

A Severe COVID-19-associated hNTPDase2 Variant is Enzymatically Inactive

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Abstract

Nucleoside triphosphate diphosphohydrolases (NTPDases) regulate extracellular nucleotide levels and thereby modulate nucleotide signalling. Increased extracellular ATP levels and altered nucleotide signalling have been reported in patients with moderate to severe COVID-19. A specific variant in the gene encoding human NTPDase2 (hNTPDase2), classified in ClinVar as likely pathogenic, has been reported in patients with severe COVID-19. Sequence analysis of this variant revealed a single G nucleotide deletion at position 1170 of the coding sequence, resulting in a frameshift and a premature stop codon. The predicted protein is truncated from 495 to 408 amino acids and lacks ACR5, the C-terminal transmembrane domain, and a few other functionally important residues. We generated the corresponding variant, referred to as hN2COV, by site-directed mutagenesis and expressed it in CHO-S cells. Expression of the FLAG-tagged hN2COV was confirmed by western blotting. Whereas the reference hNTPDase2 efficiently hydrolyzed ATP and UTP, no detectable activity was observed for hN2COV with ATP, ADP, UTP, or UDP. These findings identify hN2COV as a loss-of-function variant and suggest that loss of NTPDase2 activity may contribute to altered extracellular nucleotide signalling in individuals carrying this variant.

Introduction

Nucleoside triphosphate diphosphohydrolases (NTPDases) are a group of enzymes that hydrolyze nucleoside tri- and diphosphates¹⁻³. In humans NTPDases are comprised of two types based on their cellular localization. First group includes human NTPDase1 (hNTPDase1), hNTPDase2, hNTPDase3, and hNTPDase8 which are found at the cell surface. The second group is comprised of NTPDase4-7 which are normally found inside the cell; some of them may be also secreted¹⁻³.

The first group of hNTPDases are the most important enzymes that hydrolyse extracellular nucleotides such as ATP and UTP modulating their concentrations in extracellular environments such as bloodstream¹⁻³ which is essential for homeostasis. In the context of inflammation, studies have suggested that the accumulation of extracellular ATP and UTP promote the release of pro-inflammatory cytokines and recruitment of pro-inflammatory neutrophils and macrophages through the activation of P2 receptors^{2,4-10}. In addition, increased extracellular ATP levels in the blood and alterations in purinergic signalling have been observed in patients with moderate to severe COVID19^{11,12}.

Interestingly, a specific mutation in the gene encoding hNTPDase2 has recently been reported in the ClinVar database in patients with severe COVID-19. The reference hNTPDase2, like other members of the first group of hNTPDases mentioned above, has two transmembrane domains close to each extremity^{1-3,13}. It contains 5 apyrase-conserved regions (ACRs)^{1,3,13}. The gene encoding hNTPDase2, *ENTPD2*, is composed of 9 exons and has a coding DNA sequence (CDS) of 1488 nucleotides. The hNTPDase2 variant, hereafter referred to as hN2COV, is classified in ClinVar as likely pathogenic, and given its predicted structural consequences, we sought to determine its impact on hNTPDase2 enzymatic activity.

Materials and methods

Materials

A full list of the materials, kits and equipment is provided in the supplementary information.

In silico analysis

The *ENTPD2* variant NM_203468.3:c.1170del (ClinVar Variation ID 3256927; VCV003256927.1), predicted to cause a frameshift at Gln391, was obtained from ClinVar. The corresponding mutant protein sequence was generated from the reference *ENTPD2* transcript and aligned with the human wild-type *ENTPD2* protein sequence (UniProtKB accession Q9Y5L3) on Uniprot. The alignment was used to identify the amino-acid sequence retained upstream of the frameshift and the C-terminal region altered or absent in the variant. Coordinates of apyrase-conserved regions (ACRs) and reported post-translational modification sites were obtained from *ATP: From Neurotransmission to Cancer Therapy*³) and mapped onto the wild-type and variant sequences.³

Cell culture

CHO-S suspension cells were cultured in CHO-S freestyle expression medium supplemented with 8 mM glutamine and antibiotic. CO₂ levels were maintained at 8% and temperature at 37°C for maintenance phase and at 30°C for protein production phase. The cells were passed every 48 hours to maintain the density at around 1 to 1.5 x 10⁶ /ml.

Site-directed mutagenesis

To create the single nucleotide deletion mutation, two non-overlapping forward and reverse primers flanking the target G1170 nucleotide were designed (F1 and R1 in the supplementary information). A FLAG tag, preceded by a short Gly-Gly-Gly-Ser linker, was also added to the C-terminus of hNTPDase2 (NM_203468) cloned into pcDNA3.1 vector, and hN2COV proteins using two non-overlapping forward and reverse primers flanking the site of insertion (F2, R2, F3, R3 in the supplementary information). The mutants were generated by PCR (supplementary data) as described in the kit.

Transformation of bacteria, single colony isolation, plasmid DNA extraction, and sanger sequencing

Following the site-directed mutagenesis, the reaction products were transformed into NEB 5-alpha Competent *E. coli*, and plasmid DNA from selected colonies was isolated and verified by Sanger sequencing.

Transfection

CHO-S cells were transfected with a DNA mixture containing 95% hN2-, hN2COV-, hN2FLAG-, or hN2COVFLAG-expressing pcDNA3.1 plasmid and 5% the green fluorescent protein (GFP)-expressing plasmid (pEGFP-C3), using PEI at a DNA:PEI ratio of 1:7. Following the transfection, the cells were maintained at 30°C for 7 days until protein extraction.

RNA extraction, cDNA synthesis, and PCR

To detect gene expression at mRNA level, at day 4 post transfection the CHO-S cells transfected with hN2/hN2COV/hN2FLAG/hN2COVFLAG-coding DNA constructs were collected by centrifugation. The extraction of RNA and the synthesis of cDNA was performed as mentioned in the kit's manual (see supplementary information).

Protein extraction, quantification, SDS-PAGE, and western blot

Suspensions from transfected cells were harvested by centrifugation and membrane homogenates were prepared, supplemented by 7.5% glycerol and stored in aliquots at -80°C for further analysis as before¹⁴. The protein contents were quantified using Bradford assay. For SDS-PAGE, the membrane homogenates were mixed with the loading buffer with(out) β -mercaptoethanol and heated at 95°C for 5 minutes. SDS-PAGE in (non)reduced conditions and transfer to polyvinylidene fluoride (PVDF) membrane was done according to the Invitrogen's manual. Immunostaining was done with either mouse anti-FLAG antibody or mouse anti-hN2 antibodies as the primary antibody and anti-murine HRP-conjugated antibody as the secondary antibody.

Activity assays

Enzymatic activity was measured using ATP, ADP, UTP, and UDP as substrates with the malachite green assay, as previously described¹⁴. Activity assays were performed using samples from three independent transfection. Statistical significance between groups was assessed using a two-tailed unpaired Student's t-test. A p value < 0.05 was considered statistically significant.

Results

Sequence analysis of the hN2COV variant

hN2COV contains a single G nucleotide deletion at position 1170 of the coding sequence, located at the beginning of exon 8. This deletion causes a frameshift leading to a premature stop codon (Fig. 1A). Consequently, the predicted hN2COV protein is expected to be 408 amino acids long, compared with 495 amino acids for the wild type hNTPDase2 (Fig. 2F). The frameshift results in the loss of the last 105

amino acids of the wild-type protein, including ACR5, the C-terminal transmembrane domain, and some functionally important residues, such as: Cys399, shown to be essential for disulfide bridge formation and full enzymatic function¹⁵; Asn443, shown to be a glycosylation site and also crucial for full enzymatic activity¹⁵; and Arg394 and Tyr398 which are involved in nucleotide binding (Uniprot, Q9Y5L3 ENTP2_HUMAN) (Fig. 1B). Consequently, the deleted C-terminal sequence is replaced by a novel 18-amino-acid tail. The homology analysis of the C-terminal tail of hN2COV did not show any association of this sequence to any domain or family of proteins, minimizing the chances that this newly created sequence might confer new functions to the enzyme.

Generation and expression of hN2COV

To study the impact of the G1170 deletion on hN2 activity, we generated the hN2COV mutant by site-directed mutagenesis using our hN2 cDNA standard sequence cloned into pcDNA3.1¹⁶ (Fig. 1. B). In a second set of constructs, we added a FLAG tag (preceded by Gly-Gly-Gly-Ser linker) at the C-terminal end of both hN2 and hN2COV to ensure protein detection by western blot using anti-FLAG antibody (Fig. 2. F). The sequences obtained were confirmed by sanger DNA sequencing. The validated constructs were expressed in CHO-S cells, and membrane homogenates were extracted. As hN2COV protein expression could not be detected with our anti-hN2 antibody, we did an RT-PCR to confirm the expression at the mRNA level (Fig. 2. C). The presence of hN2COV-corresponding band on agarose gel confirms mRNA expression, however, the absence of hN2COV-corresponding band on the PVDF membrane suggests that our anti-hN2 antibody cannot detect the hN2COV, as it recognizes native hN2¹⁷ (Fig. 2. B). In contrast, the expression at protein level of the FLAG-tagged hN2 and FLAG-tagged hN2COV could be confirmed by western blot using an anti-FLAG antibody (Fig. 2. E).

Enzymatic activity of hN2COV mutants

Enzymatic activity was assessed by measuring the release of inorganic phosphate following the hydrolysis of ATP, ADP, UTP, and UDP, using non-transfected CHO cell homogenates as a negative control. As expected, the wild type hN2, with or without a FLAG tag, efficiently hydrolyzed ATP and UTP, whereas ADP and UDP hydrolysis was negligible (Fig. 2. A, E)¹⁴. In contrast, no detectable activity was observed for hN2COV or hN2COVFLAG with any of the four substrates, indicating a complete loss of enzymatic activity (Fig. 2. A, E).

Discussion

In this study a specific variant of hN2 protein that has been registered as likely-pathogenic on ClinVar database was created and studied. We found that hN2COV, a variant reported in Bulgarian patients with severe COVID-19, showed no detectable activity towards ATP, UTP, ADP or UDP.

Our findings are consistent with previous reports. Most notably, Boyer's group previously reported that Cys399, which is located just before ACR5, and absent from hN2COV, is essential for maintaining full

enzymatic function¹⁵. More specifically, this conserved cysteine residue has been reported to form a disulfide bond with Cys377, which is required for correct folding and full activity of hN2¹⁵. In the AlphaFold3-predicted structure of hN2COV it seems that in the absence of Cys399, Cys377 could instead form a disulfide bond with Cys405, a residue present in the novel C-terminal tail but absent from the reference hN2¹³. The same study also reported that Asn443, a putative N-linked glycosylation site in ACR5, is crucial for enzymatic activity¹⁵. Furthermore, chimeric studies have shown that the ACR5 and the regions between ACR4 and ACR5 are essential for determining substrate specificity of hN2¹⁸.

In the context of inflammation, elevated extracellular ATP levels have been reported in the blood of patients with COVID-19^{11,12,19}. Importantly, numerous studies have documented the pro-inflammatory effects of ATP and UTP through activation of P2 receptors, including the induction of pro-inflammatory cytokine release and the recruitment of inflammatory immune cells^{1,3,4,6}. Although increased extracellular ATP levels have been reported in the blood of patients with COVID-19, a direct contribution of NTPDase2 to the regulation of circulating ATP appears unlikely, as *ENTPD2* expression is not detected in major circulating immune cell populations^{1,3}. In the blood and vasculature, extracellular nucleotide hydrolysis is instead largely associated with NTPDase1/CD39, which is expressed by leukocytes, platelets, and endothelial cells^{1,3}. In contrast, according to the Human Protein Atlas, NTPDase2 protein expression is detected, among other sites, in the respiratory epithelium of the nasopharynx and bronchi. This is particularly relevant to SARS-CoV-2 infection, as the airway epithelium represents an important initial site of infection and ciliated epithelial cells are among the predominant cellular targets of the virus^{20,21}. Furthermore, even though currently there is no study evaluating the link between airway extracellular nucleotide levels and COVID-19, there are several studies reporting high levels of ATP and/or UTP in the airways in other respiratory infections. In mice in the context of influenza A infection, it has been reported that both ATP and UTP levels in the bronchoalveolar lavage fluid are increased, leading to overactivation of P2Y receptors and higher pulmonary edema²². In another murine study a 5-fold increase of pulmonary ATP levels was reported in the mice infected with influenza A and was associated with worse disease progression²³. Likewise, in the context of respiratory syncytial virus, high levels of UTP and ATP in the airways have been reported in infected mice and humans, respectively^{24,25}.

The prevalence of hN2COV in the patients with severe COVID-19 symptoms and the inactivity of this variant that we are reporting in this study are pointing to the fact that the perturbation of extracellular nucleotide levels in the airways and resulting dysregulated nucleotide signalling could locally aggravate inflammatory responses and thus contribute to the severity of the symptoms, which warrants further investigation^{7,16,17}.

Declarations

Consent for publication:

All authors consent to publication.

Conflict of interest:

The authors declare no competing interests.

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Author Contribution

S.R.D.: Conceptualization, investigation, in silico analysis, data analysis, writing—original draft, writing—review and editing. M.B.M. and I.T.: Investigation. J.P.: Investigation, writing—review and editing. J.S.: Conceptualization, supervision, data analysis, writing—original draft, writing—review and editing. All authors read and approved the final manuscript.

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Figures

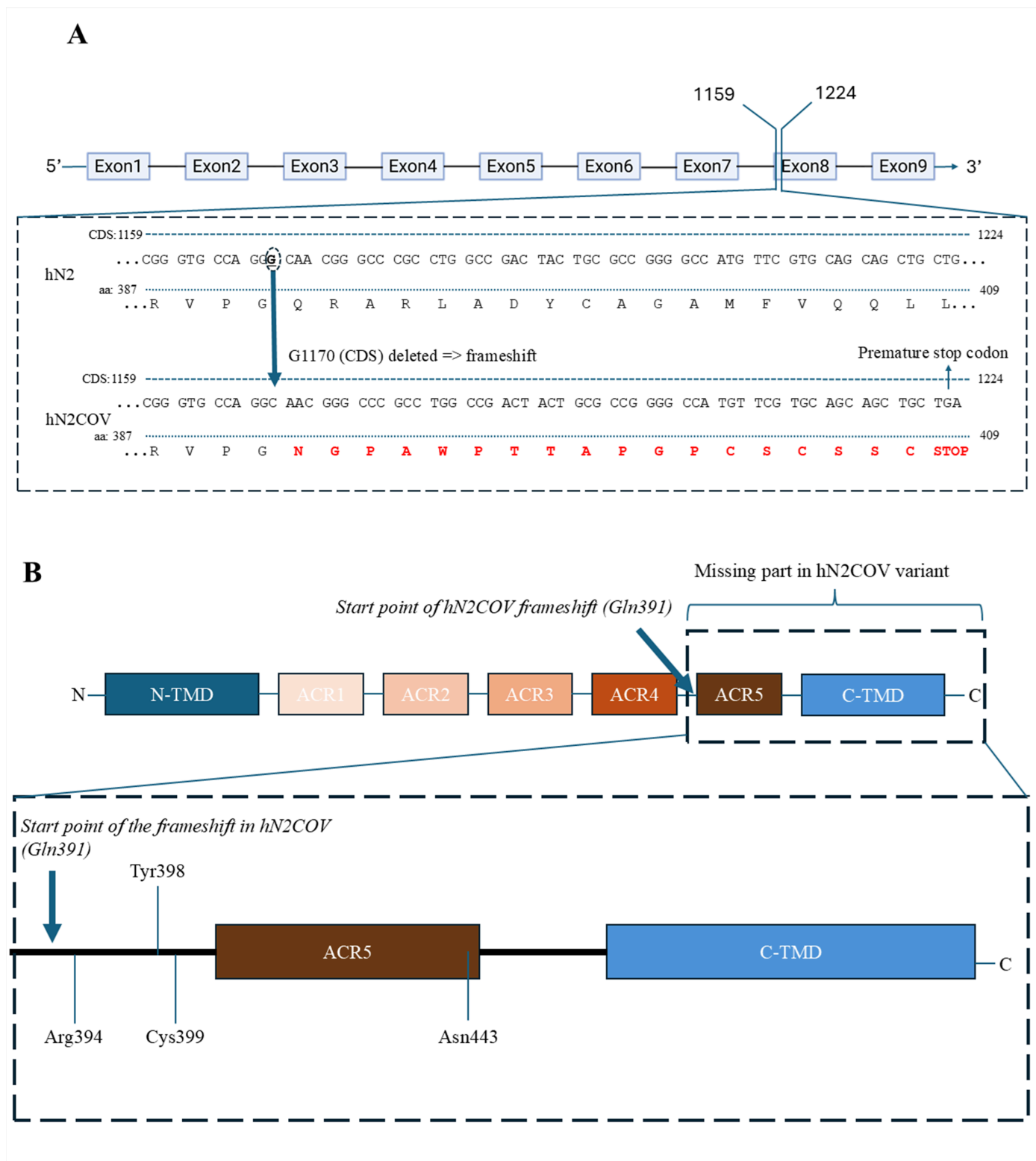


Figure 1

Sequence analysis of hN2 and hN2COV. A) Structure of hN2 and hN2COV coding DNA sequences (CDSs) and corresponding amino acid sequence around the site of mutation. The big arrow points to the G1170 nucleotide³ deleted in the hN2COV variant and the smaller arrow shows the premature stop codon created downstream of the mutation site in hN2COV. **B)** Key elements of hN2 protein and the key

regions that are present or missing in hN2COV because of the mutation. (ACR = apyrase conserved region, TMD = transmembrane domain)

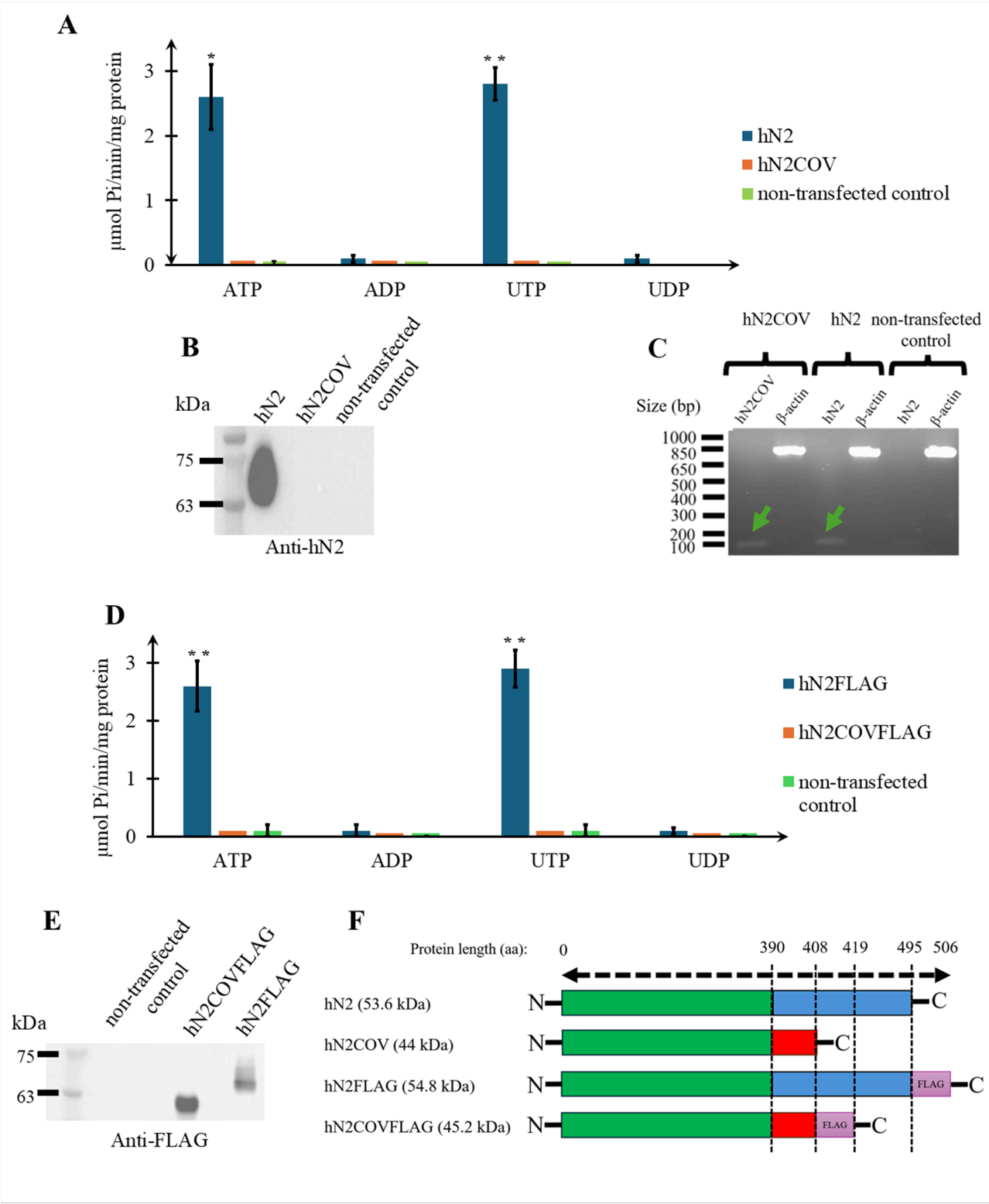


Figure 2

Detection and functional analysis of hN2 and hN2COV, with and without a FLAG tag. Specific activities of CHO-S cell membrane homogenates transfected by hN2- hN2COV- (A) hN2FLAG- or hN2COVFLAG-

expressing **(D)** DNA constructs (and non-transfected CHO-S cells as the negative control) in the presence of ATP, ADP, UTP, and UDP. The activities are reported as μmol of inorganic phosphate (Pi) released per minute per mg of total membrane protein content at 37°C and pH 7.4. Western blot of the expressed hN2 and hN2COV **(B)**, hN2FLAG and hN2COVFLAG **(E)**. **(C)** Agarose (1.8 %) gel electrophoresis with RT-PCR products to confirm the expression of the hN2 and hN2COV constructs. *B-ACTIN* was used as the housekeeping gene. The green arrows are showing the expression of hN2/hN2COV at mRNA level. The amplicon sizes; β -actin = 722 bp, hN2/hN2COV = 94 bp. **(F)** The amino acid length and molecular weight of the hN2, hN2COV, hN2FLAG, and hN2COVFLAG protein constructs. The FLAG tags are preceded by a short flexible linker Gly-Gly-Gly-Ser. Green = the common region present in all constructs (hN2 and hN2COV with and without a FLAG), red = present only in hN2COV with and without a FLAG, blue = native C-terminal sequence present only in hN2 with and without FLAG, purple = FLAG tag preceded by a short flexible linker Gly-Gly-Gly-Ser. Error bars are shown as mean $\pm$ standard deviation, * p value < 0.05, ** p value < 0.01.

Supplementary Files

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