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# Extracellular Nm23H1 stimulates neurite outgrowth from dorsal root ganglia neurons *in vitro* independently of nerve growth factor supplementation or its nucleoside diphosphate kinase activity

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ABSTRACT

The nucleoside diphosphate (NDP) kinase, *Nm23H1*, is a highly expressed during neuronal development, whilst induced over-expression in neuronal cells results in increased neurite outgrowth. Extracellular Nm23H1 affects the survival, proliferation and differentiation of non-neuronal cells. Therefore, this study has examined whether extracellular Nm23H1 regulates nerve growth. We have immobilised recombinant Nm23H1 proteins to defined locations of culture plates, which were then seeded with explants of embryonic chick dorsal root ganglia (DRG) or dissociated adult rat DRG neurons. The substratum-bound extracellular Nm23H1 was stimulatory for neurite outgrowth from chick DRG explants in a concentration-dependent manner. On high concentrations of Nm23H1, chick DRG neurite outgrowth was extensive and effectively limited to the location of the Nm23H1, i.e. neuronal growth cones turned away from adjacent collagen-coated substrata. Nm23H1-coated substrata also significantly enhanced rat DRG neuronal cell adhesion and neurite outgrowth in comparison to collagen-coated substrata. These effects were independent of NGF supplementation. Recombinant Nm23H1 (H118F), which does not possess NDP kinase activity, exhibited the same activity as the wild-type protein. Hence, a novel neuro-stimulatory activity for extracellular Nm23H1 has been identified *in vitro*, which may function in developing neuronal systems.



## 1. Introduction

Nm23H1 belongs to a family of multifunctional, nucleoside diphosphate (NDP) kinases with roles relating to signal transduction, gene expression, embryonic development, tumour progression and cell migration [1]. Early studies demonstrating an inverse correlation of *Nm23H1* expression with the aggression of a variety of tumours, e.g. breast cancer and malignant melanoma [2,3], together with inhibited tumour cell migration and metastasis following exogenous *Nm23H1* over-expression [4,5], account for its naming as Nm (non metastatic) 23H1 [6]. However, evidence suggests that Nm23H1 also plays a physiological role in the regulation of nerve growth. During mouse embryogenesis, *Nm23M1* (the murine orthologue) is expressed at high levels in central and peripheral neural tissues, including the brain, spinal cord and spinal and cranial ganglia [7]. In addition, *Nm23M1* transfection of PC12 cells, an established model of neuronal differentiation, was associated with increased neurite outgrowth, whilst anti-sense *Nm23M1* transfection inhibited neurite outgrowth [8]. Transfection of murine N1E-115 neuroblastoma cells with human *DR-Nm23*, another member of the NDP kinase family with 70% homology to *Nm23H1*, similarly enhanced neurite outgrowth [9].

Extracellular Nm23H1 is present in body fluids, where its protein levels in blood were correlated with poor prognosis in acute myelogenous leukemia (AML), malignant lymphoma and neuroblastoma [10–12]. The presence of Nm23H1 in blood may relate to lysis of effete erythrocytes [13] or of tumour cells themselves [14], but may also arise through secretion as Nm23 proteins have been detected in conditioned culture medium from established, viable tumour cell lines [15,16]. Furthermore, supplementing culture medium with recombinant Nm23H1 alters the growth and survival of normal hematopoietic cells, AML cells and embryonic stem cells [14,17–19]. Therefore, evidence suggests that extracellular Nm23H1 may regulate cell activity. Here, we have used established *in vitro* protocols to examine the effects of extracellular recombinant Nm23H1 on nerve growth.

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## 2. Materials and methods

### 2.1. Explant cultures of embryonic chick dorsal root ganglia (DRG)

Embryonic day 10 (E10) chick DRG explants were prepared according to previously described methods [20] and seeded into culture plates pre-coated with recombinant Nm23H1 or collagen type I (prepared as described below). The DRG explants were cultured in DMEM/F12 culture medium, supplemented with insulin, transferrin, and selenium (Invitrogen, Paisley, UK), non essential amino acids (Invitrogen) and with or without 50 ng/ml nerve growth factor (NGF, Sigma, Poole, UK).

### 2.2. Dissociated adult rat DRG cultures

Dissociated DRG neurons were prepared from adult male Sprague Dawley rats, as described previously [21]. About $2 \times 10^3$ cells per well were seeded into culture plates pre-coated with recombinant Nm23H1 and collagen type I and maintained in Neurobasal A medium (Invitrogen Life Technologies) supplemented with or without 50 ng/ml NGF (Sigma).

### 2.3. Preparation of recombinant wild type (WT) Nm23H1 and mutant Nm23H1 (H118F)

The Nm23H1 his-tagged clone was kindly provided by Dr. Patricia Steeg (Bethesda, MD). The clone was transformed into *E. coli* BL21 (DE3) cells (Novagen, CN Biosciences UK Ltd., Nottingham, UK) according to the manufacturer's instructions. Bacterial cultures for induction were prepared by inoculation of L-Broth media, containing 100 μg/mL Ampicillin (Sigma), grown to an $OD_{600}$ of 0.6. Recombinant Nm23H1 protein expression was induced with 1 mM IPTG (Sigma) and prepared by the Bugbuster Ni–NTA His Bind Purification Kit (Novagen). To generate the mutant form of Nm23H1 (H118F), which is NDP kinase inactive [22], complementary oligonucleotides containing the histidine to phenylalanine mutation at position 118 were synthesised such that:

3′-TTGGCAGGAACATTATACATGGCAGTGATTCTGTGGAGAGTGC-5′

3′-TTGGCAGGAATTTTATACATGGCAGTGATTCTGTGGAGAGTGC-5′

These oligonucleotides were used with the Qiagen Quikchange XL site-directed mutagenesis kit (Qiagen, Crawley, UK). The integrity of the mutant cDNAs was verified by base sequencing. The mutated plasmid was transformed into XL10-Gold ultra competent cells (Strategene, Leicester, UK) and the recombinant mutant protein produced as for the WT Nm23H1. The Kinase-Glo® Luminescent Kinase Assay (Promega, Madison, WI, USA) with substrates of ATP and UDP was used to confirm the NDP kinase activity and inactivity of recombinant WT Nm23H1 and Nm23H1 (H118), respectively (data not shown).

### 2.4. Coating of culture plates with recombinant Nm23H1

A choice assay of culture substrata has been used extensively to model the interaction of neurons with immobilised ECM molecules present within developing and injured neural systems [21,23,24]. This assay was adapted to examine the response of neurons to immobilised extracellular recombinant Nm23H1 proteins as follows: culture plates were pre-coated with a thin layer of protein-binding nitrocellulose (BA85, Schleicher and Schuell, Dassel, Germany), which was then blotted with strips of filter paper presoaked in recombinant WT Nm23H1 or Nm23H1 (H118F) proteins at various concentrations (10–1000 μg/ml). After the filter strips had dried to completion, they were removed and the plates were coated with a solution of 100 μg/ml of collagen type I (in phosphate buffered saline, PBS; both Sigma) for 5 min, and washed in PBS prior to seeding with chick DRG explants or rat DRG neurons. The immobilisation of the recombinant Nm23H1 on the culture plates was routinely visualized by inclusion of 10% (v/v) rhodamine B (Sigma) in the Nm23H1 solutions and confirmed in separate plates by Nm23H1 immunoblotting (data not shown). In separate experiments, nitrocellulose-coated culture plates were uniformly coated with solutions of laminin (40 μg/ml, Sigma) or Nm23H1 (800 μg/ml) prior to seeding with chick DRG explants in NGF-supplemented culture medium. After 48 h, the culture medium was then further supplemented with the JG22 monoclonal antibody (1/200 final concentration, Developmental Studies Hybridoma Bank, University of Iowa, USA) or with an irrelevant negative control antibody. Treatment of chick DRG neurons with the JG22 antibody has been demonstrated to specifically block neuronal integrins binding to the Asp-Gly-Asp (RGD) motif of laminin [25,26]. The extent of continued outgrowth of DRG neurites on these uniform substrata in the presence of the JG22 function blocking antibody versus control was then monitored for a further 24 h.

### 2.5. Treatment of chick DRG explants with soluble Nm23H1

E10 chick DRG explants were seeded in culture plates uniformly coated with 100 μg/ml collagen type I and then treated with 20–200 μg/ml of soluble WT Nm23H1 in serum free culture medium without NGF supplementation. Control cultures were set identically in the absence of WT Nm23H1. After 48 h, the DRG neurite outgrowth was quantitated using previously described methods [24].

### 2.6. Beta-III tubulin immunocytochemistry

Rat DRG neurons were 10% formalin-fixed, washed repeatedly in PBS, incubated with a blocking buffer, and then incubated with rabbit anti-rat beta-III tubulin antibodies (Sigma). Control immunolocalisation was performed on parallel cultures omitting the primary antisera only. Immunoreactivity was revealed with a secondary anti-rabbit Ig-Alexa 488 conjugated antibody (Invitrogen). No immunoreactivity was observed when the primary antibodies were omitted (data not shown).

### 2.7. Microscopy, image capture and analysis

Cultures were viewed using phase contrast and fluorescence microscopy (Nikon, Kingston-upon-Thames, UK). Digitized images were captured with a Hamamatsu digital camera (Hamamatsu photonics, Welwyn Garden City, UK) and analysed using IPLab software (Becton Dickinson). Adapting methods previously described [23,24], the digitised images were used to quantitate (i) the number of beta-III tubulin-immunopositive rat DRG neuronal bodies present on substrata of recombinant Nm23H1 proteins versus an equivalent area of adjacent collagen; (ii) the proportion of those DRG neuronal bodies that extended neurites, i.e. processes greater than 10 μ in length. For time-lapse microscopy, digitized images were captured using a digital video camera (JVC, London, UK) and converted into video files using Media Studio Video Editor (Ulead Systems, Karst, Germany).

### 2.8. Statistical analysis

Experiments were performed independently at least three times. Data derived from images of rat DRG neurons were pooled and analysed using the non-parametric Mann Whitney *U* test. Data on chick DRG neurite length in the presence of soluble Nm23H1 versus control were pooled and analysed using the Mann Whitney *U* test. The effects of supplementing culture medium with Nm23H1 in solution were examined using a non-parametric analysis of variance, the Kruskal–Wallis test. In all figures, levels of significance

have been indicated or given in the figure legends as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

## 3. Results

### *3.1. Extracellular Nm23H1 promoted neurite outgrowth from explants of chick DRG and was a preferred culture substratum compared to collagen type I*

Chick DRG explants were seeded in culture plates with substratum-bound recombinant Nm23H1 adjacent to areas that were coated only with collagen type I. When DRG explants settled onto the collagen substrata, and in the presence of NGF, the DRG neurites grew out from the explants to extend from the collagen onto the Nm23H1 substrata. The neurites then continued to extend over the Nm23H1 until they reached the other side of the immobilised Nm23H1 protein strip, where the Nm23H1 again bordered with the collagen-coated area. At this point, the neurites turned away from the collagen-coated substrata to remain on the Nm23H1 and continued extending on the side of the Nm23H1. This apparent "repellent" effect of the collagen substrata was dependent on the concentration of the recombinant Nm23H1 that was used to coat the culture plates. Hence, at coating concentrations of 10 μg/ml Nm23H1, very few DRG neurites turned in this manner, whereas at coating concentrations of 100 μg/ml Nm23H1, the majority of the DRG neurites turned to remain on the Nm23H1 (Fig. 1A and B). At 500 μg/ml Nm23H1 (or above), chick DRG neurite outgrowth on the Nm23H1 substrata was so favoured that once neurites had extended onto the Nm23H1, they then remained almost exclusively on the Nm23H1. DRG neurites on the Nm23H1-coated areas also extended more rapidly than those on the collagen-coated areas (Fig. 1C; see also Supplementary Video 1). Furthermore, the

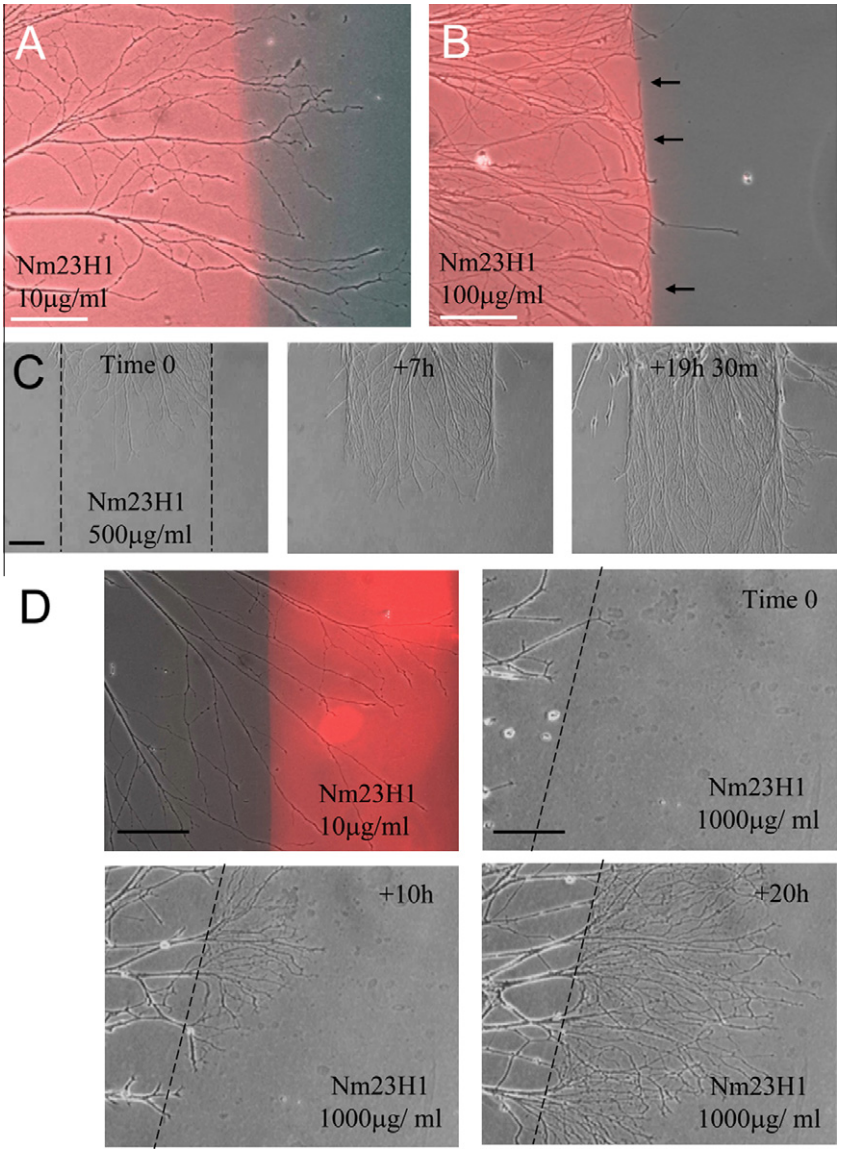


**Fig. 1.** Extracellular Nm23H1 formed a preferred culture substratum for chick DRG neurite outgrowth compared to collagen type I. (A) At low coating concentrations of Nm23H1 (10 μg/ml) there was no evidence of growth cone turning at Nm23H1 (red fluorescence): collagen (black background) borders. (B) At equivalent concentrations of Nm23H1 and collagen (100 μg/ml), extending DRG neurites turned to avoid the collagen and remained on the Nm23H1-coated areas (arrowed). (C) At high Nm23H1 concentrations (500 μg/ml), the extending DRG neurites appeared almost limited to the Nm23H1 substrata (see Supplementary Video 1). The location of the Nm23H1: collagen border is indicated by a dotted line. (D) DRG neurite bundles extending from collagen-coated areas (left of the dotted line) to encounter Nm23H1-coated areas (right of the dotted line; 1000 μg/ml) underwent extensive de-fasciculation and increased branching (see Supplementary Video 2). These effects were less evident at low concentrations of Nm23H1 (top left panel). Calibration bars A–B = 200 μm; C–D = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

DRG neurites on Nm23H1 substrata versus collagen also differed in characteristic. Fig. 1D is representative of how DRG neurite outgrowth became de-fasciculated and branched extensively as they extended from collagen-coated areas of the culture plates onto high concentrations of Nm23H1 substrata (coated with 1000 μg/ml: see also Supplementary Video 2). These effects were less evident at lower concentrations of Nm23H1, such that at coating concentrations of 10 μg/ml Nm23H1 the pattern of DRG neurite growth on adjacent collagen and Nm23H1 substrata were effectively indistinguishable.

In the absence of NGF, chick DRG neurite outgrowth onto homogenous collagen substrata was generally poor. However, neurite outgrowth over collagen substrata was moderately increased in culture medium supplemented with recombinant Nm23H1, in an Nm23H1 concentration-dependent fashion. Hence, after 48 h of supplementation of the medium with 200 μg/ml of Nm23H1 (final concentration) the mean neurite length in chick explants was ≃50% greater than that observed in non-treated chick DRG explants and also greater than that seen in chick explants treated with 20 μg/ml Nm23H1 (Fig. 2). Although moderate, these increases in neurite outgrowth in response to Nm23H1 supplementation were significant, suggesting that soluble Nm23H1 stimulated neurite outgrowth. However, it is also possible that the Nm23H1 protein added to the culture medium may have bound to the culture plates over this period to account for the effect.

Extracellular Nm23H1 promoted adult rat DRG neuronal adhesion and stimulated DRG neurite outgrowth in the presence or absence of supplementary NGF.

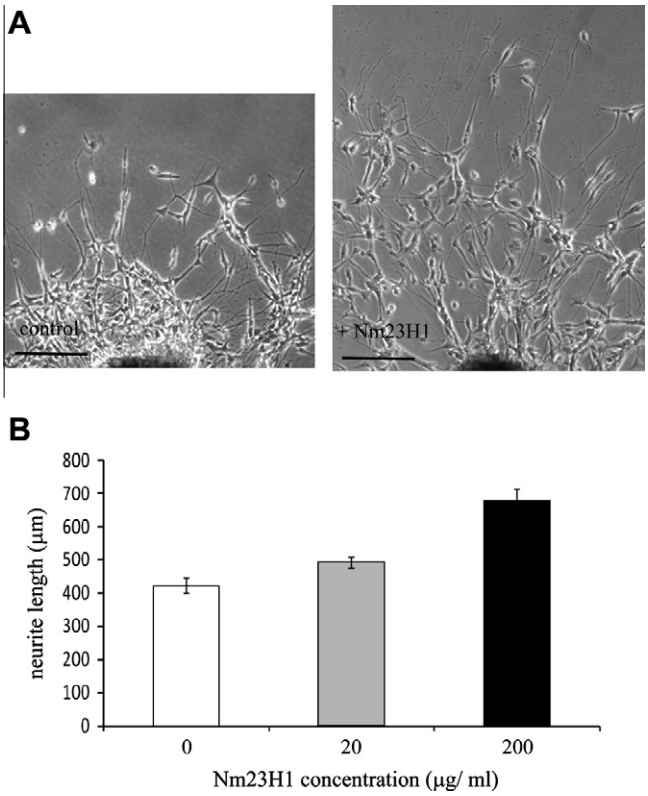


**Fig. 2.** Supplementing culture medium with recombinant Nm23H1 was associated with moderate increases in chick DRG neurite outgrowth. (A) Representative images are shown of DRG neurite outgrowth in medium supplemented with 200 μg/ml of Nm23H1 (right panel) and in non-supplemented control medium (left panel) after 48 h. (B) Although moderate compared with the effects of substrata of Nm23H1 (e.g. see Fig. 3D), the increase in DRG neurite outgrowth in Nm23H1-supplemented medium was significant and Nm23H1 concentration-dependent ($p \leqslant 0.05$ Kruskal–Wallis). Calibration bars = 200 μ. Data shown are means ± SEM.

We tested the effects of extracellular Nm23H1 protein on neurons dissociated from adult rat DRG. Hence, Nm23H1 formed a permissive substratum for rat DRG neuronal adhesion, with significantly more βIII-tubulin immunopositive neurons adhering to the Nm23H1-coated areas than to adjacent areas coated with collagen type I. In addition, a greater proportion of those adherent DRG neurons on the Nm23H1 extended neurites than those neurons on the adjacent collagen-coated areas. Similar to the effects seen with chick DRG explants, some of the rat DRG neurites turned away from the collagen at the Nm23H1: collagen border. However, many of the rat DRG neurites also stopped extending at the border, with no apparent change in direction or neurite retraction (highlighted in Fig. 3A). The significant increases in rat DRG neuronal adhesion and neurite outgrowth on Nm23H1-coated areas compared to collagen was seen both in the presence (Fig. 3B and C) and absence (Fig. 3E and F) of supplementary NGF, with no marked differences between the two conditions. Furthermore, the increased outgrowth of neurites from chick DRG explants onto Nm23H1-coated areas, and the preference of these neurites for the Nm23H1 substrata in comparison to collagen was observed also in the absence of NGF supplementation (Fig. 3D). Hence, the neuro-stimulatory effects of substratum-bound extracellular Nm23H1 were NGF independent.

### *3.2. The neuro-stimulatory effects of extracellular Nm23H1 were independent of its NDP kinase activity*

The same coating concentrations of wild type (WT) Nm23H1 and Nm23H1 (H118F) were compared (100 μg/ml) adjacent to substrata that had been coated with collagen type I (also at 100 μg/ml). The NDP kinase inactive form of Nm23H1 (H118F) stimulated chick DRG neurite outgrowth and induced neurite turning at collagen borders in a similar fashion to the WT Nm23H1 (Fig. 4A). Furthermore, Nm23H1 (H118F) was as effective as WT Nm23H1 in promoting neuronal adhesion and neurite outgrowth from dissociated adult rat DRG neurons (Fig. 4B and C).

## 4. Discussion

In non-neuronal cells, an extracellular activity for Nm23H1 protein has been demonstrated [14,17–19]. Despite not having any classical transport motifs, Nm23H1 can be detected in blood [10–12] and in cell culture conditioned medium [15,16]. Therefore, we have performed an *in vitro* study to examine the effects of extracellular Nm23H1 on nerve growth. We have demonstrated a remarkable neuro-stimulatory activity for recombinant Nm23H1 when the protein was immobilized as a culture substratum for primary chick and rat DRG neurons. This novel finding suggests that extracellular Nm23H1 may function in the regulation of neuronal growth.

The mechanisms by which extracellular Nm23H1 stimulates neurite outgrowth are currently unclear. In non-neuronal cells, the NDP kinase activity of intracellular Nm23H1 has been reported to signal through the Rho pathway [27]. Rho signalling is integral to axonal growth and path-finding mechanisms during development [28]. Other studies have demonstrated that extracellular ATP can induce neuronal growth cone turning [29]. Therefore, we hypothesized that the neuro-stimulatory activity of extracellular Nm23H1 may be dependent on its NDP kinase activity. We tested this by examining the effects of an NDP kinase inactive form of Nm23H1 (H118F) [22]. However, we found that recombinant Nm23H1 (H118F) was stimulatory for chick and rat DRG neurons and induced growth cone turning when adjacent to collagen substrata to much the same extent as recombinant WT Nm23H1. Lombardi and co-workers [30] found that transfection of PC12 cells

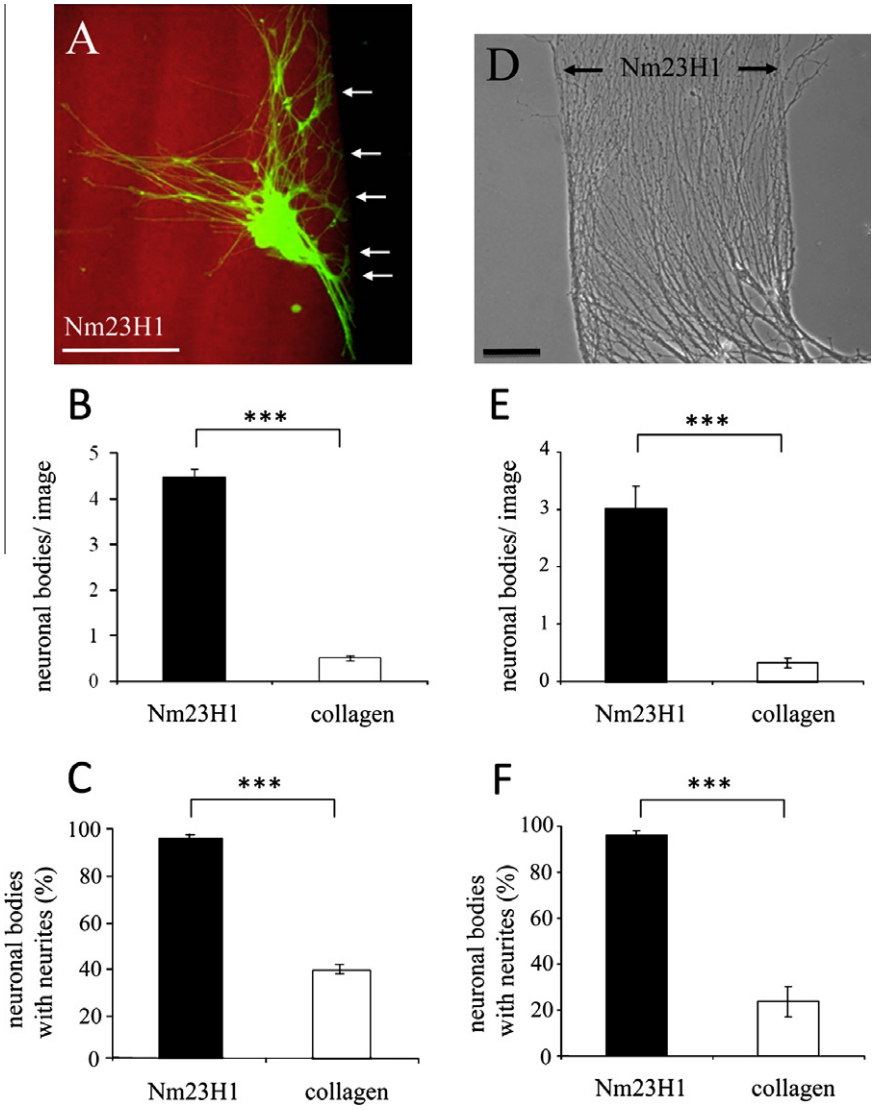


**Fig. 3.** Extracellular Nm23H1 substrata promoted rat DRG neuronal adhesion and stimulated DRG neurite outgrowth in the presence and absence of supplementary NGF: (A) Merged image where dissociated rat DRG neurons have been immunolabelled for βIII tubulin (shown in green), the Nm23H1-coated area is shown in red, and the adjacent collagen substratum is unstained (black background). DRG neurites stopped extending as they encountered the Nm23H1 border with collagen (arrowed). (B) The number of βIII-tubulin immunopositive neuronal bodies adhered to the Nm23H1-coated areas (black bar) was significantly greater than the number adhered to an equal area of the adjacent collagen substrata (open bar). (C) The percentage of adherent neurons which extended neurites onto the Nm23H1 substrata (black bar) was significantly greater than those neurons which extended neurites onto collagen substrata (open bar). The neuro-stimulatory effects of Nm23H1 were also seen in the absence of NGF supplementation. (D) A representative image of neurite outgrowth from a chick DRG explant onto an Nm23H1-coated area (coating concentration of 200 μg/ml) in the absence of NGF. Significant increases were also seen in the number of rat DRG neuronal bodies that adhered to Nm23H1 substrata compared with adjacent areas of collagen (E) and in the proportion of those neurons that extended neurites (F) in the absence of NGF. Calibration bars = 100 μ. Data shown are means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

with *Nm23H1 (H118F)* stimulated neurite outgrowth to a similar extent as transfection with WT *Nm23H1*. The capacity of induced Nm23H1 expression to inhibit the metastasis of prostrate carcinoma cells was also independent its NDP kinase activity [22], as were the effects of extracellular recombinant Nm23H1 proteins on haematopoietic cell differentiation [17]. Conversely, transfection of tumour cells with double mutated *Nm23H1*, i.e. with S120G combined with P96S, which prevents correct protein folding and also blocks the induced proteins' NDP kinase activity, abrogated its inhibitory effects on metastasis [31]. Therefore, we conclude that the neuro-stimulatory activity of extracellular Nm23H1 was independent of its NDP kinase activity, whilst considering that these various mutations remain targets in the evaluation of how extracellular Nm23H1 functions to this effect.

There are alternative means by which extracellular Nm23H1 may influence nerve growth. A putative membrane receptor for Nm23H1 has recently been identified, where the type I membrane glycoprotein of the mucin family, MUC1, functioned in regulating the growth of human embryonic stem cells [19] and various tumour cell lines [32] via its interaction with extracellular Nm23H1. This interaction depended on whether the ectodomain of the MUC1 receptor was cleaved to generate a transmembrane cleavage product, so-called MUC1*, which only then bound to extracellular Nm23H1 ligand. The specificity of the interaction between the MUC-1* receptor and extracellular Nm23H1 was demonstrated by application of a monovalent Fab against the MUC1* cleavage product [32]. A similar approach could be used to examine this putative interaction between extracellular Nm23H1 and neurons, if these cells are also found to express MUC1*. Alternatively, Nm23H1 contains a single arginine-glycine-aspartic (RGD) sequence, a well characterised cell adhesion motif for β1 integrins present on growth cones that are known to be important to axonal

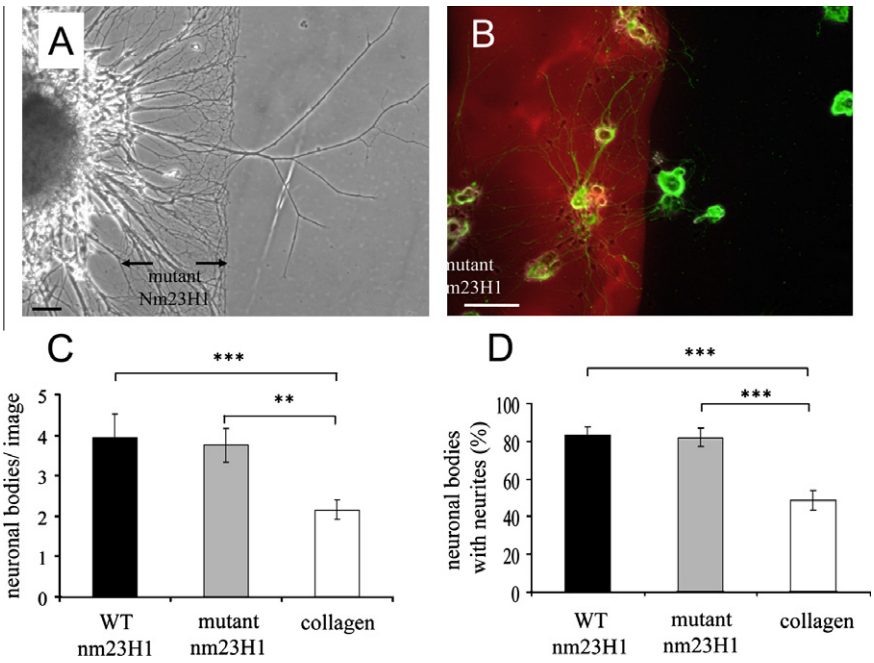


**Fig. 4.** The neuro-stimulatory effects of substratum-bound Nm23H1 were independent of its NDP kinase activity. (A) A representative image demonstrating preferential and extensive chick DRG neurite outgrowth on Nm23H1 (H118F) substrata in comparison with adjacent collagen-coated areas. This response was similar to that seen with WT Nm23H1. (B) A merged representative image of βIII-tubulin III immunopositive rat DRG neuronal bodies (green fluorescence), which preferentially adhered to substrata of Nm23H1 (H118F) (red fluorescence) compared with adjacent collagen-coated areas (black background). (C) Substratum-bound WT Nm23H1 (black bar) and substratum-bound Nm23H1 (H118F) (grey bar) promoted adult rat DRG neuronal adhesion and neurite outgrowth to a similar extent, compared to adjacent collagen substrata. In these experiments, all substrata were established with equivalent protein coating concentrations, i.e. WT Nm23H1, Nm23H1 (H118F) and collagen type I were all at 100 μg/ml. Calibration bars = 100 μ. Data shown are means ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

outgrowth and growth cone turning [33]. We found that treatment with function blocking monoclonal antibodies [25,26] to inhibit binding of neuronal integrins with the RGD motif of extracellular substrata caused neurite retraction on laminin, which also contains the RGD motif, but was ineffective on Nm23H1 substrata (data not shown). This suggests that the effects of extracellular Nm23H1 in stimulating nerve growth are not dependent on RGD: integrin interaction. However, the potential involvement of the Nm23H1 RGD motif in stimulating nerve growth may be further explored by using recombinant forms of Nm23H1 protein where the motif has been removed or altered.

When Nm23H1 was immobilized to culture plates in defined locations versus collagen type I, a clear preference of the growing DRG neurites for the Nm23H1-coated areas was observed, even when the coating concentrations of the Nm23H1 and collagen type I were equivalent. Previously, we have demonstrated that collagen type I-coated culture plates also established at the same coating concentrations of 100 μg/ml were conducive to DRG neurite outgrowth and formed a preferable substratum to other extracellular proteins present in the injured central nervous system, i.e. chondroitin sulphated proteoglycans (CSPGs), myelin associated glycoprotein (MAG) and Nogo-A [24]. In earlier studies, we found that chick DRG neurites showed no marked preferences for adjacent substrata of laminin versus collagen type I, or for collagen type I versus collagen type II [23]. Clearly, therefore, the turning of neuronal growth cones to remain on recombinant Nm23H1 at Nm23H1: collagen borders, i.e. where the collagen type I appeared nerve "inhibitory", depends on a relative "choice" between those extracellular proteins present. This "choice" resulted in an overall positive guidance effect of the Nm23H1. The mechanisms responsible for this preference are currently unclear, but the application of recombinant Nm23H1 proteins may prove useful in providing guidance cues for tissue repair of neural deficits, where collagen and CSPG deposition occurs in scar tissues to prevent axonal regeneration [34].

In conclusion, this study has demonstrated a remarkable and novel neuro-stimulatory activity for extracellular Nm23H1. The identification of this activity may provide insight into how Nm23H1 functions in developing neuronal systems. The mechanisms involved in the neurotrophic function of extracellular Nm23H1 remain unclear, although we have determined that this function does not depend on its NDP kinase activity or presence of supplementary NGF, or appear to depend on RGD: integrin interactions. A number of other potential mechanisms have been suggested and their elucidation will help target the use of Nm23H1 for therapeutic application.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.039.

## References

[1] M. Boissan, S. Dabernat, E. Peuchant, et al., The mammalian Nm23/NDPK family: from metastasis control to cilia movement, Mol. Cell. Biochem. 329 (2009) 51–62.
[2] G. Bevilacqua, M.E. Sobel, L.A. Liotta, et al., Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node

involvement and other histopathological indicators of high metastatic potential, Cancer Res. 49 (1989) 5185–5190.
[3] M.A. Caligo, P. Grammatico, G. Cipollini, et al., A low NM23.H1 gene expression identifying high malignancy human melanomas, Melanoma Res. 4 (1994) 179–184.
[4] J.D. Kantor, B. McCormick, P.S. Steeg, et al., Inhibition of cell motility after nm23 transfection of human and murine tumor cells, Cancer Res. 53 (1993) 1971–1973.
[5] E. Suzuki, T. Ota, K. Tsukuda, et al., Nm23–H1 reduces in vitro cell migration and the liver metastatic potential of colon cancer cells by regulating myosin light chain phosphorylation, Int. J. Cancer 108 (2004) 207–211.
[6] J.H. Lee, J.C. Marshall, P.S. Steeg, et al., Altered gene and protein expression by Nm23–H1 in metastasis suppression, Mol. Cell. Biochem. 329 (2009) 141–148.
[7] A. Bilitou, J. Watson, A. Gartner, et al., The NM23 family in development, Mol. Cell. Biochem. 329 (2009) 17–33.
[8] F. Gervasi, I. D'Agnano, S. Vossio, et al., NM23 influences proliferation and differentiation of PC12 cells in response to nerve growth factor, Cell Growth Differ. 7 (1996) 1689–1695.
[9] R. Amendole, R. Martinez, A. Negroni, et al., DR-NM23 gene expression in neuroblastoma cells: relationship to intergrin expression, adhesion characteristics, and differentiation, J. Natl. Cancer Inst. 89 (1997) 1300–1310.
[10] N. Niitsu, J. Okabe-Kado, M. Nakayama, et al., Plasma levels of the differentiation inhibitory factor NM23–H1 protein and their clinical implications in acute myelogenous leukemia, Blood 96 (2000) 1080–1086.
[11] N. Niitsu, J. Okabe-Kado, M. Okamoto, et al., Serum NM23–H1 protein as a prognostic factor in aggressive non-Hodgkin lymphoma, Blood 97 (2001) 1202–1210.
[12] J. Okabe-Kado, T. Kasukabe, Y. Honma, et al., Clinical significance of serum NM23–H1 protein in neuroblastoma, Cancer Sci. 96 (2005) 653–660.
[13] R. Willems, D.R. Van Bockstaele, F. Lardon, et al., Decrease in nucleoside diphosphate kinase (NDPK/nm23) expression during hematopoietic maturation, J. Biol. Chem. 273 (1998) 13663–13668.
[14] J. Okabe-Kado, T. Kasukabe, Y. Honma, et al., Extracellular NM23 protein promotes the growth and survival of primary cultured human acute myelogenous leukemia cells, Cancer Sci. 100 (2009) 1885–1894.
[15] J. Okabe-Kado, T. Kasukabe, Y. Honma, et al., Identity of a differentiation inhibiting factor for mouse myeloid leukemia cells with NM23/nucleoside diphosphate kinase, Biochem. Biophys. Res. Commun. 182 (1992) 987–994.
[16] J. Anzinger, N.A. Malmquist, J. Gould, et al., Secretion of a nucleoside diphosphate kinase (Nm23–H2) by cells from human breast, colon, pancreas and lung tumors, Proc. West Pharmacol. Soc. 44 (2001) 61–63.
[17] R. Willems, H. Slegers, I. Rodrigus, et al., Extracellular nucleoside diphosphate kinase NM23/NDPK modulates normal hematopoietic differentiation, Exp. Hematol. 30 (2002) 640–648.
[18] J. Okabe-Kado, T. Kasukabe, Y. Honma, et al., Extracellular NM23–H1 protein inhibits the survival of primary cultured normal human peripheral blood mononuclear cells and activates the cytokine production, Int. J. Hematol. 90 (2009) 143–152.
[19] S.T. Hikita, K.S. Kosik, D.O. Clegg, et al., MUC1* mediates the growth of human pluripotent stem cells, PLoS One 3 (2008) e3312.
[20] D.M. Snow, V. Lemmon, D.A. Carrino, et al., Sulphated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro, Exp. Neurol. 109 (1990) 111–130.
[21] Z. Ahmed, R.G. Dent, E.L. Suggate, et al., Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75NTR and Rho-A, Mol. Cell. Neurosci. 28 (2005) 509–523.
[22] H.Y. Lee, H. Lee, Inhibitory activity of nm23–H1 on invasion and colonization of human prostate carcinoma cells is not mediated by its NDP kinase activity, Cancer Lett. 145 (1999) 93–99.
[23] W.E.B. Johnson, B. Caterson, S.M. Eisenstein, et al., Human intervertebral disc aggrecan inhibits nerve growth in vitro, Arthritis Rheum. 46 (2002) 2658–2664.
[24] K.T. Wright, W. El Masri, A. Osman, et al., Bone marrow stromal cells stimulate neurite outgrowth over neural proteoglycans (CSPG), myelin associated glycoprotein and Nogo-A, Biochem. Biophys. Res. Commun. 354 (2007) 559–566.
[25] B. Wehrle-Haller, M. Chiquet, Dual function of tenascin: simultaneous promotion of neurite growth and inhibition of glial migration, J. Cell. Sci. 106 (1993) 597–610.
[26] J.M. Greve, D.I. Gottlieb, Monoclonal antibodies which alter the morphology of cultured chick myogenic cells, J. Cell. Biochem. 18 (1982) 221–229.
[27] M. Murakami, P.I. Meneses, K. Lan, et al., The suppressor of metastasis Nm23–H1 interacts with the Cdc42 Rho family member and the pleckstrin homology domain of oncoprotein Dbl-1 to suppress cell migration, Cancer Biol. Ther. 7 (2008) 677–688.
[28] E.E. Govek, S.E. Newey, L. Van Aelst, The role of the Rho GTPases in neuronal development, Genes Dev. 19 (2005) 1–49.
[29] W.M. Fu, Y.B. Tang, K.F. Lee, Turning of nerve growth cones induced by the activation of protein kinase C, Neuroreport 8 (1997) 2005–2009.
[30] D. Lombardi, E. Palescandolo, A. Giordano, et al., Interplay between the antimetastatic nm23 and the Retinoblastoma-related Rb2/p130 genes in promoting neuronal differentiation of PC12 cells, Cell Death Differ. 8 (2001) 470–476.
[31] Q. Zhou, X. Yang, D. Zhu, et al., Double mutant P96S/S120G of Nm23–H1 abrogates its NDPK activity and motility-suppressive ability, Biochem. Biophys. Res. Commun. 356 (2007) 348–353.
[32] S. Mahanta, S.P. Fessler, J. Park, et al., A minimal fragment of MUC1 mediates growth of cancer cells, PLoS One 3 (2008) e2054.
[33] E.L. Goh, J.K. Young, K. Kuwako, et al., Beta1-integrin mediates myelin-associated glycoprotein signaling in neuronal growth cones, Mol. Brain 1 (2008) 10.
[34] S. Hermanns, N. Klapka, M. Gasis, et al., The collagenous wound healing scar in the injured central nervous system inhibits axonal regeneration, Adv. Exp. Med. Biol. 557 (2006) 177–190.