction enzyme sites for Southern blot are indicated. (B) sSypELDTA construct. The fragment SypP-loxP-EGFP-Neo cassette-Stop-loxP-DTA-bpA from SypELDTA was cloned into pGEM-T vector. 3 kb upstream fragment of Syp was used to drive downstream gene expression.

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Table 1. Characterization of transgenic lines 141–148.

| Transgene   | Line | Male germ line transmission | Copy number | Transgene expression in prostate NE cells (%) | Transgene expression in prostate non-NE cells |
|-------------|------|----------------------------|-------------|---------------------------------------------|---------------------------------------------|
| sSypELDTA   | 141  | Yes                        | 11          | 72                                          | Yes                                        |
|             | 142  | Yes                        | 2           | 81                                          | Yes                                        |
|             | 143  | Yes                        | ND#         | ND                                          | Yes                                        |
|             | 144  | No                         | 1           | ND                                          | ND                                         |
| SypELDTA    | 145  | No                         | 1           | 88                                          | No                                         |
|             | 146  | No                         | ND          | ND                                          | ND                                         |
|             | 147  | Yes                        | 1           | 90                                          | No                                         |
|             | 148  | Yes                        | 1           | 83                                          | No                                         |

*Percentage of EGFP; SYP double positive cells within total number of SYP positive cells was determined by double immunofluorescence staining.
#Not done.

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region of prostatic ducts of PB-Cre4; R26R mice. Thus, Cre-loxP mediated recombination occurs in NE cell lineage after Cre expression directed by probasin promoter (Figure S7).

To evaluate whether prostate epithelium-specific NE cell ablation impacts prostate development and function, male PB-Cre4 transgenic mice have been crossed with female SypELDTA (lines 147 and 148) mice to get PB-Cre4; SypELDTA male offspring (Figure 6A). Both lines shown similar phenotypes and line 147 has been characterized to the fullest extent. As compared to wild-type (FVB/N) and SypELDTA (147) age-matched controls and littermates, the size of prostate of PB-Cre4; SypELDTA (AP147) was smaller (Figure 6B, S8). In agreement with earlier reports of broad PB-Cre4 transgene expression in the prostate epithelium [33], loss of floxed EGFP was reproducibly observed in all prostate lobes according to microdissection-PCR genotyping of AP147 (Figure S9). However, only the NE cell population was diminished by 60% in AP147 mice as compared to controls according to immunostaining for SYP in the proximal regions of prostatic ducts (147 vs. AP147: 1.19±0.34% vs. 0.5±0.19%, P<0.0001; Figure 6C-E). The decrease was observed consistently in various areas of the proximal region, such as ventral (1.07±0.22% vs. 0.55±0.18%, P<0.0001; Figure 6F) and dorsolateral (1.42±0.39% vs. 0.44±0.19%, P<0.0001; Figure 6G) lobes. In addition, the average diameter of prostatic ducts in distal regions was decreased, especially in the dorsolateral lobe (Figure S10). Interestingly, more NE cells were observed (1.42±0.39% vs. 1.07±0.22%, P=0.0252; Figure 6H) and higher percentage of NE cells was ablated (70% vs. 49%; Figure 6F, G) in the proximal regions of prostatic ducts of dorsolateral lobes, as compared to those of ventral lobes, which could be associated with the smaller diameter of prostatic ducts in dorsolateral lobes.

Except for the size of lumens, there were no significant changes in overall morphology of prostatic ducts in the ventral and dorsolateral lobes of AP147 mice, or in either the proximal or distal regions of prostatic ducts (Figure S10). NE cell ablation did not result in any detectable changes to the extent of luminal (CK8) and basal (CK5) differentiation in the proximal and distal regions of ducts (Figure S11). No significant cell death was observed in luminal and basal cells in any of the regions (Figure S12), which implies a tight regulation of Syp promoter in prostate non-NE epithelium cells. Taken together, these results support the notion that NE cells play an important role in prostate development.

**Discussion**

Previous report indicated that 2.6 kb but not 1.2 kb upstream fragment of Syp was sufficient to confer cell type specific expression [29]. Consistent with this observation, we have found high

### Table 2. Transgene expression in SypELDTA line 147.

| Tissue                        | Transgene expression in SYP expressing cells (%) | Transgene expression in non-SYP expressing cells |
|-------------------------------|-------------------------------------------------|-------------------------------------------------|
| Brain                         | 100                                             | 0                                               |
| Lung                          | 92                                              | 0                                               |
| Medulla of adrenal gland      | 98                                              | 0                                               |
| Islets of Langerhans          | 100                                             | 0                                               |

*Percentage of EGFP; SYP double positive cells within total number of SYP positive cells was determined by double immunofluorescence staining.*

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evolutionary conservation not only in the proximal (0 to −600 bp) but also distal (−2000 to −3000 bp) regions of the Syp gene. Based on this information, we have designed transgenic constructs preserving NRSE in the first intron of Syp gene and containing either 3 kb or 121 kb upstream regulatory sequence. While 3 kb sequence was sufficient for NE specific expression of transgene in cultured cells, it did not assure specificity of gene expression in the transgenic mice. At the same time, a longer promoter has been highly specific for NE cells. These results suggest that other cis-elements, farther than 3 kb upstream fragment, provide the cell type specific expression in the transgenic mice. At the same time, a longer promoter has been highly specific for NE cells. These results suggest that other cis-elements, farther than 3 kb upstream fragment, provide the cell type specific expression in the context of the whole organism. It is also possible that the 36 kb 3' flanking region of Syp might play a role in regulating cell specificity as well, similarly to other genes, such as human tyrosine hydroxylase [42]. Also, longer upstream and downstream regions of Syp locus may further increase the Syp promoter from position effects by other genes at the site of transgene integration [43]. However, given NE cell specific expression of 3 kb construct after its multi-copy integration in cell culture, the latter possibility is less likely. Identification of environment driven mechanisms responsible for accurate gene expression should allow much better understanding of Syp regulation.

It should be noted that in addition to NE cells, SYP is also expressed in neurons of the nervous system [19,27]. Unlike other REST regulated genes, such as BDNF and GluR2 genes, Syp is similarly regulated in neuronal and NE cells [44]. Consistently, we have observed broad expression of Syp driven EGFP and DTA in mouse neurons of the brain and spinal ganglia. Thus, our model can be used for targeted ablation of NE and/or neuronal cells depending on a particular Cre driving promoter.

Some neuropeptides, such as calcitonin gene-related peptide (CGRP) and gastrin-releasing peptide (GRP) are expressed only in subsets of NE cells [26,45,46]. Therefore, theoretically it is possible that some NE cells do not express SYP. Although we were unable to find any literature supporting this possibility, our SypELDTA mice should allow detection of such cells by using double immunofluorescence for various NE markers before and after Syp driven ablation in future.

As a proof of the utility of our model, we performed prostate epithelium-specific ablation of NE cells. The mouse prostate is composed of a series of branching ducts, each containing distal, intermediate and proximal regions relative to the urethra [47]. Each duct contains three differentiated cell types: luminal, basal and NE cells, with stem cells preferentially concentrated in the proximal region [48–51]. NE cells secrete a large number of neuropeptides which can be mitogenic and growth-promoting. Receptors for some of the NE products have been found to be expressed in benign prostate and/or prostate cancer. It has been
proposed that the NE cells may regulate the growth, differentiation and secretory activity of the prostatic epithelium, possibly through a paracrine mechanism [11]. However, studies directly addressing role of NE cells in prostate development have been lacking.

Based on co-detection of SYP and EGFP double immunofluorescence, as well as lack of Cre-mediated recombination and cell death in prostate non-NE epithelium cells of PB-Cre4; SypELDTA mice, expression of SypELDTA has been highly specific to SYP positive prostate NE cells. Consistent with the important role of NE cells in prostate biology, prostates with decreased number of NE cells were hypotrophic, with decreased sizes of prostate lobes and reduced average diameters of prostatic ducts. Notably, the most pronounced effect of NE ablation on prostate hypotrophy was in the dorsolateral prostate, where NE cells were ablated to the greatest extent. Our study also indicates that NE cells are located in the proximal regions of the prostatic ducts, the area of preferential stem cell location. Future in-depths studies should address the kinetics of NE cell ablation effects and explore if the effect of NE cells on prostate size can be explained by their proximity to prostate stem cells.

It should be noted that in spite of 90% gene expression specificity in prostate NE cells of SypELDTA line 147 mice, we observed only 60% decrease in number of prostate NE cells in PB-Cre4; SypELDTA mice. Consistent with previous study describing compartmentalization of gene expression between prostate lobes [52], our results support a possibility that efficiency of Cre-mediated homologous recombination is different in NE cells of a particular region. We also cannot exclude that there are intrinsic differences in regulation of gene expression in individual NE cells, which may lead to the reduction of DTA expression in some of them. Thus, further improvements in design of constructs for highly efficient NE cell ablation may yield even more dramatic effect on prostate hypotrophy.

NE cell differentiation is positively correlated with prostate cancer progression, castrate-resistance and poor prognosis [12,53]. Previous reports have indicated that NE cells can stimulate cell proliferation, invasion, and apoptosis resistance of cultured prostate cancer cells [12]. However, specific mechanisms involved in the pathogenesis of NE differentiation are not well known. Crossing PB-Cre4; SypELDTA transgenic mice with Pten knockout mice [41,54] or other established mouse prostate cancer models...
with preferential NE differentiation, such as TRAMP or LADY [53,56], should decisively determine the role of NE cells in prostate carcinogenesis. More generally, $S_{ypELDTA}$ mice should be useful for studying other NE neoplasms, such as small cell lung carcinoma and NE tumors in the gastrointestinal tract [1,2,12].

Taken together, we have identified a region of $S_{yp}$ gene sufficient for faithful expression of genetic constructs. We have also successfully generated the $S_{ypELDTA}$ mouse model suitable for flexible organ-specific detection and ablation of NE cells. This model system should provide an important tool for studies of NE cell functions in development and carcinogenesis in various tissues.

**Supporting Information**

**Figure S1** Generation of the BAC targeting construct. (A) The targeting strategy was to replace exon region of the $S_{yp}$ locus with loxP-EGFP-Nos cassette-Stop-loxP-DTA-bpd by homologous recombination. Exon 1 and intron 1 have been preserved because of the NRSE sequence, which is essential for silencing activity in non-neuronal cells. Start codon ATG in exon 1 was mutated to alanine codon GCA. $S_{ypF}$-synaptophysin promoter. NRSE: neuro-restrictive suppressor element. (B) The backbone of RP23-267C15 (pBACe3.6 vector) contains a loxP site which has been replaced with $\beta$-lactamase sequence by homologous recombination. The modified BAC construct was named $S_{ypELDTA}$.

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**Figure S2** Functional testing of s$S_{ypELDTA}$ in cultured prostate NE cells and E. coli. (A) $S_{YP}$ expression in PCN1-PCN3 cells by western blotting. GAPDH, internal control. (B-G) Detection of EGFP expression by BAC transgene in prostate cancer cell line with NE differentiation (PCN3, C, E, G, arrows), but not in line without NE differentiation (PCN1, B, D, F). (B, C) Light microscopy, (D, E) green fluorescence, (F, G) merged images. Calibration bar: 200 μm (B-G). (H) Detection of Cre-loxP mediated recombination in $sS_{ypELDTA}$ transgenic construct in the bacterial system EL350. EL350 bacteria, which contain endogenous arabinose-inducible Cre, were transformed with $sS_{ypELDTA}$ transgenic construct and induced with arabinose. PCR genotyping was performed with F1/F2/R1 primers without (lane 2) and with (lane 3) arabinose induction. 256 bp and 617 bp fragments are diagnostic for internal control (primer F2/R1) and Cre-mediated recombination (primer F1/R1), respectively. Lane 1: marker (M).

**(TIF)**

**Figure S3** The transgene copy number in mice of $sS_{ypELDTA}$ lines 141-143 and $S_{ypELDTA}$ lines 144-148. (A) Experimental design. (B) Southern blot analysis of transgenic line 141. Genomic DNA digested with restriction enzymes AsCl (Lane 2), PacI (Lane 3), and AsCl, PacI and AlflI (Lane 4), Lane 1: DNA marker (M). AlflI but not AsCl and PacI restriction sites are present within the transgene. 8,466 bp band in lane 4 is diagnostic for multiple transgene copies integrated into a single genomic site, whereas top band (arrow) is diagnostic for single copy of transgene. EGFP, DNA probe. (C) Quantification of transgene copy number in transgenic lines by quantitative PCR. Transgenic line 141 carrying 11 copies of transgene was used as a reference to estimate copy number of other lines. (D) Genotyping analysis of transgenic line 147. The upper bands are diagnostic for EGFP (lane 2, E, 546 bp), Nmecras (lane 3, N, 342 bp), and DTA (lane 4, D, 348 bp) fragments of transgene. 196 bp band in lanes 2-4 is internal control (endoogenous Rb). Lane 1: DNA marker (M).

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**Figure S4** Transgene expression in $sS_{ypELDTA}$ lines 141-143. (A-H) Detection of EGFP (B, D, F, H, green) and SYP (C, D, G, H, red) expression (arrows) in non-NE (A-D) and NE cells (E-H) of the prostate epithelium. Yellow color in overlay (D, H) indicates co-localization of EGFP and SYP fluorescent signals. Counterstaining with DAPI (A, D, E, H, blue). Calibration bar: 50 μm (A-D), 18 μm (E-H).

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**Figure S5** $S_{ypELDTA}$ transgene expression has high specificity in SYP expressing cells. (A-T) Detection of EGFP (B, D, F, H, J, L, N, P, R, T, green) and SYP (C, D, G, H, K, L, O, P, S, T, red) expression (arrows) in prostate NE cells (A-D), lung NE cells (E-H), medulla of adrenal gland (I-L), islets of Langerhans in pancreas (M-P), and brain (Q-T) of $S_{ypELDTA}$ line 147 transgenic mice. Yellow color in overlay (D, H) indicates co-localization of EGFP and SYP fluorescent signals. Counterstaining with DAPI (A, D, E, H, I, L, M, P, Q, T, blue). Calibration bar: 25 μm (A-D), 50 μm (E-T).

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**Figure S6** NE cells are mostly located in the proximal region of prostatic ducts. (A, B) SYP expression in NE cells in proximal (A) and distal (B) regions of prostatic ducts of the prostate (n = 6). NE cells and nerve terminals are indicated by arrows and arrowheads, respectively. Calibration bar: 50 μm (A), 100 μm (B). (C) Quantification of SYP positive NE cells. Distal regions of prostatic ducts contain no NE cells. Error bar denotes SD.

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**Figure S7** Cre recombinase under the control of probasin promoter is expressed in prostate NE cells. (A-D) Detection of SYP (B, D, green) and $\beta$-galactosidase (C, D, red, indicative of Cre-loxP mediated recombination) expression (arrows) in the prostate NE cells in PB-Cre4; R26R mice harboring Probasin-Cre and lacZ reporter gene. Yellow color in overlay (D) indicates co-localization of SYP and $\beta$-galactosidase fluorescent signals. Counterstaining with DAPI (A, D, blue). Calibration bar: 50 μm (A-D).

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**Figure S8** Reduced size of prostate lobes in PB-Cre4; $S_{ypELDTA}$ mice. (A-D) Quantification of size of anterior (A), dorsal (B), lateral (C), and ventral (D) lobes among age-matched FVB/N (FVB, n = 8), $S_{ypELDTA}$ (147; n = 4), and PB-Cre4; $S_{ypELDTA}$ (AP147; n = 4) mice. *P<0.05. **P<0.01. Error bar denotes SD.

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**Figure S9** PB-Cre drives Cre-loxP recombination in the prostate of PB-Cre4; $S_{ypELDTA}$ mice. (A-F) Microdissection-PCR. Proximal (A) and distal (ventral, B, dorsolateral, C, and anterior, D) regions of prostatic ducts and the muscular layer of the prostate (E) of age-matched $S_{ypELDTA}$ (147) and PB-Cre4; $S_{ypELDTA}$ (AP147) mice before and after microdissection. Hema-toxylin and eosin. Calibration bar: 50 μm (A-E). (F) PCR design and detection of Cre-loxP mediated recombination in microdissected proximal region (P, lane 2 and 7), and ventral (V, lane 3 and 8),
dorsolateral (DL, lane 4 and 9), anterior (A, lane 5 and 10) distal regions, and muscular layer (ML, lane 6) of prostates from SypELDTA/lanes 2-5 and PB-Cre4; SypELDTA (lanes 6-10) mice. 346 bp fragment is generated with primers Flank R1and is diagnostic for EosP (present before GreloxP mediated recombination). 196 bp fragment (endogenous Rb) is internal control. Lane 1: marker (M).

**Figure S10** NE cell ablation results in proportional reduction of prostatic duct diameters in distal regions. (A-I) Histology (A, B, D, E, G, H) and quantification of diameters (C, F, I) of proximal (A, B, C) and distal (ventral, D, E, F, and dorsolateral, G, H, I) regions of prostatic ducts of age-matched SypELDTA (147; n = 6; A, D, G) and PB-Cre4; SypELDTA (API47, n = 6; B, E, H) mice. Hematoxylin and eosin. Calibration bar: 50 μm (A, B), 100 μm (D, E, G, H). *P < 0.05, **P < 0.01. All error bars denote standard deviation.

**Figure S11** NE cell ablation does not affect luminal (CK8+) or basal (CK5+) cell differentiation. (A-L) Detection of CK8 (A-F) and CK5 (G-L) expression (brown) in epithelial cells (TIF) of proximal (A, B, C) and distal (ventral, D, E, F, and dorsolateral, C, F, I, L) regions of prostatic ducts of age-matched SypELDTA (147, A-C, G-I) and PB-Cre4; SypELDTA (API47, D-F, J-L) mice. ABC Elite method. Hematoxylin counterstaining. Calibration bar: 50 μm (A-L).

**Figure S12** No significant cell death is observed in prostate epithelium non-NE cells in PB-Cre4; SypELDTA mice. (A-F) cleaved Caspase-3 expression in proximal (A, B), and distal (ventral, C, D, and dorsolateral, E, F) regions of prostatic ducts in age-matched SypELDTA (API47, A, C, E) and PB-Cre4; SypELDTA (API47, B, D, F) mice. The staining of embryonic dorsal root ganglia (Figure 5) served as a positive control for cleaved Caspase-3 immunostaining. ABC Elite method. Hematoxylin counterstaining. Calibration bar: 50 μm (A-F).

**Materials and Methods S1.** Detailed protocol descriptions for generation of the targeting construct and BAC recombineering, genotyping, cell culture experiments, transgene copy number quantification, antibody sources and dilutions of immunohistochemical analyses, and microdissection-polymerase chain reaction. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: AYN CYC. Performed the experiments: CYC ZZ. Analyzed the data: AYN CYC ZZ. Contributed reagents/materials/analysis tools: AYN CYC ZZ. Wrote the paper: AYN CYC.

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