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Thymosin Beta 4 has tumor suppressive effects and its decreased expression results in poor prognosis and decreased survival in multiple myeloma

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Abstract

Thymosin beta 4 (Tβ4) is a polypeptide involved in cellular proliferation, differentiation, and migration, overexpressed in several tumor-entities. We evaluated its expression and function in 298 newly diagnosed multiple-myeloma patients and the murine 5TMM model. Mean-*Tβ4*-expression was significantly lower in myeloma cells compared to normal plasma cells ($P<.001$). The same observation can be made in the 5TMM-mouse model by qRT-PCR and ELISA. Here, *Tβ4*-overexpression by lentiviral transduction of 5T33MMvt-cells led to a significantly decreased proliferative and migratory capacities and increased sensitivity to apoptosis-induction. Mice injected with *Tβ4*-overexpressing myeloma-cells showed a longer survival compared to mice injected with controls (88,9 vs. 65,9 days, $P<.05$). In 209 MM-patients treated with high-dose therapy and autologous stem cell transplantation, expression of *Tβ4* below the median was associated with a significantly shorter event-free survival (37.6 vs. 26.2 months, $P< .05$). In conclusion, our results indicate a possible tumor suppressive function of Tβ4.

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Introduction

Beta-thymosins are a family of small peptides that were originally proposed to be thymic hormones (1). They were identified as actin monomer binding proteins, controlling the availability of actin for polymerization. They therefore may have a crucial role in regulating cellular functions involving actin polymerization/depolymerization cycles. Currently, 15 β-thymosins have been identified and characterized as highly conservative 5-kDa peptides containing 40 to 44 amino acid residues. In most mammalian tissues, thymosin-β 4 (Tβ4), the most abundant thymosin peptide, Tβ10 and Tβ15, have been studied as important members of the β-thymosin family (2). Several studies reported that these genes are overexpressed in solid tumors, which could be correlated to the angiogenic and metastatic potential of the studied tumors (3).

Multiple Myeloma (MM) is a hematological malignancy characterized by the accumulation of monoclonal plasma cells (PC) in the bone marrow (BM). MM cell biology can be dissected into the interactions of MM cells with their surrounding stroma (matrix proteins, cytokines and BM cells) and in the acquisition of essential changes in cell behavior such as self-sufficiency in growth signals, evasion of apoptosis and acquisition of invasive and spreading capacities (4). Earlier reports indicated that Tβ4 was downregulated in RNA from primary human MM cells and cell lines (5). This observation is in contrast to the results obtained in most solid tumors where an upregulation is often seen in malignant cells compared to their normal counterparts. Cha

et al showed that overexpression of Tβ4 resulted in an increased metastatic capacity of lung cancer cells and increased angiogenic response (6). Since migration, invasion and associated angiogenesis are key features in MM biology, we were interested in studying Tβ4 expression in a large panel of MM patients and its functionality in the murine 5TMM model.

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Material and methods

Gene expression analysis on human myeloma cells.

Tβ4 expression was analyzed in purified PCs from BM samples obtained from 14 healthy donors, 11 patients with monoclonal gammopathy of unknown significance (MGUS) and 298 previously untreated multiple MM patients at the University Hospitals of Heidelberg or Montpellier (7). Of these, 209 MM-patients were treated by high dose therapy and autologous stem cell transplantation (ASCT). Biotinylated complementary RNA (cRNA) was amplified according to the Affymetrix labeling protocol (Affymetrix, Santa Clara, CA, USA). cRNA from a first group of patients (7 normal donors, 7 MGUS- and 65 MM-patients) was hybridized to the human U133 A and B. This group is termed hereafter the HM1-group. A second independent validation group of patients (7 normal donors, 16 MGUS- and 233 MM-patients) was termed the HM2 group. For these patients the U133 2.0 GeneChip was used. These micro-array data were previously used for several analyses, but thymosin beta 4 expression was never analyzed before (8, 9). HM2 data were corrected for batch effect due to the usage of different labelling kits according to Johnson et al (10). Expression data were gcrma-normalised and analyzed by the bioconductor packages for R. For patient characteristic see supplementary table 1.

The 5T2MM and 5T33MM murine models of myeloma.

The 5TMM models originated in elderly C57Bl/KaLwRij mice (11). The 5T33MM*vivo* (5T33MM*vv*) cells grow *in vitro* stroma-dependently with a limited survival while the 5T33MM*vitro* (5T33MM*vt*) cell line is a clonally identical variant that originated from an *in vitro* culture of 5T33MM*vv* cells, growing BM stroma-independently in RPMI-1640

supplemented with 10% bovine serum 1% natriumpyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all from Biowhittaker, Verviers, Belgium) (12).

Quantification of intracellular protein levels of Tβ4 and F-Actin -GActin

Enzyme-Linked Immunosorbent Assays (ELISA) for measuring Tβ4 concentrations were performed according to the manufacturer's instructions (Immundiagnostik, Bensheim, Germany). Cells (10^7) were lyzed in a phosphate buffer containing 0.14 M NaCl, 2.6 M KCl, 8 mM Na2HPO4, 1.4 M KH2PO4 and 1% Triton X100 and sonicated with an ultrasound finger. Protein levels and ratios between F-Actin and G-Actin were determined using the G-actin / F-actin *in vivo* assay kit (Cytoskeleton Inc, Denver, USA).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using the ABI Prism 7700 Sequence Detection System. For the detection of both human and mouse T*β*4 mRNA and the endogenous reference gene *GUS*, Assays on Demand (Applied Biosystems) were used. To verify the results obtained with the microarrays studies, *Tβ4* expression was measured in 3 cell lines and in 3 patient samples and their correlations statistically verified using a Spearman correlation test.

Generation of 5T33MMvt cells overexpressing T*β*4

A lentiviral transferplasmid encoding mouse *Tβ4* (m*Tβ4*) was constructed. The *mTβ4* gene was obtained from H.J. Cha (NIDCR, NIH, Bethesda, USA (6)) and inserted into the transferplasmid pHR'tripCMV-IRES-tNGFR-SIN (13). m*Tβ4*-encoding lentiviral vector

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particles were produced in 293T cells, collected, ultracentrifugated and their viral titer determined (14). After transduction, 5T33MM*vt* cells were surface stained using a house made biotinylated anti-tNGFR antibody and purified by FACS sorting into a 6-well plate (Becton Dickinson, FACSVantage). Next, they were analyzed for *Tβ4* expression by RT-PCR. The 5T33MMvt cells overexpressing *Tβ4* are termed in the following $5T33MMvt^{T\beta4+}$.

In vitro and in vivo effects of T*β*4-overexpression

In vitro proliferation was assessed by measuring DNA synthesis using a ^{3}H-thymidine incorporation assay, as described earlier (15). Apoptosis sensitivity of the MM cells was analyzed by staining with FITC labeled-annexin V and propidium iodide according to the manufacturers' instructions (BD Biosciences, Erembodegem, Belgium). *In vitro* migration studies were performed using Transwell chambers and 10% fatal calf serum as chemoattractant and were quantified through flow cytometry. To determine the effect of *Tβ4* overexpression on survival, groups of 10 C57BLKaLwRij mice were intravenously injected with either $5x10^{5}$ $5T33MMvt^{T\beta4+}$ or wild type 5T33MMvt cells. Animals were sacrificed when they showed signs of morbidity, namely hind limb paralysis. Kaplan-Meier analysis was used to determine a difference in the survival.

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Results and Discussion

Different studies indicated a pivotal role of T*β*4 in the metastatic process of solid tumors (16, 17). An adenoviral-based overexpression of *Tβ4* was applied in a colon cancer and melanoma model showing increased growth, motility and invasive capacities *in vitro* and a larger tumor load *in vivo* (18, 19). Since proliferation, migration and invasion are part of the hallmarks in the biology of MM, we were interested in investigating an involvement of T*β*4 in this disease. We first investigated the *Tβ4*-expression pattern in 298 primary MM-cell samples and 14 normal plasma cell samples from healthy donors. *Tβ4* is significantly lower expressed in MM cells of the HM1 group ($P<.05$) and HM2 group ($P<.001$) compared to normal plasma cells. This holds true for a significantly lower *Tβ4* expression in its pre-malignant stage (MGUS), its early (Durie Salmon stage I) or late stage (Durie Salmon II and III) in both HM1 and HM2 groups ($P<.001$) (Figure 1.a). No relevant correlation could be found between *TB4*-expression and percentage of plasma cell infiltration in the bone marrow smear. Gene expression assessed by DNA-microarray correlates well with qRT-PCR performed on MM patient samples (coefficient of correlation $r = 0.993$, $P< .001$). These data are in agreement with results from Gondo et al. showing an decreased *Tβ4*-expression in a small number of MM samples by northern blot analysis (5).

Given the differential *Tβ4* expression in MM patients, we subsequently investigated a possible prognostic value and influence on event free (EFS) and overall survival (OS) in our patient population. As *Tβ4* was expressed in all MM-cells, we examined the survival of 209 patients by comparing patients with *Tβ4* expression above ($T\beta4^{high}$) and below

($T\beta 4^{low}$) the median (Figure 1.b). Patients with $T\beta 4^{high}$ vs. $T\beta 4^{low}$ show a significantly longer median EFS (n=209, 37.6 months vs. 26,2, $P < .05$), but only a trend regarding OS ($P = .1$). Concerning EFS, multivariate analyses on *Tβ4* expression with either ISS or β2-microglobulin indicated an independent ($P = .04$) prognostic value of *Tβ4* expression regarding ISS ($P < .001$), but not ($P = .07$) regarding β2-microglobulin ($P = .003$) levels. In multi-variate analyses for OS, ISS and β2-microglobulin appear as significant ($P = < .001$) variables, whereas *Tβ4* expression fails to reach independence ($P = .09$ and $P = .1$, respectively).

A supervized analysis of expression data comparing the $T\beta 4^{high}$ vs. $T\beta 4^{low}$ group identified over 300 significantly differentially expressed genes. These genes are listed in supplementary table 2. Analysis of their biological function allowed separation in main functional categories and this distribution is illustrated in figure 1.c. Signal transduction, protein metabolism and nuclear functions were the largest categories, but 19 genes were implicated in cytoskeletal organization and 32 genes in lymphoid differentiation and immunoglobulin processing. In general these gene clusters indicate a biological difference between MM cells of the two patient groups .

The data obtained in MM-patients were also seen in the 5T33MM murine MM model by qRT-PCR demonstrating a decreased mRNA expression in 5TMM cells compared to normal BM cells ($P < .001$, Figure 1.d). Competitive ELISA confirmed these results on protein level (results not shown). To study functional effects of T*β*4, the gene was

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overexpressed using a lentiviral expression vector. The 5T33MM*vt* cell line was stably transduced and after subcloning, a 99% pure clone with strong t-NGFR expression was obtained. qRT-PCR confirmed the overexpression of *Tβ4* compared to control cells (Figure 2a).

To assess the functional involvement of differential *Tβ4* expression we used the 5T33MMvt and 5T33MMvt$^{T\beta4+}$ cell lines. In a ^{3}H thymidine assay, 5T33MMvt$^{T\beta4+}$ cells showed a significant decrease in DNA synthesis compared to control cells (P<.05). 5T33MMvt$^{T\beta4+}$ cells showed an significantly increased sensitivity to vinca-alkaloids (vinblastin) and bortezomib (Figure 2.b P <.001 for both bortezomib and vinblastin).

Likewise, bortezomib induced apoptosis was higher in 5T33MMvt$^{T\beta4+}$ vs. 5T33MMvt cells (P<.05, Figure 2.c). In addition to affect survival pathways, *Tβ4* overexpression reduced migratory capacities of 5T3MM cells; the percentages of cells that migrated in basal conditions and in 10%FCI was significantly lower in 5T33MMvt$^{T\beta4+}$ compared to control cells (P<.05, Figure 2.d). The relative increase after stimulation (compared to basal conditions) was however similar in both populations. We further examined the effects of *Tβ4* expression on tumor development and survival of diseased mice by injecting mice intravenously with 5T33MMvt$^{T\beta4+}$ or control cells. In this study, the mean survival of mice injected with control cells was significantly shorter 65,9 days (SD 6.6 days), compared to 88,9 days (SD 9,3 days) for mice injected with 5T33MMvt$^{T\beta4+}$ cells (P< .05, Figure 2.f). These *in vivo* results confirm data obtained using the *in vitro* proliferation and apoptosis assays.

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In solid tumors, Tβ4 expression is frequently upregulated in malignant and metastatic cells. In these cancers, higher Tβ4-expression resulted in increased metastatic and invasive capacities of tumor cells, while proliferation remained unaffected (6). In hematological disorders, malignant plasma cell disorders, such as plasma cell leukemia and MM were the rare disorders that showed a decreased Tβ4-expression.(5, 20). In contrast to solid tumors, publications on the function of Tβ4 in hematological conditions are scanty but indicate some inhibitory activity. Tβ4 was initially isolated and purified from a thymic protein preparation, called thymosin fraction-5. Addition of this protein fraction to different leukemic cell lines resulted into decreased growth responses (21). Similar inhibitory effects were recently described for Tβ4 on hematopoietic stem cells (22), bone marrow derived mast cells (23) and human promyelocytic leukemia cells (24), in agreement with the results presented here. Whereas a mechanistic explanation of this discrepancy is beyond the scope this paper, further investigations are clearly merited.

Since T*β*4 has been shown to bind G-actin in a 1:1 manner and thus affects the polymerization of G-Actin into F-Actin, we analyzed in a semi-quantitative way, intracellular G-actin and F-Actin. This quantification showed a lowered G- Actin-F-Actin ratio after *Tβ4* overexpression (Figure 2.e). F-Actin is of particular importance in cytoskeleton changes involved in cellular migration and in microtubuli organization controlling the mitotic spindle (25, 26). In line with these results, vinca-alkaloids (e.g. vinblastine used here) with micro-tubulin [polymerization] inhibitory activity, had more affect on the proliferation capacities of 5T33MMvt$^{T\beta4+}$ cells than on control cells (fig 2.b). Since immunohistochemical studies also showed a nuclear staining of T*β*4 in

5TMM cells (results not shown), involvement of other pathways might also be implicated. Supervized gene analysis comparing *Tβ4*^{high} vs. *Tβ4*^{low} found different groups of genes differently expressed, included genes involved in cytoskeleton organization, nuclear homeostasis, lymphocyte differentiation and protein metabolism, which might indicate that the role of Tβ4 is more complicated as initially supposed.

In conclusion, our results propose a tumor suppressive function of *Tβ4*-expression in MM with impact on survival. *Tβ4* was downregulated in MM cells of patients compared to the normal BM plasma cells and studies with the murine 5T33MM model show a decreased *in vitro* and *in vivo* tumor growth for cells overexpressing the *Tβ4* gene.

Authorship

JC and DH were the principal investigators and took primary responsibility for the paper. JC, DH, IK, TJB, EDB and EM participated in the laboratory work for this study. BVC, EVV, BK and KV coordinated the research. HG and BK were responsible for patient recruitment and patient data. JC, DH, TJB, BK and KV wrote the paper. The authors reported no potential conflicts of interest.

Running heads

J Caers et al. Thymosin beta 4 as tumor suppressor in MM

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Characteristic	HM1 n=48	HM2 n=161
Age	58,5 [37-72]	57 [27-73]
Monoclonal protein		
IgG	25	97
IgA	11	36
Bence Jones	10	25
Asecretory	2	2
IgD	0	1
NA	0	0
Myeloma in Durie and Salmon stage		
I	4	16
II	5	27
III	39	118
Myeloma in ISS stage		
I	15	80
II	26	50
III	7	28
NA	0	3
Serum β2-microglobulin	3.55 [1.3-[illegible]	3.0 [1.3-53.6]
Plasma cells in bone marrow	45 [5-100]	38 [1-100]

Supplementary Table S1. Clinical data for patients undergoing high-dose chemotherapy and autologous stem cell transplantation. Clinical patient data for age, serum-β2-microglobulin, and plasma cell infiltration in the Heidelberg/Montpellier-group 1 (HM1) and -2 (HM2). Median value and range are given. NA, not available; ISS, International Staging System.

Supplementary Table S2. Supervised gene analysis resulted in identification of differently expressed genes in myeloma cells from $T\beta 4^{high}$ and $T\beta 4^{low}$ patients from the validation group. After identification of the biological function of the gene, these were grouped into different classes.

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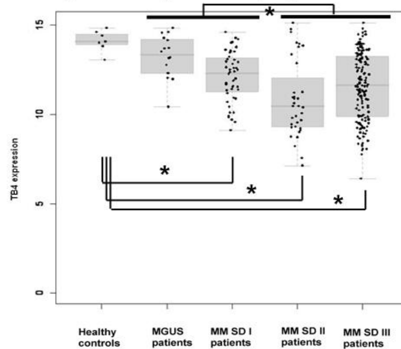
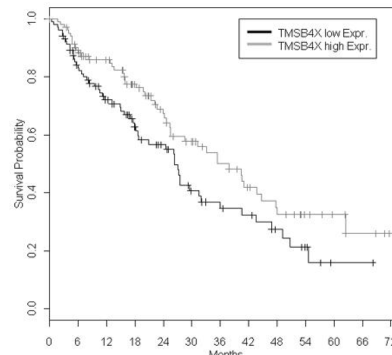
Figure legends

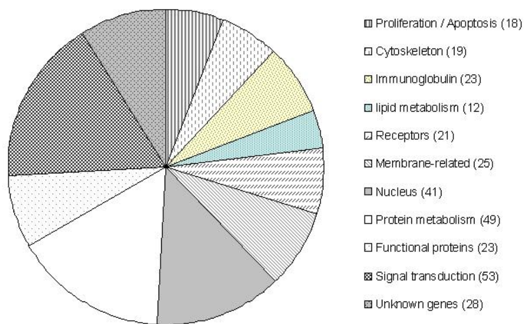
1.a Micro-array data on TB4 expression in a myeloma patient population



1.b Event free survival of highly TB4 expressing patients vs low expressing patients.



1.c Supervised analysis of differential gene expression



1.d TB4 expression in the 5TMM cells

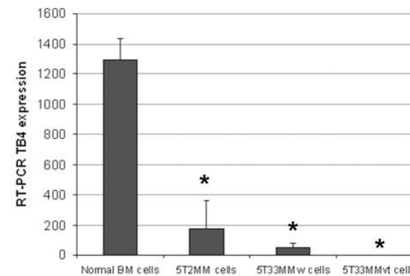


Figure 1.a shows the micro-array data obtained for the *Tβ4* expression in CD138+ sorted BM plasma cells from healthy donors and MM patients. These results were validated by quantitative RT-PCR. MGUS: monoclonal gammopathy of undetermined significance, MM multiple MM, SD: Salmon & Durie Stage **Figure 1.b** illustrates the event free survival of *Tβ4*^{high} and *Tβ4*^{low} patients. Patients with *Tβ4*^{low} MM had statistically significantly decreased event free survivals compared to patients with *Tβ4*^{high} MM (P<.001), while also their overall survival tended to be shorter. **Figure 1.c** illustrates the differently expressed genes between *Tβ4*^{high} and *Tβ4*^{low} patient groups. After

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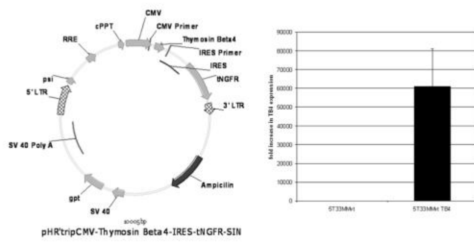
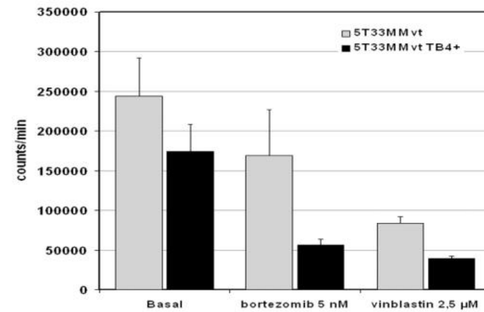
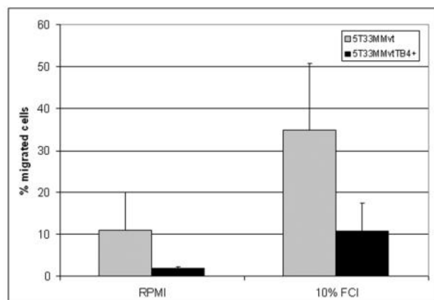
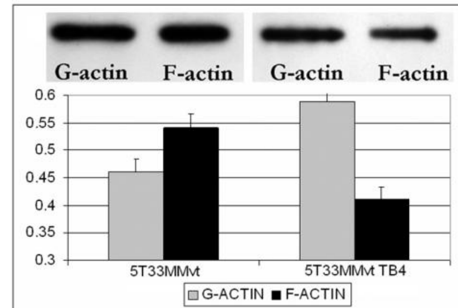
identification of the gene, these were grouped into similar biological function. A complete list of the genes can be found in supplementary table 2. **Figure 1.d** A similar gene expression pattern was observed in the murine 5TMM models where *Tβ4* mRNA expression in 5T33MM and 5T2MM invaded BM was lowered compared to normal BM cells (**Figure 1.d**).









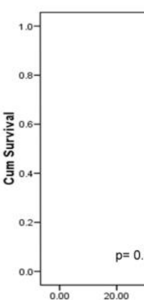


Figure 2.a Schematic representation of the modified lentiviral transfer plasmid and results of RT-PCR and qRT-PCR indicating the presence of the inserted *Tβ4* gene in cultured 5T33MMvt$^{Tβ4+}$ cells.

Figure 2.b. 3-H thymidine uptake revealed a decreased DNA synthesis rate in 5T33MMvt$^{Tβ4+}$ cells compared to wild type cells. Incubation with the anti-MM agent

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bortezomib (5nM) or the micro-tubuli inhibitor vinblastine (2,5 μM) had significantly (*P*<.001) stronger effects on 5T33MMvt$^{T\beta4+}$ cells than on control cells. A similar observation was made in apoptosis studies **(Figure 2.c)**, where 5nM of bortezomib resulted in a significantly (P< .05) increased apoptotic cell population after 18h incubation. **Figure 2.d** illustrates the effects of *Tβ4* overexpression on migration of 5T33MMvt cells: using 10% fetal calf serum as chemo-attractant, only 10,8% (SD 6,6%) of 5T33MMvt$^{T\beta4+}$ cells migrated compared to 34,7% (SD 15,9%) of the control 5T33MMvt cells (*P*< .05). The upper part of **Figure 2.e** shows the F-actin and G-actin bands of 5T33MMvt and 5T33MMvt$^{T\beta4+}$ cells, whereas the graph illustrates the ratios of quantified F-actin and G-actin. In 5T33MMt cells actin is present in it polymerized form, whereas *Tβ4* overexpression results in decreased F-actin formation and a greater pool of G-actin. **Figure 2.f** C57Bl/KaLwRij mice were injected with 5T33MMvt wild type and 5T33MMvt$^{T\beta4+}$ cells. Kaplan-Meier analysis showed a significantly different survival between these 2 groups with a mean survival of mice injected with 5T33MMvt wild type of 65,9 days (SD 6.6 days), compared to 88,9 days (SD 9,3 days) for mice injected with 5T33MMvt$^{T\beta4+}$ cells. (*P* < .05). Abbreviations: LTR: long terminal repeat; Δgag: frame-shifted gag gene; RRE: rev-responsive element; CMV: cytomegalovirus promotor trip: central polypurine tract + termination sequence; Ires: internal ribosomal entry site; tNGFR: truncated form of the nerve growth factor receptor

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