# The nucleolar protein PES1 is a marker of neuroblastoma outcome and is associated with neuroblastoma differentiation

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**Summary**

Neuroblastoma (NB) is a childhood malignant tumor that arises from precursor cells of the sympathetic nervous system. Spontaneous regression is a phenomenon unique to NBs and is caused by differentiation of tumor cells. PES1 is a multifunctional protein with roles in both neural development and ribosome biogenesis. Various kinds of models have revealed the significance of PES1 in neurodevelopment. However, the roles of PES1 in NB tumorigenesis and differentiation have remained unknown. Here we demonstrated that NB cases with *MYCN* amplification and clinically unfavorable stage (INSS stage 4~~, but not 4S~~) express higher levels of *PES1*. High *PES1* expression was associated with worse overall and relapse-free survival. In NB cell lines, *PES1*-knockdown suppressed tumor cell growth and induced apoptosis. This growth inhibition was associated with the expression of NB differentiation markers. On the other hand, when the differentiation of NB cell lines was induced by the use of all-trans retinoic acid, there was a corresponding decrease in *PES1* expression. *Pes1* expression of tumorspheres originated from *MYCN* transgenic mice also diminished after the induction of differentiation with growth factors. We also reanalyzed the distribution of PES1 in the nucleolus. PES1 was localized in the dense fibrillar component, but not in the granular component of nucleoli. After treatment with the DNA-damaging agent camptothecin, this distribution was dramatically changed to diffuse nucleoplasmic. These data suggest that PES1 is a marker of NB outcome, regulates NB cell proliferation and is associated with NB

differentiation.

## Introduction

Neuroblastoma (NB) is the most common extracranial solid malignant tumor of childhood and is derived from precursor cells of the sympathetic nervous system (1). Unlike in the case of other malignant tumors, a subset of patients with NBs show spontaneous regression without any treatment. This phenomenon is caused by differentiation and programmed cell death of tumor cells (2). Differentiation induction and proliferation inhibition with 13-cis-retinoic acid is thus one of the effective therapies for high-risk NBs (3). *MYCN* is a well-known driver gene of NBs. Twenty to thirty percent of NB cases exhibit *MYCN* amplification and poor prognosis (4). Although MYCN, which has a helix-loop-helix domain and belongs to the MYC family of transcription factors, activates the transcription of various kinds of growth signals, recent data show that MYCN is also required for the differentiation program (5).

The gene *PES1* (and its zebrafish ortholog pes) is highly conserved from yeast to humans. *PES1* was originally identified in zebrafish in an insertional mutagenesis screen. *PES1* mutant embryos exhibited brains with reduced volumes and other developmental abnormalities (6). *PES1* mutant overexpression disrupted oligodendrocyte development, oligodendrocyte migration and axon extension (7). In the developing *Xenopus laevis*, *pes1* is expressed in the migrating cranial neural crest cells. Inhibition of *pes1* using morpholino

oligonucleotide caused neural crest migration impairment (8). In developing mice, *Pes1* displayed a distinct spatial and temporal pattern of gene expression with high levels in the germinal zone and other specific brain regions that contain neural progenitor cells and postmitotic neurons (9). In addition to these neurodevelopmental roles, PES1 plays a crucial part in ribosome neogenesis (10, 11), particularly in pre-ribosomal RNA (pre-rRNA) processing. In interphase cells, PES1 is localized in the nucleolus, an intranuclear body where ribosome neogenesis takes place (12, 13). Previous reports revealed that *PES1* was upregulated in glioblastoma, breast cancer and head and neck squamous cell carcinoma (9, 14, 15). The close relationship between ribosome biogenesis and carcinogenesis could be the reason for these up-regulations. *PES1*-knockdown has been shown to inhibit the proliferation and tumorigenicity of breast cancer cell lines (16).

As described above, PES1 has two important roles, namely neural development and oncogenesis. These two key processes are themselves characteristics of NBs. Therefore in the current study we investigated the relationship between PES1 and NBs. We show here that *PES1* is a prognostic marker of NBs and regulates NB proliferation and apoptosis. Reduced *PES1* expression is associated with NB differentiation in NB cell lines and tumorsphere models. In addition, our analysis with confocal microscopy demonstrated that PES1 is distributed in the dense fibrillar component (DFC) in the nucleolus, and its distribution is radically changed after treatment with the DNA-damaging agent camptothecin (CPT).

## Material and Methods

### Mice

The *MYCN* transgenic (Tg) mice (17) were maintained in the animal facility at Nagoya University Graduate School of Medicine, where they were housed in a controlled environment and provided with standard nourishment and water. Normal ganglia and precancerous and tumor tissues from wild-type (WT), hemizygous, and homozygous *MYCN* Tg mice were dissected and minced, and then total RNA was extracted. This study was approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine, Nagoya, Japan.

### Gene expression profiling

Gene expression profiling was carried out as previously described (18). Samples were analyzed with a GeneChip Mouse Genome 430 2.0 array (Affymetrix). The data have been deposited in the Gene Expression Omnibus database of NCBI (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE43419.

### Human neuroblastoma tumor profile

Kaplan-Meier analysis of the prognosis of NB patients was performed on the R2 website (http://r2.amc.nl). The dataset was from the AMC cohort study of 88 NB patients (GSE16476).

This dataset was profiled on the Affymetrix HGU133 plus2.0 platform and normalized using the MASS5.0 algorithm.

**Cell culture**

The human neuroblastoma cell lines NB39 and TNB1 were obtained from RIKEN Cell Bank (Tsukuba, Japan). SH-EP and *MYCN*-inducible SH-EP cell line (SH-EP$^{MYCN}$) cells were gifts from Dr. Schwab of the Division of Tumor Genetics, German Cancer Research Center (19). They were cultured with Dulbecco's Modified Eagle's Medium (D5796; Sigma-Aldrich) or RPMI-1640 (R8758; Sigma-Aldrich) or a mixture of some other media following the manufacturer's instructions. Induction of *MYCN* was regulated by removal of tetracycline (1μg/ml). The media were supplied with 10% heat-inactivated FBS in an incubator with humidified air at 37°C with 5% $CO_2$. They were routinely authenticated on the basis of their viability, growth rate, and morphology by microscopic examination.

**RNA interference**

To deliver short hairpin RNA (shRNA) expression construct into NB cell lines, commercially available lentiviral shRNA vectors were used. Nontargeting shRNA (Sigma) and specific shRNAs for *PES1* (Open Biosystems) were used. The shRNA sequences are listed in Table S1.

## Plasmids and their transfection

Human *MYCN* construct was generated by PCR using the Gateway recombination system. *MYCN* construct was cloned into CSII-CMV-RfA-IRES-venus plasmid. This plasmid and CSII-CMV-venus control plasmid were kindly provided by Dr. Miyoshi of RIKEN. For producing lentiviral particles, HEK293T cells were co-transfected with shRNA plasmids or overexpression plasmid in addition to psPAX2 (Addgene) and pMD2.G (Addgene) plasmids using FuGENE HD reagent (Promega) according to the manufacturer's instructions. The neuroblastoma cell lines were infected in the presence of 8 μg/ml polybrene (Sigma).

## Quantitative reverse transcription PCR

RNA extraction from cultured cells and tumorspheres, and quantitative reverse transcription PCR (qRT-PCR) were carried out as previously described (20). Quantitative PCR analyses were performed using an MX3000P or MX3005P Real-time QPCR System (Agilent). qPCR was carried out with ReverTra Ace (Toyobo) and THUNDERBIRD SYBR qPCR Mix (Toyobo). The relative expression levels were acquired according to the manufacturer's instructions. The primers used are listed in Table S2.

## Immunocytochemistry

Immunocytochemistry was carried out as previously described using the following primary antibodies: rabbit anti-Pescadillo (A300-902A; Bethyl Laboratories, Montgomery, TX), mouse anti-Pescadillo (#ab88543; Abcam), rabbit anti-Nucleolin (#ab22758; Abcam), mouse anti-phospho-Histone H2AX (Ser139) (#05-636; Millipore), and mouse anti-neuronal class III β-Tubulin (tuj1) (MMS-435p; Covance). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG were used as secondary antibodies. Hoechst 33258 was used to visualize nuclei.

**Primary culture of tumorspheres from the primary tumors of *MYCN* transgenic mice**

The procedure was carried out as previously described (21). Briefly, dissected tumor tissue was minced and digested with 0.25% trypsin (Sigma). To obtain tumorspheres from the primary tumors, the cells were suspended in DMEM/F12ham (Sigma) plus EGF 10 ng/ml, bFGF 15 ng/ml (Peprotech), 2% B27 supplement, 1% penicillin/streptomycin (PS; GIBCO), 15% FBS (Hyclone), 1% non-essential amino acid (NEAA), 1% sodium pyruvate and 55 μM beta-mercaptoethanol. Cells were cultured in a nontreated Petri dish in a 37°C, 5% $CO_2$ tissue culture incubator. To induce differentiation, spheres were collected and re-suspended with medium containing DMEM/F12ham, 1%FBS, 2% B27 supplement, 1% sodium pyruvate, $10^3$ units/ml LIF, and 1% PS. The spheres were seeded into a poly-D-lysine/laminin/fibronectin pre-coated dish. After 12 hours the medium was changed to

differentiation medium: DMEM/F12 Ham, 1%FBS, 2% B27 supplement, 1% N2 supplement, 50 ng/ml nerve growth factor (NGF), 50 ng/ml neurotrophin-3(NT3) and 1% PS.

**TUNEL assay**

The TUNEL assay was performed using the APO-DIRECT kit (556381; BD Pharmingen). Cell fixation and staining was performed following the manufacturer's instruction. The cells were analyzed with BD FACS Calibur (BD Pharmingen) and BD CellQuest Pro software (BD Biosciences).

**Data analysis**

Results are expressed as the mean +-SD. Their homoscedasticities were checked by *f* test. Statistical significance was evaluated with a 2-tailed, unpaired *t* Student's *t*-test.

## Results

### *PES1* expression was increased with NB tumor development in the *MYCN* Tg mouse model

To examine the relationship between NB formation and *PES1* expression, we utilized a *MYCN* transgenic (Tg) mouse model (17). In this model, human *MYCN* is overexpressed under the tyrosine hydroxylase promoter, which is active in migrating neural crest cells. At 2 weeks of age, the superior mesenteric ganglion (SMG) of wild-type (wt) mice and hemizygous or homozygous *MYCN* Tg mice are similar in size. However, compared to those of 2-week-old wt mice, the SMGs of 2-week-old homozygote *MYCN* Tg mice exhibit accumulation of immature neuroblasts microscopically. These SMGs can be regarded as “precancerous” or “hyperplastic” lesions (18, 20, 22, 23). Finally, at age 6-14 weeks, 100% (for homozygotes) or about 70% (hemizygotes) of Tg mice develop NB and die. Histology of tumors of both hemizygous and homozygous *MYCN* Tg mice are similar and equivalent to human neuroblastomas (23). We first examined the expression profiles of the SMGs of 2-week-old wt mice, the SMGs of 2-week-old homozygous *MYCN* Tg mice, and the terminal tumors of 6-week-old hemizygous or homozygous *MYCN* Tg mice (GSE43419). The precancerous lesions (2-week homozygous MYCN Tg mice SMG) showed higher *Pes1* expression than the normal ganglia, and the terminal tumors of hemizygous or homozygous *MYCN* Tg mice showed even higher *Pes1* expression than the precancerous ganglia or wt ganglia (Fig. 1).

This suggests that *Pes1* expression increased as the NBs grew in the *MYCN* Tg mouse model.

### ***PES1* expression was associated with poor prognosis of NB**

We then investigated *PES1* expression in human NB cases. For this analysis we used the database from an AMC cohort study of 88 NB patients (GSE16476) and the R2 genomics analysis and visualization platform. Expression of *PES1* was significantly higher in the *MYCN*-amplified cases ($p=6.9 \times 10^{-10}$; Fig. 2a). *MYCN* amplification has been associated with rapid disease progression and unfavorable prognosis (4). Since *PES1* promotor region has an E-box, the MYCN binding sequence, we examined whether *MYCN* induction enhance *PES1* expression. *MYCN* induction in SH-EP$^{MYCN}$ (19), SY5Y and SH-EP cell lines significantly increased *PES1* expression (Supplementary Fig. S1). This result was consistent with Fig. 2a. Tumor stage is also an important prognostic factor of NBs. NBs were previously classified based on the International Neuroblastoma Staging System (INSS) (24). Although this classification is widely accepted, in 2009, the International Neuroblastoma Risk Group (INRG) proposed a new staging system (25). Both classifications include the designations 4S or MS, in addition to 4 and M, which indicate metastatic diseases. The S is short for “special,” and indeed the 4S and MS stages are unique to NBs. 4S (or MS) cases show favorable prognosis and spontaneous regression in spite of multiple metastasis of the skin, liver or bone

marrow. We investigated the *PES1* expression in each of the INSS stages using the same cohort. Stage 4 cases exhibited higher levels of *PES1* expression than stage 1-3 cases (p=0.008; Fig. 2b). In addition, ~~Surprisingly,~~ compared to stage 4S cases, stage4 cases also exhibited significantly higher *PES1* expression (p=0.016; Fig. 2b). The correlation of *PES1* expression with *MYCN* status or clinical stage raised the possibility that *PES1* may be a good prognostic marker. We therefore analyzed the prognostic value of *PES1* expression using Kaplan-Meier survival curves. High *PES1* expression was significantly associated with poorer overall survival ($p=8.4 \times 10^{-5}$ after Bonferroni correction; Fig. 2c) and relapse-free survival ($p=5.4 \times 10^{-3}$ after Bonferroni correction; Fig. 2d). Previous reports have demonstrated that malignant tumors express higher *PES1* levels than normal tissues or benign tumors in other types of cancers (9, 14-16, 26). In line with previous findings, these results indicate that *PES1* expression is correlated with *MYCN* status and clinical stage, and that *PES1* is a prognostic marker of NBs. ~~However, the difference in *PES1* expressions between the INSS 4 and INSS 4S cases suggests that *PES1* has additional activities in NBs, which will be addressed later.~~

**_PES1_ knockdown suppressed NB cell growth and induced apoptosis**

Since NB cases with high *PES1* expression are associated with unfavorable prognosis, we next investigated the role of PES1 in NB cell lines. We knocked down *PES1* expression using two different shRNAs against human *PES1*. The shRNAs efficiently knocked down *PES1*

expression at both the mRNA and protein level (Fig. 3a,b). shRNAs suppressed the growth of both a *MYCN*-amplified cell line (NB39) and a *MYCN* non-amplified cell line (SH-EP) (Fig. 3c,d). We next performed a TUNEL assay to examine whether these growth suppressions were caused by apoptosis. Compared to non-targeting shRNA, shRNAs against *PES1* dramatically increased the percentage of TUNEL-positive cells (Fig. 3e, f). These data suggest that *PES1* knockdown inhibited NB cell growth and this inhibition was caused by apoptosis.

As described in the Introduction, tumor cell differentiation is both a characteristic and an important treatment strategy of NB (3). Accordingly, we next investigated whether *PES1* knockdown cause the differentiation of NB cells. Differentiated cells possess more and longer neurites and express higher levels of differentiation markers (GAP43, RET) (27). Although *PES1* knockdown did not induce clear increase in neurite outgrowth (data not shown), the results of qPCR showed an increase in the expression of differentiation-related genes (Fig. 3g). Since the cell shape was not remarkably changed, we could not conclude that *PES1* knockdown caused the differentiation. But this result raised the possibility that *PES1* expression is associated with differentiation status.

**Differentiation induction in the NB cell lines decreased *PES1* expression**

We then examined whether the induction of differentiation can change the *PES1* expression.

Historically, various methods have been used to induce NB differentiation, including serum starvation and treatment with all-trans retinoic acid (ATRA) (28-31). ATRA, a derivative of vitamin A, is essential for development and differentiation. ATRA-induced differentiation of leukemic cells has been shown to dramatically improve the prognosis of patients with acute promyelocytic leukemia (32). ATRA can also differentiate NB cells. We induced differentiation in NB cell lines with serum reduction (from 10% fetal bovine serum in original culture to 1%) and ATRA (1 μM). This treatment increased the number and length of the neuritic extensions of NB39 and TNB1 cells (Fig. 4a; Supplementary Fig. S2), and these neurites were positive for class III beta-tubulin (Tuj1), a neuronal marker. This differentiation induction was further verified by the increased expression of differentiation-related gene (Fig. 4b; Supplementary Fig. S2) (33). Interestingly, the differentiation induction decreased *PES1* expression (Fig. 4b; Supplementary Fig. S2). This result suggests the possibility that ATRA-induced differentiation modulates *PES1* expression.

**Differentiation induction in the mouse NB model also decreased *PES1* expression**

To further verify the association of *PES1* with tumor cell differentiation, we tried another NB model. We used tumorspheres derived from an *MYCN* Tg mouse primary tumor (21). Differentiation induction with NGF, NT3 and N2 supplement caused radial neurite outgrowth (Fig. 5a). Concurrently, the expressions of differentiation marker (Tuj1) increased and

stemness markers (Snail and Musashi) decreased (Fig. 5b). In line with the results in the NB cell lines, *Pes1* expression was reduced with the differentiation induction (Fig. 5b). These results suggest that *Pes1* expression was negatively correlated with the differentiation conditions in two kinds of different NB models.

**PES1 was localized in the dense fibrillar component in the nucleolus and DNA damage induced redistribution.**

As previously reported, PES1 was localized in nucleolus in the nucleus, where ribosome neogenesis takes place (9, 11). Since one of the major roles of PES1 is 32S pre-ribosomal RNA (pre-rRNA) processing (10, 34), this distribution is reasonable. The nucleolus consists of three distinct subnucleolar compartments called the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC) from inside to outside (35), and ribosome maturation also proceeds from inside to outside. A previous analysis using tagged PES1 showed its GC distribution (11). However, we found that endogenous PES1 is surrounded by nucleolin, a major nucleolar protein which is found in DFC and GC (36), with nucleolin showing “donut-like” fluorescence (Fig. 6a). Considering the fact that pre-rRNA resides in the DFC, this result suggest that PES1 is mainly localized in DFC of nucleolus, not in GC.

In addition to its principal role, ribosome neogenesis, nucleolus also works as a stress sensor

of DNA damage (37, 38). Previous report found that *PES1* knockdown induced the expression of phosphorylated H2AX (γH2AX), a DNA damage marker, and delayed the recovery from DNA damage (39). The authors indicated some association between PES1 and DNA damage response, but the relationship is still unclear. Therefore, we next investigated the dynamics of PES1 under DNA damage. Camptothecin (CPT) is a topoisomerase-1 inhibitor that causes DNA damage and is used to treat cancers. (37, 40). In the interphase TNB1 cells, PES1 is located in the nucleolus. After treatment with CPT (1 μM) for 5hours, γH2AX was expressed strongly in the nucleus and formed γH2AX foci (41) (Fig. 6b). PES1, together with nucleolin, exhibited a radical change in distribution to a diffuse nucleoplasmic distribution (Fig. 6b, c). Since DNA-damaging agents are known to cause nucleolar disruption (42), this result was not surprising. The reason and its biological significance of this distribution change remains to be investigated. This result suggests that DNA damage can redistribute PES1 as well as nucleolin. PES1 expression partially, but not completely, overlapped with γH2AX (Fig. 6d).

## Discussion

The present study addressed the roles of PES1 in NB tumorigenesis by using a clinical NB database of mRNA expression and survival, human NB cell lines, an NB model in MYCN Tg mice, and tumorspheres derived from *MYCN* Tg mice. *MYCN* Tg mice are a reliable NB model that recapitulates the clinical features of NB, histology, and molecular changes (17, 43). Our study revealed that PES1 is involved in NB cell growth, survival and differentiation. Its high expression is associated with poor prognosis. *PES1* knockdown suppresses NB tumor cell growth and induces apoptosis. In their differentiated state, the NB cell lines and tumorspheres exhibit lower *PES1* expression. On the other hand, *PES1* knockdown induce differentiation features. In addition, we found PES1 distribution in DFC in the nucleolus and diffuse redistribution into the nucleoplasm after CPT treatment.

Our study demonstrated that *PES1* is a prognostic marker of NBs. NB cases with unfavorable prognosis (*MYCN* amplified or INSS 4) exhibit high *PES1* expression. Previous reports indicated that cancerous tissue expressed more *PES1* than normal tissue or benign tumors (9, 14-16, 26). However, these results were based on the results of tissue microarray or cell lines. We showed the relationship between *PES1* expression and prognosis in NBs in this study for the first time. ~~The fact that *PES1* expression is higher in patients with INSS stage 4 NB than those with INSS 4S disease suggests that *PES1* expression is more highly correlated to prognosis than to metastatic status (INSS 4S is characterized by multiple~~

~~metastases of skin, liver or bone marrow)~~ As previously shown in colon cancer cell lines (26), PES1 knockdown strikingly suppressed NB cell growth in our study.

We showed that *PES1* knockdown upregulated some differentiation-related genes. To further elucidate the relationship between *PES1* expression and differentiation, we used two different kinds of models to induce NB differentiation. One is ATRA treatment of NB cell lines, and the other is tumorsphere differentiation with growth factors. We previously reported a new tumorsphere culture condition (21), and found that this condition worked well in the present experimental scheme. In both models, we successfully induced differentiation as demonstrated by morphology change (neurite growth), and differentiation gene expression. Under these conditions, *PES1* was downregulated. However, we could not successfully rescue these phenomena by *PES1* overexpression (data not shown). These results suggest that PES1 is required but not sufficient to suppress NB cell differentiation.

We also reanalyzed the PES1 distribution in the nucleoli using confocal microscopy. In our analysis, PES1 was encircled by nucleolin. Nucleolin and nucleophosmin are the two major protein components of the nucleolus. Previous reports have described multiple functions of nucleolin from rRNA splicing to ribosome assembly. Our results indicate that PES1 is mainly distributed in DFC, but not GC, while nucleolin is localized in DFC and GC. Since DFC is an rRNA-rich lesion in nucleoli, the DFC distribution of PES1 makes sense. We also observed a dynamic change of PES1 distribution after treatment with the DNA-damaging

agent CPT. The relationship between PES1 and DNA damage response is still unclear. One previous study indicated that PES1 works protectively against DNA damage and PES1 knockdown induces γH2AX expression (39). From our results and previous knowledge, we can hypothesize that PES1 knockdown cause DNA damage and accumulated DNA damage drives cells to apoptosis.

Another interesting fact is that PES1 has been known to have a BRCA1 C-terminal domain (BRCT domain)(12). BRCT domains is a phosphoprotein binding domain and an integral signaling module in the DNA damage response(44). Since the role or binding partners of the BRCT domain of PES1 is not known, we investigated the binding partners of PES1 using Liquid chromatography–mass spectrometry with peptide mass fingerprinting (LC-MS/MS). At this stage, NONO and SFPQ are among the list of identified proteins as binding partners (data not shown). These proteins form heterodimer and enhance DNA strand break rejoining (45, 46). These results also suggested the possible close association of PES1 with DNA damage. But we need further study to elucidate the role of PES1 in DNA damage response. ~~Our results highlighted the close association of PES1 with DNA damage induced by an anticancer drug.~~

PES1 is a multifunctional protein. In this article, we also described multiple functions of this protein. Classical and the best known role of PES1 is ribosomegenesis (10, 11). Since ribosome is an essential intracellular organelle to protein synthesis, its dysfunction cause

severe cellular disturbance. The growth inhibitions and apoptosis observed in our study (Fig. 3) might be caused by this ribosomal dysfunction. In addition, as mentioned above, DNA damage caused by PES1 knockdown could also be the cause of apoptosis and growth inhibitions. Arrest of cell division is a prerequisite to enter a program of differentiation (47). Therefore, the phenotypes related to differentiation (Fig. 3-5) are closely linked to growth inhibition.

In summary, we demonstrated that PES1 is a good prognostic marker of NBs. As a multifunctional protein, PES1 is involved in NB cell survival and differentiation. DNA damage might contribute to apoptosis regulation in NB cells.

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**Disclosure Statement**

The authors have no conflicts of interest.

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**Figure legends:**

**Fig. 1.** *Pes1* expression in NB mice model. Results of a microarray analysis of the relative expression levels of *Pes1* in superior mesenteric ganglia of wild-type mice, 2-week homozygous *MYCN* Tg mice (precancerous lesions) and terminal tumors (hemizygous and homozygous *MYCN* Tg mice). wt, wild-type mice; homo, homozygous *MYCN* Tg mice; hemi, hemizygous *MYCN* Tg mice.

**Fig. 2.** *PES1* expression in human NB cases and patient prognosis (AMC cohort, GSE16476). (a) *PES1* expression in *MYCN* non-amplified/ amplified cases (n=72, 16 each). (b) *PES1* expression in each INSS stages (INSS 1-3: n=36, INSS 4: n=40, INSS 4S: n=12). (c, d) Kaplan-Meier curves and log-rank test for the overall survival and relapse free survival (c, d respectively).

**Fig. 3.** *PES1* knockdown inhibited NB cell growth and induced apoptosis. (a, b) RT-qPCR analysis and Western blot analysis of PES1 expression in NB cell lines (NB39 and SH-EP) after shRNA treatments (n=3). (c, d) Cell proliferation analysis of NB cell lines after treatment of non-target shRNA or shRNA against *PES1* (NB39: n=4, SH-EP: n=3). (e, f) Quantification of TUNEL positive apoptotic cells. (n=3) (g) RT-qPCR analysis of differentiation-related gene expressions of NB39 (n=4). *p<0.05 non-target versus shRNA against *PES1* (Student's t test).

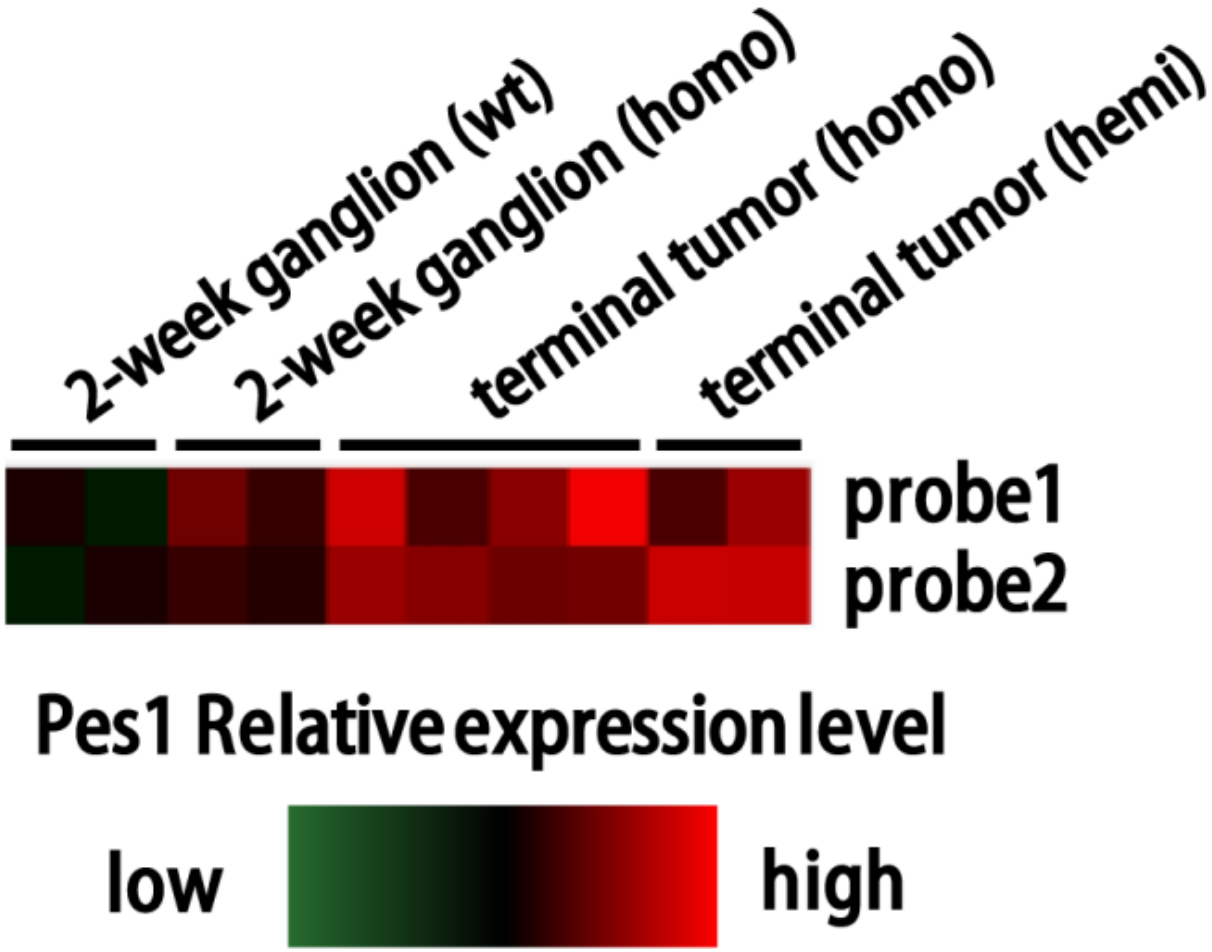
**Fig. 4.** Induced differentiation attenuated *PES1* expression in NB cell line. (a) Phase contrast images (top panels) and confocal image of fluorescence immunostaining for Tuj1 (red, bottom

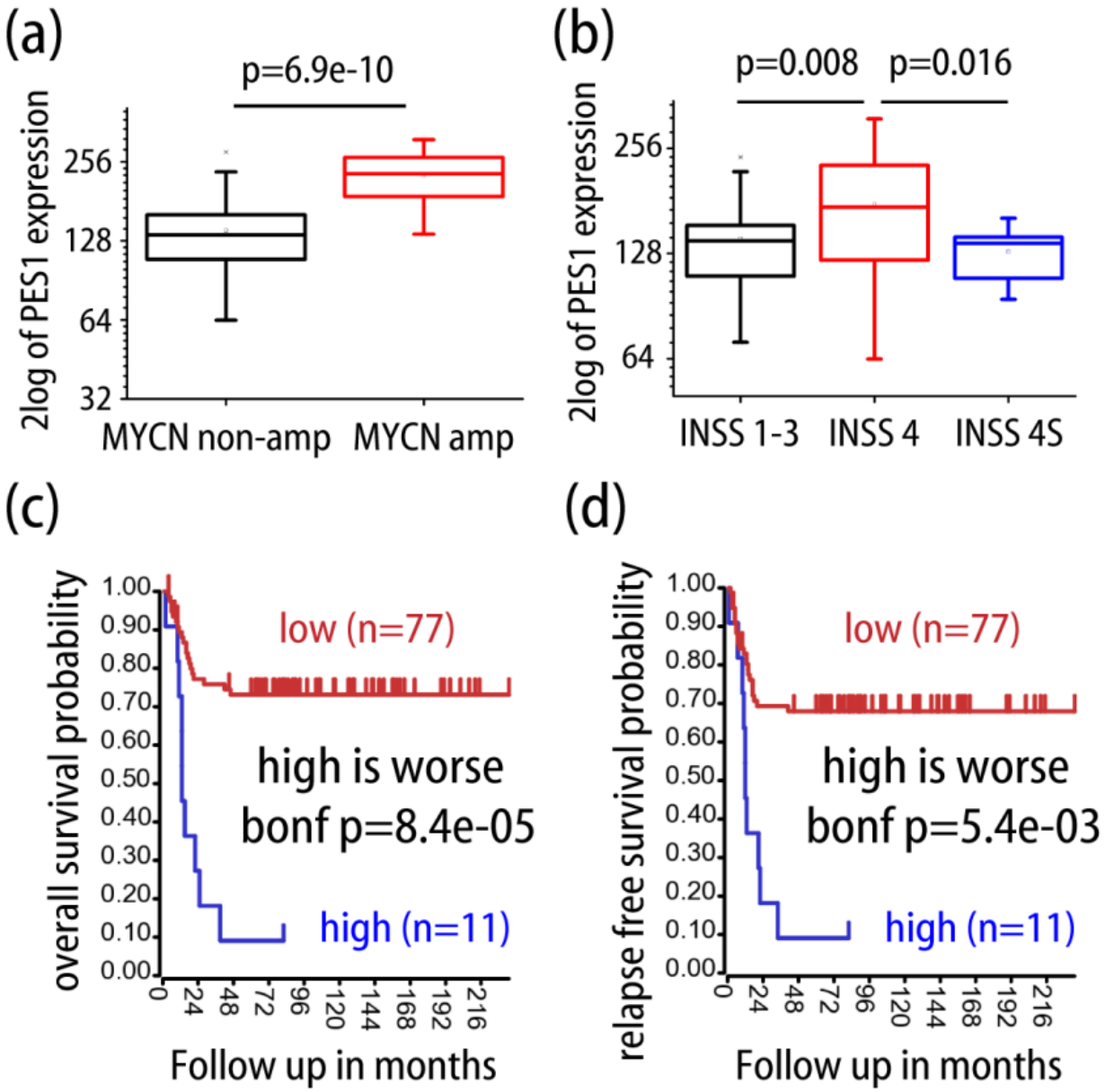
panels) of NB39 cells under normal culture condition (10% FBS) and differentiation condition (1% FBS + 1 μM ATRA). Hoechst 33258 was used to visualize nuclei. Scale bar, 100μm. (b) RT-qPCR analysis of differentiation-related gene (RET) and *PES1* expressions (n=4). *p<0.05 normal culture condition versus differentiation condition (Student's t test).

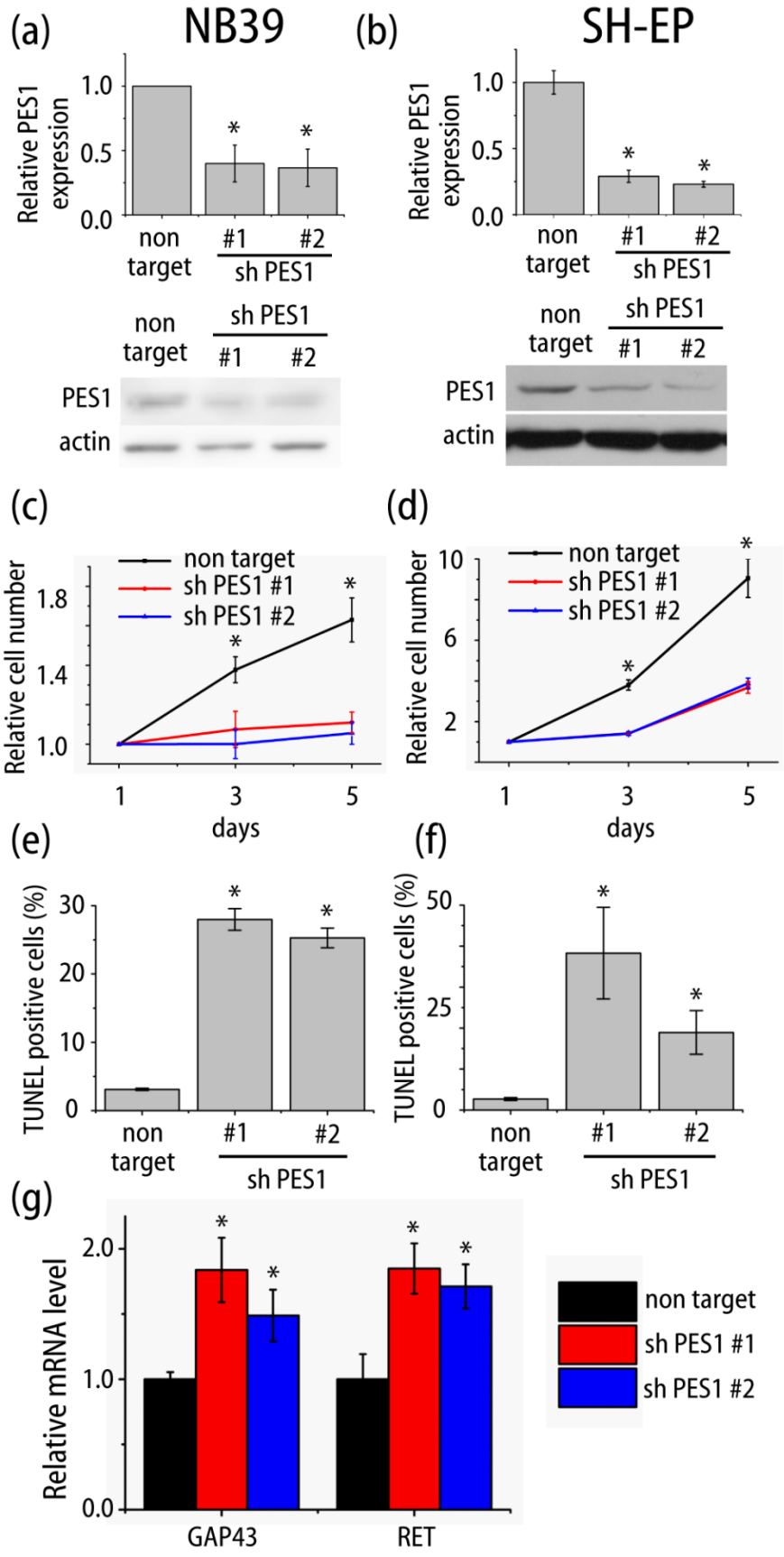
**Fig. 5.** Induced differentiation decreased *Pes1* expression in *MYCN* Tg mice model tumorsphere. (a) Phase contrast images of tumor spheres established from *MYCN* Tg mice primary tumor under normal culture condition and differentiation condition (NGF, NT3 addition). Scale bar, 100μm. (b) RT-qPCR analysis of differentiation (Tuj1) or stemness related gene (Snail and Musashi) and *Pes1* expressions (n=4). Only the values of differentiation condition are shown (normal culture condition = 1.0). The result is the average of four independently established tumorsphere samples. *p<0.05 normal culture condition versus differentiation condition (Student's t test).

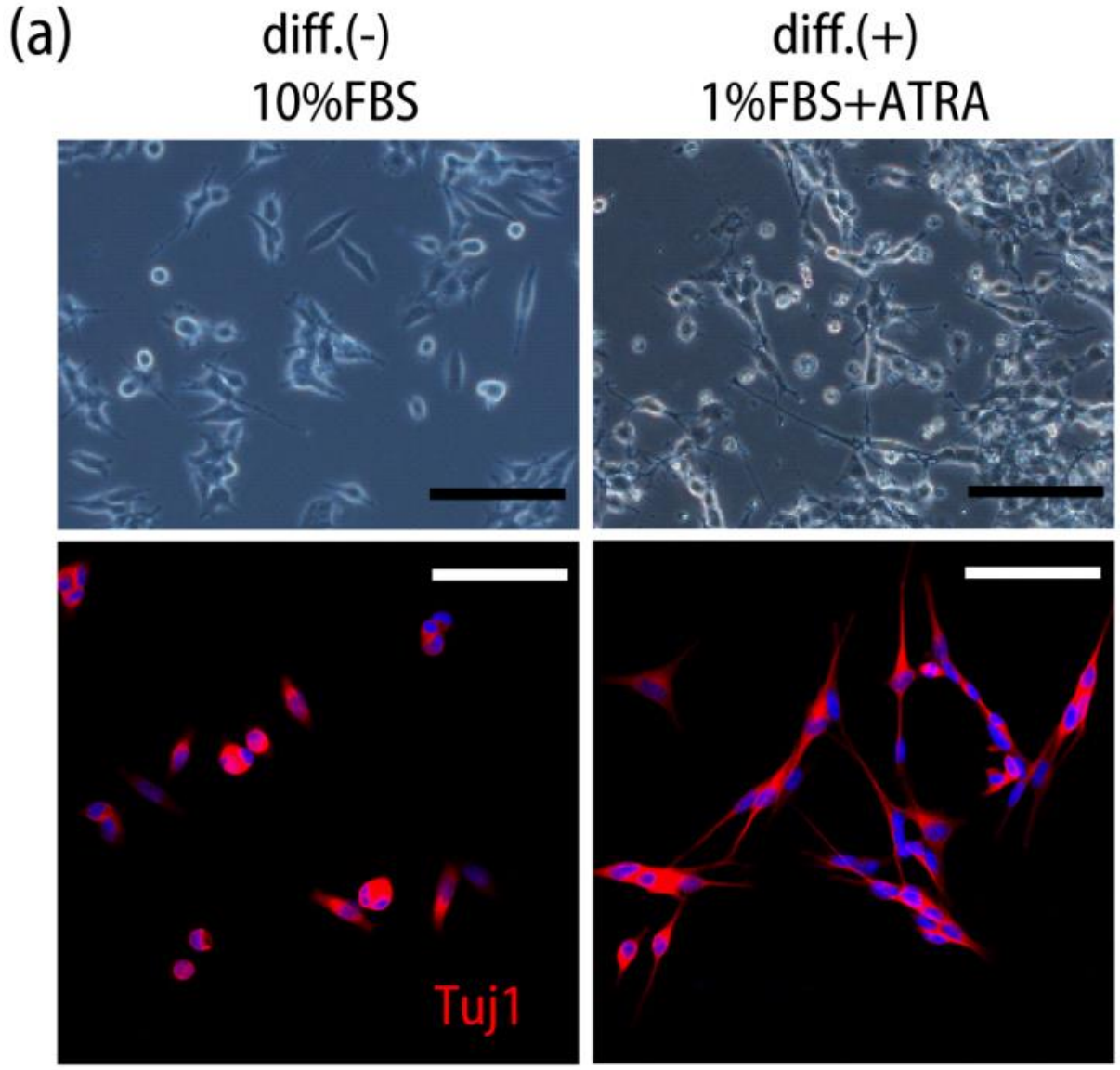
**Fig. 6.** Endogenous PES1 localization and its redistribution after DNA damage. (a) Confocal images of TNB1 cells immunostained for PES1 (green), Nucleolin (red) with nuclear stain (Hoechst33258, blue). PES1 is encircled by nucleolin, which shows 'donut-like' staining. (b, c) Confocal images of TNB1 cells after DMSO or CPT (1μM) treatment. Scale bar, 15μm. (d) Enlarged confocal images of TNB1 cells after CPT (1μM) treatment. One nucleus was enlarged.

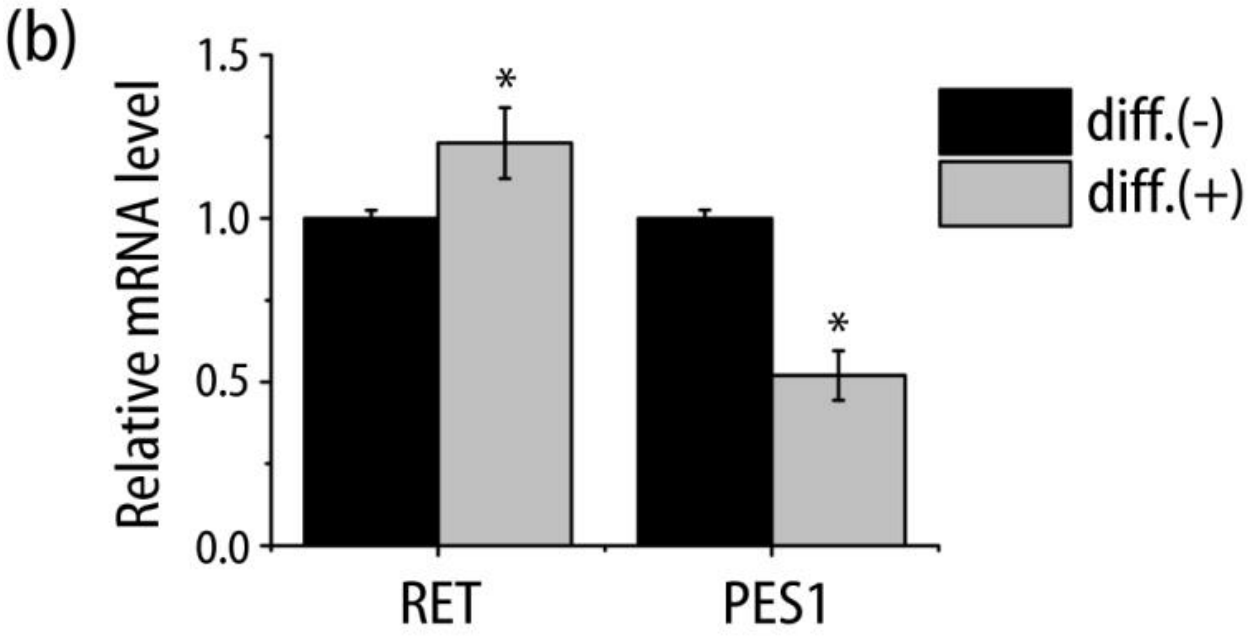
**List of Supporting Information**

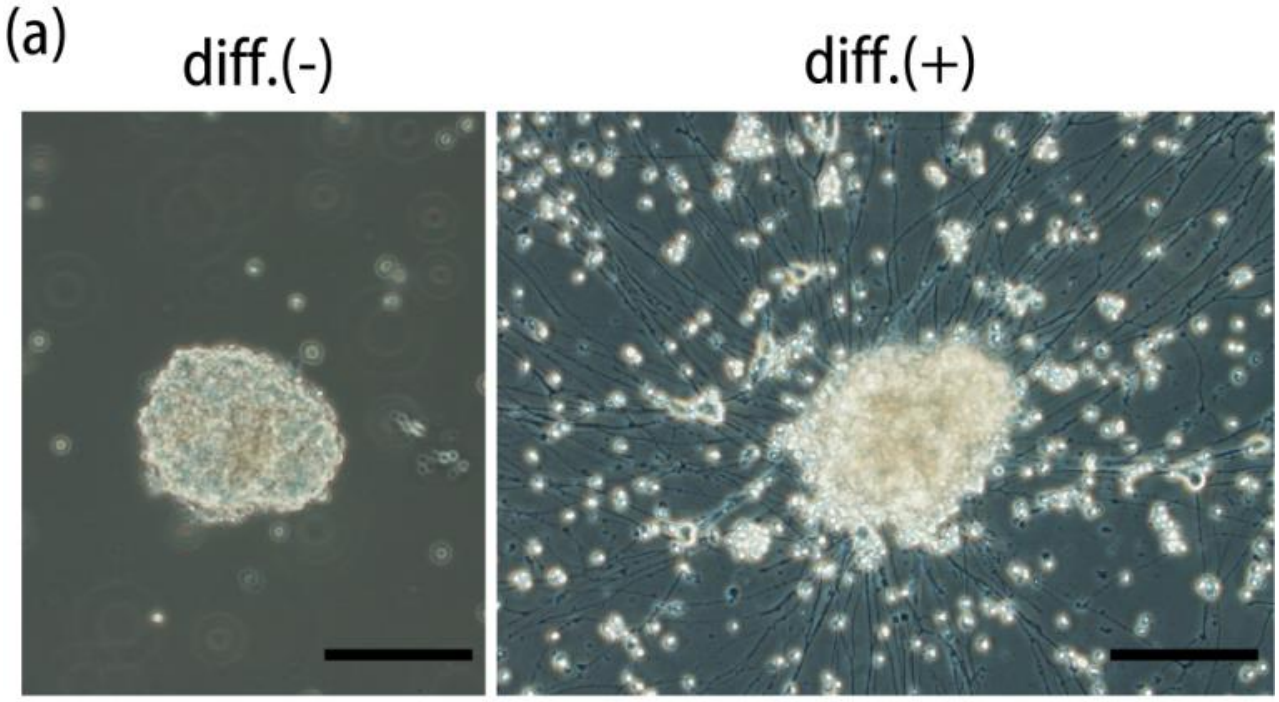

2-week ganglion (wt)
2-week ganglion (homo)
terminal tumor (homo)
terminal tumor (hemi)
probe1
probe2
Pes1 Relative expression level
low
high

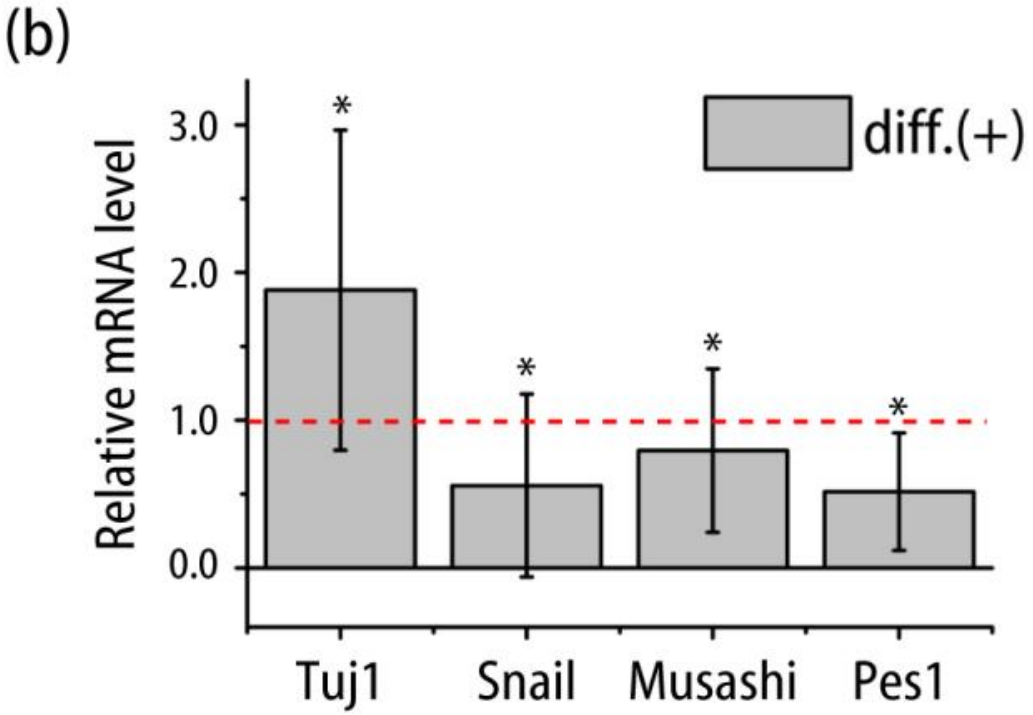

(a)
p=6.9e-10
2log of PES1 expression
256
128
64
32
MYCN non-amp
MYCN amp
(b)
p=0.008
p=0.016
2log of PES1 expression
256
128
64
INSS 1-3
INSS 4
INSS 4S
(c)
overall survival probability
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
low (n=77)
high is worse
bonf p=8.4e-05
high (n=11)
0
24
48
72
96
120
144
168
192
216
Follow up in months
(d)
relapse free survival probability
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
low (n=77)
high is worse
bonf p=5.4e-03
high (n=11)
0
24
48
72
96
120
144
168
192
216
Follow up in months

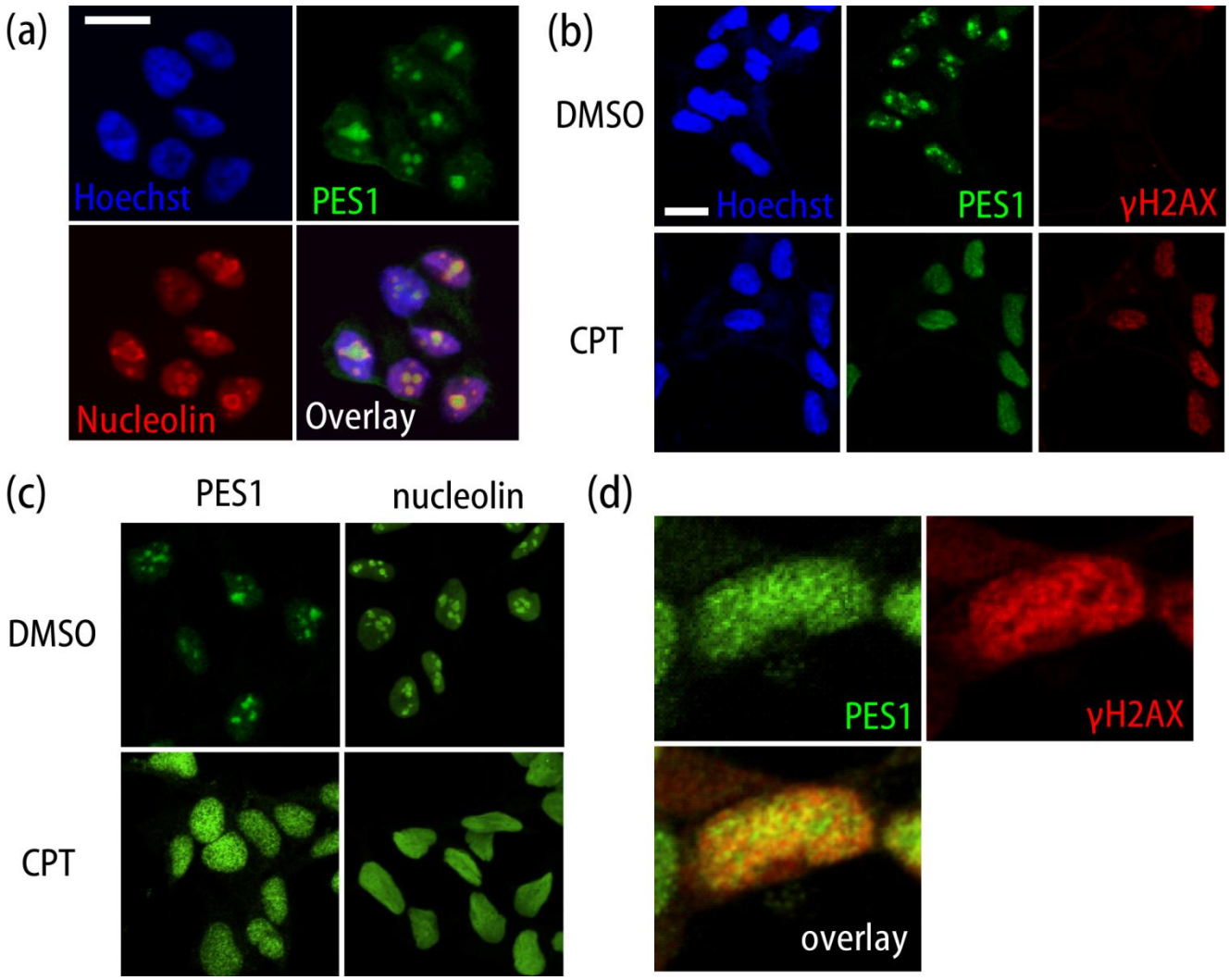

(a)
NB39
Relative PES1 expression
1.0
0.5
0.0
*
*
non target
#1
#2
sh PES1
non target
sh PES1
#1
#2
PES1
actin
(b)
SH-EP
Relative PES1 expression
1.0
0.5
0.0
*
*
non target
#1
#2
sh PES1
non target
sh PES1
#1
#2
PES1
actin
(c)
Relative cell number
1.8
1.4
1.0
non target
sh PES1 #1
sh PES1 #2
*
*
1
3
5
days
(d)
Relative cell number
10
6
2
non target
sh PES1 #1
sh PES1 #2
*
*
1
3
5
days
(e)
TUNEL positive cells (%)
30
20
10
0
*
*
non target
#1
#2
sh PES1
(f)
TUNEL positive cells (%)
50
25
0
*
*
non target
#1
#2
sh PES1
(g)
Relative mRNA level
2.0
1.0
0.0
*
*
*
*
GAP43
RET
non target
sh PES1 #1
sh PES1 #2

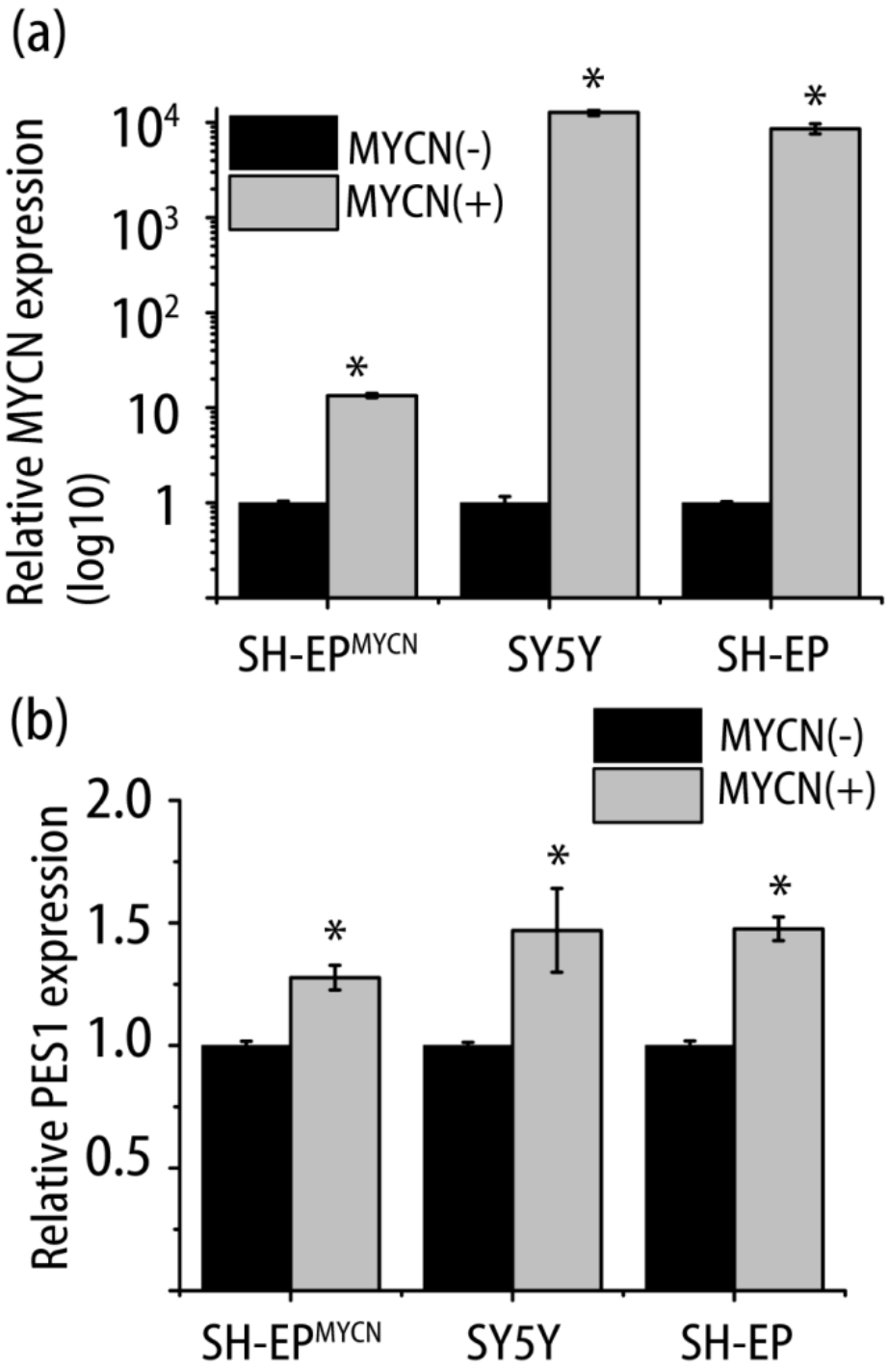

(a)
diff.(-)
10%FBS
diff.(+)
1%FBS+ATRA
Tuj1


(b)
Relative mRNA level
1.5
1.0
0.5
0.0
*
*
diff.(-)
diff.(+)
RET
PES1


(a)
diff.(-)
diff.(+)


(b)
Relative mRNA level
3.0
2.0
1.0
0.0
diff.(+)
*
*
*
*
Tuj1
Snail
Musashi
Pes1

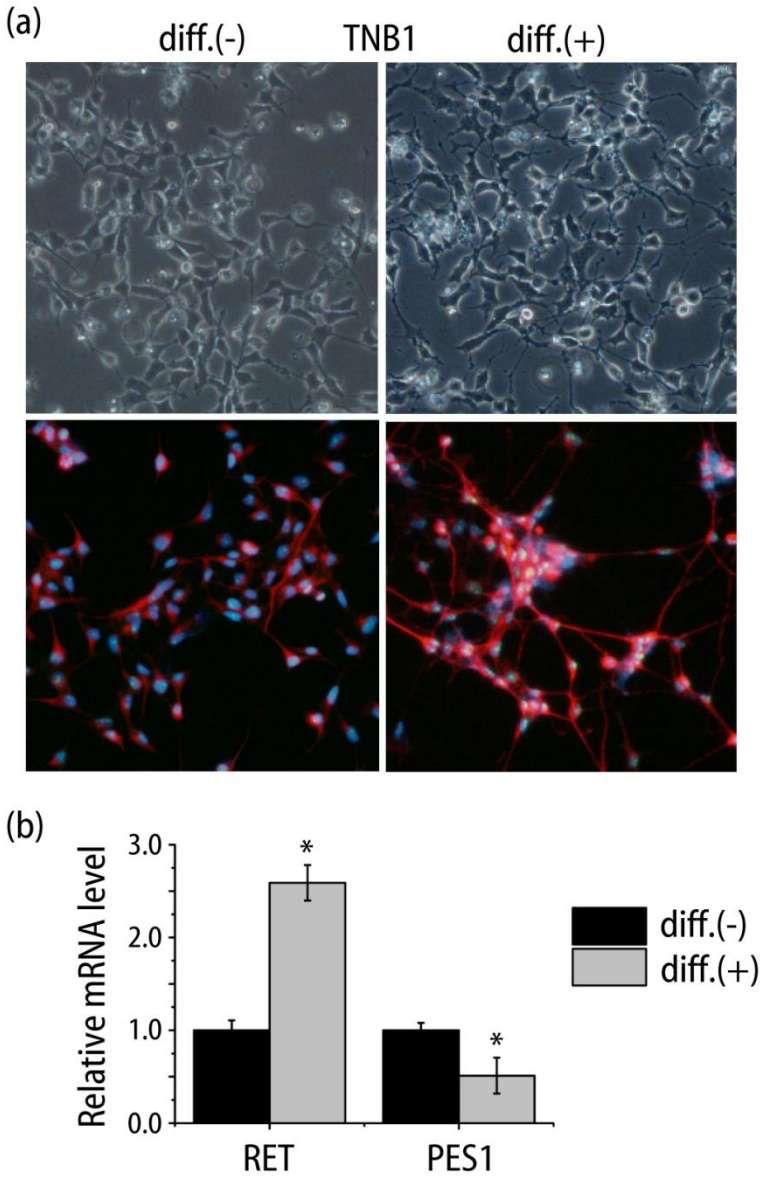

(a)
Hoechst
PES1
Nucleolin
Overlay
(b)
DMSO
CPT
Hoechst
PES1
γH2AX
(c)
PES1
nucleolin
DMSO
CPT
(d)
PES1
γH2AX
overlay




Supplementary Figure 1

MYCN induction in MYCN single-copy cell lines.

MYCN was induced in each cell lines using tetracycline-off system (SH-EP$^{MYCN}$) or MYCN over expression vector (SY5Y, SH-EP). RT-qPCR analysis of MYCN (a) and PES1 expressions (b) (n=3).







Supplementary Figure 2

(a) Phase contrast images (top panels) and fluorescence immunostaining for Hoechst 33258 (blue) and Tuj1 (red) (bottom panels) of TNB1 cells under normal culture condition (10% FBS) and differentiation condition (1% FBS + 1 $\mu$g/ml ATRA).
(b) RT-qPCR analysis of differentiation-related gene expressions (n=4).
(c) RT-qPCR analysis of PES1 expressions(n=4).
*$p<0.05$ normal culture condition versus differentiation condition (Student' s t test).

**Supplemental Table 1**. shRNA target sequences (PES1)

| | |
|---|---|
| shRNA1 | AAAGGCGGAAGTTGACAAAGC |
| shRNA2 | ATACCGTTCCTTGATGATGTG |

**Supplemental Table 2.** Primer sequences for PCR experiments

| | | |
|---|---|---|
| human GAPDH | Forward | ATCATCCCTGCCTCTACTGG |
| | Reverse | CCCTCCGACGCCTGCTTCAC |
| human PES1 | Forward | CATCACCCATCAGATTGTCG |
| | Reverse | AGCAGCTTCAGCTTCTCAGG |
| human GAP43 | Forward | AGTGAGCAAGCGAGCAGAA |
| | Reverse | GTTGCGGCCTTATGAGCTT |
| human RET | Forward | CATGGGCGACCTCATCTC |
| | Reverse | GAAATCCGAAATCTTCATCTTCC |
| mouse Gapdh | Forward | GGAGGCCATGTAGGCCATGA |
| | Reverse | GGTGGTGAAGCAGGCATCTG |
| mouse Pes1 | Forward | TCACCCACCAGCTTGTTGAC |
| | Reverse | GCTTCTCAGGGGGAATGTAA |
| mouse Snail | Forward | GAGGACAGTGGCAAAAGCTC |
| | Reverse | GGAGAATGGCTTCTCACCAG |
| mouse Musashi | Forward | ATGGTGGAATGCAAGAAAGC |
| | Reverse | TAGGTGTAACCAGGGGCAAG |
| mouse Tuj1 | Forward | TGAGGCCTCCTCTCACAAGT |
| | Reverse | AGCACTGTGTTGGCGTACAG |