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DUSP5 expression associates with poor prognosis in human neuroblastoma

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Abstract

Regulation of growth and differentiation of neuroblastoma (NB) cells is the rational of some maintenance therapies for high-risk NB. MAP kinase phosphatases (MKPs) are potential physiologic regulators of neuronal differentiation and survival, but their expression patterns in NB are scarcely known. Here, an expression analysis of the MKP family has been performed using human NB tumor samples and human NB cell lines (SH-SY5Y, SMS-KCNR, and IMR-32) undergoing retinoic acid (RA)-induced differentiation or subjected to stimuli that activate the MAPK ERK1/2 pathway. We have identified candidate MKPs that could modulate differentiation and growth of NB cells. pERK1/2 high expression correlated with high expression of the MKP DUSP5 in NB tumors, and was associated with poor prognosis. ERK1/2 activation on SH-SY5Y cells was accompanied by increased cell proliferation, and correlated with the expression levels of DUSP5. Accordingly, siRNA knock-down of DUSP5 augmented proliferation of SH-SY5Y cells. Our findings provide insights into the dynamic expression of MKPs in NB cells, disclose DUSP5 as a potential marker of NB poor prognosis, and suggest a role for DUSP5 in limiting ERK1/2-mediated NB proliferation.

Keywords: MAP kinase phosphatase; neuroblastoma differentiation; neuroblastoma proliferation; metastatic neuroblastoma
1. Introduction

Neuroblastoma (NB) is the most commonly diagnosed extracranial solid tumor in infants. It develops in the sympathetic nervous system, from simpatico adrenal lineage cells from the developing neural crest, and clinically manifests as primary tumors in the abdomen, cervix, thorax, and pelvis, which frequently progress to local- and distant site-metastasis (Maris et al., 2007). A property of NB is the frequent spontaneous regression of the low-risk tumors, which takes place in young children and is thought to represent a switch back of the tumor cells to the neuronal differentiation program (Brodeur and Bagatell, 2014; Mohlin et al., 2011). High-risk NB tumors are highly aggressive and currently incurable, constituting the cause of most of the cancer related deaths in infants. The major molecular marker of high-risk NB is the gene amplification of the transcription factor MYCN, which correlates with a neuronal undifferentiated molecular and morphological phenotype, and MYCN target genes are current under scrutiny for treatment of high-risk NB (Bassiri et al., 2015; Evageliou et al., 2016). Additional oncogenes and tumor suppressors have been involved in the etiology of high-risk NB, although their use in prognostic and patient stratification is still limited (Matthay et al., 2016; Navarro et al., 2012; Pinto et al., 2015). Importantly, recurrent RAS/MAPK pathway mutations have been found in relapsed NB tumors obtained after chemotherapy, highlighting the relation of MAPK activation with NB therapy resistance (Eleveld et al., 2015; Padovan-Merhar et al., 2016).

NB tumor differentiation can be recapitulated in vitro by NB cell lines grown in the presence of differentiating agents, including neurotrophic factors, phorbol esters, or the vitamin A-derivative retinoic acid (RA) (Edsjo et al., 2007). Importantly, RA compounds are used as a maintenance therapy for minimal residual disease in high-risk NB patients after surgery and chemo-, radio- and/or myeloablative therapy, although it remains uncertain the beneficial effects of RA for these patients (Peinemann et al., 2015; Reynolds et al., 2003). Many patients relapse after current therapies, making important therapeutic improvements, which include appropriate patient risk classification and more effective combination therapies (Cheung, 2015; Pinto et al., 2015). Major molecular alterations of NB cells after RA treatment include down-regulation of MYCN and upregulation of Trk receptors, associated with
activation of the ERK1/2 MAP kinases (MAPK) and PI3K/Akt pathways (Niles, 2004; Westermark et al., 2011). In NB cells, RA also downregulates the expression of ALK protein (Futami and Sakai, 2010), a receptor tyrosine kinase (RTK) that is mutated in the germline of familial NB patients as well as in NB tumors (Ogawa et al., 2011).

The extended protein tyrosine phosphatases (PTP) superfamily (PTPome) (Alonso and Pulido, 2016) includes redox-dependent phosphatases which are major regulators of tyrosine phosphorylation and play a relevant role in hereditary and non-hereditary human disease, including cancer and neurodevelopmental diseases (Hendriks et al., 2013; Hendriks and Pulido, 2013). MAPK phosphatases (MKPs) constitute a PTPome subfamily of dual-specificity phosphatases that dephosphorylate specifically the Thr- and Tyr-activating residues of the MAPKs (Dickinson and Keyse, 2006; Kidger and Keyse, 2016; Nunes-Xavier et al., 2011). The expression of several MKPs is inducible upon specific activation of MAPK pathways, and they display a well-tuned substrate specificity towards the ERK1/2, JNKs, and p38s major MAPKs. For instance, DUSP5, DUSP6, DUSP7, and DUSP9 target preferentially ERK1/2 and control spatially and temporarily the effects of these MAPKs in cell proliferation and survival, whereas DUSP1, DUSP8, DUSP10, and DUSP16 target preferentially JNKs and p38s regulating the cellular stress-response. MKPs are involved in oncogenesis and resistance to cancer therapies, and have been proposed as suitable anti-cancer targets (Haagenson and Wu, 2010; Keyse, 2008; Low and Zhang, 2016; Nunes-Xavier et al., 2011; Rios et al., 2014). Although the involvement of the MAPK pathways in NB cell growth, survival, and differentiation has been widely documented (Chen et al., 2016; Eleveld et al., 2015; Nalluri et al., 2015; Tanaka et al., 2016; Umapathy et al., 2014), studies addressing the global expression and function of MKPs in NB are scarce. Global inhibition of PTPs by vanadium-based compounds enhances RA-triggered differentiation of NB cells, suggesting an active role for members of the PTPome in controlling proliferation and senescence of NB cells (Clark et al., 2013). More specifically, DUSP4, DUSP5, and DUSP6 have been found up-regulated in ALK-mutated NB cell lines (Lambertz et al., 2015), and DUSP6 transcription has been found to be repressed by MYCN in NB cells as part of a positive feedback mediated by activated ERK1/2 (Marshall et al., 2011).
The aim of this study was to identify MKPs that might represent new biomarkers or potential regulators of NB cell growth, performing a comprehensive reverse transcription (RT)-quantitative real time PCR (qPCR) screening and siRNA knock-down experiments of MKPs on human NB cell lines, and immunohistochemistry (IHC) analysis on NB tumor samples.

2. Materials and methods

2.1 Cell lines and reagents

Human NB cell lines SH-SY5Y (ALK F1174L), SMS-KCNR (ALK R1275Q, MYCN amplified), and IMR-32 (MYCN amplified) are from ATCC. Cells were grown at 37 °C in a humidified 5% CO2, 95% air incubator. SH-SY5Y and IMR-32 cells were grown in DMEM/F12 supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml of penicillin, 0.1 mg/ml of streptomycin, and 1% non-essential amino acids. SMS-KCNR were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin. The three cell lines differentiate upon RA treatment (Gaetano et al., 1992; Pahlman et al., 1984; Sidell et al., 1983). Cell differentiation was induced by adding 10 µM all-trans retinoic acid (RA) (Sigma) to the cultures, followed by 7, 10, or 14 days incubation (media was changed after 5 days).

2.2 RNA isolation and RT-qPCR

RT-qPCR was performed using total RNA from SH-SY5Y, SMS-KCNR, and IMR-32 cells treated or not with the distinct agents, isolated with the IllustraRNAspin mini purification kit (GE Healthcare Life Sciences). 1 µg of total RNA was reverse transcribed using RevertAidTM reverse transcriptase, oligo(dT)18 primers, and RiboLock and RNase inhibitor (all from Fermentas). qPCR was performed as previously described (Nunes-Xavier and Pulido, 2016) using validated primer sets (Qiagen) specific for the MKPs. All quantifications were normalized to the hypoxanthine phosphoribosyltransferase 1 (HPRT1) reference gene data. Relative quantification was performed using the comparative ΔΔCt method. Significant upregulation or down-regulation was considered with a threshold of 2 or -2 fold change (Log2 scale), respectively. Non-significant upregulation and non-significant
down-regulation were considered between 1 and 2 fold change or between -1 and -2 fold change, respectively.

2.3 DUSP5 knock-down and MTS cell proliferation assays
DUSP5 knock-down was performed by transfection of specific siRNAs using Lipofectamine 3000 or RNAiMAX reagents (Thermo Fisher) following manufacturer’s protocol. DUSP5 (siDUSP5 #1, SI03194282; siDUSP5 #2, SI02655828) siRNAs were from Qiagen, and siNS (non-specific) and siGAPDH siRNAs were from Ambion. DUSP5 and GAPDH knock-down was verified 72 h post-transfection at mRNA level by isolation of RNA and RT-qPCR as described above. SH-SY5Y cell proliferation was analyzed 24 h after cell stimulation (Fig. 3) or 72 h post-transfection (Fig. 4) using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) following manufacturer’s protocol. For statistical analysis, student t-test was performed, and significant differences were considered for two-tailed P values below 0.01.

2.4 Immunoblot analysis
SH-SY5Y cells were incubated in the presence of epidermal growth factor (EGF; 50 ng/ml), retinoic acid (RA, 10 µM), phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), or nerve growth factor (NGF, 50 ng/ml) for 2 h, and whole cell protein extracts were prepared by cell lysis in ice-cold M-PER™ lysis buffer (ThermoFisher Scientific) supplemented with PhosSTOP phosphatase inhibitor and cOmplete protease inhibitor cocktails (Roche, Switzerland), followed by centrifugation at 15200 g for 10 min and collection of the supernatant. Proteins (50 µg) were resolved in 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes (Merck Millipore). Immunoblotting was performed using anti-ERK1+ERK2 (1/1000, sc-271269+sc-1647, Santa Cruz Biotechnology), anti-pERK1/2 (1/1000, #9106, Cell Signaling) and anti-GAPDH (1/1000, sc-32233, Santa Cruz Biotechnology) antibodies, followed by IRDye secondary antibody (LI-COR) and visualization by Odyssey® CLx Imaging System.

2.5 Patients, tissue specimens, and immunohistochemistry
The characteristics of the patients included in the study are shown in Table 1. 48 retrospectively-obtained tumors were included (44 neuroblastomas and 4 ganglioneuromas), collected from the archives of the Department of Pathology from Cruces University Hospital. The selected samples had representative and viable tumoral foci, and included small diagnostic biopsies and surgical resections. Histological sections of tissue microarrays (TMA; 2mm core tumor specimens; 21-22 cores per TMA) or routine paraffin blocks were used for immunohistochemistry (IHC). The antibodies and dilutions used for IHC were: DUSP5 (1/200, HPA055143, Sigma-Aldrich), DUSP7 (1/100, ab135690, Abcam), DUSP9 (1/100, ab152119, Abcam), and pERK1/2 (1/250, ab50011, Abcam). Immunostainings were performed in automated immunostainers (EnVision FLEX, Dako Autostainer Plus; Dako, Glostrup, Denmark) following routine methods. The analysis was done blind by LZ and performed using a Nikon Eclipse 80i microscope (Tokyo, Japan). The IHC evaluation considered positive those cases with intense nuclear or granular cytoplasmic staining, and negative those with weak or non-existent staining. Each examined core and routine paraffin block contained a minimum of 200 tumoral cells. The authors declare that all the experiments carried out in this study comply with current Spanish and European Union legal regulations, and have been approved by the Ethical and Scientific Committees of the Basque Country Public Health System (Osakidetza) (INC+CES BIOEF 2015-03).

2.6 Statistical analysis of immunohistochemistry data
Statistics of IHC staining included Chi-squared two tailed P value analysis. For classification and feature selection methods, we used the Waikato Environment for Knowledge Analysis (WEKA) (Frank et al., 2016). In particular, antibodies were considered as features and the four variables (MYCN, Risk, Stage, and Regression) were considered as response variables. Feature selection was performed using three different algorithms (a best-first search, a rank-search, and a random-search). We also performed a classification strategy using the CART (classification and regression tree) algorithm over individual response variables after coding them as binary variables.
3. Results

3.1 MKP mRNA expression profile in RA-treated NB cells
To explore the potential role of MKPs in NB differentiation, we performed a quantitative RT-qPCR analysis of the mRNA expression of the 11 MKP family members on human NB cell lines differentiated in the presence of all-trans RA. The cell lines included SH-SY5Y, SMS-KCNR, and IMR-32 cell lines, which show different genetic backgrounds in terms of ALK mutational status and MYCN amplification (see under Materials and Methods). The cell lines were grown in the absence or in the presence of RA for 10 days, and mRNA was isolated and subjected to RT-qPCR using a MKP oligonucleotide primer set collection (Nunes-Xavier and Pulido, 2016). Fig. 1 shows the relative changes of mRNA expression from RA-treated cells in comparison with untreated cells. A complex pattern of mRNA MKP up- and down-regulation was obtained, suggesting the existence of different MAPK regulatory networks driven by MKPs in NB cells during RA-differentiation. For instance, DUSP16 was up-regulated in the three cell lines, whereas DUSP5 and DUSP9 were up-regulated and down-regulated, respectively, in two of the cell lines (Fig. 1). Together, these results illustrate selectivity in the regulation of MKP expression in NB cells differentiated by RA.

3.2 MKP and pERK1/2 immunohistochemical analysis of NB tumor samples
Next, histological sections of TMAs or routine paraffin blocks containing NB and ganglioneuroma (as an example of differentiated disease) tumor samples were stained with a panel of anti-MKP antibodies, selected on the basis of our RT-qPCR results and the availability of antibodies suitable for IHC analysis. These included anti-DUSP5, -DUSP6, -DUSP7, -DUSP9, and -DUSP16 antibodies although anti-DUSP6 and -DUSP16 antibodies did not provide reliable immunostaining, and the IHC detection of these MKPs was not pursued further. We also analyzed by IHC the expression of phosphoERK1/2 (pERK1/2), as a potential MKP substrate and effector. A summary of our results is shown in Table 2, and illustrative NB immunostained sections are shown in Fig. 2. DUSP5 and DUSP7 were positive or weakly positive in most of the cases, whereas DUSP9 was negative in most of the cases, and the few positive samples did not show relations to any parameters. DUSP5 immunostaining was mostly nuclear, whereas DUSP7 immunostaining was mostly cytoplasmic..
granular. pERK1/2 was positive in about 25% of NB tumor samples. Remarkably, DUSP5 high expression associated positively with amplified MYCN, high risk, and metastasis, and negatively with total regression. Feature selection confirmed the association of stage and risk to DUSP5, and CART classification algorithms provided good accuracies with regression and risk as response variables by a classification tree in which the only existing variable was DUSP5. Classification accuracies were 81.82% for regression and 79.54% for risk. Finally, expression of pERK1/2 in tumor samples associated with expression of DUSP5 (pERK1/2 high/DUSP5 high, 9 samples; low-low, 18 samples; high-low, 6 samples; low-high, 1 sample; Chi-squared two tailed P = 0.0005). Together, these findings argue for DUSP5 high expression as a potential marker for NB poor prognosis, and indicate an association between pERK1/2 and DUSP5 expression.

3.3 ERK1/2 activation correlates with NB cell proliferation and DUSP5 upregulation
Our IHC results suggest a functional relation between expression of pERK1/2 and DUSP5 in NB tumors. To further analyze this, we investigated the impact of ERK1/2 activation in the cell proliferation and in the regulated expression of DUSP5 in SH-SY5Y NB cells in vitro. SH-SY5Y cells were treated with different ERK1/2-activating stimuli, including EGF, RA, PMA, and NGF, and DUSP5 mRNA expression and cell proliferation were determined. As shown, a good correlation between ERK1/2 activation (as indicated by pERK1/2 levels) and DUSP5 mRNA upregulation in SH-SY5Y cells was observed (Fig. 3). Of notice, our analysis also revealed a correlation between the extent of ERK1/2 activation and the increase in SH-SY5Y cell proliferation (as determined by MTS assays) under the different stimulation conditions (Fig. 3). These results illustrate and association in SH-SY5Y NB cells between ERK1/2 activation, cell proliferation, and DUSP5 mRNA upregulation.

3.4 DUSP5 knock-down and proliferation of NB cells
We next analyzed whether changes in DUSP5 expression could affect the proliferation of SH-SY5Y cells. SH-SY5Y cells were transiently transfected with siRNAs targeting DUSP5, and MTS proliferation assays were performed 72 h after transfection. As shown, down-regulation of DUSP5 expression by specific siRNAs
resulted in increased cell proliferation, when compared to non-specific or GAPDH silencing conditions (Fig. 4). This is in agreement with a negative regulation of ERK1/2 activation by DUSP5. These results suggest the involvement of DUSP5 in the regulation of growth of NB cells.

4. Discussion

High-risk NB treatment demands new and more effective therapies, which may arise from a better molecular definition of NB cell growth and differentiation, as well as from more precise patient risk stratification based on the expression of novel biomarkers (Bagatell and Cohn, 2016; Berlanga et al., 2017). Our results suggest that a complex pattern of MKPs mRNA expression occurs in NB cells undergoing differentiation. This highlights the potential of MKPs in the response of NB patients to consolidation and maintenance high-risk NB therapies, and suggests that specific MKPs behave selectively as MAPK terminating-signal enzymes during the long-term differentiation of NB cells. In NB tumor samples, we detected high expression of the MKPs DUSP5 and DUSP7, and low expression of DUSP9. Importantly, we show an association of DUSP5 high expression with NB poor prognosis, as indicated by their enrichment in tumor samples from high-risk NB patients and from patients which did not have disease regression. This may have important clinical implications for a better patient stratification and for the implementation of future novel targeted therapies. Noticeably, siRNA knock-down of DUSP5 resulted in increased cell proliferation of SH-SY5Y cells, indicating that DUSP5 expression is important to regulate NB cell growth. DUSP5 is a major negative regulator of ERK1/2 activity (Mandl et al., 2005), and we show that ERK1/2 activation correlates with cell proliferation in SH-SY5Y NB cells. Together, these findings suggest that DUSP5 could regulate NB cell growth by dephosphorylating and inactivating ERK1/2. Additional DUSPs may coordinately target ERK1/2 and cooperate in this process in a compensatory manner, as it has been suggested in other cancers (Buffet et al., 2017). In this regard, inhibitors of the ERK1/2 activator, MEK1, block the growth of NB cells displaying high pERK1/2 levels (Tanaka et al., 2016). These findings also suggest that DUSP5 high expression in NB tumors might not be instrumental for NB cell transformation, but rather arise as a consequence of NB tumor progression. In fact, DUSP5 mRNA expression also
displayed an upregulation trend upon RA-differentiation of NB cells, which we attribute to RA-mediated ERK1/2 activation. In line with this, a correlation was also observed between pERK1/2 and DUSP5 protein expression in NB tumors, as well as between pERK1/2 and DUSP5 mRNA levels in SH-SY5Y cells. This is in accordance with the ERK1/2-activation dependent upregulation of DUSP5 in other cell lines (Buffet et al., 2015; Kucharska et al., 2009; Nunes-Xavier et al., 2010), as well as with the increased DUSP5 mRNA levels found in ALK-activated NB cell lines (Lambertz et al., 2015). Since the oncogenic role attributed to DUSP5 is uncertain (Kidger and Keyse, 2016), further work is required to understand the potential involvement of DUSP5 in NB and to validate DUSP5 as a novel NB prognostic biomarker.

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Conflict of interest statement
The authors declare no conflict of interest.
Table 1. Clinic-pathologic characteristics of study population

Patients (n=48)  

<table>
<thead>
<tr>
<th>Neuroblastoma (n=44)</th>
<th>n</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>52</td>
</tr>
<tr>
<td><strong>Age at diagnosis (months)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;18</td>
<td>29</td>
<td>66</td>
</tr>
<tr>
<td>&gt;18</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td><strong>Risk</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>19</td>
<td>43</td>
</tr>
<tr>
<td>Medium</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>High</td>
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</tr>
<tr>
<td><strong>Stage</strong></td>
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<td></td>
</tr>
<tr>
<td>Non-metastatic</td>
<td>33</td>
<td>75</td>
</tr>
<tr>
<td>Metastatic</td>
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<td>25</td>
</tr>
<tr>
<td><strong>NMYC</strong></td>
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</tr>
<tr>
<td>Non-amplified</td>
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<td>57</td>
</tr>
<tr>
<td>Amplified</td>
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<tr>
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<tr>
<td>No/partial</td>
<td>29</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>34</td>
</tr>
<tr>
<td><strong>Dead</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Ganglioneuroma (n=4)  

| Gender | | |
| Male | 3 | 75 |
| Female | 1 | 25 |
| **Age at diagnosis (months)** | | |
| <18 | 1 | 25 |
| >18 | 3 | 75 |
Table 2. Immunostaining of MKPs and pERK1/2 from NB tissue sections

<table>
<thead>
<tr>
<th>Expression</th>
<th>DUSP5</th>
<th>DUSP7</th>
<th>DUSP9</th>
<th>pERK1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hig h</td>
<td>Low/no</td>
<td>Hig h</td>
<td>Low/no</td>
</tr>
<tr>
<td>MYCN</td>
<td>0.7</td>
<td>0.0047</td>
<td>0.6</td>
<td>0.7405</td>
</tr>
<tr>
<td>Amplified (8)</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Non-amplified (25)</td>
<td>9</td>
<td>14</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>RISK</td>
<td>0.83</td>
<td>0.0001</td>
<td>0.63</td>
<td>0.1991</td>
</tr>
<tr>
<td>High (16)</td>
<td>14</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Intermediate/Low (28)</td>
<td>7</td>
<td>20</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>STAGE</td>
<td>0.67</td>
<td>0.0033</td>
<td>0.6</td>
<td>0.6250</td>
</tr>
<tr>
<td>Metastatic (11)</td>
<td>8</td>
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<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Non-metastatic (33)</td>
<td>13</td>
<td>18</td>
<td>11</td>
<td>20</td>
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<tr>
<td>REGRESSION</td>
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<td>0.49</td>
<td>0.7426</td>
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<tr>
<td>No/partial (29)</td>
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<td>6</td>
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<td>16</td>
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<tr>
<td>Total (15)</td>
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<td>13</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>GANGLIONEUROMA</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

The number of samples for each condition is indicated. Note that information on MYCN amplification is not available for all samples (see Table 1). In addition, the number of samples for some PTPs is inferior to the total number of samples in some cases, due to lack of tissue to perform the analysis or to non-informative immunostaining results. The boxes with a diagonal slash indicate the accuracy values for each PTP in relation with the variables (top number, number of cases matching the hypothesis/total number of cases) and the significance of the non-random distribution (bottom number, Chi-squared two tailed P value).

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Figure legends

**Fig. 1.** mRNA expression analysis of the MKPs from human NB cell lines treated with RA. Cell lines were kept untreated or were treated for 10 days with RA, RNA was extracted and RT-qPCR was performed using a set of specific MKP primers. Relative mRNA expression values are shown in Log$_2$ as fold change ± SD of treated cells versus untreated cells, from at least two independent experiments.

**Fig. 2.** Immunostaining of DUSP5 and pERK1/2 from selected NB tissue sections. In the two upper panels, representative DUSP5 immunostaining patterns are shown from two different tumors. In the two lower panels, high DUSP5 and pERK1/2 immunostainings are shown from the same tumor (magnification: X200).

**Fig. 3.** Response of SH-SY5Y cells to ERK1/2-activating agents. **A.** Cells were treated with the indicated agents for 2h, followed by cell lysis and immunoblot analysis with the indicated antibodies. Blot images from a representative experiment are shown. **B.** Cells were treated with the indicated agents for 24 h, followed by cell lysis and RT-qPCR analysis with DUSP5-specific primers; **, p < 0.0056 with respect to control; *, p < 0.056 with respect to control. **C.** Cells were treated with the indicated agents for 24 h, followed by MTS cell proliferation assay; **, p < 0.0001 with respect to control; *, p < 0.002 with respect to control. Data are shown as the mean ± SD from technical quadruplicates from a representative experiment.

**Fig. 4.** Proliferation of SH-SY5Y cells upon DUSP5 or PTPN1 siRNA knock-down. Cells were transfected with control (siNS, non-specific; siGAPDH) siRNAs or with different siRNAs targeting DUSP5 (siDUSP5 #1, siDUSP5 #2). **A.** The silencing efficiency of the distinct siRNAs is shown, as monitored by RT-qPCR using specific primers. **B.** MTS cell proliferation assays were performed 72 h after transfection. Data are shown as the mean ± SD from two technical replicates from a representative experiment; *, p < 0.0076 with respect to siNS.