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Effects of mutations in the post-translational modification sites on the trafficking of hyaluronan synthase 2 (HAS2)


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ABSTRACT

Vesicular trafficking of hyaluronan synthases (HAS1-3) from endoplasmic reticulum (ER) through Golgi to plasma membrane (PM), and either back to endosomes and lysosomes, or out into extracellular vesicles, is important for their activities. We studied how post-translational modifications affect the trafficking of HAS2 by mutagenesis of the sites of ubiquitination (K190R), phosphorylation (T110A) and O-GlcNAcylation (S221A), using Dendra2- and EGFP-HAS2 transfected into COS1 cells. Confocal microscopy showed HAS2 wild type (wt) and its K190R and S221A mutants in ER, Golgi and extracellular vesicles, while the T110A mutant remained mostly in the ER. HA synthesis was reduced by S221A, while completely blocked by K190R and T110A. Cell-surface biotinylation indicated that T110A was absent from PM, while S221A was close to the level of wt, and K190R was increased in PM. TIRF microscopy analysis gave similar results. Rab10 silencing increased HA secretion by HAS2, likely by inhibiting endocytosis of the enzyme from PM, as reported before for HAS3. Green-to-red photo-conversion of Dendra2-HAS2 constructs suggested slower decay of K190R and S221A than HAS2 wt, while T110A was barely degraded at all. S221D and S221E, the phosphomimetic mutants of this site, decayed faster and blocked hyaluronan synthesis, suggesting alternative O-GlcNAc-/PO₄ substitution to regulate the stability of the enzyme. Probing the role of dynamic O-GlcNAcylation at S221 by adding glucosamine increased the half-life of only HAS2 wt. The Dendra2-HAS2 disappearance from Golgi was slower for K190R. Of the two inactive constructs, K190R co-transfected with HAS2 wt suppressed, whereas T110A had no effect on HA synthesis. Interestingly, the HAS2-stimulated shedding of extracellular vesicles was dependent on HAS residence in PM but independent of HA synthesis. The results indicate that post-translational modifications control the trafficking of HAS2, and that trafficking is an integral part of the post-translational regulation of HAS2 activity.

KEYWORDS

hyaluronan, hyaluronan synthase, monoubiquitination, O-GlcNAcylation, phosphorylation, protein traffic.

INTRODUCTION

Hyaluronan (HA) was originally described as an inert structural scaffold for tissues. There is now increasing evidence for its widespread activities in cellular signaling, differentiation, proliferation, and migration in internal organs, peripheral tissues and even the central nervous system [1–4].

HA is distinct from other glycosaminoglycans due to its huge size (up to 10 MDa, several µm extended length), lack of core protein and sulfate esters, and its site of synthesis in plasma membrane [5]. The latter property makes a growing chain of HA a part of the cell surface glycocalyx
of many cell types [6]. HA binds to specific receptors like CD44, and also to proteoglycans, thus organizing and controlling the properties of the pericellular and extracellular matrix [7].

There are three homologous enzymes (HAS1-3) in mammals that synthesize HA [8,9]. HAS2 is the major producer of HA in adult vertebrates, and the only one indispensable for embryonic development [10]. HA produced by HAS2 is an important contributor to inflammatory responses [11–13] and the progression of cancer [14–17]. A number of signaling pathways have been found that upregulate the transcription of the HAS2 gene [16,18]. Indeed, most growth factors have direct or indirect impacts on HAS2 expression, and the same applies to cytokines, and also other danger signals [19], suggesting that HAS2 is an integral part of the reactions aiming at restoring tissue homeostasis. The multiple regulatory factors contributing to HAS2 expression obviously reflect the need for a fine balance of hyaluronan synthesis and its concentration around the cells.

In addition to the transcriptional regulation, recent findings have indicated that HAS2 is also subject to post-translational modifications that control its activity [20]. The threonine at position 110 of HAS2 has been suggested to be phosphorylated by AMPK and suppress the enzymatic activity of HAS2 [21]. While phosphorylation appears to have an inhibitory influence on HAS2 activity, O-GlcNAc modification at serine 221 enhances the stability of HAS2, and thus stimulates hyaluronan synthesis [22]. Moreover, monoubiquitination at lysine 190 is absolutely required for the enzymatic activity of HAS2 [23].

We have shown previously that O-GlcNAcylation increases the activity of HAS3, and that the activation is largely mediated by its effects on HAS3 traffic to PM and lysosomes [24]. In the present work we aimed at testing whether the known post-translational modifications of HAS2 also influence its activity through changes in the intracellular trafficking of the enzyme. Indeed, the S221 O-GlcNAcylation site mutated to S221A increased the lifetime of HAS2. In contrast, substitution of this site with the acidic mutants S221D and S221E enhanced the turnover, suggesting a possibility of enhanced decay by phosphorylation at S221. Unexpectedly, replacing the T110 phosphorylation site with the T110A mutant made it completely inactive in live cells due to blocking its traffic from ER to Golgi. The enzymatically inactive K190R monoubiquitination mutant was present in PM at levels exceeding those of the wt, which could explain its dominant negative effect on hyaluronan synthesis, while the inactive T110A remaining in the ER showed no such effect.

RESULTS

The HAS2 mutants show different hyaluronan secretion rates and surface coats

Human HAS2 wt, and its previously established K190R, T110A and S221A mutants were transiently transfected into COS1 cells, known for their minimum production of HA. For comparison, a plasmid with HAS3 (wt) was also transfected. The relatively high expression of the transfected HAS2 and its mutants did not significantly affect the cellular homeostasis, as indicated by unchanged expression of the unfolded response genes and the rate of apoptosis (Supplemental Fig. 1)

Withholding ubiquitination (K190R) or phosphorylation (T110A) completely prevented the production of HA, as indicated by the lack of HA coat on cell surface (Fig. 1A,B) and missing HA in the medium (Fig. 1C), while blocking O-GlcNAcylation (S221A) reduced the HA secretion and coat size (Fig.1B,C). None of the constructs changed the expression of CD44 that could potentially affect the coat size or HA metabolism (data not shown). The results thus demonstrate the strong effects of post-translational modifications on the synthesis of HA by HAS2.

The mutant HAS2 proteins show different subcellular distributions

To learn how intracellular trafficking of HAS2 might be involved in the HA synthesis of the post-translational modification site mutants, we first studied the subcellular distributions of the HAS2 mutants. The Dendra2 alone without HAS2 gave a diffuse signal consistent with its cytosolic localization. The fluorescence of HAS2 wt, K190R and S221A suggested its origin in the endoplasmic reticulum (ER), cytoplasmic vesicles, plasma membrane (PM), and Golgi, the latter the dominant site. In contrast, no signs of T110A were seen in cytoplasmic vesicles and PM, while the
reticular pattern and strong signal at the nuclear membrane suggested that it mainly resided in ER (Fig. 2A, supplemental Fig. 1A). The fluorescent markers BODIPY TR ceramide for Golgi and a calnexin antibody for ER were used to check the colocalizations of the mutant proteins. Calculation of Manders correlation coefficients confirmed that HAS2-T110A was more co-localized with the ER marker, and less co-localized with the Golgi marker, as compared to that HAS2 wt, K190R and S221A (Fig. 2B and supplemental Fig. 2B). Moreover, when we calculated the difference of the co-localization values from the red (M1) and the green (M2) channels for both Golgi and ER markers, a significant difference towards ER was found (Supplemental Fig. 2C). This means that T110A co-localized better in ER than in Golgi. The finding that there was some co-localization also between the Golgi marker and T110A is explained by the fact that these compartments are functionally and anatomically closely connected and therefore difficult to separate completely. As expected, Dendra2 showed no preference for either ER or Golgi since in cytosol it is close to both compartments. The above findings suggest that except HAS2-T110A, all other mutants matured in Golgi. The inability of HAS2-T110A to travel from ER to Golgi (and further to PM) was presumably the main reason for its inactivity in HA synthesis (Fig. 1).

The overall half-lives of the HAS2 mutants are different

Like most proteins in PM, HAS2 is eventually endocytosed and degraded in lysosomes. The rate of HAS2 catabolism was studied by transfecting HAS2 fused with Dendra2, the green fluorescence of which can be photo-converted to red, and measuring the disappearance of the red signal (Fig. 3A,B). Chloroquine, an inhibitor of lysosomal degradation, strongly delayed the disappearance of the photo-converted Dendra2-HAS2 wt, confirming the importance of lysosomes as the site of HAS2 degradation (Fig. 3C). The decreasing intensity data were fitted to a first-order decay function, or a plateau first-order decay, to estimate the degradation rate constant, which was then used to calculate when the half-life is achieved [22,24,25]. The calculation resulted a half-life ($t_{1/2}$) of 1h 48 min for HAS2 wt (Figs. 3B and 4E). While the decay curve of the K190R mutant showed considerable overlap with HAS2 wt, its slope was clearly different, resulting in a longer $t_{1/2}$ (Figs. 3B and 4E). Half-life for the HAS2-T110A mutant could not be calculated as it did not follow a first-order decay function, and actually did not decay much at all. However, the accumulation was not severe enough to raise ER stress at least through a classical unfolded protein response, since the expression of ERN1 and CTFR, components and indicators of the response, were not significantly increased, neither was apoptosis (supplementary Fig. 2) [26]. The HAS2-S221A mutant showed a different behavior, first exhibited a plateau, or a period when the intensity stayed constant, which was followed by a first-order decay (Fig. 3B), which was only slightly longer than that of HAS2-wt (Fig. 4E).

All the mutants (K190A, T110A and S221A) thus tended to increase the overall time reaching the half-life ($t_{1/2}$), but for different reasons, as the T110A obviously never reached the normal traffic cycle of HAS2, and S221A although its decay was faster, the protein was stable for a period of 1h 30 min before starting its degradation (Fig. 3B). When the decay was evaluated by the Dendra2HAS2 red signal remaining at the end of the 5 h follow-up, those of S221A and T110A, but not K190R, stayed significantly higher than HAS2 wt (Fig. 3B).

The supply of UDP-GlcNAc differentially affects HAS2 mutant half-lives

UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetyl glucosamine (UDP-GlcNAc) are required as substrates for HA synthesis. The cellular content of UDP-GlcNAc also controls the O-GlcNAc modification levels of HAS2 and HAS3. This modification increases the overall stability of HAS2 and HAS3 [22,24]. Feeding cells with glucosamine (GlcN) increases the cellular content of UDP-GlcNAc and consequently protein O-GlcNAcylation [24,27]. Indeed, GlcN changed the $t_{1/2}$ of HAS2 wt from 1 h 48 min to 2 h 24 min (Fig. 4,E), and significantly increased the amount of the protein remaining after 5h (Fig. 4,A). A similar trend was observed for K190R but its $t_{1/2}$ could not be calculated since the curve shape not fit with a first order decay, and the remaining protein at 5 h, while appearing higher, did not reach significance (Fig. 4B,E). Interestingly, addition of GlcN to the mutant with blocked O-GlcNAcylation site (S221A) showed a tendency to decrease the end point level of S221A (Fig.4D). This was associated with elimination of the initial plateau in the decay curve, while the $t_{1/2}$ remained unchanged (Fig 4E). GlcN also appeared to reduce the $t_{1/2}$ of T110A (Fig. 4C,E). This
could be associated with generally increased cellular O-GlcNAcylation, relieving the ER stress caused by T110A accumulation.

**Slower forward traffic of HAS2 mutants through Golgi**

The microscopic images shown above (Figs. 1-3) indicate that except the T110A mutant, Golgi usually had the largest deposit of HAS2, while at any time just a small proportion appeared at PM. This suggested that the traffic from Golgi to PM is one of the regulatory points of HAS activity.

The level of fluorescent HAS2 in PM, unlike that of HAS3 [24,28], was too small for microscopic quantification. We therefore estimated the HAS2 forward traffic from Golgi by assaying the rate of signal decrease in Golgi after Dendra2-HAS2 photo-conversion in the perinuclear region corresponding the Golgi (Fig. 5A). A 60 min follow-up period revealed that the intensities of the photo-converted Dendra2-HAS2 S221A and K190R mutants appeared to decrease at a rate slower than that of HAS2 wt, the difference was significant for HAS2-K190R. This suggests that “the renovation rate” of HAS2 K190R and S221A in Golgi was lower (Fig. 5B,C).

**Silencing of Rab10 increases HA synthesis by HAS2 wt and HAS2 S221A**

PM residence of HAS2 could be influenced by Rab10, a member of the GTPase family proteins that regulate vesicular trafficking. Rab10 enhances the endocytosis of HAS3 from PM and therefore inhibits HA synthesis [28]. Indeed, when Rab10 was specifically silenced with siRNA (Fig. 6A) HA synthesis by both HAS2 wt and HAS3 was enhanced, presumably through their increased abundance in PM due to an inhibition of their retrograde traffic [28]. A similar increase of HA production by Rab10 siRNA was seen in S221A, while K190R remained enzymatically inactive (Fig. 6B). The results suggest that the retrograde trafficking of HAS2 back from PM is stimulated by Rab10 in the same way as that of HAS3.

**Increased plasma membrane residence of HAS2 K190R and absence of T110A**

For measuring HAS2 residence in PM we used a strategy in which cells transfected with EGFP-HAS2 plasmids with the mutations, and EGFP-HAS3 for comparison, were subjected to biotinylation of proteins on cell surface. Among all lysine residues, 7 are predicted to be present in the extracellular domains of HAS2, while HAS3 is supposed to have 5 lysines in such positions (http://www.uniprot.org/uniprot/Q92819). After labelling the extracellular domains of the proteins present on the PM, using DTT-cleavable biotin, a pull-down against EGFP, biotin and EGFP was performed in succession using anti-GFP streptavidin-coated and anti-GFP magnetic beads, as reported previously [24]. Western blotting revealed that HAS2 (and HAS3) proteins were biotinylated as shown by their binding to the streptavidin beads, indicating their presence on plasma membrane (Fig. 7A). The non-biotinylated EGFP-HAS proteins are shown in the adjacent GFP beads blot image. Quantification of the western blots showed that in K190R and S221A a higher proportion of total HAS2 resided in the PM, as compared to HAS2 wt, the increase of K190R was statistically significant. In contrast, T110A completely prevented the access of HAS2 to PM (Fig. 7B), which is in line with its inability to exit ER (Fig. 2). Using this technique in similar experimental conditions, a larger proportion of HAS3 than HAS2 was recovered from PM (Fig. 7B).

Additionally, EGFP-HAS2 and its mutants were analyzed in COS1 cells using Total Internal Reflection Fluorescence (TIRF) microscopy, a technique with limited detection of anything beyond ~200 nm from PM. After subtracting the background fluorescence and bleed through from the strong Golgi, vesicles in the size range of 10-500 pixels were specifically visualized using ImageJ (Fig. 7C), and the resulting count mask particles were quantified. Compared to HAS2 wt, there were higher numbers of cell surface HAS vesicles in HAS2 K190R and HAS3 transfected cells, while vesicle numbers in HAS2 S221A were not different from HAS2 wt (Fig. 7D). The results are well in line with the HAS PM residence data from the western blots of the immunoprecipitates (Fig. 7A,B).

**Co-transfection of the HAS2 mutants influence HA synthesis by HAS2 wt and HAS3**

All HAS isoforms have the potential to form homo- and heteromeric complexes [23,29] Accordingly, the enzymatically inactive HAS2 K190R has been reported to quench the activity of HAS2 wt and HAS3 [23]. We wanted to study whether similar quenching occurred with the other
mutants. Co-transfection of the enzymatically inactive K190R with HAS2 wt caused a concentration dependent inhibition of HA synthesis (Fig. 8A), as before [23]. In contrast, T110A, the other enzymatically inactive construct, did not exhibit a significant decrease in HA synthesis. Co-transfection of S221A, a mutant that retains the capacity to produce HA, had no effect on the synthesis of HA by HAS2 wt. Co-transfection of all HAS2 mutants with HAS3 showed a tendency for a gradual decrease of HA (Fig. 8B).

**HAS2wt-induced stimulation of extracellular vesicle secretion is not affected by K190R and S221A**

A fraction of HAS enzymes are secreted from PM into extracellular vesicles (EVs), often covered by HA [30–32]. We studied whether the mutations blocking post-translational modifications also affected this branch of HAS2 trafficking by measuring the size and number of EVs with a nanoparticle tracking analyzer. Most of the EVs secreted by the COS-1 cells ranged in size (diameter) between ~50 and 300 nm, averaging about 150 nm (Fig. 9A), and no significant differences were noted between the mutants and controls, as evident from their mean sizes (Fig. 9B). Non-transfected cells and mock controls (empty plasmid) secreted similar numbers of EVs, 4.5 x 10^11 particles/ml (Fig. 9C). In contrast, the cultures transfected with HAS2 wt secreted ~70% more EVs as compared to non-transfected and mock controls (Fig. 9C). A similar stimulation of EV secretion was found with K190R and S221A, while no increase of EVs occurred with T110A. These results allow two interesting conclusions. First, PM residence of HAS2 is likely required for the stimulation of EV secretion, since T110A did not reach PM. And second, HA synthesis is not necessary for the stimulation of EV secretion since K190R is enzymatically inactive (Fig. 5).

The finding that K190R, while unable to synthesize HA, stimulated the shedding of MVs lead to the question whether K190R itself enters the EVs. This was checked by growing the cells in a 3D matrix that immobilizes EVs close to the cells, then allowing fluorescent imaging of the HAS2, if present in the EVs. The data show that HAS2 wt, S221A and even K190R, but not T110A were present in the EVs (Fig. 9D). This indicates that the mechanism of HAS2 stimulation of EVs involves HAS2 incorporation in them, and without involvement of HA. The role of the HA synthesis in EV shedding was further probed by the HA synthesis inhibitor 4-MU, which is known to suppress the expression of the endogenous HAS2 gene and deplete its UDP-glucuronic acid substrate. Unexpectedly, 4-MU blocked the shedding of all transfected HAS2 and its mutants, even that of the K190R (Fig. 9D).

**Phospho-mimetic and phospho-deficient S221 mutants suggest a Yin-Yang position**

There are several examples of protein Ser/Thr residues that can be either phosphorylated or O-GlcNAcylated, the alternatives showing strong functional consequences [33–37]. This made us to consider the possibility that the increased t1/2 of S221A could be due to the inability of this site for phosphorylation, phosphorylation being a signal to limit HAS2 lifetime and activity. To start probing this idea we prepared phosphomimetic mutants in which S221 was mutated to aspartic (S221D) or glutamic acid (S221E) to mimic constitutive phosphorylation. The previously established S221A then served as a phospho-deficient control, mimicking the unphosphorylated state. The newly generated S221D, and especially S221E mutation tended to enhance the decay (Fig. 10A) and reduce the half-life (Fig. 10C) as compared to S221A. The remaining protein after 5 h was also significantly decreased in S221D and S221E, as compared to the phospho-deficient S221A. In line with their suggested instability, the synthesis of HA by S221D and S221E was blocked (Fig. 10D). There was no reason to suspect that the inactivity would be due to a missing transport signal, as with T110A, since the subcellular locations of S221D and S221E could not be distinguished from that of HAS2 wt, mostly in the Golgi (Fig. 10B). The findings are consistent with, but do not prove, that HAS2 phosphorylation at S221 is involved in the regulation of its lifetime and activity.
DISCUSSION

The present data indicate that preventing certain post-translational modifications of human HAS2 by site-directed mutagenesis influences the trafficking of this protein at many transport points. The results stress the role of trafficking for its enzymatic activity. Trafficking, controlled by certain post-translational modifications, may actually be a major regulator of HAS2 activity. Specifically, intact T110 is required for transport from ER to Golgi, K190 slows down the forward traffic of HAS2 through Golgi and likely from PM to endosomes, and different substituents of S221 have opposite effects on the overall decay of HAS2 by lysosomes. A schematic illustration of the post-translational modifications and their effect on HAS2 trafficking is given in Fig. 11.

Why does T110A remain in the ER? - The HAS proteins, like all integral membrane proteins, are first inserted in endoplasmic reticulum (ER), checked for correct folding, and taken to Golgi by bulk flow or receptor-enhanced uptake into transport vesicles. Immunohistochemical assays and fluorescent tags (Figs. 1 and 2) demonstrated that only a small part of HAS2 wt is normally present in the ER [38,39]. Therefore, the mutation most likely induces a defect in the proper folding or maturation of HAS2 or, alternatively, in the recognition site for active transport to Golgi [40]. In any case, the membrane insertion should not be affected, since all the mutation sites are in the cytoplasmic domain.

Originally T110 has been described as a target of AMPK, and its phosphorylation suggested to inhibit HAS activity. The T110A mutation made HAS2 unresponsive to AMPK-mediated inactivation [21]. Importantly, in the cited study the activity of the mutant was then assayed only in membrane preparations of homogenized cells in vitro [21]. This explains why the transport defect in live cells has not been recognized before.

Since the T110A mutant is still active in vitro, major folding problems, which would probably disrupt the activity, are unlikely, making a specific fault in the trafficking between ER and Golgi more probable. This was also supported by the fact that no change in the indicator of unfolded protein response, or the rate of apoptosis took place. The sustained activity of T110A in vitro offers an interesting piece of information concerning the enigma of HAS2 activation mechanism, or actually the mechanism of keeping it inactive before insertion in PM. These findings suggest that the normal block on premature, intracellular HA synthesis is lifted by something associated with disruption of the cells.

What makes the K190R mutant capable of inhibiting HA synthesis? - HAS2 K190R increased in PM but was enzymatically inactive. It has been shown that HAS3 is subject to continuous endocytosis and recycling back to PM unless engaged in HA synthesis, which keeps the enzyme in PM [24]. The same probably occurs with HAS2, as suggested by the increase of HA synthesis when the endocytosis facilitated by Rab10 was blocked. The high level of K190R in PM might thus be attributed to defective endocytosis rather than enhanced Golgi to PM transport, which was actually slower for K190R.

The inactivity of K190R was unlike that of T110A, which can be explained by ER retention. In this respect the T110A resembled the inactive HAS3 constructs C-terminally truncated by R539stop and S10stop, both of which also remained in ER [38]. D216A, another inactive mutant of HAS3, could travel to Golgi, but did not reach PM [38]. HAS2 K190R is thus the only known enzymatically inactive vertebrate HAS mutant that reaches PM. Of the mutants studied here it is also the only one that inhibits HA synthesis in a dominant negative manner, probably due to formation of homomers with intact HASs [23].

Native, enzymatically active HAS isoenzymes can bind to each other and change the hyaluronan synthesis of their partner [29]. However, even severely truncated, inactive HAS3 constructs can dimerize with HAS3 wt [29] but have no effect on HA synthesis (Deen et al, unpublished data). However, those constructs remain in the ER, like HAS2 T110A, and do not reach PM. The dominant negative effect may actually be caused by the high access of K190R to PM where HA synthesis takes place. This would mean that the formation of homo- and heteromers between HASs is dynamic, not fixed throughout the trafficking.

HAS2 stability is regulated by substitution of S221 - This site is known to be subjected to O-GlcNAcylation which increases the stability of the enzyme [22]. This is consistent with the current findings that the S221A mutation made HAS2 unresponsive to the life-extending effect of GlcN. In
fact, addition of GlcN enhanced the degradation as judged by the reduced content of S221A after the 5 h follow-up. This was probably caused by effects mediated by O-GlcNAcylation of other targets involved in protein catabolism, in the same way as the faster decay observed for S110A with added GlcN.

Phosphorylation and O-GlcNAcylation can be alternative substitutions of certain protein Ser/Thr residues [34]. We therefore studied whether there could also be another dimension in the stability control of HAS2 by the S221 site. This appeared quite feasible since changing the neutral amino acid (A) in this site to acidic (D) canceled the life extension by S221A and inhibited HA synthesis. Moreover, a further change of S221 to an acidic amino acid one carbon longer (E), induced even faster decay of the enzyme. The subcellular localizations of S221D and S221E were similar to that of HAS2 wt, suggesting no major problems in folding that could block trafficking at least up to Golgi. The data on these phospho-mimetic mutants are thus in line with an idea that S221 is a Yin-Yang site, subject to phosphorylation unless O-GlcNAcylated. Confirming this will have to await direct identification of the substituents of S221 in vivo.

PM abundance of HAS2 stimulates EV secretion in a way not dependent on hyaluronan synthesis - HAS3 expression has been associated with the secretion of HAS3-positive, HA covered EVs in the culture medium [30], and the secretion is stimulated by raising the PM level of HAS3 [24]. The role of HA synthesis in the enhanced EV secretion could not be defined previously since HA synthesis has always been tightly associated with the PM level of HAS3 [38].

The present discovery of an inactive HAS2 residing in PM offered a perfect opportunity to distinguish between the contributions of the HAS protein itself, and its HA synthesis, to EV secretion. Tightly packed HA on cell surface is expected to support the PM curvature in the protrusions of cells [6,41], and EVs are shed from the tips of such protrusions [30,31]. It was therefore a surprise that the enzymatically inactive K190R enhanced EV secretion to the same extent as HAS2 wt, while it did not induce the PM protrusions. Just the insertion of HAS2 in PM must therefore trigger a signal or structural alteration in the membrane that facilitates its inclusion in, and shedding of the EVs. The present finding thus adds another curious, HA-unrelated function to HAS2. Previously, the TGFβ-induced EMT (epithelial to mesenchymal transition or trans-differentiation) of mammary epithelial cells has been shown to depend on HAS2 but be resistant to hyaluronidase treatment and CD44 knockdown [42].

The secretion of EVs with enzymatically inactive, dominant negative HAS2 K190R offers an interesting tool in the future to probe the functions of HAS and HA in the communication between adjacent cells and distant tissues, EVs with HAS2 wt and K190R could be compared in their ability to find and fuse with target cells and influence their phenotype.

Another interesting finding was that while HA was not necessary for EV formation, the HA synthesis inhibitor 4-MU blocked HAS2 insertion in the EVs. This may represent yet another mechanism of HA synthesis inhibition by 4-MU, previously shown to involve downregulation of HAS2 gene expression, depletion of UDP-glucuronic acid [43], loss of CD44, and induction of hyaluronidases [44]. Exploring the mechanism of the block and its importance in HA synthesis and EV shedding will be interesting targets of future studies, especially in cancer epidemiology.

Differences and similarities in the subcellular trafficking of HAS2 and HAS3 - Microscopic images of cells transfected with EGFP-HAS3 and EGFP-HAS2 in the present work and in those published before [28,45] suggest that HAS3 is more abundant in PM than HAS2. This was supported by the cell surface biotinylation data in the present work. The hyaluronan coat of HAS2 appeared wider than that of HAS3, while adjacent to PM the coat of HAS3 was more dense; this difference could be explained by a higher molecular mass of the hyaluronan synthesized by HAS2 than HAS3 [46]. A higher concentration of HA chains on cell surface is thus consistent with the higher density of HAS3 in PM. The swelling pressure of the tightly packed HA on the outer surface of PM can be one of the factors driving the membrane curvature required in the formation and maintenance of the microvilli. The difference in PM abundance of the HASs could then explain the higher numbers of microvilli-like protrusions in cells transfected with HAS3 as compared to HAS2 [45].

It has been shown that HAS3 consumes UDP-sugars faster than HAS2, to produce HA [45,46]. This, and the difference in PM residence suggest that the HA synthesis of HAS2 is post-translationally more tightly regulated than that of HAS3. However, more experimental evidence is required to confirm this.
Rab10 influenced HA synthesis of HAS2 apparently by enhancing its endocytosis from PM, as shown previously for HAS3 [28]. This is actually expected, based on the formation of heteromers between HASs [29], probably transported in the same vesicular bodies with shared regulators like Rab10. Another feature common to HAS2 and HAS3 is their O-GlcNAc modification which stabilizes the proteins and prevents their lysosomal degradation [22,24]. The enzymatic activity of both HAS3 and HAS2 depends on the availability of UDP-GlcUA and UDP-GlcNAc [22,24], although HAS2 is more sensitive to the depletion of these substrates [45]. Both HASs are also incorporated into EVs shed into the extracellular space. Thus, while there are similarities in the vesicular trafficking, stabilization by O-GlcNAcylation, and sensitivity to the substrate supply, significant differences exist between HAS2 and HAS3 in their regulation, especially by ubiquitination and phosphorylation which have not been demonstrated in HAS3. Another currently unknown issue in HAS trafficking is the possible interaction of ubiquitination with O-GlcNAcylation or phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Reagents** — The following general reagents were used: Lipofectamine 3000 (Invitrogen), Dynabeads® Protein G (Novex), Dynabeads® MyOne™ Streptavidin T1 (Invitrogen), EZ-Link™ Sulfo-NHS-SS-Biotin (Thermo Scientific, NY 14072, USA), Pierce BCA protein assay Kit (Thermo Scientific), bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma).

**Antibodies and markers** — Rabbit polyclonal anti-calnexin (Cell Signaling, Danvers, MA), rabbit monoclonal anti-GM130 (Cell Signaling), rabbit polyclonal anti-GFP (Invitrogen, Life Technologies Ltd, Paisley, UK), goat polyclonal anti-rabbit and anti-mouse Dylight 800 (Thermo Scientific), anti-rabbit Texas Red-labeled secondary antibody (Vector Laboratories, CA, USA), CellMask™ Deep Red plasma membrane indicator (Molecular Probes, Invitrogen), and the Golgi marker BODIPY TR ceramide (Molecular Probes, Leinen, The Netherlands) were used.

**Plasmids** — The cDNAs encoding the open reading frames of (wild type) human HAS2 gene [45], the human HAS2 K190R [18], and human HAS2 T110A [21] and HAS2 S221A [22] were prepared as described before. The cDNAs were excised by XhoI/HindIII digestions, followed by ligation in Dendra2-C1 (Evrogen) and EGFP-C1 (Clonetech) plasmids. They were amplified and purified with Plasmid Mini kit (Fermentas) and sequenced to confirm that the insertions were in-frame. pcDNA3 (Clonetech) was used as a mock vector, and monomeric Cherry (Clonetech) as a control of the transfections.

**Cell culture** — The SV40-transformed African green monkey kidney cell line COS1 was used in all experiments, and grown in DMEM high Glucose (Euroclone, Milan, Italy), 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-Glutamine (Sigma), 100 µg/ml streptomycin, and 100 IU/ml penicillin (Sigma). The cells were subcultured when 90% confluent.

The confocal experiments were performed in 8-well Ibidi µ-slides (IbiTreat, Ibidi, GmbH, 82152 Planegg/Martinsried, Germany), seeded with 15,000 cells/well, and for the immunoprecipitation and western blot experiments in 10 cm dishes with 900,000 cells/dish. Fetal bovine serum (FBS) and PBS, depleted of extracellular vesicles (EV), was used for EV isolation experiments. Briefly, the reagents were purified by 16 h centrifugation at 100,000 x g and sterile-filtered with 0.22 µm filters (Minsart, Sartorius Stedim, Biotech, Goettingen, Germany).

**Transfection** — The transfections were done with Lipofectamine 3000 (Invitrogen). The transfection mix was prepared separately and dropped in the medium of each well 24 h after splitting the cells. The replacement for the medium was made with fresh complete medium 16 h later. The mix for each Ibidi plate well (15,000 cells/well) contained: 0.2 µg of DNA (0.1 µg plasmid of interest and 0.1 µg mock plasmid), 0.4 µl P3000 and 0.3 µl Lipofectamine in 20 µl of the DMEM. Co-transfections of the different HAS constructs were performed in 24 well plates (30,000 cells/well). The mix was prepared with either 0.04 µg of HAS2 wt or 0.02 µg of HAS3, with increasing amounts of the different mutants (0.04, 0.1, and 0.2 µg), 0.8 µl P3000 and 0.6 µl Lipofectamine in 40 µl of DMEM. The transfections for the pull-down assays were made in 10 cm dishes (900,000 cells) with 6 µg of the plasmids, 24 µl P3000, and 18 µl Lipofectamine in 600 µl of DMEM.
Confocal Microscopy and Live Cell Imaging — The fluorescent images were obtained with 40x 1.3 NA oil objective (512 x 512-pixel resolution) on a Zeiss Axio Observer microscope equipped with a Zeiss LSM 700 confocal module (Carl Zeiss Microimaging). For live cell imaging, the Zeiss XL-LSM S1 incubator module was used to control temperature and CO₂.

Hyaluronan secretion assay — COS1 cells were split in 96 well plates (6,000 cells/well) to measure the hyaluronan content in the media. After 24h, the cells were transfected with the different plasmids. The transfection mix was left with the cells overnight, and the medium was changed after 24h. The new medium was collected 24 h later and cell numbers were counted. The culture media were stored at -20°C before hyaluronan assay by an enzyme-linked sorbent assay [47]. The results were calculated as ng hyaluronan/10,000 cells.

Hyaluronan Coat Analysis — For visualization of HA surface coat on live cells, a HA-binding complex (HABC) conjugated to Alexa Fluor 568 was used as described previously [45]. Briefly, for quantitative analysis cells were imaged with a 20x objective from 15 random fields, using z-stacks of 1.93-µm thickness. The mean intensity of HA coat (Alexa Fluor 568, red) from the images was quantified from the compressed z-stacks of 320 x 320-µm area, divided by the mean intensity of EGFP fluorescence. Representative images were taken with a 40x oil objective.

Immunocytochemistry — COS1 cells were transfected with the different plasmids. After 24 h, the cells were washed with 0.1 M sodium phosphate buffer, pH 7.4 (PB), and then fixed with 4% paraformaldehyde in PB for 20 min and washed again. The fixed cells were permeabilized for 15 min with 0.1% Triton X-100 with 1% BSA, blocked with 1% BSA for 20 min, all at room temperature, and then incubated overnight with the primary antibody; Rabbit polyclonal anti-calnexin (1:200), or rabbit monoclonal anti-GM130 (1:1,000) in 1% BSA at 4 °C. After washing, the cells were incubated with a Texas Red -labeled secondary antibody (Vector Laboratories, CA, USA) (1:500 in PB) for 1 h, washed with PB, and stored at 4°C.

Analysis of HAS2 present in plasma membrane — COS1 cells were plated in 10 cm dishes and transfected the next day. After 24h of transfection, plasma membrane protein biotinylation was done with a modification of the procedure described by Sun et al [48]. The cells were washed thrice with ice-cold PBS and incubated with 1 mM of freshly prepared EZ-link Sulfo-NHS-SS-biotin for 30 min at room temperature. The unreacted biotin was quenched with 50 mM Tris in ice-cold PBS (pH 8.0) for 10 min and washed twice with ice-cold PBS. The cells were then lysed and scrapped in 0.3 ml of lysis buffer (5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1:300 phosphatase inhibitor cocktail from Sigma, 1:200 protease inhibitor cocktail from Sigma, and 1% Triton X-100 in PBS, pH 7.4). The lysates were homogenized with a UP50H ultrasonic processor (Hielscher, Boutersem, Belgium) with three cycles of 15 seconds, and the concentration of protein was measured. All the steps during the cell lysis procedure were done in ice-cold conditions.

Protein G magnetic beads (Dynabeads®, Life Technologies AS, Norway) were incubated with an anti-GFP antibody following the manufacturer’s instructions to get the maximum binding capacity. Briefly, the amount of antibody used in the incubation was 8 µg per mg of beads in PBS with 0.01% Tween. For each condition the amount of beads was 1.5 mg. The resulting supernatant from two 10 cm dishes was added to the anti-GFP conjugated beads and incubated overnight under vertical rotation. The immunoprecipitated EGFP, fused with the different HAS2 protein constructs, were eluted using 40 µl of 50 mM glycine-HCl buffer, pH 2.8. The eluted protein fractions were immediately diluted with 600 µl of PBS, pH checked, and protein inhibitors added. Then an immunoprecipitation was performed with 50 µl of Streptavidin M-280 Dynabeads®, incubated overnight. The Streptavidin beads were eluted at 65°C for 15 min with 4X SDS sample buffer for western blotting. The supernatant not bound to Streptavidin beads was concentrated with a second anti-GFP immunoprecipitation and the beads eluted as before. The western blots of the streptavidin bound and non-bound HAS2 constructs were visualized by the anti-GFP antibody.

TIRF imaging and analysis — COS1 cells were transfected with EGFP-tagged plasmids of HAS2 wt, HAS3, HAS2 K190R and HAS2 S221A. An Olympus cell-R TIRF system microscope equipped with a 100x oil immersion objective with a high numerical aperture (1.45) was used to reach TIRF illumination. The laser source used was 488nm/30mW. The 8-bit images were processed using
ImageJ software. Using a threshold of 15-25 au, background fluorescence was subtracted from the actual signal. To exclude the bleed through of fluorescence from Golgi, a cut-off value of 10-500 pixels was used while analyzing the number of EGFP-positive particles. The resulting particle counts were divided by the number of transfected cells in the field.

**Co-localization Analysis** — For colocalization analysis, images were taken using a 40x oil objective, and the analysis was done with the ImageJ software. The autothreshold function was used to process and analyse the co-localized pixels. Manders coefficient was used to measure the degree of co-localization in the split channels (M1, red channel; M2, green channel), with 7 images/group. Co-localization thresholds of the images shown aim at visualizing the co-localised pixels in a representative image.

**Western Blotting** — Immunoblotting was carried out using 10% SDS-PAGE and transfer to nitrocellulose membranes (Protran, Whatman, GE Health Care Biosciences, Pittsburg, PA 15264-3065, USA), blocked with 3% BSA in Tris buffer containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated overnight with the anti-GFP antibody (1:2000, in 1% BSA-TBST). The blots were then washed thrice with TBST and incubated with the anti-rabbit Dylight 680 antibody (1:5000, in 1% BSA-TBST) for 1 h at room temperature and washed thrice with TBST. The blots were then imaged using LI-COR Odyssey Infrared imaging system (LI-COR Biosciences, Lincoln, NE).

**Analysis of HAS2 degradation** — COS1 cells transfected with Dendra2-HAS2 wt and Dendra2-HAS2 mutants were photoconverted from green to red using a 405 nm UV laser, as described before [24,28]. A region of interest (ROI) was drawn around the whole cell and the disappearance of the red Dendra2 was analysed as an indicator of the degradation of the protein. The decay was followed for 5 h with an interval of 20 min between images. Transmitted light was used as a reference signal to correct for a possible drift in the optical focus. For calculating the half-life \( t_{1/2} \) of the different proteins, the percentage of photoconverted Dendra2 vs time was plotted and a first order decay was fitted. In the cases where there was a plateau before the decay, the first order decay was fitted after that. The point of discontinuity between the plateau and the decay was called X0. The time when half-life was achieved was calculated by adding together X0 and the \( t_{1/2} \) of the subsequent decay. The time-point immediately after photo-conversion was set as “0”.

**Analysis of HAS2 flux in Golgi** — Just before imaging, a plasma membrane (PM) indicator (Deep mask red dye, Invitrogen) was added to the transfected COS1 cells for better orientation. A laser pulse at 405 nm (10% laser strength, 10 iterations per second) was used to photoconvert the green Dendra2-HAS2 to red in the perinuclear region of the cell, corresponding to Golgi. The intensities at 488, 555 nm (Dendra2 green and red) and 680 nm (PM marker) were measured every 10 min during 1h to track the traffic of red Dendra2-HAS2 from Golgi towards PM. The time of photoconversion was set as point “0”. The fluorescence values of the red Dendra2 at each time point was divided by the green Dendra2. The results were calculated as the percentage of photoconverted Dentra2 fluorescence ratio related to time point “0”.

**siRNA Silencing of Rab10** — The Rab10 (Sense: GCCGAAGAUAUCCUUCGAG; Antisense: UUCGAAGGAUUCUCAGC) and scrambled siRNAs (Eurogentec reference no: SR-CL000-005) were obtained from Eurogentec (Seraing, Belgium). About 20,000 cells were seeded on 24-well plates and 24h later treated with 60 nM siRNA using Lipofectamine RNAiMax reagent (Invitrogen), according to the manufacturer’s instructions. After 6h the medium was changed and 24h later 0.1 µg of the HAS vectors were transfected with lipofectamine 3000 in fresh medium. 24 h after transfection the media were collected for HA quantification and cells counted for normalization.

**Quantitative real-time PCR (qRT-PCR)** – Cells were lysed and total RNA was isolated using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH). Complementary DNAs (cDNAs) were synthesized using the Verso cDNA kit (Thermo Scientific). The qRT-PCR was performed with Fast Start Universal SYBR Green mix (Roche Applied Science) using the Stratagene Mx3000P real-time PCR system (Agilent Technologies). Relative mRNA expression levels were compared by using the 2\(^{-ΔΔC(T)}\) method, with GAPDH as a control. The primer sequences used for real-time PCR were;
Catalog no: V35113, 5'GGAGGCTTGTGATGGTGAAA TCGC-3';
Rab35 5'-CCTGTGGGCAGAAGCAGTTTAC-3';
GAPDH 5'-AGAGGCTGGGCTCATTG-3';
ER1N 5'-GGCCTGGATTTTGGAAA-3';
CFTR 5'-GCTCCTACCCAGCCATT-3';
HAS2 wt and mutants
(Has2) and targeted to Dendra2
— site directed mutagenesis — Primers for HAS2 S221D (forward: 5'-CCTTACATCTCCAGAATCGGC-3');
reverse: 5'-CTTTTACCCTCTCCAGAATCGGC-3'); and HAS2 S221E (forward: 5'-CCTTACATCTCCAGAATCGGC-3');
reverse: 5'-CTTTTACCCTCTCCAGAATCGGC-3'); were designed using PrimerX
(Site-directed mutagenesis: see [31]). Nanoparticle Tracking Analyzer (Malvern Instruments Ltd., Malvern, UK)
using NTA 3.1 software (NanoSight, Amesbury, UK).

Analysis of extracellular vesicles (EV) secreted by HAS2-transfected COS1 cells — The COS1
cells were transfected with HAS2 wt and mutants were grown to 80% confluency in 10 cm culture plates
and the culture medium was subjected to centrifugation at 1000 x g for 5 min to remove cell debris.
The supernatant was centrifuged at 11,000x g for 90 min at 4°C and the pellet obtained was
suspended in PBS [31]. Nanoparticle Tracking Analyzer (Malvern Instruments Ltd., Malvern, UK)
was used to measure the concentration and size distribution of EV. The data for each sample was obtained from four technical replicates with constant equipment settings
as follows: camera level 13, acquisition time 30 s and detection threshold 3. Data analysis was done
using NTA 3.1 software (NanoSight, Amesbury, UK).

Annexin V staining and flowcytometry analysis of COS1 cells — COS1 cells transfected with
EGFP-expressing HAS3 or HAS2 wt and mutants were grown to 80% confluency in 6-well plates,
along with non-treated cultures and a chlorpromazine (Sigma) positive control. To induce apoptosis
in the positive control, 10 µg/ml of chlorpromazine was added to the growth medium and the cells
were treated for 24 h. Annexin V conjugated with APC (Catalog no: V35113, Molecular Probes, ThermoFisher scientific) was used to stain the apoptotic cells, as per manufacturer’s instructions.
Briefly, 1 x 10^6 cells were pelleted down and resuspended in 100 µl of 1X Annexin V binding buffer.
5 µl of Annexin V-APC conjugate was added in each group and incubated at 37 °C for 15 min.
After incubation, an additional 400 µl of the binding buffer was added. A no stain control was used as a
negative control for Annexin V staining. The cells were then analyzed in a CytoFlex S flowcytometer
(Beckman Coulter) using CytExpert software, with 10,000 events per group.

Analysis of EVs in 3D cultures — COS1 cells were transfected with EGFP expressing HAS3 or
HAS2 wt and mutants, 24 h later, cells were trypsinized and a small fraction of 100,000 per group
was pelleted down. The cells were then resuspended in growth medium and mixed with the
basement membrane extract, Cultrex® (Trevigen Inc., Gaithersburg, MD) in a 1:3 ratio.
A homogenous suspension of 35-40 µl containing 5 x 10^3 cells per group were added in the center of the
wells in an 8-well µ-slide plate (ibidi GmbH) and incubated for 1h @ 37 °C. Once the matrix was
solidified, 200 to 250 µl of growth medium with or without 0.5 mM of 4MU was added in the wells. 24
h later, the cells were fixed in 4% PFA in PB, washed with PB and stored in +4 °C until use. Release
of EVs was analyzed using LSM 800 confocal microscope, 40x objective, with z-stacks of 1 µm
optical thickness. 3D images were compressed and then analyzed using Zen 2012 software.

Statistical Analysis — All statistical analyses were done in GraphPad Prism version 6.0. One-way
ANOVA with Tukey’s multiple comparisons post-hoc test and student’s t-test were used, with a p
value < 0.05 considered significant.

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Hospital (MIT), The UEF Spearhead Funds - Cancer Center of Eastern Finland (MIT), The Finnish Cultural Foundation (AJD) and EATRIS, European Infrastructure for Translational Medicine (NTA facility, UEF).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


FIGURE LEGENDS

Fig. 1. Hyaluronan surface coats on, and hyaluronan secretion by COS1 cells transfected with different HAS constructs. (A) Representative images of the coats (red) visualized by Alexa Fluor-labeled hyaluronan binding complex in cells transfected with different GFP-HAS2 constructs (green). Cells transfected with GFP-HAS3 are shown for comparison. Scale bar = 10 μm. (B) Image analysis of the HA coat sizes produced by the constructs. The data were obtained using a 20x objective and represent means ± S.E. of 3 independent experiments from 15 random fields in each. *p<0.05; **p<0.01; ***p<0.001 (one-way ANOVA, Tukey's test). NT, not transfected (C) HA production into culture medium during a 24 h incubation starting two days after transfection. Means ± S.E. of 4 experiments are shown, statistical significances as above.

Fig. 2. Subcellular distributions of the HAS2 mutated at different post-translational modification sites. The fluorescent markers for Golgi (BODIPY TR ceramide) and endoplasmic reticulum (ER; calnexin) are colocalized with the HAS2 constructs indicated above the panels. White signal indicates co-localized pixels. Manders’ correlation coefficients for the co-localizations of the red channel (Golgi or ER marker) are shown below. 1 = maximum correlation. Means ± S.E. of 7 images/group are shown. Scale bar = 10 μm.

Fig. 3. Post-translational modifications affect the decay of HAS2. (A) Left: COS1 cells transfected with Dendra-HAS2-wt. Right: the same cells after exposure to a pulse of intense light at 405 nm which photo-converted a part of the green into the red form of Dendra2. Signals from the green and red channels of the image are shown at top and bottom, respectively. Scale bar 10 μm. (B) The intensity of the red protein was measured every 20 min. (C) The lysosomal inhibitor chloroquine was used as a positive control for an increased t1/2 of HAS2. Means ± S.E. of 5 independent experiments are shown. The statistically significant differences in the proportion of the remaining protein after 5 h are indicated by the asterisks, *p<0.05 ***p<0.001; ns, not significant (One-way ANOVA, Tukey's test).

Fig. 4. Glucosamine supplementation effects on the decay of the different HAS2 constructs. (A-D) The decay of the photoconverted Dendra2-HAS2 constructs shown in Fig. 3B are compared to those of identical cultures treated with 2 mM glucosamine (GlcN) to increase the cellular content of UDP-GlcNAc. (E) The initial lag periods and the following t1/2 (X0+t1/2) of the HAS2 constructs in the presence and absence of GlcN. The half-life could not be calculated for K190R +GlcN and T110A since their curve shapes did not fit with a first order decay. Means ± S.E. of 5 independent experiments are shown. Significant differences in the proportion of the remaining protein after 5 h are indicated by the asterisks, **p<0.01; ns, not significant (One-way ANOVA, Turkey's post-hoc test).

Fig. 5. Traffic rates of HAS2 from Golgi. (A) The forward traffic from Golgi was studied by the decay rate in Golgi of the photo-converted Dendra2-HAS2 constructs. (B) The intensity of the red signal in Golgi was assayed at 10 min intervals up to 60 min, time 0 set as 100%. Means ± S.E. are shown of 8 independent experiments. (C) The decay followed first order kinetics with the rate constants shown. ***p< 0.001; ns, not significant (t-test).

Fig. 6. Silencing of Rab10 increases hyaluronan in the medium of HAS2-wt and HAS2-S221A. (A) SiRNA was used to silence Rab10. The decline of its mRNA was examined by qRT-PCR, using Rab35 mRNA as a negative control. Means ± S.E. of 4 experiments. (B) Hyaluronan content in the medium (ng/10,000 cells) of cultures transfected with the indicated constructs, and either scrambled or Rab10 siRNAs, normalized to cultures with scrambled siRNA. The response of HAS3 is shown for comparison. Means ± S.E. of 4 independent experiments. *p<0.05; ns, not significant (t-test).

Fig. 7. Plasma membrane residence of EGFP-HAS2 is increased by K190R, while completely prevented by T110A. (A) Proteins on the surface of the cells transfected with the EGFP-HAS constructs were biotinylated. The cells were lysed and all EGFP-HAS proteins were immunoprecipitated with an anti-GFP antibody. The EGFP-HAS2 subjected to biotinylation on cell surface was specifically recovered by immunoprecipitation with streptavidin. Then the EGFP-HAS2 not biotinylated was recovered from the supernatant with anti-EGFP magnetic beads. Representative western blots of both pull-downs are shown. The stacking gels are also shown since the HAS proteins tend to form insoluble aggregates. (B) The percentage of HAS proteins present on PM, calculated from total HAS protein constructs (GFP westerns of HAS bound to Streptavidin beads and their supernatants). Means ± S.E. of 4 independent experiments. Control represents a non-biotinylated sample. (C) The original TIRF microscopy images of the different EGFP-HAS proteins are shown on the left panels, while those on the right represent the same cells processed with ImageJ to produce count masks, filtering the bleed from Golgi and demonstrating the number of EGFP-positive vesicles adjacent
to PM. (D) Quantification of the EGFP-positive vesicles per cell in the TIRF zone. Means ± S.E. from 4-11 images/group. *p<0.05, **p<0.01; ns, not significant, by Student’s t-test.

Fig. 8. Suppression of hyaluronan synthesis by co-transfection of HAS2 K190R. (A) Increasing quantities of plasmids containing the indicated mutants were co-transfected with HAS2 (wt) into COS-1 cells and the content of HA/cell was measured from medium. (B) Similar co-transfections of increasing amounts of the HAS2 mutants were done with HAS3 (wt), and HA synthesis determined as above. The data represent means ± S.E. of 4 independent experiments. The differences of K190R to mock transfection controls were evaluated with on t-test, **p<0.01; ***p<0.001

Fig. 9. The stimulation of extracellular vesicle (EV) secretion by HAS2 and its mutants. (A) The size distribution of EVs secreted in the growth medium by COS-1 control cells. (B) The mean size of EVs secreted by cells transfected with the different HAS constructs. (C) The numbers of vesicles secreted by the constructs. Means ± S.E. of 4 experiments are shown. Significances of the differences between the constructs: *p<0.05; **p<0.01; ***p<0.001; one-way ANOVA, Tukey’s test. (D) Representative confocal images from a 3D matrix culture of transfected cells treated with or without 0.5 mM 4MU.

Fig. 10. Phospho-deficient and phosphomimetic S221 mutants induce opposite effects on HAS2 stability. (A) Decay plots of Dendra-HAS2 with the mutations S221A (phosphodeficient), S221D, and S221E (phosphomimetic). Significance of the differences between protein levels remaining at 5 h are shown by the asterisks. The brackets and asterisks on the left indicate differences vs. control, those on the right vs. S221A. *p<0.05, **p<0.01, ***p<0.001 (means ± S.E. of 4 independent experiments, one-way ANOVA, Tukey’s test). (B) Subcellular localizations (green) of S221D and S221E, with nuclei staining as purple. (C) Comparison of the initial lags and half-lives of the constructs. (D) Hyaluronan in the growth medium of wt and phosphomimetic HAS2 mutants. Means ± S.E. of 4 independent experiments. **p<0.01 as compared with HAS2 wt, with one-way ANOVA, Tukey’s test. NT, not transfected.

Fig. 11. Schematic summary of changes in subcellular trafficking due to post-translational modifications in HAS2. Through the conventional ER-Golgi route, HAS2 is transported to PM, where it synthesizes HA. Rab10 inhibits HA synthesis, most likely due to regulation of HAS2 endocytosis. The scheme shows the changes in trafficking when the normal posttranslational modifications are prevented by site directed mutations. As compared to HAS2 wt, the phosphorylation mutant T110A is unable to reach Golgi and is therefore not seen in PM, produce HA, or exit into extracellular vesicles (EV). The ubiquitination mutant K190R, while slow in passing through Golgi, accumulates in PM, shows slightly longer half-life, and stimulates EV secretion like HAS2 wt. Still, it is enzymatically inactive, even in a dominant negative manner. The O-GlcNAcylation mutant S221A tends to pass slower out from Golgi, has increased half-life, resides in PM, produces HA and stimulates EV secretion. On the contrary, the phospho-mimic S221E mutant of the same site reaches Golgi but is rapidly degraded in lysosomes and does not produce HA. Increased and decreased traffic, activity, or abundance are indicated by the green and red arrows, respectively.

Suppl. Fig. 1. (A) Co-localization images of the different Dendra2 constructs are shown for both Golgi and ER markers. (B) Manders coefficients for co-localizations in the green Dendra2 channel (M2). (C) Difference between the co-localization values of the red channel for ER and Golgi markers (from Fig. 2B) and that of the green Dendra2 channel in panel (B) above, expressed as a natural number.

Suppl. Fig. 2. (A) Expression of the unfolded protein response genes ERN1 and (B) CTFR 24 h after transfection of the indicated constructs, and non-transfected cells (control). (C) Apoptosis assay 24 h after transfection of the indicated constructs and controls using Annexin V Conjugates for Flow Cytometry. 10 μl of Chlorpromazin was used as a positive control of apoptosis.
FIGURES

Figure 1

A

B

C

- HAS3
- wt
- K190R
- T110A
- S221A

HA coat intensity
(RED/GREEN fluorescence)

- NT
- HAS2
- Dendra
- Dendra - HAS2

HA (ng/10,000 cells)
Figure 2

A. Images showing the localization of Dendra2-HAS2 in control and mutated forms (HAS2-wt, HAS2-K190R, HAS2-T110A, HAS2-S221A) in different cellular compartments (ER, Golgi, ER/Golgi). The merge images show the combined localization of Dendra2 and HAS2.

B. Graph showing the quantification of the M. Manders coefficient (red channel) for Dendra2-HAS2 in different conditions. The graph includes error bars indicating the standard deviation.
Figure 4

(A) HAS2-wt

(B) HAS2-K190R

(C) HAS2-T110A

(D) HAS2-S221A

(E) Time (h) where t₁/₂ is achieved (X₀ + t₁/₂)

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Control</th>
<th>HAS2-K190R</th>
<th>HAS2-T110A</th>
<th>HAS2-S221A</th>
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<tr>
<td>Control</td>
<td>0.0 ± 1.8</td>
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<td>1.5 ± 0.9</td>
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<td>Glucosamine</td>
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Figure 5

(A) Dendra-HAS2 photo-switching

(B) % red/green Dendr2 in Golgi

(C) Constructs | Rate constant, K (min⁻¹ ± SE)
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<td>HAS2-wt</td>
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<td>HAS2-S221A</td>
<td>0.048 ± 0.024 ns</td>
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Figure 6

A

Relative quantity

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Control
scrambled siRNA
Rab10 siRNA

B

% HA in media (normalized to scrambled siRNA)

200
150
100
50
0

Empty vector
HAS3
wt
K190R
HAS2
S221A

ns
*
*
ns
*

Scrambled siRNA
Rab10 siRNA
Figure 7

A) Staining and protein banding patterns for different HAS variants.

B) Bar graph showing protein on plasma membrane (% of total protein) for various HAS variants.

C) TIRF images and count masks for different HAS variants.

D) Bar graph showing TIRF vesicles per cell for different HAS variants.
Figure 8

A

B

% HA in media (normalized to 0.04 µg HAS2-wt)

HAS2 mutants (µg) 0.04 0.04 0.04 0.04 -

HAS2-wt (µg)

0.04 0.04 0.04 0.04 -

K190R T110A S221A

** *** *** ***

% HA in media (normalized to 0.02 µg HAS3)

HAS2 mutants (µg) 0.04 0.04 0.04 0.04 -

HAS3 (µg) 0.02 0.02 0.02 0.02 -
Figure 9

A

![Graph showing distribution of particles in different size categories](image)

B

![Bar chart comparing EV size across different conditions](image)

C

![Graph comparing EV secretion across different conditions](image)

D

![Images showing untreated and 4MU treated samples](image)

Figure 10

A

![Graph showing phototransformation over time](image)

B

![Images comparing HAS2-S221D and HAS2-S221E](image)

C

<table>
<thead>
<tr>
<th>Condition</th>
<th>t(1/2) (min)</th>
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<tr>
<td>HAS2-wt</td>
<td>0.6 ± 1.8</td>
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<tr>
<td>HAS2-S221A</td>
<td>1.5 ± 0.9</td>
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<td>HAS2-S221D</td>
<td>0.3 ± 0.7</td>
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<tr>
<td>HAS2-S221E</td>
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D

![Graph showing HA levels](image)
Figure 11
Highlights

- A mutation preventing phosphorylation (T110A) blocks HAS2 exit from the endoplasmic reticulum, hence entry to the plasma membrane and hyaluronan synthesis.
- A neutral mutation in the O-GlcNAcylation site (S221A) inhibits HAS2 turnover, while mimicking phosphorylation in the same site (S221E) enhances its turnover and inhibits hyaluronan synthesis.
- Withholding ubiquitination (K190R) increases plasma membrane residence of HAS2, but nevertheless blocks hyaluronan synthesis, even in a dominant negative manner.
- Silencing of Rab10 enhances hyaluronan synthesis by HAS2, likely by suppressing its endocytosis from the plasma membrane, as previously shown for HAS3.
- Transfection of HAS2 K190R stimulates extracellular vesicle shedding to the same extent as HAS2 wild type, indicating that hyaluronan synthesis is not required for the HAS-induced vesicle shedding.