Mix and match of the tumor metastasis suppressor Nm23 protein isoforms in vitro and in vivo

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Nm23/NME was identified 30 years ago as the first metastatic gene suppressor family. Despite extensive studies, the mechanism of action behind the observed antitumour potential of Nm23 has remained largely unresolved. Human Nm23 is present in various isoforms, of which Nm23-H1 and Nm23-H2 are by far the most dominant. Both isoforms are multifunctional enzymes involved in important cellular processes, through their nucleic acid binding ability, their protein–protein interactions and/or their histidine kinase activity. Although Nm23-H1 and Nm23-H2 exhibit 88% sequence homology, they often are considered to have distinct biological functions. Here, we developed an efficient and robust purification protocol to pull-down Nm23 isoforms in their native state. We applied this protocol to purify both overexpressed isoform pure as well as endogenous Nm23 proteins from several human cell lines and mouse brain tissue. Subsequent native mass spectrometry (MS) analysis revealed that all purified Nm23 samples form hexamers, whereby the endogenous protein assembly is primarily present as heterohexamers formed by statistical association of the Nm23-H1 and Nm23-H2 isoforms. Therefore, we conclude that isoform-pure hexameric Nm23 complexes scarcely exist in vivo. We also used native and top-down MS to investigate the histidine autophosphorylation activity of purified Nm23 assemblies. Our data in fine challenge the biological relevance of studying the genes/proteins Nm23-H1 and Nm23-H2 individually.

Introduction

The human Nm23/NME gene family is composed of (at least) 10 isoforms, the two most abundant (Nm23-H1 and Nm23-H2) being both highly and ubiquitously expressed [1]. These two isoforms code for nucleoside diphosphate kinase (NDPK) A and B, initially considered as housekeeping enzymes whose main cellular function is to preserve the balance between different nucleoside triphosphates within the cell [2,3]. In 1988, Nm23 was identified as the first metastatic suppressor gene [4] and by now a correlation between the expression of Nm23-H1 and Nm23-H2 and the metastatic potential of numerous cancer types has been well documented [5–7]. Over the years, both Nm23-H1 and Nm23-H2 isoforms have been extensively studied and while underlying molecular mechanisms of their metastasis suppressor potentials remain to this day largely unresolved, it appeared in the last three decades that Nm23 proteins are multifunctional proteins, involved in the regulation of a multitude of cellular processes [1]. Nm23-H1 and Nm23-H2 share an 88% amino acid sequence homology and available crystal structures of isoform pure Nm23-H1 or isoform pure Nm23-H2 revealed that both form stable hexamers with strikingly similar structures [8,9] (Fig. 1). It is then

Abbreviations
EThcD, electron-transfer/higher energy collision dissociation; MS, mass spectrometry; NDPK, nucleoside diphosphate kinase.
remarkable that both isoforms are mostly studied separately and described in the literature as having little in common in terms of protein interactors or substrates, and thus have distinct biological functions. One notable difference between these highly alike isoforms reside in their distinct isoelectric point, explained by the
presence of few highly basic external residues in Nm23-H2 (Fig. 1), some of these nonconserved residues being linked to its ability to bind DNA [10].

Changes in either Nm23-H1 [11,12] or Nm23-H2 [13,14] expression have been shown to regulate an important set of genes, related to cell adhesion, invasion, metastasis, proliferation and/or differentiation. The Nm23 main isoforms are both involved in gene regulation through DNA binding or via interactions with various transcription factors. However, it has been proposed that few of the mechanisms of transcriptional regulation are thought to be shared by the two isoforms [15], which have even been described as having antagonistic effects in the case of the modulation of estrogen response element-mediated transcription [16,17]. Among the other numerous important biological roles of Nm23 proteins, Nm23-H1 was described as having a granzyme A-activated exonuclease activity [18], as inhibiting the TGFβ [19] and ERK [20] signaling pathways, and/or as being involved in GTPase regulation [21]. The less-studied Nm23-H2, on the other hand, has been described as a transcriptional activator of c-MYC [22] or as regulating the ion channel KCa3.1 [23]. The Nm23 gene family became even more fascinating when its two most abundant protein isoforms Nm23-H1 and Nm23-H2 were identified as the first – and to this day only known – human histidine kinases [24], capable of transferring a phosphate group to a substrate’s histidine residue after autophosphorylation of its histidine active site. This class of enzyme was thought to have been lost in evolution, histidine phosphorylation being considered as an archaic phosphorylation event bearing essential roles in lower single-cell organism’s metabolism [25] or primitive cell signaling as part of the well-known two-component system [26]. Concerning the histidine kinase activity of Nm23 proteins, little overlap currently exists between the handful of identified substrates of both Nm23-H1 and Nm23-H2.

Several factors hamper the unraveling of the molecular mode(s) of action of Nm23 protein/isoforms, as for example the lack of suitable analytical tools to study the acid-labile protein histidine phosphorylation. Recently reported strategies could help to further decipher the biological functions related to the histidine kinase activity of Nm23 proteins through the potential identification of new substrates [27,28]. Moreover, difficulties related to the Nm23 proteins pull-down have led to the identification of misleading protein interactions when these proteins are expressed above endogenous levels [29]. Here, we present a robust purification protocol of Nm23 proteins combined with subsequent high-resolution native mass spectrometry (MS), allowing us to clearly differentiate Nm23-H1 and Nm23-H2 based on their accurate masses. The presented purification approach can be applied to all Nm23 isoforms, enabling specific purification of overexpressed as well as of endogenous Nm23 proteins from cell lines or tissue. Native MS and 1D gel analysis revealed that overexpressed isoform-pure endogenous Nm23-H1 and Nm23-H2 form exclusively hexamers. In contrast, endogenous purification of Nm23 from cells and/or tissue revealed that Nm23 is in vivo present as isoform-mixed hexamers following statistical association. According to the human protein atlas [30], the two isoforms are ubiquitous but can exhibit differential expression, depending on the cell type. Nevertheless, in many cells both Nm23-H1 and Nm23-H2 are highly expressed and as a result, the cellular abundance of isoform-pure Nm23 hexamers in vivo will be marginal. This observation suggests that mixed hexamers are responsible for the main molecular functions of Nm23 proteins. Therefore, this finding challenges the biological relevance of studying the Nm23-H1 and Nm23-H2 isoforms individually.

Interestingly, purified (Nm23-H1)6 and (Nm23-H2)6 do not exchange subunits in vitro, suggesting that formed hexamers are not dynamic complexes, which will not readjust if the expression levels of the two isoforms are differentially altered, allowing tight regulation of the Nm23 hexameric complexes population. Here, we show that Native MS is the method of choice to precisely monitor the different Nm23 hexameric species abundance distribution. Finally, we demonstrate that both the purified isoform-pure and isoform-mixed hexamers exhibit autophosphorylation activity. The hexamer is phosphorylated six times, and as revealed by top-down proteomics, exclusively at the known histidine phosphate-acceptor residue.

Results and Discussion

Nm23 isoforms H1 and H2 are enzymes known to form hexamers, whose main cellular functions are to maintain the equilibrium between different nucleotide triphosphates, and as such Nm23 possess one nucleotide-binding site per monomer. In an earlier study using affinity chromatography to purify the cAMP- and cGMP-regulated kinases PKA and PKG in heart tissue, we observed and reported the abundant copurification of Nm23 proteins [31]. Two interesting observations concerning Nm23 proteins could be extracted from these data: using cyclic GMP coupled with beads as a bait allowed efficient capture of Nm23 H1 and H2 isoforms, which could then be eluted from the beads by addition of ADP. Here, we adopt a similar affinity purification approach by reversing the order of competitive binding/
elution, to specifically retain Nm23 proteins while eliminating cyclic nucleotide-binding proteins (e.g., PKA, PKG, and cyclic nucleotide phosphodiesterases). This pull-down strategy takes advantage of the multiplicity of nucleotide binding sites of Nm23 proteins and their higher affinity toward ADP over cAMP/cGMP. The amount of purified protein and its degree of purity are critical factors to obtain satisfactory samples for subsequent analysis by Native MS. To optimize our purification protocol, we first focused on HEK293T cells wherein Nm23-H1 or Nm23-H2 was separately overexpressed. A look at the SDS/PAGE profiles of the total cell lysates and pull-down flow-through indicated an efficient, quasi-quantitative capture of Nm23-H1 and Nm23-H2 (Fig. 2A). After optimization of the protocol, high purity overexpressed Nm23-H1 and Nm23-H2 proteins were obtained, as attested by both the SDS/PAGE (Fig. 2A) and the Native MS analysis (Fig. 3A,B). As expected, both isoforms were detected exclusively as isoform pure homohexamers. The determined accurate masses of 102358.5 Da [(Nm23-H1)₆] and 103254.5 Da [(Nm23-H2)₆] exactly correspond to the theoretical mass of the isoform pure hexamers, taking into account that the initiator methionine is cleaved off and that monomers are N-terminal acetylated. Notably, the highly resolved native mass spectra allow us to unambiguously distinguish the highly alike two isoforms. No protein phosphorylation or any other post-translational modification was detected in these spectra. Next, we incubated the two isoform-specific homohexamers together at room temperature for 24 h. Interestingly, the Native MS data revealed that purified (Nm23-H1)₆ and (Nm23-H2)₆ do not exchange subunits in vitro, suggesting a high stability of the purified hexameric protein complexes (Fig. 3C).

The high degree of purity of the purified overexpressed Nm23 proteins spurred us to attempt to purify endogenous Nm23 proteins. The same pull-down protocol was, therefore, applied to nontransfected HEK293T cells, and SDS/PAGE again revealed the excellent purification of Nm23 proteins (Fig. 2B). Subsequent analysis by Native MS revealed that purified endogenous Nm23 proteins are also present as hexameric complexes, albeit composed of mixed and matched isoforms (Figs 3E and 4A). To further validate these observations, we analyzed isoform pure purified (Nm23-H1)₆ and (Nm23-H2)₆ as well as endogenous Nm23 by Native PAGE (Fig. 2C). (Nm23-H1)₆ and (Nm23-H2)₆ displayed different electrophoretic mobilities in the native gel, probably due to the intrinsic different pI value of the two monomers (pI ~ 5.83 for Nm23-H1 vs. 8.52 for Nm23-H2). Endogenous Nm23 hexamers exhibited an intermediate electrophoretic mobility profile, confirming their heterohexameric nature. To ensure that the formation of isoform-mixed hexamers was not an artifact arising from potential denaturation during the pull-down process, cells overexpressing only Nm23-H1 or Nm23-H2 were mixed and lysed together prior to the Nm23 purification. In this control experiment, exclusively isoform-pure...
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hexamers were observed by Native MS analysis (Fig. 3D). When looking closely at the distribution and abundance of the different observed endogenous heterohexamers, it appeared that the distribution followed a hypergeometric model, revealing a statistical association, based on the in vivo abundance of the two

**Fig. 3.** Native mass spectrometric analysis of Nm23 hexamers. Native mass spectrometric analysis of purified overexpressed recombinant Nm23-H1 (A) and Nm23-H2 (B). Both isoforms are uniquely present as homohexamers; (Nm23)₆. Measured MW of the hexamers are in agreement with the expected MW, as calculated from the used gene sequences and thus in these purifications the Nm23 proteins harbor no post-translational modifications. (C) Remarkably, no formation of mixed hexamers was observed when the two isoform pure homohexamers were incubated together, for as long as 24 h. (D) Cells overexpressing either Nm23-H1 or Nm23-H2 were mixed prior to purification. Also here, isoform pure hexameric complexes were the sole observed species, indicating again no exchange of monomers in between the isoform pure complexes. (E) Endogenous Nm23 proteins are present in vivo as isoform-mixed heterohexamers.

**Fig. 4.** Nm23 proteins are present in vivo as isoform-mixed heterohexamers after statistical association. (A) Relative abundances of the different hexameric species present in different cell lines or tissue (HEK293T, U2OS, PC9, and MCF7 cell lines as well as mouse brain tissue). The observed distributions point out to a random association of the two main Nm23 isoforms and abundances of the different protein complexes seem to directly depend on the level of expression of Nm23-H1 and Nm23-H2. (B) Purified overexpressed Nm23-H1 and Nm23-H2 complexes were mixed at a ratio close to 1 : 1, thereafter denatured via acidic treatment, followed by renaturation/refolding into hexamers. Following this protocol, arithmetical formation of heterohexamers is observed, which is confirmed when the same complexes were mixed at a 1 : 10 ratio, and again denatured and renatured/refolded (C).
isomer monomers. Notably, both homohexamers were present only in marginal proportions and barely visible in the mass spectra, with a total intensity representing < 2% of the measured species. The existence of such mixed isoform species has been proposed before, when it was shown that Nm23 proteins purified from human erythrocytes under denaturing conditions followed by renaturation presented intermediate ion exchange chromatographic profiles when compared with profiles of isoform pure Nm23-H1 and Nm23-H2 [32], similar to our Native PAGE analysis (Fig. 2C). Nevertheless, our study provides the first direct evidence of the formation of heterohexameric complexes after random association of endogenous Nm23 proteins in vivo. Indeed, Nm23 proteins are here purified in their native conformation as no denaturing conditions are necessary to disrupt the bait/protein interactions, and MS analysis allows precise quantitation of the different species abundance within the cell. To further validate the statistical formation of isoform-mixed hexamers, both overexpressed isoform-pure hexamers were mixed in different ratios, disassociated through acidic treatment, before renaturation. The formation of isoform-mixed hexamers was again observed, with distributions reflecting the mixing ratio (Fig. 4B,C).

It is well known that the expression of different Nm23 isoforms is cell and tissue dependent [30]. From our Native MS data, it appears that both isoforms are quasi equally expressed in HEK293T cells. We then sought to determine the distribution of the different Nm23 isoform-mixed hexamers present in tissue or cell lines exhibiting differential expression of Nm23-H1 and Nm23-H2. Nm23-H1 is known to be highly expressed in brain tissue [30]; consequently, we purified Nm23 proteins from mouse brain tissue using the same protocol. The distribution of the different hexameric species observed by Native MS followed the expected trend, that is, presented a shift toward a higher stoichiometry of Nm23-H1 (Fig. 4A). In contrast, Native MS analysis of Nm23 proteins purified from U2OS, PC9, and MCF7 cells revealed a distribution shifted to a higher stoichiometry of Nm23-H2 (Fig. 4A), as a consequence of a higher expression of the second Nm23 isoform. Overall, these data demonstrate that the abundance of the different mix and matched isoform in the hexamers directly reflects the level of expression of the Nm23 isoforms.

The physiological relevance of the random association of Nm23 isoforms remains to be determined. In the literature, reported biological functions of Nm23-H1 and Nm23-H2 are mostly different, a consequence of their different intrinsic biochemical properties. One could hypothesize that heterohexameric Nm23-H1/H2 complexes, of intermediate biochemical properties, could have distinct interactors, substrates, and/or cellular localization. Altering the ratio of expressions of the two main Nm23 isoforms in order to study the role of Nm23 proteins could then potentially result in physiologically irrelevant conclusions. In an effort to investigate the impact of the H1/H2 complex stoichiometry on cellular localization, we isolated U2OS cells’ nuclei and performed the Nm23 proteins’ pull-down. Native MS analysis showed that nuclear Nm23 complexes exhibit a shift toward a higher Nm23-H2 stoichiometry when compared to Nm23 complexes purified from the whole cell lysate (Fig. 5). This observation is in accordance with reports of higher nuclear localization of Nm23-H2 isoform when compared to Nm23-H1 [33]. Interestingly, we also observed low abundant Nm23 complexes in which one Nm23-H1 or Nm23-H2 monomer was substituted by one Nm23-H3 monomer. This is the first report of the existence of such complexes in vivo, an observation once again conform with the reported nuclear localization of the Nm23-H3 isoform [30], notably involved in DNA repair [34], as well as with the in vitro association of Nm23-H3 with Nm23-H1 [35].

To monitor whether our Nm23 purification under native conditions indeed led to active Nm23 protein hexamers, the Nm23 hexamers were incubated with ATP and magnesium to investigate histidine kinase autophosphorylation activity. Both isoform-pure hexamers displayed very fast autophosphorylation reactions and were able to reach a nearly fully phosphorylated state (i.e., harboring six phosphate groups), corresponding to the single phosphorylation of ~70% of the monomers, in < 5 min at room temperature (Fig. 6A,B). Addition of EDTA as a chelating agent did not compromise the autophosphorylation reaction, confirming the previous observation that an extremely low magnesium concentration suffices for maximum autophosphorylation [36]. Furthermore, phosphorylation had no noticeable influence on the stability of the hexamers as no formation of heterohexamers was observed upon addition of ATP to

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**Fig. 5.** Native MS analysis of U2OS nuclear Nm23 complexes reveal a shift of the distribution toward a higher stoichiometry of Nm23-H2. (A) Native mass spectrum of endogenous Nm23 purified from U2OS whole cell lysate. (B) Native mass spectrum of endogenous NM23 purified from isolated U2OS nuclei. (C) Comparison of the relative abundances of the observed hexamers in spectra displayed in panels A and B.
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the mixed isoforms (Fig. 6C). Top-down MS (Fig. 6D), as well as the hydrolysis of the observed phosphorylation at acidic pH pointed out to a single His118 histidine phosphorylation per monomer. In Uniprot the nearby residues Ser 120, 122, and 125 have been reported to be potentially phosphorylated. Here, we show that these serine phosphorylations are likely not the results of Nm23 autophosphorylation, but may become phosphorylated by other kinases. Finally, endogenous or in vitro formed isoform-mixed hexamers exhibited the same autophosphorylation behavior than isoform-pure hexamers, in terms of reaction kinetics and final phosphorylation status (Fig. 5E). The fact that the endogenous purified Nm23 hexamers (Figs 3 and 4) were not phosphorylated may indicate a lower cellular Nm23 phosphorylation stoichiometry or, more likely, the known facile hydrolysis of the histidine phosphorylation during the purification procedure.

In summary, Nm23 proteins are fascinating, highly and ubiquitously abundant, multifunctional enzymes impacting crucial cellular processes. Nevertheless, much effort remains to be made to decipher the multitudes of biological functions of Nm23 proteins, especially concerning the elusive histidine kinase activity. By presenting a robust and straightforward purification protocol of endogenously expressed Nm23, we believe our work could represent a valuable technological contribution. Our work reveals that the two main isoforms of Nm23 are present in vivo as mixed hexamers and that the stoichiometry within the hexamers will be directly dependent on the expression level of the Nm23 isoforms. The conclusion that Nm23-H1 and Nm23-H2 homohexamers are marginally present in vivo question the biological relevance of studying these two proteins separately. Indeed, the fact that Nm23-H1/2 are predominately present as heterohexameric complexes could have important impacts on the understanding of biomolecular mechanisms governing Nm23 biological functions, as it could very well influence protein–protein interactions or the relation between the histidine kinase and its substrates. Overall, we expect that our cost-effective and efficient purification protocol will allow researchers to better disentangle the intriguing properties of endogenous Nm23.

Materials and methods

Cell culture, overexpression, and mouse brain tissue

HEK293T, PC9, U2OS, and MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium (Lonza, Basel, Switzerland) containing 10% heat-inactivated FBS (Gibco, Waltham, MA, USA), 2 mM l-glutamine (Lonza), and 1% penicillin-streptomycin (Lonza). Cells were washed twice with ice-cold PBS on-plate, detached by trypsin (Lonza), and collected by low-speed centrifugation for 5 min. Cells were subsequently washed three times with ice-cold PBS and pelleted at low speed for 5 min. HEK293T overexpressing tag-less Nm23-H1 (Uniprot ID P15531, isoform 1) or Nm23-H2 (Uniprot ID P22392, isoform 1) were obtained from U-protein Express (Utrecht, the Netherlands). Mouse brain tissue was obtained as previously described [37].

Nuclei isolation

For the nuclei purification U2OS cells were resuspended at a concentration of 2 million cells·mL⁻¹ in 25 mM HEPES pH 7.5, 50 mM NaCl, 5 mM KCl, protease inhibitors (Roche, Basel, Switzerland), 10 μM MG132. The cells were then left to swell for 20 min on ice before addition of digitonin to the suspension at a final concentration of 40 μg·mL⁻¹. The suspension was passed 100 times through a dounce tissue grinder (pestle B; Sigma, St. Louis, MO, USA) and subsequently the nuclei pelleted for 10 min at 400 g. The pelleted nuclei were washed once in resuspension buffer before lysis. The nuclei were lysed in 50 mM Tris pH 7.4, 300 mM NaCl, 1% Triton X-100 and protease inhibitors, incubated 15 min on ice, and centrifuged at 200 00 g for 30 min. The soluble fraction was used to enrich the Nm23 hexamers.

Purification protocol

Cell pellets (corresponding to 2e⁷ and 1e⁸ cells for overexpressed or endogenously expressed Nm23, respectively) or mouse brain tissue were resuspended in five volumes of lysis buffer composed of PBS, 0.1% Tween, phosphatase inhibitors (Roche) and complete-mini EDTA-free protease inhibitors (Roche). Cells/tissue in suspension were transferred into a dounce-homogenizer and thoroughly lysed on ice as follows: two 30-s cycles with pestle A before two 30-s cycles with pestle B with 1 min of rest in between cycles. After transfer into Eppendorf tubes, the lysate was centrifuged at 20 000 g for 10 min at 4 °C. The supernatant was then filtered on a 0.45-μm filter, the protein concentration was estimated by Bradford assay and the lysate diluted to a concentration of 2 mg·mL⁻¹. Cyclic nucleotides (cAMP and cGMP; Biolog, Hayward, CA, USA) were then added to the lysate at a final concentration of 15 μM each to block binding of cyclic nucleotide binding proteins to the beads. The lysate was subsequently incubated at 4 °C for 30 min. 8-AET-cGMP agarose beads (Biolog) were then added to the lysate at a ratio of 10 μL of dried beads per mg of proteins. The mixture was incubated for 2 h at 4 °C and transferred...
into an empty micro bio-spin polypropylene column (Bio-
rad, Hercules, CA, USA). Beads were washed twice with
1 mL washing buffer composed of PBS, 0.1% Tween and
10 μm cyclic nucleotides, then twice with 1 mL of PBS.
Sequential elutions were performed by addition of
2 × 100 μL elution buffer A (PBS + 10 mM ADP) and

Fig. 6. Autophosphorylation reaction of Nm23 proteins. Overexpressed isoform pure hexameric Nm23-H1 (A) and Nm23-H2 (B) exhibited
fast autophosphorylation reaction, reaching a maximum phosphorylated state corresponding to phosphorylation of 70% of monomers after
only 5 min at room temperature. No addition of magnesium divalent cation was necessary to the reaction. (C) Phosphorylation did not have
any significant effect on the stability of the two homohexameric complexes, as no formation of mixed hexamer was observed when both
homohexamers were mixed in the presence of ATP. (D) Top-down electron-transfer/higher energy collision dissociation (ETnCVD)
disintegration of in vitro phosphorylated Nm23-H1 confirmed the phosphorylation of the histidine 118 residue. The presence of the highly
intense and clearly resolved discriminant ion Z4 points to a single phosphosite, as no other phosphosite could be identified. (E)
Endogenous heterohexamers exhibited the same behavior than homohexamers when incubated with ATP, in term of autophosphorylation
reaction kinetics or maximum phosphorylation state.
2 × 100 μL elution buffer B (PBS + 100 mM ADP). The pH of both elution buffers was adjusted to a value of 7.4 with sodium hydroxide (Sigma). The different elution fractions were pooled and subjected to buffer exchange into 200 mM ammonium acetate (Sigma) and 1 mM DTT (Sigma-Aldrich) adjusted to a pH of 7.4, by eight sequential dilution/concentration steps using 10 kDa molecular weight cutoff columns (Millipore, Burlington, MA, USA).

Autophosphorylation reaction
Magnesium chloride (Sigma-Aldrich) and ATP (Sigma-Aldrich) and were added to the purified overexpressed or endogenous Nm23 complexes at a final concentration of 2 and 5 mM, respectively. In the case of the autophosphorylation reaction without magnesium divalent cation, ATP and EDTA (Sigma-Aldrich) were added to the purified complexes at a concentration of 5 and 10 mM, respectively. After incubation at room temperature, samples were buffer exchanged into 200 mM ammonium acetate and 1 mM DTT adjusted to a pH of 7.4, by five sequential dilution/concentration steps using 10 kDa molecular weight cutoff columns.

Denaturation/renaturation of overexpressed Nm23-H1/2
Purified overexpressed isoform pure Nm23-H1 and Nm23-H2 were mixed at different ratios and buffer exchanged to 1% formic acid (FA; Sigma-Aldrich). Ten percent FA was then added to the sample, at a final concentration of 5%. Following incubation for 5 min at room temperature, samples were buffer exchanged into 200 mM ammonium acetate and 1 mM DTT adjusted to a pH of 7.4, by five sequential dilution/concentration steps using 10 kDa molecular weight cutoff columns.

Native mass spectrometry analysis
Samples were ionized by electrospray and introduced into the exactive EMR (Thermo Fisher Scientific, San Jose, CA, USA) mass spectrometer through the nanospray ionization interface using doubly gold-coated borosilicate needles prepared in house. Spray voltage was set to 1300 V in positive ion mode and the transfer capillary temperature set to 250 °C. The ion transfer optics settings (injection flatapole, interflatapole lens, bent flatapole, transfer multipole, and C-trap entrance lens) were set to 7, 6, 6, 7, and 5.7 V, respectively. Automatic gain control and resolution settings were optimized for each sample. Spectra deconvolution and calculation of the intensities of the different species were performed by the software PROTEIN INTACT (Protein Metrics, San Carlos, CA, USA).

SDS/PAGE and native gel electrophoresis
For SDS/PAGE each sample was loaded and run at constant voltage onto Criterion XT 4–12% precast gels (Biorad). The gels were subsequently washed twice in MilliQ water, stained 1 h with Gel Code Blue Stain Reagent (Thermo Scientific) and destained with MilliQ water. For the native electrophoresis analysis, each purified Nm23 sample was mixed with Native PAGE sample buffer, then loaded and run onto Native PAGE 4–16% Bis-Tris precast gels (Thermo Fisher Scientific) with a cathode running buffer containing the native PAGE Cathode Additive according to the manufacturer’s instructions. The gels were fixed for 1 h shaking with a solution of 40% methanol, 10% acetic acid, and destained with 8% acetic acid.

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Author contributions
CMP and DF performed the experiments and analyzed the data. CMP and AJRH conceived and initiated the study. All authors wrote the manuscript.

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