Human HINT1 Mutant Proteins that Cause Axonal Motor Neuropathy Exhibit Anomalous Interactions with Partner Proteins

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Short report

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Abstract

The 14 kDa histidine triad nucleotide-binding protein 1 (HINT1) is critical to maintain the normal function of motor neurons. Thus, a series of human HINT1 mutants cause autosomal recessive axonal neuropathy with neuromyotonia. HINT1 establishes a series of regulatory interactions with signaling proteins, some of which are enriched in motor neurons, such as the type 1 sigma receptor or intracellular domain (ICD) of transmembrane teneurin 1, both of which are also implicated in motor disturbances. In a previous study, we reported the capacity of HINT1 to remove the small ubiquitin-like modifier (SUMO) from a series of substrates and the influence of HINT1 mutants on this activity. We now report how human HINT1 mutations affect the interaction of HINT1 with the regulator of its SUMOylase activity, calcium-activated calmodulin, and its substrate SUMO. Moreover, HINT1 mutants exhibited anomalous interactions with G-protein coupled receptors, such as the mu-opioid, and with glutamate *N*-methyl-D-aspartate receptors as well. Additionally, these HINT1 mutants showed impaired associations with transcriptional regulators such as the regulator of G protein signaling Z2 protein and the cleaved N-terminal ICD of teneurin 1. Thus, the altered SUMOylase activity of human HINT1 mutants and their anomalous interactions with partner proteins may disrupt signaling pathways essential to the normal function of human motor neurons.

Background

Histidine triad nucleotide-binding protein 1 (HINT1) is highly phylogenically conserved [1] and belongs to the histidine triad (HIT) family. This 14 kDa protein exhibits zinc- and calmodulin (CaM)-regulated protease activity to remove small-ubiquitin-like modifier (SUMO) from target proteins [2]. In recent years, a rare form of hereditary peripheral neuropathy named autosomal recessive axonal neuropathy with neuromyotonia (ARAN-NM) has convincingly being related to mutations in the *HINT1* gene. These patients present muscle weakness, wasting and sensory loss, which starts in the distal parts of the limbs and slowly progresses in a length-dependent manner [3, 4]. HINT1 neuropathy is worldwide distributed and is particularly prevalent in populations in central and southeastern Europe. To date, the majority of diagnosed individuals are of European origin, and among the 15 HINT1 mutations reported, the most common are R37P, C84R and H112N [5]. Notably, the targeted disruption of the *HINT1* gene does not promote neuropathy-related phenotypes, at least in mice [6]. This finding suggests that human HINT1 mutant proteins, by interacting with third-partner signaling proteins, cause the disease, and some of these interactions may play a relevant role in motor neuron function.

HINT1 is widely expressed in the central nervous system and in other tissues [7, 8]. At the cellular level, this protein is found in the plasma membrane, cytoplasm and nucleus [1]. HINT1 behaves as a scaffold and/or chaperone in its regulatory interactions with a variety of signaling proteins. HINT1 binds simultaneously to the cytosolic C-terminal of the mu-opioid receptor (MOR), to protein kinase C (PKC) and to regulators of G protein signaling (RGS) proteins of the Rz family, such as RGSZ1 and RGSZ2, when they are coupled to neural nitric oxide synthase (nNOS) [9, 10]. With the aid of nitric oxide (NO) from nNOS and zinc ions from cysteine-rich domains of RGSZ2 proteins [11, 12], HINT1 binds the regulatory

domain of Raf-1 and conventional PKCγ and PKCα and inhibits their activity [12, 13]. HINT1 also binds to NR1 subunits of inotropic glutamate *N*-methyl-D-aspartate receptors (NMDARs) [12, 14] and, together with type 1 of sigma receptors (σ1Rs), coordinates the activity of G-protein coupled receptors (GPCRs) with that of NMDARs [14].

HINT1 interacts in the plasma membrane and in the nucleus with potential transcription factors such as RGSZ2 [15, 16], the Pontin/Reptin complex [17], and the cleaved N-terminal intracellular domain (ICD) of transmembrane protein teneurin 1, which induces the activity of the microphthalmia-associated transcription factor [18]. Previous results show that HINT1 acts as a transcriptional repressor; it is recruited by the DNA damage response [19, 20], triggers apoptosis [21] and exhibits tumor-suppressive activity [22–24]. RGSZ2 and ICD teneurin 1 incorporate SUMO [2, 25], which modifies protein association and transcriptional regulation [26]. HINT1 removes SUMO from these proteins, and this SUMOylase activity appears to be altered in all the ARAN-NM-related HINT1 mutants described so far [2].

Because HINT1 establishes weak interactions with σ1Rs, in this study, we investigated HINT1 interactions with SUMO and calcium-activated CaM, the substrate and the regulator of HINT1 SUMOylase function. We also analyzed HINT1 binding to the C-terminal cytosolic regions of the MOR, to the NR1 subunit of glutamate NMDARs, to the RGSZ2 protein and to ICD teneurin 1. Our data indicate that most human HINT1 mutants display much weaker interactions with these essential proteins than does the wild-type protein.

Methods

Recombinant protein expression

The coding region of human full-length HINT1 (NM_005340: residues 1-126) and its mutated sequences, full-length RGSZ2 (NM_019958.4: residues 1-210), the ICD region of teneurin 1 (NM_011855: residues 2–317), the C-terminal region of MOR1 (AB047546: residues 286–398) and C0-C1-C2 region of the glutamate NMDAR NR1 subunit (NM_008169: residues 834–938), were amplified by RT-PCR using total RNA isolated from the mouse brain as the template.

Specific primers containing an upstream Sgf I restriction site and a downstream Pme I restriction site were used, as described previously [12]. The PCR products were cloned downstream of the Glutathione S-transferase (GST) coding sequence (for NR1 and RGSZ2) or HaloTag coding sequence (for HINT1, MOR, and ICD teneurin 1) (Flexi® Vector, Promega) and the Tobacco Etch Virus (TEV) protease site. All the sequences were confirmed by automated capillary sequencing, and they were identical to the GenBank™ sequences. The vector was introduced into the *E. coli* BL21 (KRX #L3002, Promega) and clones were selected on solid medium containing ampicillin. After 3 h of induction at room temperature (RT) in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.1% rhamnose, the cells were collected by centrifugation and maintained at -80° C.

The GST fusion proteins were purified under native conditions on GSTrap FF columns (#17-5130-01, GE Healthcare), and further purification was achieved by high-resolution ion exchange (#780-0001 Enrich Q, BioRad). The HaloTag fusion proteins were purified under native conditions with HaloLink Resin (G1915, Promega), and they were cleaved in bulk with ProTEV protease (#V605A, Promega); further purification was achieved by high-resolution ion-exchange chromatography (#780-0001 Enrich Q, BioRad). Sequences were confirmed by automated capillary sequencing.

In vitro interactions between recombinant proteins: pull-down of recombinant proteins

The recombinant HINT1 protein (200 nM) or HINT1 mutants were incubated either with Sepharose 4B (#17-0120-01, GE Healthcare; negative control) or together with the immobilized proteins CaM-agarose 4B (#17-0529-01, GE Healthcare), SUMO1-agarose (#UL-740, Boston Biochem) or RGSZ2, ICD teneurin 1, the C-terminus of MOR1 and the C0-C1-C2 region of NMDAR NR1 subunit, which were covalent attached to NHS-activated Sepharose 4 fast flow (4FF, #17-0906-01, GE Healthcare) according to the manufacturer's instructions.

The interactions were studied in 300 µL of a buffer containing 50 mM Tris-HCl, pH 7.5, and 0.2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in the presence of 2.5 mM $CaCl_2$ and mixed by rotation for 30 min at RT. After incubation, the pellets were recovered by centrifugation, washed three times in the presence of 2.5 mM $CaCl_2$, solubilized in 2x Laemmli buffer, and analyzed by Western blotting.

Western blotting

The detached HINT1 proteins recovered in the aforementioned procedure were resolved with SDS-PAGE in 4–12% Bis-Tris gels (#NP0341, Invitrogen, Fisher Scientific), with MES SDS as the running buffer (#NP0002, Invitrogen). The proteins were transferred onto 0.2 µm PVDF membranes (#162–0176, BioRad) and probed overnight at 6º C with primary antibodies diluted in Tris-buffered saline (pH 7.7) (TBS) + 0.05% Tween 20 (TTBS). The primary antibody was detected using the appropriate horseradish peroxidase-conjugated secondary antibody, which was visualized by chemiluminescence (#170–5061, BioRad) and recorded on an ImageQuant™ LAS 500 (GE Healthcare). Because all the assays were performed with recombinant proteins, the immune-signals provided a single band of the expected size, which was used for the subsequent densitometry analysis. Accordingly, no other regions of the blots provided information and were routinely excluded from the analysis. The software automatically calculated the optimal exposure time for each blot to provide the strongest possible signal, from which the labelling could be accurately quantified. For each group of samples, protein immunosignals were measured using the area of the strongest signal (average optical density of the pixels within the object

area/mm^2; AlphaEase FC software). The gray values of the means were then normalized within the 8 bit/256 gray levels [(256-computed value)/computed value].

Antibodies

The primary antibodies to detect the recombinant proteins were anti-MOR Ct aa 387–398 (GenScript Co.), anti-Calmodulin (#05-173, Millipore,), anti-GST (#2622, Cell Signaling), anti-RGSZ2(1) (#PA1-25695, Thermo Scientific), anti-RGSZ2(2) (aa 192–215; GenScript Co), anti-teneurin1 (#NBP2-41315, Novus Biologicals), anti-SUMO1 (#BML-PW9460, Enzo), anti-SUMO-2/3 (#BML-PW9465, Enzo). The anti-HINT1 antibody was raised in rabbits (Immunostep) against the peptide sequence GYRMVVNEGADGGG (93–106). All primary antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies.

Statistical analyses

All graphs and statistical analyses were generated and made using the Sigmaplot/SigmaStat v.14 package (SPSS Science Software, Erkrath). Experiments were performed at least twice on separate experimental days. Western blot signals were expressed as the change relative to the controls (wild-type HINT1 interaction), which were assigned an arbitrary value of 1. Data using recombinant proteins were analyzed using one-way analysis of variance (ANOVA) followed by the Holm-Sidak multiple comparisons test. Statistical significance was defined as $p < 0.05$.

Results

Human HINT1 sequence has 126 amino acids forming three helices, seven β-sheets and the rest linear sequences (DNASTAR NovaFold v15, Madison, USA). Currently, 15 HINT1 mutants have been reported to cause ARAN-NM and protein analysis indicates that these mutants show limited localization in alpha helices (Fig. 1). The CaM-binding motif is located in the HINT1 N-terminal region (12–31 QPGGDTIFGKIIRKEIPAKI) [27], and in a β-sheet close to the C-terminus, a series of hydrophobic amino acids (110–116: HIHLHVL) form the typical composition of a SUMO-interacting motif (SIM) [28] (Fig. 1A). HINT1 SUMOylase activity is triggered by calcium-activated CaM and/or NO [2], and in *in vitro*, HINT1 binds calcium-activated CaM and SUMO1 [2]. All but the shortest HINT1 mutant Q62* were included in the study. Thus HINT1 mutants, except R37P, G89V and G93D, showed weak or no association with SUMO1 (Fig. 2A), and mostly reduced their interactions with CaM (Fig. 2B).

While, HINT1 directly associates with the cytosolic C0-C1-C2 region of the NR1 subunit of the glutamate NMDA receptor, human HINT1 mutants showed diminished interactions with this NR1 subunit, except of C38R and G89V mutants, which exhibited a strong and a very strong association, respectively, with this protein (Fig. 3A). HINT1 binds to an amino acid sequence at the beginning of the MOR cytosolic tail [29],

which comprises the 354–357 (TSST) cluster. As observed for the association of HINT1 mutants with NR1 subunits, all but G89V diminished their binding to the MOR C-terminal sequence (Fig. 3B).

The RGSZ2 protein is a sumoylated regulator of G-protein signaling and is found in the plasma membrane and in the nucleus [25]. HINT1 exhibits *in vitro* SUMOylase activity on sumoylated RGSZ2 [2], and establishes stable complexes with RGSZ2 in the C-terminal of the MOR [29]. Human HINT1 mutants showed a disparate pattern of interacting with RGSZ2, while some exhibited a strengthened association (C38R, G89V, etc.) other mutants interacted weakly with RGSZ2 proteins (Fig. 4).

Protein analysis revealed the almost absence of secondary structure in ICD teneurin 1 (Fig. 5). This protein interacts with HINT1, while calcium promotes and calcium-activated CaM diminishes this association [2]. Human HINT1 mutant proteins showed mostly weakened associations with ICD teneurin 1, except C84R and G89V, which showed increased associations (Fig. 5).

Discussion

Our present study shows that human HINT1 mutants exhibit anomalous interactions with the partner proteins examined. These proteins include CaM, which regulates HINT1 SUMO protease activity, RGSZ2 and ICD teneurin 1, which bind to HINT1 for transportation from the plasma membrane to the nucleus, and GPCRs such as MOR and ionotropic glutamate NMDAR, both of which are regulated by HINT1 function. With a few exceptions, the human HINT1 mutant proteins showed reduced associations with the analyzed proteins compared with the HINT1 wild-type. Because, the phylogenetically conserved HINT1 protein participates in a series of physiological processes at the plasma membrane, in the cytosol and in the nucleus, HINT1-regulated signaling pathways may be substantially altered by the mutations that cause ARAN-NM.

It has been proposed that HINT1 SUMO protease activity plays a relevant role in the control of transcription [2]. In wild-type HINT1, this activity is prevented by zinc ions binding to Cys84 in the catalytic domain and promoted by their removal by calcium-CaM [30, 31] or NO. The 12–31 (QPGGDTIFGKIIRKEIPAKI) N-terminal region of HINT1 binds CaM, and accordingly the non-human T17A mutation prevents the CaM-mediated activation of HINT1 SUMOylase activity but not NO-mediated activation, which releases zinc bound to Cys84 [2]. The availability of NO necessary to disrupt the zinc-Cys interaction is facilitated by the colocalization of HINT1 with nNOS in the MOR environment in which HINT1 is associated with RGSZ2–nNOS complexes [32]. Thus, the proapoptotic and tumor-suppressive role of HINT1 may depend on its SUMOylase function regulated by CaM, nNOS/NO and RGSZ2. Unfortunately, HINT1 protease activity is altered in all human HINT1 mutants, which exhibit mostly weak interactions with CaM and the substrate SUMO.

We may consider that human HINT1 mutations are distributed into two arbitrary regions of the sequence, the first cluster from F33S to K57N and the second from C84R to W123*. Some of the mutations within the first region result in isopeptidase activity, albeit deregulated; however, none of the mutations in the second cluster exhibit this activity. The second region contains the catalytic Cys84-Asp87-His114 core,

and the conserved histidine triad (HIT), which alternates with the hydrophobic amino acids of SIM (110–116, HIHLHVL).

The HINT1 protein in the plasma membrane also behaves as a scaffold and binds simultaneously to cytosolic regions of GPCRs such as MOR [32], protein kinases such as PKCγ and PKCα, Raf-1 [33, 34], and proteins of the RGS family (RGSZ1 and RGSZ2) [10, 29]. HINT1 competes with calcium-activated CaM and σ1R for their binding to the cytosolic C0-C1-C2 region of NR1 subunits of neural glutamate NMDAR [12]. As a result, σ1Rs promote NMDAR activity, while HINT1 and CaM diminish this activity. A tight functional relationship exists between HINT1 and σ1R in the neural membrane. The interplay between these proteins promotes physical coupling and uncoupling between MOR and the NR1 subunit of NMDAR [14]. Indeed, in mice lacking HINT1 or σ1R, the functional regulation between MOR and NMDAR is lost, morphine does not recruit NMDAR function, and the direct activation of NMDARs does not reduce morphine analgesia [12, 29].

Our study indicates that except for a pair of mutations, human HINT1 mutant proteins bind poorly to MORs and NMDAR NR1 subunits. HINT1 regulation of NMDARs may be relevant to the development of ARAN-NM. In this context, human HINT1 mutants deregulate NMDAR interactions with other signaling proteins contributing to neurological disorders, including neurodegenerative diseases [35], and probably alterations in motor coordination. In fact, the progression of amyotrophic lateral sclerosis (ALS) is delayed by drugs such as riluzole, which diminishes the function of these NMDARs [36].

The association of HINT1 proteins with ICD teneurin 1 and σ1Rs is relevant to the normal functioning of motor pathways. HINT1 couples with ICD teneurin 1, which acts as a transcription factor in the nucleus [18]. Teneurins (four members in humans, Ten1-4) promote neurite outgrowth, cell adhesion, dendritic morphology, axonal guidance and synapse formation [37]. The two teneurins present in *Drosophila*, Ten-m and Ten-a, regulate neuromuscular synapse organization and target selection [38]. Ten-a is presynaptic, while Ten-m is mostly postsynaptic; neuronal Ten-a and muscle Ten-m form a complex *in vivo*. Elevated Ten-m expression regulates target selection in specific moto neurons and muscles via homophilic matching and functions with additional molecules to mediate precise neuromuscular connectivity. Pre- or postsynaptic teneurin perturbations cause severe synapse loss and impair many facets of organization transynaptically and cell-autonomously. In addition, three missense mutations have been identified in the human *TEN4* gene, which are associated with patient families displaying essential tremor movement disorder [39]. Ten4 is a regulator of oligodendrocyte differentiation and plays a critical role in the myelination of small-diameter axons in the central nervous system [40]. Moreover, Ten4$^{-/-}$ mice exhibit a tremor-like phenotype, and a missense mutation in *TEN1* gene was predicted to be potentially pathogenic for cerebral palsy, a clinically heterogeneous group of disorders affecting movement and posture [41]. On the other hand, the HINT1-associated protein RGSZ2 is involved in multiple human cancers, such as lung, prostate, breast, and liver [15, 16]. While almost every mutation in the human HINT1 protein weakened the interactions with associated proteins, the interactions with RGSZ2 were more evenly altered, with almost half increasing and half decreasing the strength of their binding. Because HINT1 couples with ICD teneurin 1, which acts as a transcription factor in the nucleus

[18], disruptions in HINT1-teneurin 1 or HINT1-RGSZ2 interactions together with the impairment of HINT1 isopeptidase activity may affect the function of these proteins in the nucleus, regulating gene expression, which may underlie the pathophysiology of ARAN-NM.

The σ1R is a transmembrane protein mostly located in the endoplasmic reticulum (ER) [42] but also at the plasma membrane [43, 43–45] and at the nuclear envelope [46, 47]. Notably, σ1R is highly expressed in motor neurons [48, 49], and autosomal recessive loss-of-function mutations in σ1R are primarily associated with distal hereditary motor neuropathy [47, 50, 51] and amyotrophic lateral sclerosis/frontotemporal dementia (ALS/FTD) [52–54]. Indeed, the σ1R E102Q mutation that causes juvenile ALS [55] rapidly aggregates and accumulates in the ER and associated compartments in transfected cells, provoking alterations in proteasomal degradation and calcium homeostasis [56]. Moreover, the lack of σ1R exacerbates ALS progression in G93A-SOD1 mice, and $\sigma1R^{-/-}$ mice showed motor neuron degeneration pathology [57]. HINT1 does not stablish strong interactions with σ1Rs [14], but both proteins cooperate to bring certain GPCRs under the regulation of glutamate NMDARs [14]. Given the high expression of σ1Rs in motor neurons and the functional relation between HINT1 and σ1R, mutations in any of these proteins may impair a series of physiological pathways, provoking the onset of motor neuron pathology.

Conclusions

As mentioned, the targeted deletion of the *HINT1* gene does not promote significant motor disturbances or peripheral neuropathy in mice [6]. Notwithstanding, HINT1 mutants may have affected different signaling pathways during development and thus compromise the potential beneficial effects of blocking expression of HINT1 mutants to alleviate ARAN-NM. In summary, HINT1 is highly phylogenically conserved, suggesting that it is a protein with high biological relevance. The human HINT1 mutations reported to cause ARAN-NM all exhibit altered interactions with other proteins in the nervous system. These results suggest that the role of HINT1 in the pathogenesis of this human disease may be related to the impairment of motor neuron signaling pathways in which HINT1 is implicated.

Abbreviations

ARAN-NM = autosomal recessive axonal neuropathy with neuromyotonia

CaM = calmodulin

GPCR = G protein-coupled receptor

HINT1 = histidine triad nucleotide-binding protein 1

ICD = N-terminal intracellular domain of teneurin 1

Icv = intracerebroventricular

MOR = mu-opioid receptor

NMDA = *N*-methyl-D-aspartate

nNOS = neural nitric oxide synthase

NO = nitric oxide

PKC = protein kinase C

RGSZ2 = regulator of G protein signaling 17 (Z2)

σ1R = type 1 sigma receptor

SIM = SUMO-interacting motif

SUMO = small ubiquitin-like modifier

Declarations

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Author Contributions

The JGN, ECM & MRM designed the research. ECM & JGN wrote the manuscript. JGN & PSB obtained the funding. ECM & MRM performed the experiments and the statistical analysis of data.

Ethics approval

N/A

Consent for publication

All authors approved the final manuscript.

Availability of data and materials

All relevant data that support the finding of this study are available upon reasonable request.

Competing interests

The authors declare that all the research presented here was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

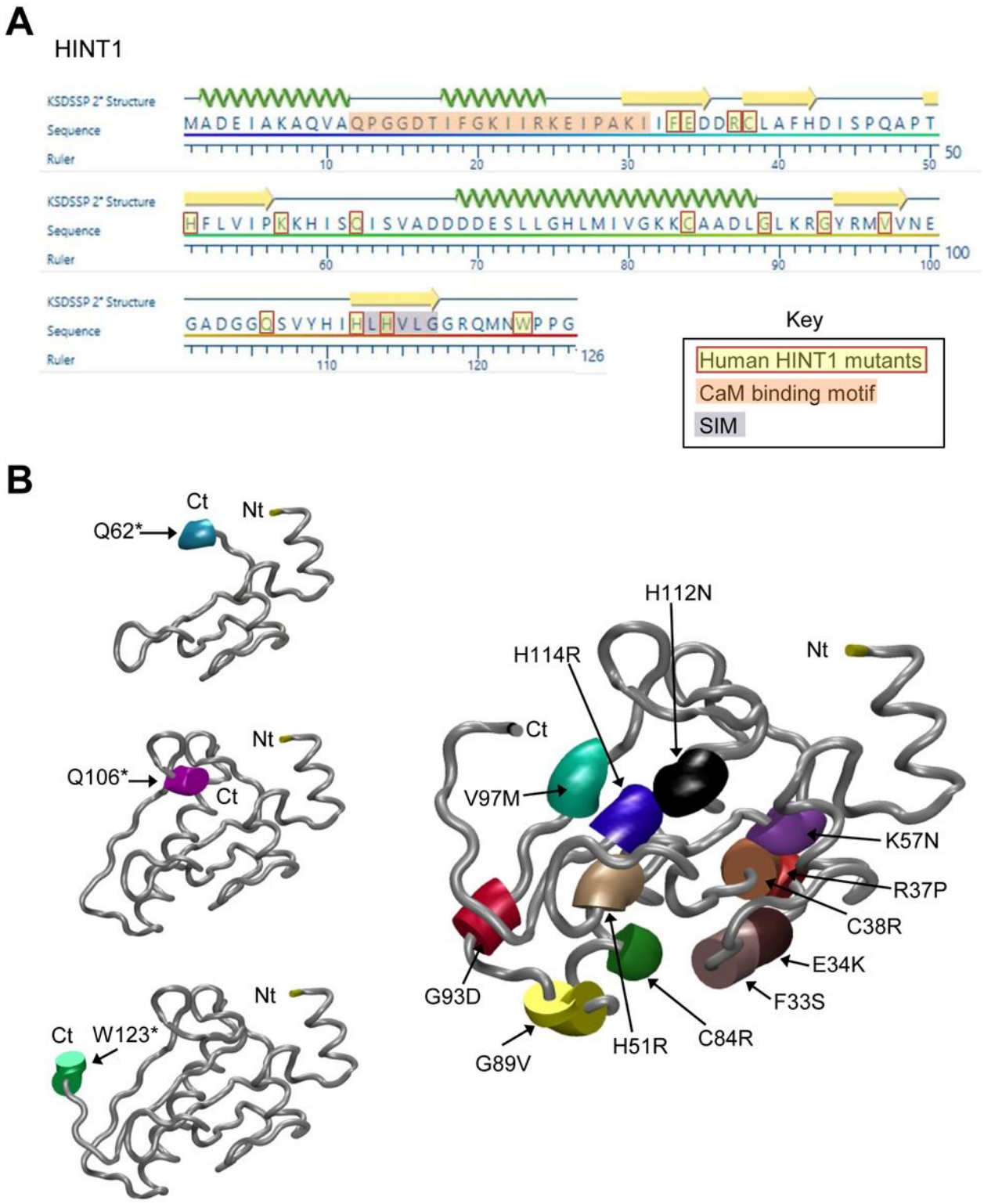

Figure 1

Secondary structure and 3D model of HINT1. (A) Protein sequence and secondary structure of HINT1 with helices represented as green waves and β-sheets as yellow arrows. HINT1 protein contains a putative CaM-binding motif (12-31) and a SUMO interacting motif (SIM) (110-116). The amino acids changed in the human HINT1 mutants causing ARAN-NM are indicated. (B) HINT1 3D structure shows the mutated amino acids causing ARAN-NM as colored tube occupancy. Left panel: human HINT1 truncating

mutations Q62*, Q106* and W123*. Right panel: the missense human HINT1 mutations. HINT1 structural model shown herein and its secondary structure were constructed by Novafold (DNASTAR, Inc., Madison, WI). Inset: The HINT1 3D structure is shown as a tube with reference amino acids as colored cylinders (tube occupancy). Key: 3D, three-dimensional; CaM, calmodulin; HINT1, histidine triad nucleotide-binding protein 1; SIM, SUMO interacting motif; SUMO, small ubiquitin-like modifier.

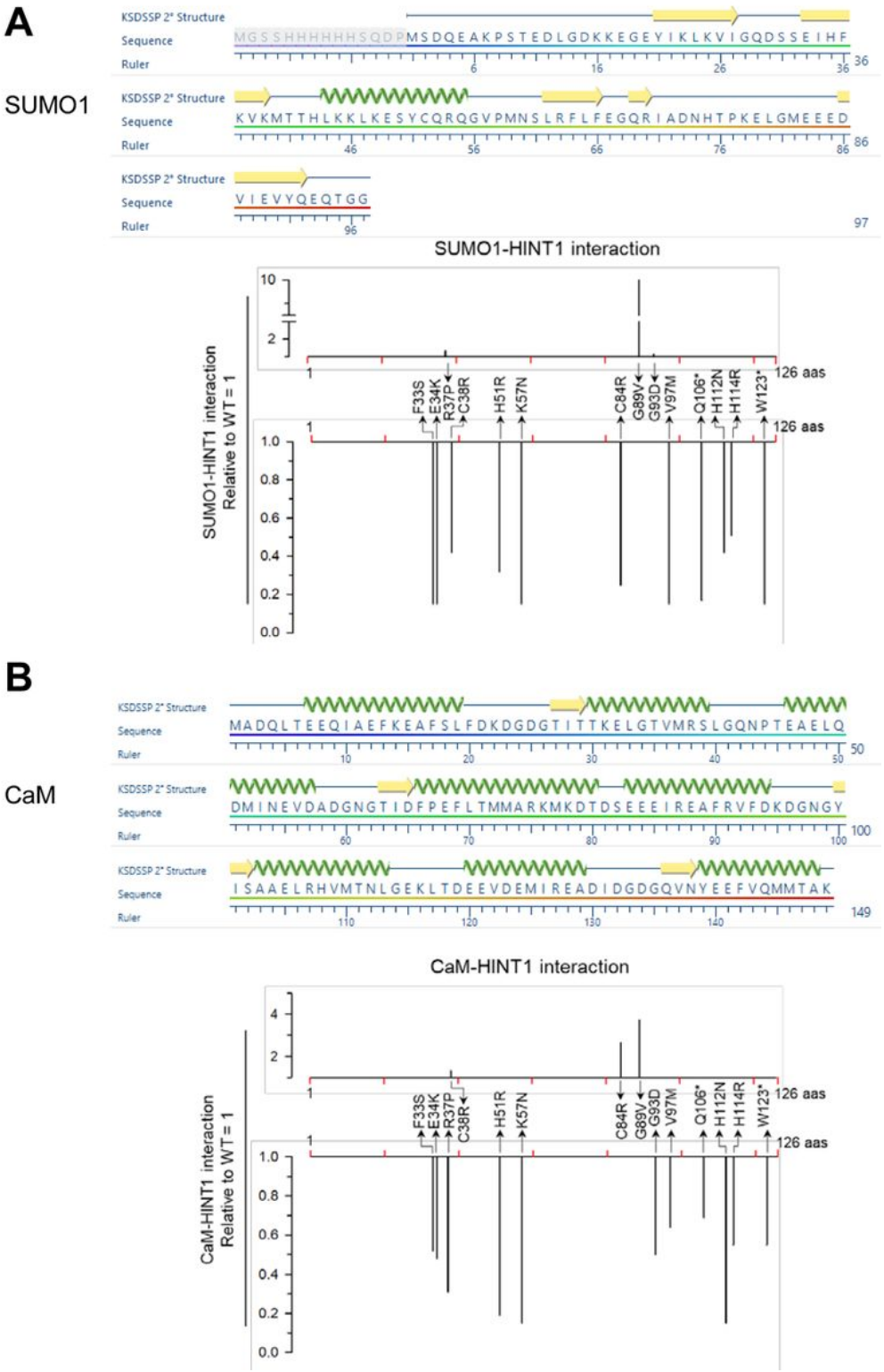

Figure 2

Disrupted binding of human HINT1 mutants to SUMO1 and calcium-activated CaM. (A) Secondary structure of SUMO1 and graphical representation of binding of different HINT1 mutants to SUMO1 and (B) CaM. Sequence of SUMO1 and of CaM showing their secondary structure with helices represented as green waves and β-sheets as yellow arrows. In vitro assay: recombinant SUMO1 (100 nM) or CaM (100 nM) covalently attached to agarose were incubated in the presence of 2.5 mM $CaCl_2$ with 200 nM HINT1 wild-type (WT) or the corresponding HINT1 mutant. Prey proteins alone did not bind to the agarose (negative control) [2]. The agarose pellets were processed as described in the Methods, and the recovered HINT1 proteins were measured by Western blotting (see the Methods section). The assays were repeated at least twice, producing comparable results. For each interaction of HINT1 wild type or human mutan, SUMO1-HINT1 and CaM-HINT1, binding was analyzed using ANOVA followed by the Holm-Sidak multiple comparisons test, $p<0.05$. Further details of the assay, immunoblot detection in Methods and supplemental Figs. 1, 2 & 3. Key: CaM, calmodulin; SUMO, small ubiquitin-like modifier; WT, wild-type. Data of HINT1 human mutants are shown relative to the data obtained using the HINT1 WT with an arbitrary value of 1.

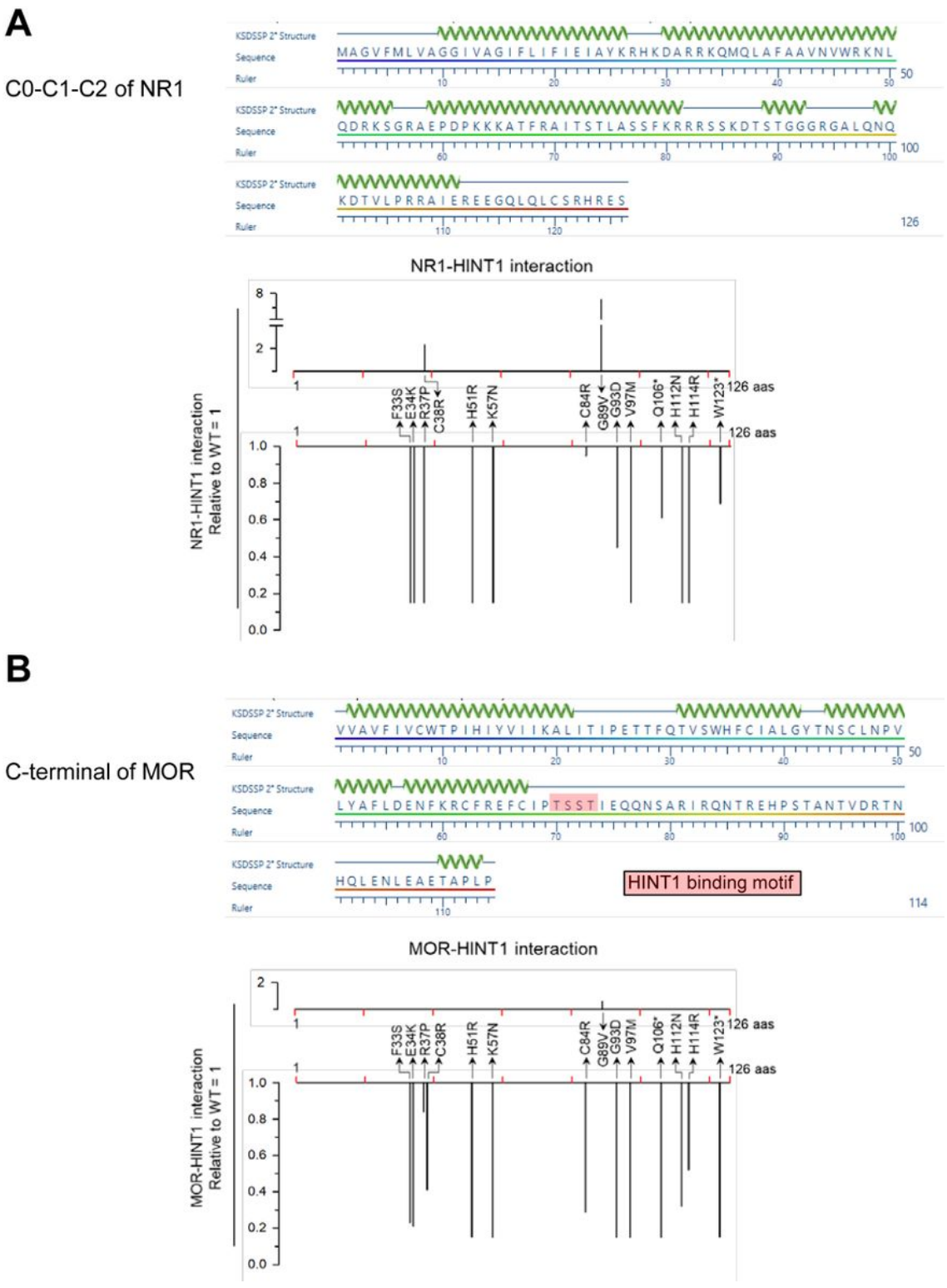

Figure 3

Disrupted binding of human HINT1 mutants to the NMDAR NR1 subunit and C-terminus of MOR. (A) Sequence of the cytosolic C-terminal region C0-C1-C2 of the NMDAR NR1 subunit showing its secondary structure. Graphical representation of the binding of HINT1 WT and mutants to NR1 subunits of the glutamate NMDA receptor. (B) Secondary structure of the C-terminus of MOR and graphical representation of the binding of HINT1 WT and HINT1 mutants to this region of the MOR. Recombinant

cytosolic C0-C1-C2 region of the NMDAR NR1 subunit or MOR Ct region (100 nM) was covalently attached to agarose and incubated with HINT1 WT or the mutants (200 nM) in the presence of 2.5 mM CaCl2 (details as in Figure 2). Further details of immunoblot detection in Methods and supplemental Figs. 4 & 5. Key: MOR, mu-opioid receptor; NMDAR, N-methyl-D-aspartate receptor, WT, wild-type.

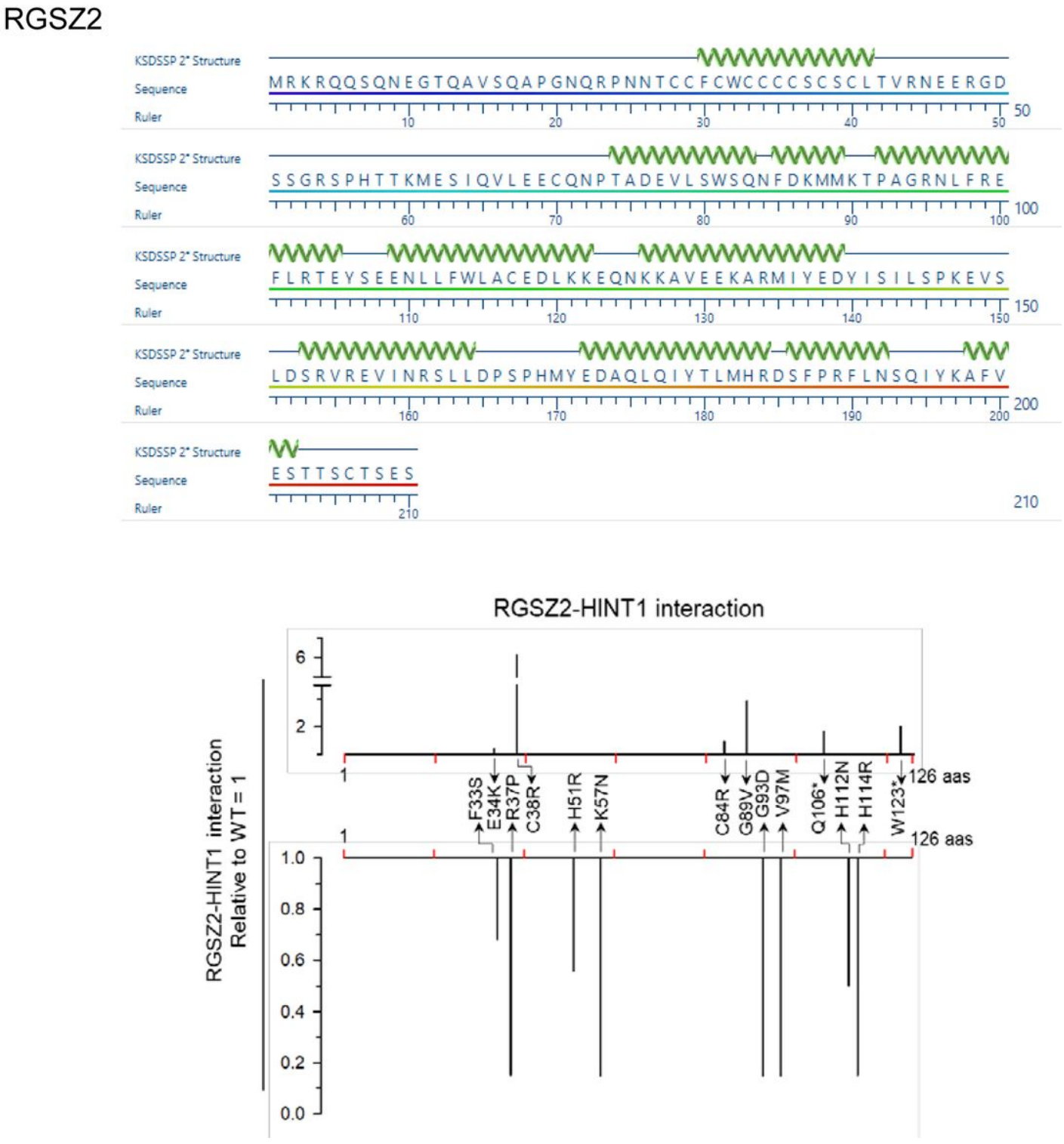

Figure 4

Disrupted binding of human HINT1 mutants to RGSZ2. Sequence of the RGSZ2 protein showing its secondary structure and graphical representation of the association of human HINT1 mutants with RGSZ2 protein. Recombinant RGSZ2 (100 nM) covalently attached to agarose was incubated with HINT1 WT or the corresponding mutant (200 nM) in the presence of 2.5 mM CaCl2 (details as in Figure 2). Further details of immunoblot detection in Methods and supplemental Fig. 6. Key: RGSZ2, regulator of G protein signaling Z2, WT, wild-type.

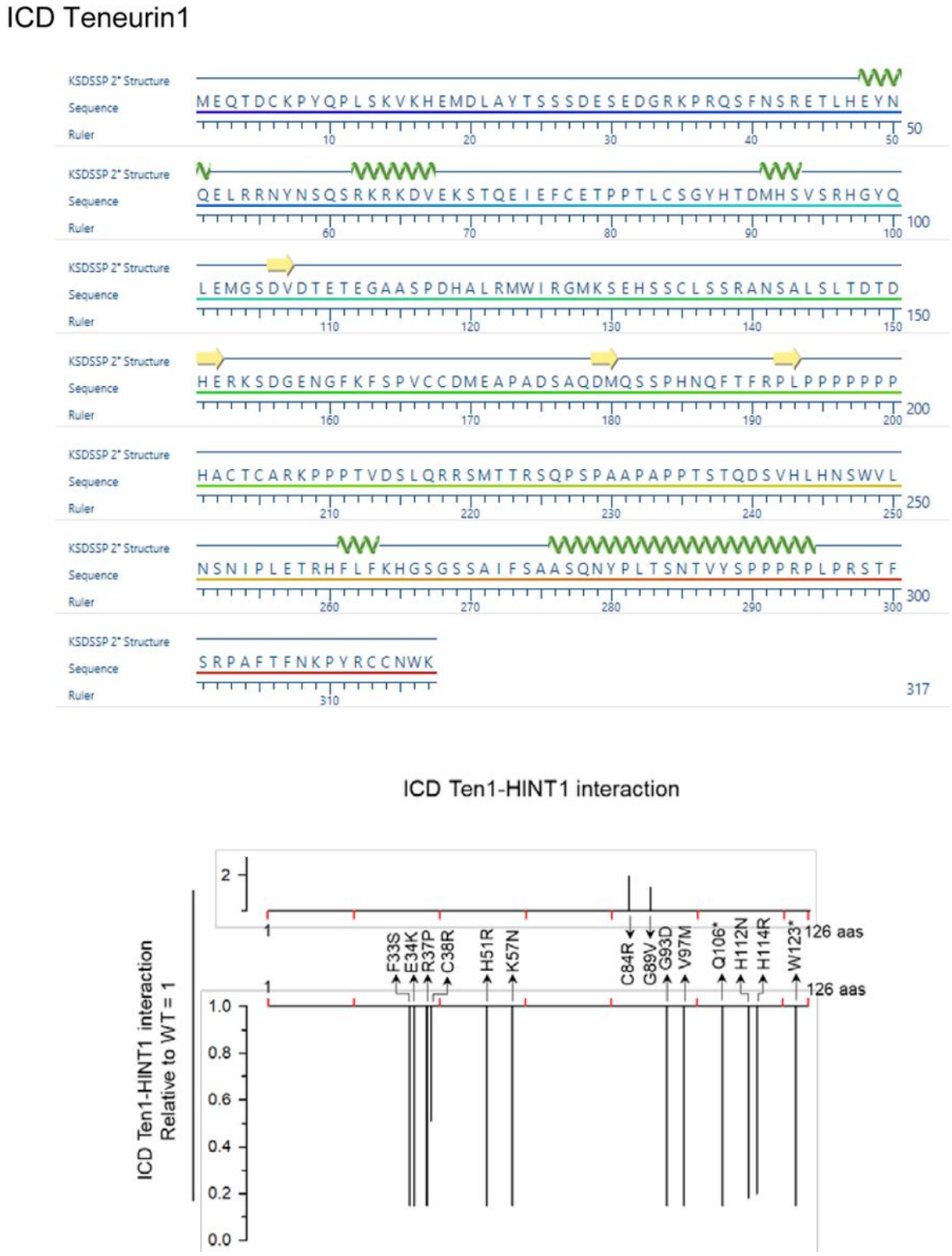

Figure 5

Disrupted binding of human HINT1 mutants to ICD teneurin 1. Sequence of ICD teneurin 1 showing the secondary structure and graphical representation of the binding of HINT1 WT and mutants to the ICD region of teneurin 1. Recombinant ICD teneurin 1 (100 nM) covalently attached to agarose was incubated with HINT1 WT and the different human HINT1 mutants (200 nM) in the presence of 2.5 mM CaCl2 (details as in Figure 2). Further details of immunoblot detection in Methods and supplemental Fig. 7. Key: ICD, intracellular domain, WT, wild-type.

Supplementary Files

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