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The Synthesis and Secretion of Versican Isoform V3 by Mammalian Cells: A Role for N-linked Glycosylation

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Abstract

Versican is a large extracellular matrix (ECM) chondroitin sulfate (CS) proteoglycan found in most soft tissues, which is encoded by the *VCAN* gene. At least four major isoforms (V0, V1, V2, and V3) are generated via alternative splicing. The isoforms of versican are expressed and accumulate in various tissues during development and disease, where they contribute to ECM structure, cell growth and migration, and immune regulation, among their many functions. While several studies have identified the mRNA transcript for the V3 isoform in a number of tissues, little is known about the synthesis, secretion, and targeting of the V3 protein. In this study, we used lentiviral generation of doxycycline-inducible rat V3 with a C-terminal tag in stable NIH 3T3 cell lines and demonstrated that V3 is processed through the classical secretory pathway. We further show that N-linked glycosylation is required for efficient secretion and solubility of the protein. By site-directed mutagenesis, we identified amino acids 57 and 330 as the active N-linked glycosylation sites on V3 when expressed in this cell type. Furthermore, exon deletion constructs of V3 revealed that exons 11-13, which code for portions of the carboxy region of the protein (G3 domain), are essential for V3 processing and secretion. Once secreted, the V3 protein associates with hyaluronan along the cell surface and within the surrounding ECM. These results establish critical parameters for the processing, solubility, and targeting of the V3 isoform by mammalian cells and establishes a role for V3 in the organization of hyaluronan.

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Keywords

Versican; N-linked glycosylation; Extracellular matrix; Hyaluronan

Introduction

Versican is a large extracellular matrix (ECM) chondroitin sulfate (CS) proteoglycan (PG) which is found in most soft tissues [1] and is synthesized by a variety of different cell types (see reviews [2–5]). In humans, it is coded from a single gene locus on chromosome 5q14.3 [6]. Versican is essential in development, as it is necessary for guiding embryonic cell migration during formation of the heart [7–10] and for outlining the path for neural crest cell migration [11, 12]. Versican is also a key player in inflammation, interacting with adhesion molecules on the surfaces of inflammatory leukocytes [13–15] and with chemokines that are involved in recruiting inflammatory cells [15–18]. In normal tissues, versican content is low, but it increases in diseases of the vascular system and heart [5, 10, 19–21], lung [15, 22, 23], and in many forms of cancer [24, 25]. Inhibiting the accumulation of versican in the lungs of mice in a poly I:C-driven injury response significantly reduces the inflammation associated with this Toll-like receptor 3 (TLR3) agonist [26].

Versican is structured into multiple molecular domains (Fig. 1A). The amino-terminal globular domain (G1) contains two link modules and has the ability to bind hyaluronan (HA) [27]. The carboxy-terminal globular domain (G3) contains epidermal growth factor (EGF) repeats, a C-type lectin domain, and a complement regulatory protein (CRP)-like domain [28, 29]. This G3 domain is also highly interactive, binding cytokines, growth factors, and cell surface receptors as well as interacting with components of the ECM to regulate assembly and organization of the ECM. The middle region between the G1 and G3 domains of the versican core protein is encoded by two large exons that specify the CS attachment regions of versican [28, 29]. These CS chains interact with soluble factors, such as cytokines and growth factors, regulating cell growth and behavior [2]. The interactive nature of all three molecular domains of versican are the basis for the name given to this “versatile” molecule. Alternative splicing of the *VCAN* gene generates at least four major isoforms (V0, V1, V2, and V3), each of which is temporally and spatially regulated and distinguished by different arrangements of the CS chain attachment regions [30, 31]. The biological significance of alternative splicing for versican is yet to be determined.

The spatiotemporal expression patterns and accumulation of the larger isoforms (V0, V1, and V2) have been well characterized. V0 is transiently expressed at high levels during embryogenesis [32], whereas V1 is most abundant in adult tissues [33]. V1 is also the isoform most prominent in repair and remodeling associated with injury and disease [2], except for the nervous system where V2 is most abundant [4]. The V3 isoform, on the other hand, has been less well characterized due to its unique structure. V3 mRNA is expressed in a variety of tissues [4, 33], but detection of V3 protein in tissues or cells has been a challenge due to the lack of a functional antibody that specifically recognizes only V3 and not the other isoforms [34]. This challenge arises from the fact that V3 contains only the G1 and G3 molecular domains common to all other versican isoforms but lacks a

glycosaminoglycan (GAG) binding domain. Other methods such as proteomic analysis have detected peptides of V3 protein made by primary human skeletal muscle cells [35]. Also, some success has been achieved in localizing V3 in tissues using tagged primer constructs in forced expression systems, such as in the myocardium proximal to the outflow track in developing heart [36], as well as in chondrogenesis [37].

Other studies demonstrated that expression of V3 can have prominent effects on the proliferation and migration of different cell types such as chondrocytes [37], arterial smooth muscle cells (SMCs) [38, 39], fibroblasts [40, 41], melanoma cells [42–44], and fibrosarcoma cells [45]. One particularly impressive consequence of expressing V3 in a variety of cells is the remodeling of the ECM, including promoting the synthesis of tropoelastin and the assembly of elastic fibers [40, 46–49]. Additionally, several point mutations in a splice acceptor site of the *VCAN* gene have been identified as the cause of erosive vitreoretinopathy and Wagner disease [50]. The most often detected exon 7 point mutation 1Z8861540309529236c.4004-5T→C, results in an imbalance of versican splice variants in the eye, with a strong expression of V2 and V3 and an insufficiency in expression of V0 and V1, indicating possible involvement of V3 in these diseases [51].

While there is abundant information on the expression and accumulation of the CS-containing isoforms of versican, little is known about the V3 isoform. Therefore, in this study, we aimed to explore the synthesis, secretion, and processing of V3 in a mammalian *in vitro* system and to determine if V3 plays a role in the organization of HA at the surface of the cell and surrounding ECM. Using lentiviral generation of doxycycline (Dox)-inducible V3 expression in stably transduced NIH 3T3 cell lines, we demonstrated that V3 is secreted through the classical secretory pathway and uses N-linked glycosylation to establish efficient secretion and maintain solubility. We determined the active sites of N-linked glycosylation and established that exons 11-13 are critical for V3 secretion. Once secreted, V3 both associates with, and organizes, the HA present at the surface of the cell, as well as within the surrounding ECM. These results establish critical parameters for the processing, solubility, and targeting of this versican isoform and should enhance our ability to distinguish the specific roles of this enigmatic form of versican.

Results

V3 is secreted through the classical secretory pathway

Due in part to its low level of expression under normal conditions and a lack of effective specific immunohistochemistry (IHC) tools, native V3 has yet to be specifically located in cells and tissues. However, viral expression systems using C-terminally tagged V3 result in the protein being localized to the extracellular space [36, 37], supporting the speculation that V3 is secreted in the same manner as the other versican isoforms. Therefore, to more specifically determine the synthesis and secretion mechanisms, as well as the cellular localization of V3, we employed a lentiviral approach to generate a stable cell line which overexpresses rat V3. Initial attempts to create stable cell lines constitutively expressing V3 driven by viral (cytomegalovirus [CMV], spleen focus forming virus [SFFV]) or mammalian (EF-1 alpha) promoters using lentiviral transduction were unsuccessful. After strong initial expression, the polyclonal lines showed a rapid loss of V3 expression, even under persistent

and stringent selection pressure, resulting in almost undetectable protein levels after two to three weeks (data not shown). This made achieving consistent levels of V3 expression between experiments impossible. Therefore, to achieve greater control over V3 expression levels, a tetracycline-inducible expression system with constitutive co-expression of the selection marker hygromycin (pSLIK-Hygro) was used [52]. NIH 3T3 fibroblasts were transduced with pSLIK-rV3-Hygro (Fig. 1B) or pGL11-rV3-GFPzeo (Fig. 1C) and were induced to express c-terminal hemagglutinin-tagged (Hag-tag) V3 with doxycycline (Dox). Consistent levels of V3 mRNA were apparent up to 8 days after each induction with Dox (Fig. 1D).

In order to determine whether V3 is secreted through the classical secretory pathway, V3-expressing cells were treated with Brefeldin A (BrefA). BrefA inhibits protein transport from the endoplasmic reticulum (ER) to the Golgi indirectly by preventing the formation of COPII-mediated transport vesicles, thereby preventing the movement of secretory proteins out of the ER. Secretion of V3 into the culture medium in response to Dox treatment was detected via western blotting using an antibody directed against the c-terminal Hag-tag. As expected, when the cells were treated with Dox alone, V3 protein could be detected (Fig. 2A), however treatment of Dox-induced cells with BrefA inhibited the secretion of V3 into the culture medium. In the absence of Dox treatment, no secreted V3 protein could be detected.

The presence of V3 protein in the ECM was confirmed by IHC using the same antibody against the c-terminal Hag-tag (Fig. 2B–F). NIH 3T3 cells were grown on glass coverslips and treated with and without Dox and BrefA, as described above. The cells were then probed for Hag-tagged V3 (orange) and F-actin (green), and the nuclei were counterstained (blue). The untreated cells showed no positive staining for V3 (Fig. 2B), whereas the Dox-induced cells showed a strong extracellular staining of secreted V3 (Fig. 2C, E). This prominent extracellular V3 staining could not be observed in the BrefA-treated cells, where positive V3 staining could only be detected in intracellular vesicles, confirming retention in the ER/Golgi (Fig. 2D, F). These results support the conclusion that V3, similar to other versican splice variants, is secreted via the classical secretory pathway into the extracellular space, where it remains soluble such that it can be detected in conditioned culture media.

G3 domain contains critical elements for V3 expression and secretion

To determine which exons are critical for generating soluble, secreted V3 in stable NIH 3T3 cell lines, we tested different exon-deletion mutants expressed using the same Dox-inducible lentiviral system as described for the native, full-length rat V3, including the addition of the c-terminal Hag-tag for detection. The mutants generated by site-directed mutagenesis of native V3 contained deletions of exon 3 (rE3), exons 4-6 (rE456), and exons 11-13 (rE1113) (Fig. 3A) and expressed similar levels of mRNA under Dox-induced conditions (not shown). As anticipated, full length V3 protein was expressed and was present in both the cell layer and the conditioned media, as were mutants rE3 and rE456 (Fig. 3B, C, respectively). Only rE1113 failed to express any V3 protein, indicating that these exons, as part of the G3 domain of the molecule, contain critical elements for successful V3 protein expression and

secretion. More in depth analysis of the G3 domain will determine the specific sequences critical for effective navigation through the secretory pathway.

V3 is N-glycosylated

Since the lack of GAG side chains on V3 does not affect either its secretion or solubility, we next determined the glycosylation status of V3. Attempts to express V3 in a bacterial system in which proteins are not glycosylated resulted in the production of a completely insoluble product located exclusively in the inclusion bodies (Suppl. Fig. 1). The primary amino acid sequence of V3 predicts that it is likely N-glycosylated, however this is the first attempt to experimentally determine its specific glycosylation pattern.

In order to test if V3 is N-glycosylated, tunicamycin (Tun) was used to block N-glycosylation during V3 synthesis. Tun inhibits, among others, the enzyme GlcNAc phosphotransferase, which catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate in the first step of glycoprotein synthesis. In this experiment, V3 expression was induced in V3-transduced NIH 3T3 fibroblasts by treatment with Dox, with or without addition of Tun. Cellular V3 protein was detected via western blotting with an antibody directed against the c-terminal Hag-tag. We found that cells treated with Dox and Tun express V3 at a lower molecular weight than that which is made by cells treated with Dox alone (Fig. 4A), indicating that N-glycosylation was successfully blocked. A portion of the V3 produced was also found in higher molecular weight bands which we have interpreted to be multimers of V3 (Fig. 4A, lanes 2 and 3) and are also reduced in size following Tun treatment. These results support an involvement of N-linked glycosylation when V3 is expressed in a mammalian system, as predicted.

We further investigated whether V3 is O-glycosylated. Cellular protein harvested from V3-expressing NIH 3T3 fibroblasts was subjected to enzymatic deglycosylation with PNGase F, sialidase, and O-glycanase for 24 h (Fig. 4B). Western blotting and antibody detection against the c-terminal Hag-tag revealed that enzymatic removal of all N-glycosylation moieties with PNGase F reduces the V3 band by approximately 6 kDa compared to non-digested cell lysate from Dox-induced cells. No further shift in the V3 band size was detected after digestion with sialidase and O-glycanase in combination with PNGase F, indicating that V3 is exclusively N-glycosylated in this expression system.

N-glycosylation enhances V3 secretion

Transduced NIH 3T3 cells were grown on glass coverslips treated with Dox to induce V3 expression and Tun to block N-glycosylation. Using an immunocytochemical approach, the cells were probed for Hag-tagged V3 (red), F-actin (green), and nuclei (blue) (Fig. 5A–C). Cells receiving Dox showed strong positive extracellular V3 staining (Fig. 5B; orange). When the cells were treated with both Dox and Tun (Fig. 5C), the extracellular staining pattern for V3 was minimally altered, suggesting that V3 was reaching the outside of the cell. However, cells transduced with a lentivirus engineered to maximize secreted recombinant protein (Fig. 1C) still showed a slight 12% reduction in secreted V3 in the presence of Dox and Tun, compared to Dox alone, as measured by digital pixel densitometry of the western blot (Fig. 5D). These data indicate that N-glycosylation is not essential for

extracellular targeting of V3, but does have an impact on the quantity of protein secreted from the cell, which could suggest an impact on protein stability and/or solubility.

Native V3 is N-glycosylated at amino acids 57 and 330 in the G1 domain

As the Tun treatment of the cells revealed that V3 is N-glycosylated, we wished to determine the exact location of the glycosylation sites. Sequence analysis of full-length rat V3 revealed that it contains a total of twelve possible N-glycosylation sites. Three of these sites, at amino acid (AA) positions 57, 330, and 613 relative to the initial methionine, were predicted to be highly likely N-glycosylation sites by NetNGlyc, a web-based N-glycosylation prediction algorithm (Suppl. Fig. 2). Of these predicted attachment sites, only AA position 57 was expected to be glycosylated by two other prediction algorithms (GlycoEP and NGlycPred).

To determine if AA position 57 is the only N-glycosylated attachment point, rat V3 was mutated at position 57 from an asparagine to a glutamine (N57Q) and expressed in Dox-inducible NIH 3T3 fibroblasts. As a comparison, we also mutated AA 330 in the same fashion (N330Q). When cell lysates from Dox-treated cells were analyzed by western blotting, mutant N57Q V3 ran at a lower apparent molecular weight compared to native V3, indicating that this is indeed an active N-glycosylation site (Fig. 6). Further treatment of the N57Q mutant with Tun resulted in a V3 band of even lower molecular weight, suggesting that V3 is glycosylated at more than just AA 57. Similar results were found with the N330Q V3 mutant, indicating that V3 is N-glycosylated at both sites.

Total inhibition of glycosylation with Tun treatment resulted in a modest reduction in the production (1.8–5.3-fold; Figs. 4A and 6) and secretion (0.12–1.9-fold; Figs. 5D and 7A) of native V3 as measured by western blot densitometric analysis. However, in the absence of Tun treatment, even partial loss of N-glycosylation was able to reduce cellular production as well as secretion of V3. Cell lysates from N57Q and N330Q mutants displayed a reduction in overall V3 production (2.2- and 11.8-fold, respectively; Fig. 6, lanes 5 and 8), suggesting that N-glycosylation is important for protein stability during processing. Furthermore, western blotting demonstrated a marked reduction in soluble V3 in the conditioned media of the N57Q and N330Q mutant V3 cultures (10.2- and 37.6-fold, respectively; Fig. 7A, lanes 5 and 8). Dox-induced cells, grown on coverslips and probed for Hag-tagged V3 (red) clearly demonstrate this marked reduction in V3 production and secretion by the glycosylation mutants compared to native V3 (Fig. 7B), which manifested as reduced accumulation of V3 in the extracellular space. Further treatment with Tun for 24 h nearly abolished the presence of pericellular V3 in the glycosylation mutant cultures (Fig. 7A, lanes 6 and 9; and Fig. 7B, right panels). Taken together, these results demonstrate that V3 is N-glycosylated at multiple sites in its G1 domain and that proper glycosylation at these sites is necessary for maximal secretion of the protein into the ECM.

Expression of V3 increases the amount of exogenous HA bound at the cell surface

V3 has the same G1 domain common to all versican splice variants, containing link domains that allow versican to bind HA, which is an important process for ECM organization during development, inflammation, and disease progression [2, 15, 16, 22]. However, the specific ability of V3 to bind HA has not been previously demonstrated and it is unknown if the close

proximity of the G3 domain to the G1 domain in V3 has an impact on the ability of the G1 domain to actively bind HA.

In order to determine the binding capacity of V3 for HA, NIH 3T3 cells transduced with native V3 were grown on glass coverslips for 24 h in the presence or absence of Dox and incubated with high molecular weight HA (> 10^6 kDa) labeled with fluorescein for an additional 24 h. In the absence of Dox, no positive staining for V3 (red) was detected, as expected, and the exogenous HA (green) was not retained in the pericellular ECM (Fig. 8A, C). In contrast, when the cells were treated with Dox to induce V3 expression, the pericellular ECM showed a bright, positive co-staining for V3 and HA confirming that the link domains in the G1 region of V3 retain their ability to bind and organize HA near the cell surface. Cells expressing N57Q glycosylation mutant V3, which secreted ~10-fold less V3, did not retain any more HA at the cell surface than the – Dox controls, which expressed no V3 (Fig. 8B, D), indicating that the quantity of secreted V3 had a direct impact on the ability of the cells to organize the exogenous HA in their pericellular ECM. Whether the lack of glycosylation also impacted the ability of V3 to bind HA could not be determined using this approach.

Discussion

In this study, we used lentiviral generation of Dox-inducible V3 with C-terminal Hag-tag in NIH 3T3 stable cell lines to demonstrate that V3 travels through the classical secretory pathway to the extracellular space and uses N-linked glycosylation to establish efficient secretion and stability. By site-directed mutagenesis, we verified predictions that AA 57 and 330 are active N-linked glycosylation sites in V3 and that loss of glycosylation at either site markedly reduced V3 production and secretion. Furthermore, exon deletion constructs revealed that exons 11-13, which encode the lectin binding region of the G3 domain, were essential for V3 processing and secretion. Once outside the cell, V3 associates with HA in the pericellular matrix. Hyaluronan is a critical component influencing cell phenotype in development and disease [53, 54]. These results establish critical parameters for proper processing and targeting of the versican V3 isoform in mammalian cells.

Although V3 contains no GAG chains, and is N-glycosylated, it should technically be considered a glycoprotein. However, as it is an isoform of versican, it is commonly grouped with PGs and is characterized as such [34]. RNA splicing of versican occurs in the two large exons, 7 and 8, which encode the GAG attachment regions, giving rise to the distinct isoforms of versican [31]. These variants differ in the length of their core proteins and number of attached GAGs [55]. Regulation of versican core protein and CS GAG synthesis can be transcriptional [56] or post-transcriptional [56–58] and may involve similar or different signaling pathways regulating core protein and CS synthesis independently [57]. The majority of studies have focused on the synthesis and secretion of the CS-carrying isoforms of versican, V0, V1, and V2. The fact that V3 contains no CS chains indicates that this isoform may be regulated and function very differently than the other isoforms.

We and others have shown that V3 expression by a variety of cell types induces significant changes in cell phenotypes. For example, V3 expression significantly decreased proliferation

of melanoma cells and cardiomyocytes [36, 42–44] and increased cell-cell association [36]. In melanoma cells, this reduction in cell proliferation was partly due to the inhibition of EGF-dependent signaling pathways [42, 43]. In Splotch mice, which have mutations in the Pax3 gene, defective neural crest migration was associated with altered expression of V3 [11, 59]. In chondrocytes, V3 expression reduced the expression of aggrecan, the principal PG present in cartilage [37]. Expression of V3 in tumor cells in a fibrosarcoma tumor model inhibited tumor cell growth [45]. Similarly, we have shown that controlled expression of V3 alters phenotypes of fibroblasts and arterial SMCs such that cell growth and migration are reduced while cell adhesion is enhanced [39, 40, 48].

Moreover, V3 expression in arterial SMCs induced a significant change in ECM composition, including a comcomitant reduction in the production of the larger V0/V1 isoforms of versican and HA, as well as an increase in elastic fiber deposition [39, 40, 46, 48]. One potential consequence of this altered ECM is the finding that V3-expressing arterial SMCs exhibit a reduced capacity to bind monocytes both *in vitro* and *in vivo* [47], by altering TGFβ-, EGF-, and NFκB-dependent signaling pathways [46]. Despite these mechanistic and functional experiments, definitive data showing V3 protein secretion and localization in these experimental systems has been lacking.

V3, like other members of the lectican family of PGs, which includes versican, aggrecan, brevican, and neurocan, is characterized by having N-terminal (G1) and C-terminal (G3) globular domains, with variable GAG attachment domains in between [60]. The V3 molecule consists of the N- and C-terminal globular domains of versican spliced together with the variable region of protein carrying the CS chains spliced out [34]. The globular domains of the lecticans each have distinctive motifs with their own folded structures [61, 62]. The G1 domain contains two PG binding tandem repeats and one immunoglobulin motif [63]. The folded domain in the tandem repeat of G1 defines the HA binding site [64]. This site engages HA after the PG is secreted. The G3 domain bears homologies to EGF, C-type lectin and sushi or CRP [65, 66] with the number of EGF repeats variable among the lecticans. A number of early studies showed that each of the globular domains can have different activities [61, 62, 67–70]. For example, the importance of the globular domains of the lecticans was recognized in nanomelia, a lethal autosomal recessive mutation in chickens caused by a premature stop codon in aggrecan. The truncated aggrecan protein accumulates within the cell due to the absense of the G3 domain [68, 70]. Other studies have focused on the importance of the globular domains in the secretion of aggrecan and versican. *In vitro,* the G1 domain tended to inhibit secretion, while G3 tended to promote secretion [61, 62, 67, 69, 71]. Our findings in the current study highlight exons 11–13 of V3, which code for a portion of the C-type lectin domain, as critical for V3 secretion and processing. This finding supports the importance of this portion of the G3 region in facilitating versican secretion similar to what has been reported for aggrecan [71]. Likewise, the loss of exon 3 in the G1 region enhanced V3 secretion, verifying that this region of G1 inhibits secretion. The mechanisms by which these regions modulate V3 secretion are still being elucidated.

Exon 3 encodes the Ig-like domain (Fig. 1). Hatano *et al.* postulated that this region was involved in versican aggregation and essential for proper heart development [72]. However, we propose that its role in V3 may be different, as the cell layer of the rE3 mutant clearly

demonstrates the presence of higher molecular weight bands (Fig. 3B) similar to those seen with cellular and secreted native V3 (Figs. 4A and 7A) which are of the correct molecular weight to be multimers of V3. The apparent increase in production and secretion of the rE3 mutant can be due to a multitude of factors including increased rate of protein production, enhanced protein stability and/or reduced protein breakdown. Further analysis will be needed to fully understand the function of exon 3 in regulating V3 production and secretion.

When expressed independently, the two globular domains of versican appear to have different effects on cellular phenotypes such as influencing cell proliferation, migration, and cell survival [2, 71, 73–76]. Interestingly, when expressed separately in cultured cells, the G1 and G3 domains each promote proliferation [76, 77]. In contrast, expressing V3, which contains the G1 and G3 domains linked together, reduces proliferation [39]. Such opposing activities between individually expressed domains and intact V3 may also have an impact on inflammation. For example, through its ability to self-associate into multimers via intermolecular disulfide bonds, G3 expression enhances the production of an ECM which promotes leukocyte interaction and aggregation [74]. Contrastingly, we have shown that expression of intact V3 organizes an ECM which resists leukocyte binding and dampens inflammatory signalling [46, 47, 49].

In other ways, the individual globular domains have activity similar to intact V3 while opposing the activity of full-length, CS-containing forms of versican. Recently, we found that adding recombinant G1 to dermal fibroblast cultures promoted the formation of cable-like structures of HA, whereas V0/V1 had no effect on HA organization [78]. Our data presented herein likewise show that expression of V3 enhances HA organization at the cell surface. Furthermore, we have previously shown that V3 expression reduces the expression and accumulation of the CS-containing isoforms of versican, V0/V1 [46], which may be a partial explanation for the opposing effects that V3 and V0/V1 have on cell phenotypes. Also, the tendency of V3 to self aggregate points to the possibilty that V3 directly interacts with other versican isoforms, and perhaps other lecticans, thus altering the organization of the ECM and changing its interaction with the cell.

The importance of N-linked glycosylation of glycoproteins is well established [79–83]. However, little is known about the importance of N-linked oligosaccharides in the processing and trafficking of PGs. Our earlier studies revealed that the protein core of the CS-carrying isoforms versican, V0 and V1, also contained N-linked and O-linked oligosaccarides in addition to the CS GAG chains [84], but it was not clear at the time if the V3 isoform contained N- and O-linked sugars. It was also not clear what role these N- and O-linked oligosaccharides played in the processing and trafficking of V0/V1. The results from the current study clearly establish that V3 does contain N-linked sugars but not O-linked sugars and that the N-linked sugars have a role in establishing proper trafficking and stability of this isoform. Previously, Yang *et al.* [85] suggested that a truncated portion of G1 containing the HA binding domain expressed alone in COS-7 cells might be glycosylated in some form based on western blot data, without confirming such. Interestingly, Seyfried *et al.* [86] showed that N-linked glycosylation of G1 was not necessary for binding to HA or for the formation of ternary complexes; however we find that it does appear to be neccessary for efficient secretion. It remains to be determined if this is a general property of all versican

isoforms, or whether it reflects differences in the structure of the isoforms which relate to differences in function.

While it is well known that the CS-bearing isoforms are degraded by a variety of proteases, V3 lacks consensus sequences for the major proteases that degrade versican, the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) proteases [87]. The major versican cleavage product generated by ADAMTS1 has been termed versikine [88], which has proinflammatory activity via its ability to stimulate production of IL-1β, IL-6 and CCL2 [89, 90]. Whether V3 is non-cleavable awaits further study, however should this be the case, there are interesting possibilities for V3 to be an effective therapeutic reagent. In fact, as the CS-containing variants of versican are emerging as potential targets for therapeutic intervention across a variety of conditions, V3 acts to counter pathological changes induced by the parent GAG-containing versican isoforms, V0, V1 and V2, highlighting the potential therapeutic importance of V3 in the treatment of disease [16, 22, 49, 91].

Experimental Procedures

Plasmid construction

The open reading frame of rat V3 (NM_001170559.1) was PCR-amplified with primers rV3SpeIF and rV3XbaIR (Table 1) introducing SpeI and XbaI restriction sites and a c-terminal Hag-tag and cloned into pEN_TTGmiRc2 (Addgene #25753) cut with SpeI and XbaI to remove the green fluorescent protein and CDDB. The resulting expression cassette consisting of the TRE-tight promoter and rV3 flanked by attL sites was cloned into pSLIK-Hygro (a gift from Iain Fraser; Addgene #25737) by gateway cloning to produce pSLIK-rV3-Hygro (Fig. 1B). This construct was further modified to generate the pGL11-rV3-GFPzeo configuration (Fig. 1C) by inserting a Gateway destination cassette amplified from pSLIK-Hygro into the pCVL-A backbone (a generous gift from David J. Rawlings of Seattle Children's Research Institute) upstream of the SFFV promoter [52, 92]. The third generation doxycycline reverse transcriptional transactivator (rtTA3) from pSLIK-Hygro was linked to a GFP Sh ble fusion protein (conferring Zeocin resistance; GFPzeo) using the P2A peptide and cloned downstream of the SFFV promoter using XhoI and XbaI restriction sites [93]. To confer additional resistance to silencing of the tetracycline-inducible CMV promoter, a minimal matrix attachment region (MAR) derived from the Ig kappa locus and the murine anti-repressor element 40 (ARE) residing upstream of the IL17R gene were fused and inserted upstream of the attR site by In-Fusion® cloning (Clontech) [94, 95]. All plasmid and insert sequences were verified by sequencing. Plasmids and plasmid sequences are available from the authors upon request.

All exon deletion and asparagine-to-glutamine mutants of rat V3 were generated by PCR using the Agilent QuikChange Mutagenesis Kit. Because the N- and C-terminal sequences are retained in all deletion construct primers, rV3SpeIF and rV3XbaIR (Table 1) were used to amplify and subclone the deletions shown in Figure 3 with the same strategy that was used for the full length V3.

Viral production and transduction

HEK 297 LTV cells (Cell BioLabs) were plated 48 h prior to transfection in Dulbecco's modified Eagle medium high glucose supplemented with non-essential amino acids, GlutaMAX, pen-strep antibiotics (Life Technologies) and 10% v/v fetal bovine serum (FBS; Atlanta Biologicals) (DMEM 10%). Cells were transfected with 25 kDa PEI-based transfection reagent (made in-house) and a mixture of the transfer vector, psPAX2 and pMD2G at a ratio of 3:2:1. Transfected cells were cultured in DMEM 10% supplemented with 8 mM butyrate overnight (~16 h), and then cultured further in DMEM 10% with 1 mM butyrate for an additional 24 h. The virus-containing conditioned media was harvested and cells and debris were removed by centrifugation. Virus was concentrated by centrifugation at 13,000 x g through a 10 % sucrose cushion for 4 h and transferred to target cultures of 50% confluent NIH 3T3 cells in DMEM 10% supplemented with 1 mg/ml Synperonic F-108 and 5 μg/ml DEAE-dextran for 6–12 h. Successfully transduced cells were then selected with 500 μg/ml hygromycin for 7 days.

Cell culture

NIH 3T3 cells (ATCC) transduced with pSLIK-rV3-Hygro were grown in DMEM 10% and induced to express Hag-tagged rV3 by treatment with 500 ng/ml Dox (Sigma-Aldrich) for 48 h. In some experiments, movement of secreted proteins through the secretory pathway was inhibited with the addition of 50 μM BrefA (Sigma-Aldrich) to the culture medium together with Dox for 24–48 h. In an independent set of experiments, N-glycosylation was blocked by the addition of 10 μM Tun (Sigma-Aldrich). Fluoresceinated HA was added to some cultures at a concentration of 1 μg/ml.

Western blotting

Cells were lysed in Laemmli buffer containing 100 mM DTT and equal protein amounts for each condition were separated on 10% SDS-PAGE gels. Volumes of conditioned media normalized to equal cellular protein were ethanol precipitated, dissolved in Laemmli buffer containing 100 mM DTT and separated on 10% SDS-PAGE gels. After semi-dry transfer of protein gels to nitrocellulose, membranes were blocked with 1% fish serum [96] in Tris-buffered saline containing 0.02% Tween 20 (TBST) and incubated with polyclonal rabbit anti-Hag-tag antibody (1:3000; Sigma-Aldrich) and, in the case of cell layer protein, mouse anti-β-actin (1:4000; Abcam) at 4 °C overnight. Membranes were washed three times in TBST and incubated with fluorescently-labeled secondary antibodies (1:20,000; Li-Cor Biosystems) for 1 h. Membranes were washed again and scanned in a Li-Cor Odyssey CLx. Desitometric analysis of Hag-tag-positive V3 bands was carried out using the Image Studio software provided by Li-Cor. Cellular protein loading was normalized to β-actin.

Enzymatic digestion of glycans

Cultures of V3-expressing cells were lysed with RIPA buffer, and 100 μg of total protein were subjected to deglycosylation with PNGase F, sialidase and O-glycanase (ProZyme) under denaturing conditions for 24 h according to the manufacturer's instructions. After enzymatic digestion, samples were analyzed for V3 via western blotting using the anti-Hag-tag antibody.

Gene expression analysis

Cultured cell monolayers were lysed in 0.5 ml Trizol followed by the addition of 0.1 ml chloroform and vigorous mixing. The solution was incubated at room temperature for 5 min and spun at 14,000 RPM for 10 min at 4 °C. The aqueous phase was collected, mixed with equal volume of 70% ethanol, and RNA was purified using EconoSpinTM columns (Epoch Life Science). cDNA was prepared from the isolated RNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to manufacturer's instructions. Real-time PCR was carried out with SYBR Select Master Mix (Life Technologies), as directed by the manufacturer, on an Applied Biosystems 7900HT Fast Real-Time PCR System. For each sample, assays were run as technical duplicates. cDNA levels were then expressed as estimated copy numbers of mRNA using the master-template approach [97]. SYBR® primer information is listed in Table 2.

Immunocytochemistry

Cells were grown on glass coverslips and fixed with neutral buffered formalin (NBF) for 2 h. Cells were washed with TBST and stained with a polyclonal rabbit anti-Hag-tag antibody (1:1000; Sigma-Aldrich). Alexa fluor-labeled secondary antibodies were obtained from Life Technologies. Affinity histochemistry of HA was performed with biotinylated HA binding protein (made in-house according to the method of Green SJ et al. [98]; and used at a concentration of 3 μg/ml), and detected with Alexa fluor-labeled streptavidin (1 μg; Life Technologies). For standard fluorescence microscopy, nuclei were labeled with DAPI (ThermoFisher), for images taken with a confocal microscope (Leica TCS SP5), TO-PRO (Life Technologies) was used instead of DAPI.

Web-based N-glysosylation site prediction

Rat V3 protein sequence (NP_001164030.1) was analyzed by NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/), GlycoEP (www.imtech.res.in/raghava/glycoep/), and NGlycPred (https://exon.niaid.nih.gov/nglycpred/) to determine the sites most likely to be N-glycosylated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	amino acid
BrefA	Brefeldin A
CMV	cytomegalovirus
CRP	complement regulatory protein
CS	chondroitin sulfate
DMEM	Dulbecco's modified Eagle medium
Dox	doxycycline
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
FBS	fetal bovine serum
G1	amino-terminal globular domain of versican
G3	carboxy-terminal globular domain of versican
GAG	glycosaminoglycan
HA	hyaluronan
Hag-tag	hemagglutinin-tagged
IHC	immunohistochemistry
NBF	neutral buffered formalin
PG	proteoglycan
SFFV	spleen focus forming virus
SMC	smooth muscle cell
TBST	Tris-buffered saline with Tween
TBS	Tris-buffered saline
Tun	tunicamycin

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Highlights

- V3 versican is secreted by mammalian cells and associates with the cell surface and throughout the extracellular matrix.
- N-linked glycosylation is required for efficient secretion and solubility of V3 protein.
- Native V3 is N-glycosylated at amino acids 57 and 330 in the G1 domain.
- Expression of V3 increases the amount of exogenous hyaluronan bound at the cell surface.

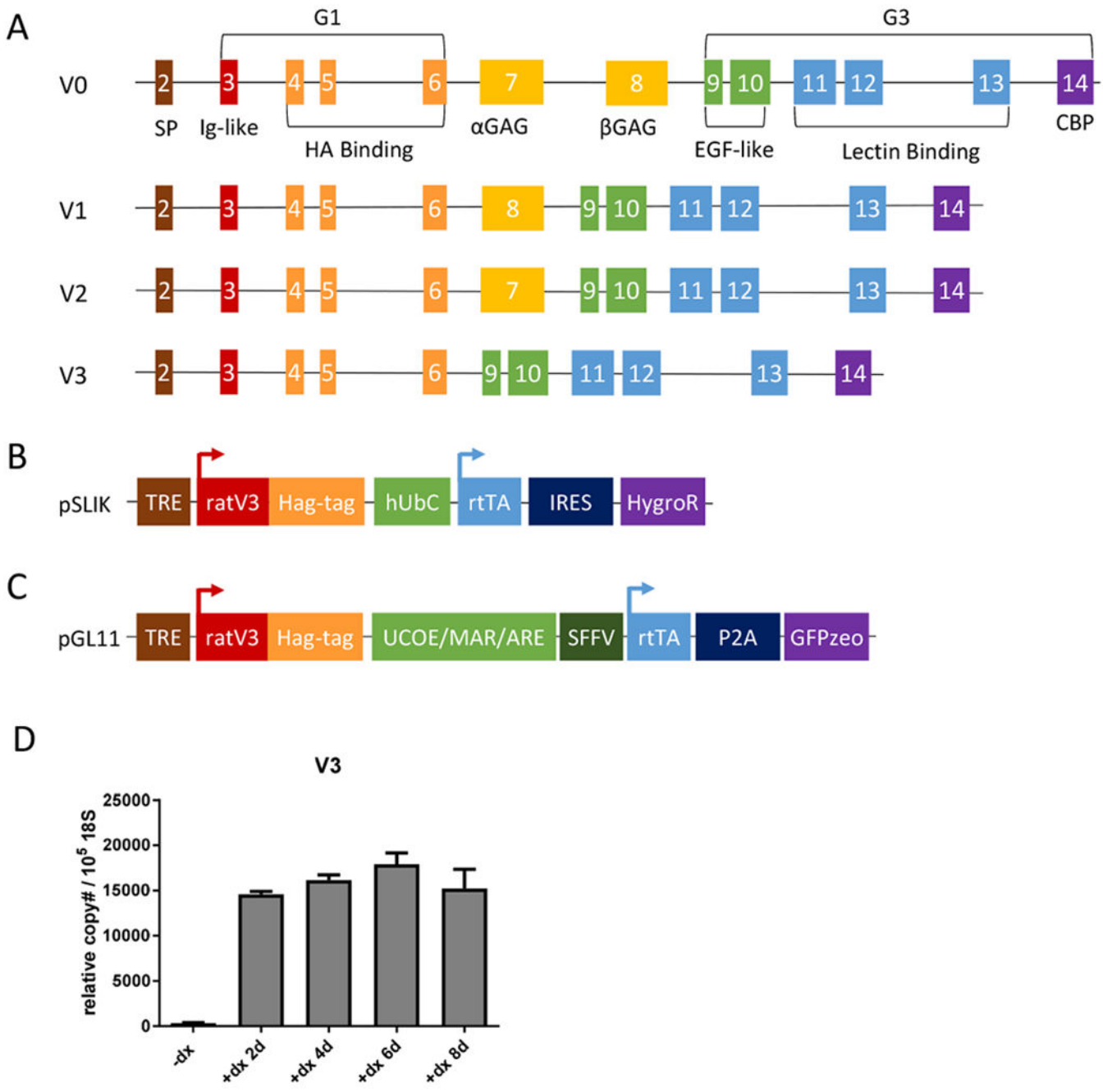

Figure 1. Structure of the lentiviral transfer plasmid used to generate stable cell lines with doxycycline-inducible V3 expression.

A) Domain structure of versican isoforms. **B)** pSLIK-rV3-HygroR lentiviral construct confers tetra/doxycycline-inducible expression of Hag-tagged rat V3 and constitutive hygromycin resistance (HygroR) under control of the human ubiquitin C (hUbC) promoter. Used in Figures 1C, 2, 3 and 8A. **C)** pGL11, a modified pSLIK construct substituting a spleen focus forming virus (SFFV) promoter for the UbC, a GFP Sh ble fusion protein conferring Zeocin resistance (GFPzeo) for HygroR and a P2A self-cleaving peptide for an internal ribosomal entry site (IRES). Stable production of Hag-tagged rat V3 is enhanced by

placement of ubiquitous chromatin opening element (UCOE), matrix attachment region (MAR) and anti-repressor (ARE) elements upstream of the promoter. Used in Figures 4, 5, 6, 7, and 8B. **D)** Expression of rat V3 RNA transcripts in NIH 3T3 cells transfected with pSLIK measured after 2, 4, 6, and 8 days post-treatment with 500 ng/ml doxycycline (Dox), demonstrating consistent expression of V3 over time using this lentiviral approach.

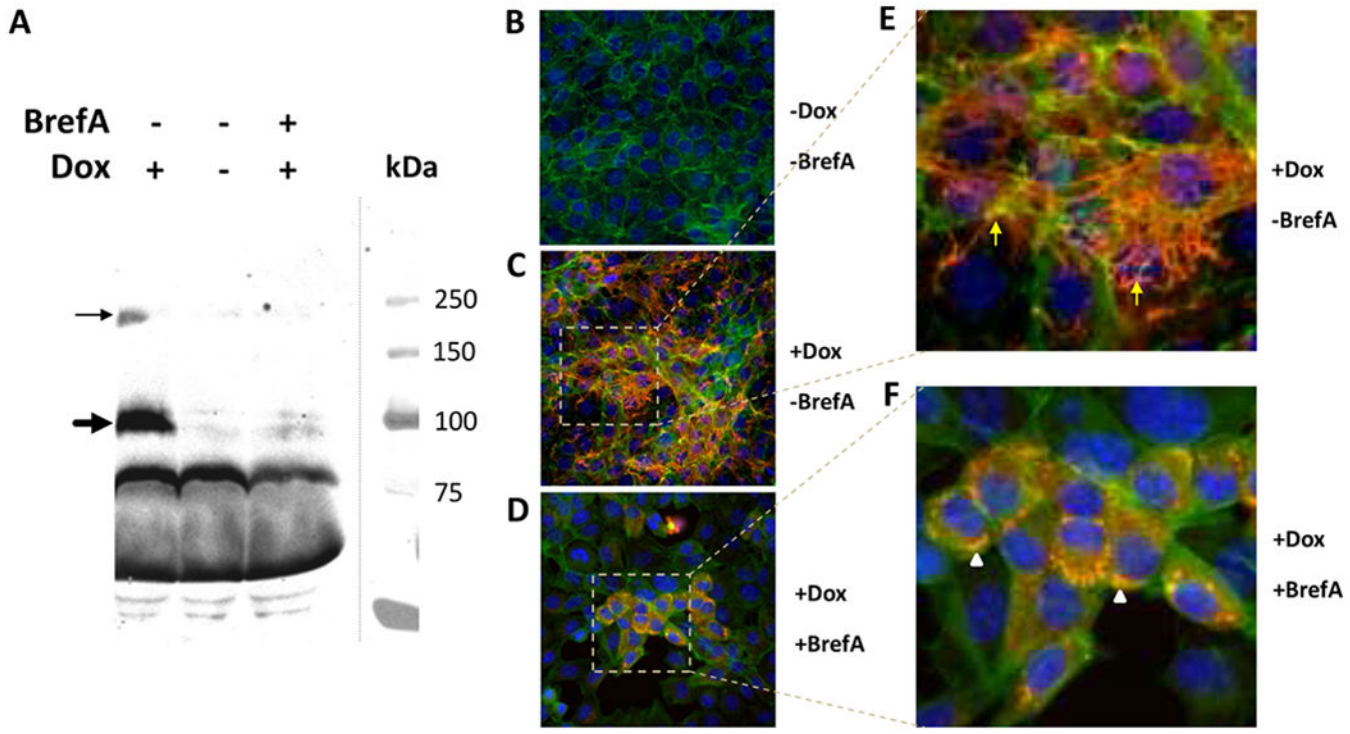

Figure 2. V3 is secreted into the culture medium through the classical secretion pathway.

A) V3 expression in transduced NIH 3T3 fibroblasts was induced with 500 ng/ml Dox for 48 h ± 50 μM Brefeldin A (BrefA) added to the medium. Conditioned medium was collected and proteins were separated on a 10% SDS-PAGE gel under denaturing conditions. After transfer to a nitrocellulose membrane, V3 secreted into the culture medium was detected with an antibody directed against the c-terminal Hag-tag. The large bands below V3 are non-specific antibody binding to serum albumin in the culture medium. Heavy arrow indicates soluble monomeric V3, whereas thin arrows indicate high molecular weight, likely multimeric, V3. Dashed line indicates where lanes were digitally rearranged from original image for clarity of presentation. **B-D)** The same NIH 3T3 cells used in **A** were seeded on coverslips, fixed with 10% NBF and stained for V3 (anti-Hag-tag; orange), F-actin (phalloidin; green) and nuclei (DAPI; blue). **E** and **F**) Higher magnification of the images shown in **C** and **D**, revealing the extracellular localization of V3 in the Dox only control cells (**E**, yellow arrows) and the intracellular and vesicular localization in the cells treated with Dox and BrefA (**F**, white arrowheads).

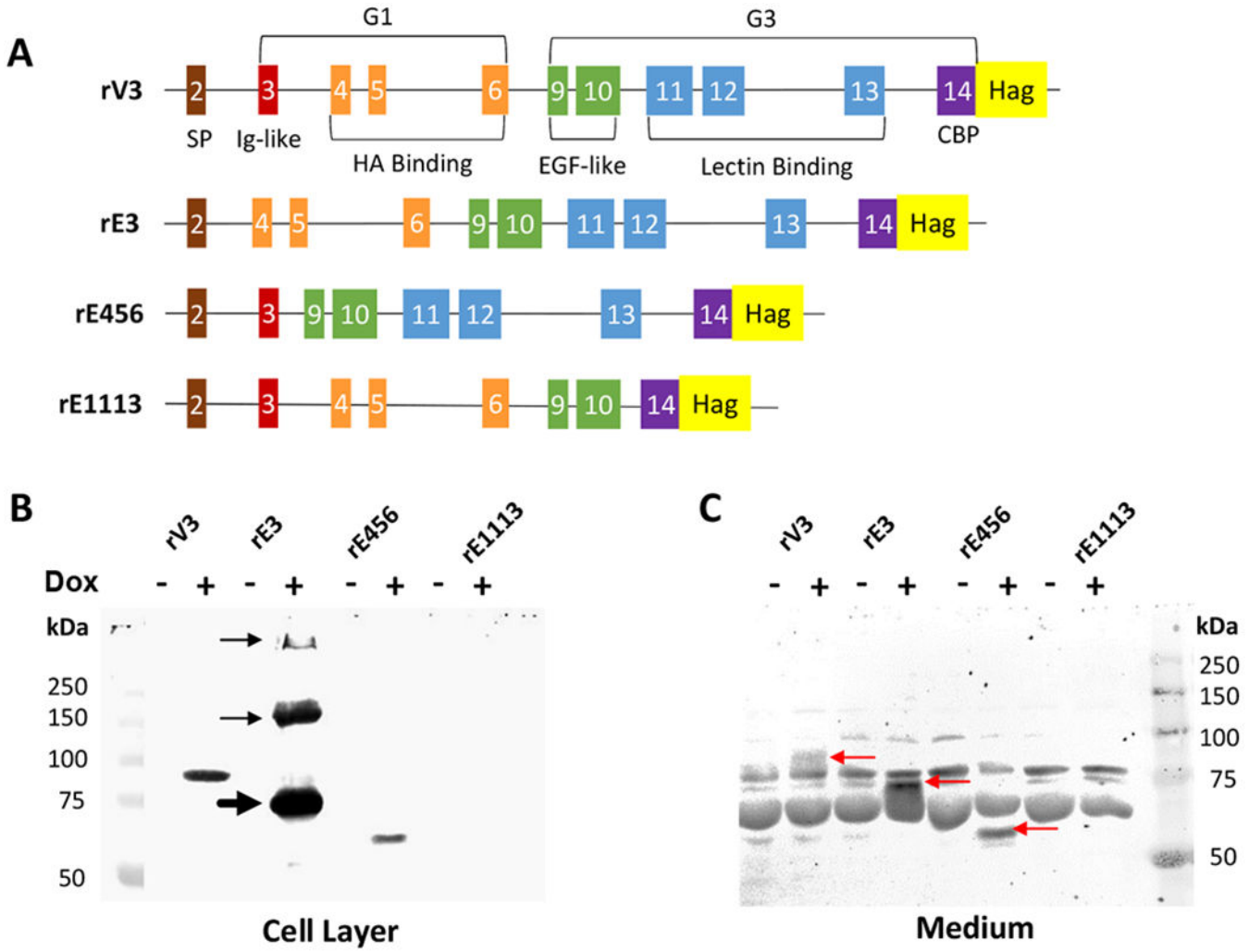

Figure 3. Exon deletion mutants highlight G3 domain as critical for expression and secretion.

V3 mutants were generated containing deletions in exon 3 (rE3), exons 4-6 (rE456), and exons 11-13 (rE1113) and were expressed in NIH 3T3 cells in a Dox-inducible manner. **A)** Domain maps of Hag-tagged V3 exon deletion mutants. **B)** Western blot of cell lysates probed for Hag-tagged V3. Heavy arrow indicates soluble monomeric V3, whereas thin arrows indicate high molecular weight, likely multimeric, V3. **C)** Conditioned media probed for Hag-tagged V3. Red arrows indicate secreted V3, all other bands are non-specific due to presence of 10% fetal bovine serum in the conditioned media.

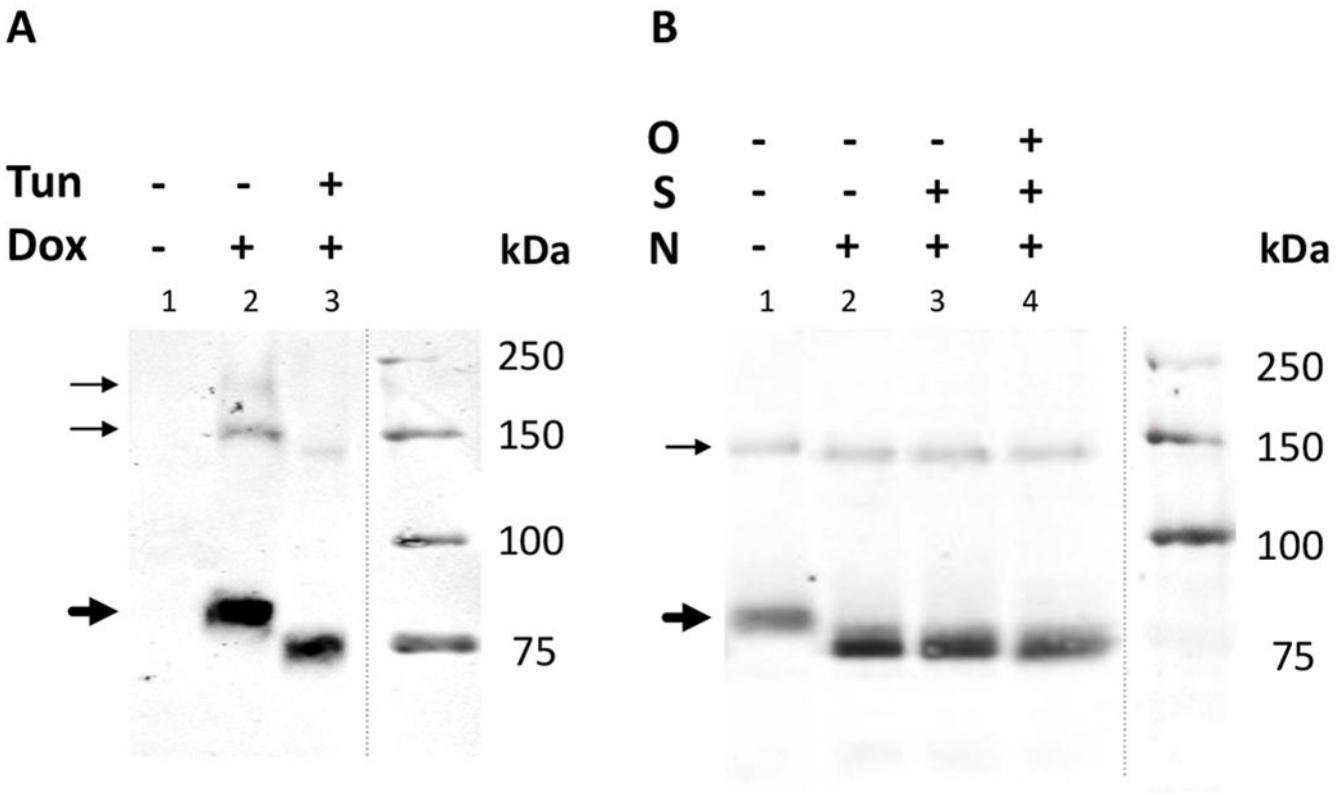

Figure 4. V3 is N-glycosylated.

A) V3 expression in transduced NIH 3T3 fibroblasts was induced by addition of 500 ng/ml Dox for 48 h ± 10 μM tunicamycin (Tun) to block N-glycosylation. Cellular protein was collected and proteins were separated on a 10% SDS-PAGE gel under denaturing conditions. After transfer to a nitrocellulose membrane, V3 was detected with an antibody directed against the c-terminal Hag-tag. **B)** Cellular protein harvested from V3-expressing cells was subjected to enzymatic deglycosylation with PNGase F (N), sialidase (S) and O-glycanase (O) for 24 h. Heavy arrow indicates soluble monomeric V3, whereas thin arrows indicate high molecular weight, likely multimeric, V3. Dashed lines indicate where lanes were digitally rearranged from original image for clarity of presentation.

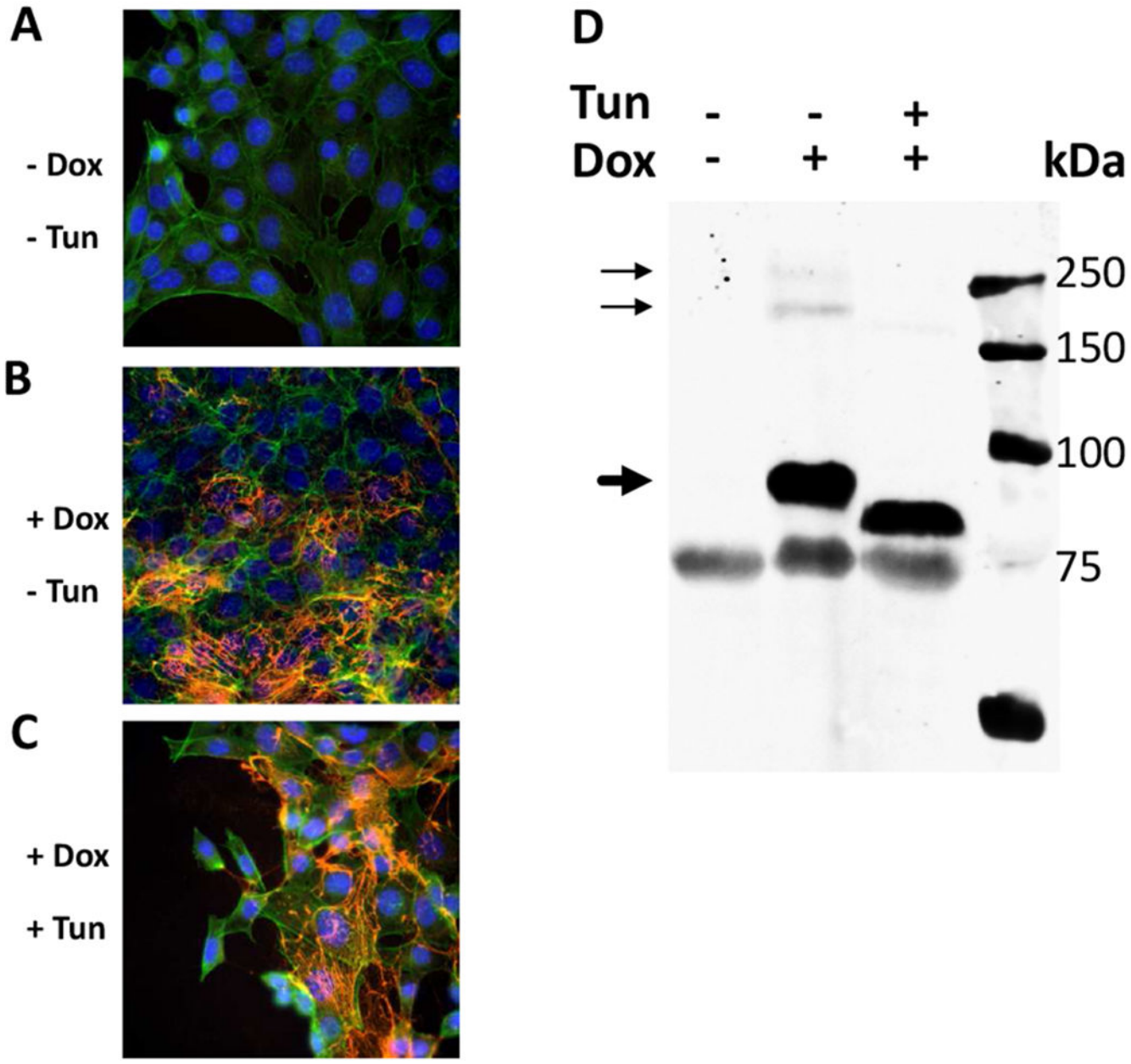

Figure 5. N-Glycosylation enhances secretion of V3.

A-C) NIH 3T3 cells seeded on coverslips were fixed in formalin after induction of V3 expression for 48 h ± 10 μM Tun to block N-glycosylation. Cells were stained for Hag-tagged V3 (orange), F-actin (green), and nuclei (blue). **D)** Medium from the cells treated the same as in **A–C** was collected and proteins were separated on a 10% SDS-PAGE gel under denaturing conditions. Conditioned media volumes normalized to equal cellular protein were loaded in each lane. After transfer to a nitrocellulose membrane, V3 was detected with an antibody directed against the c-terminal Hag-tag. Heavy arrow indicates soluble monomeric V3, whereas thin arrows indicate high molecular weight, likely multimeric, V3.

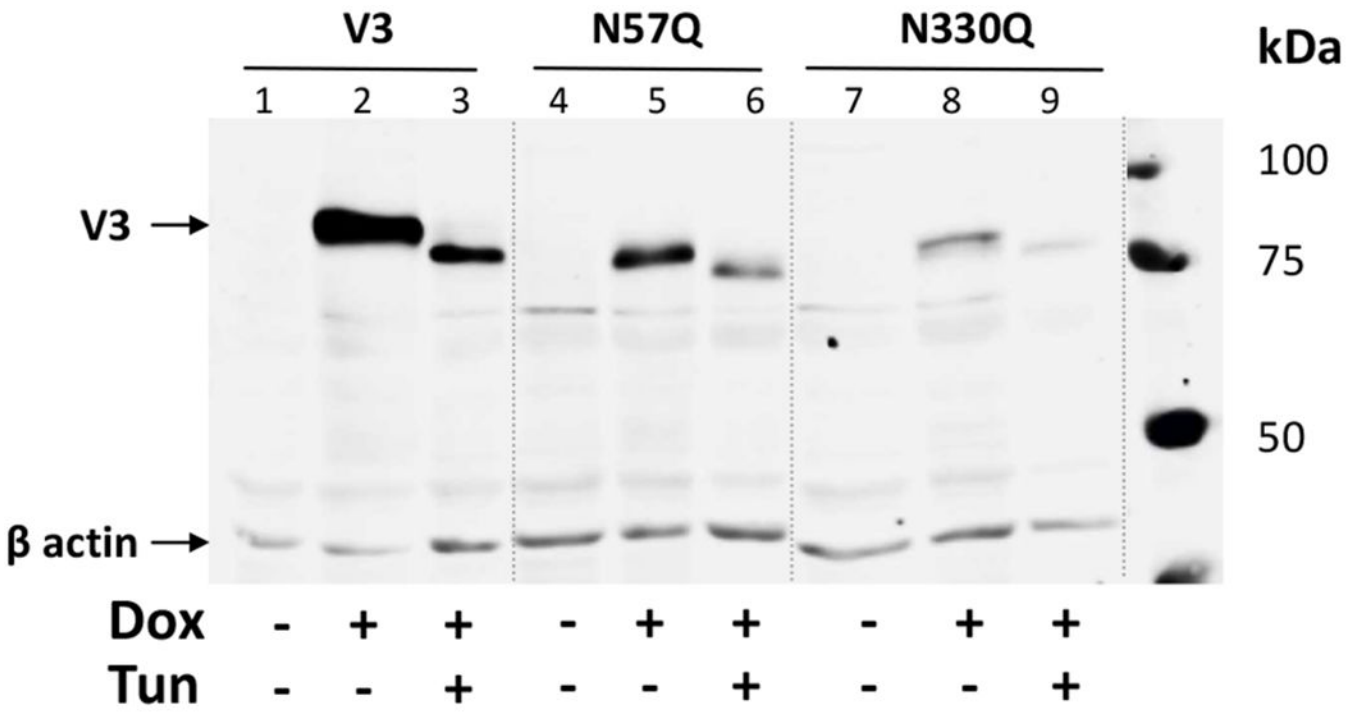

Figure 6. Mutation of putative N-glycosylation sites in V3 prevents glycosylation.

Rat V3 sequence modified at AA 57 in the Ig-like V-type domain and AA 330 in the HA-binding domain were mutated to glutamine (N57Q and N330Q, respectively) and expressed in a Dox-inducible fashion in NIH 3T3 fibroblasts treated ± Tun. Equal amounts of cellular protein were subjected to western blotting and probed for V3 using an antibody directed against the c-terminal Hag-tag and for β actin. Dashed lines indicate where lanes were digitally removed from original blot image and rearranged for clarity of presentation.

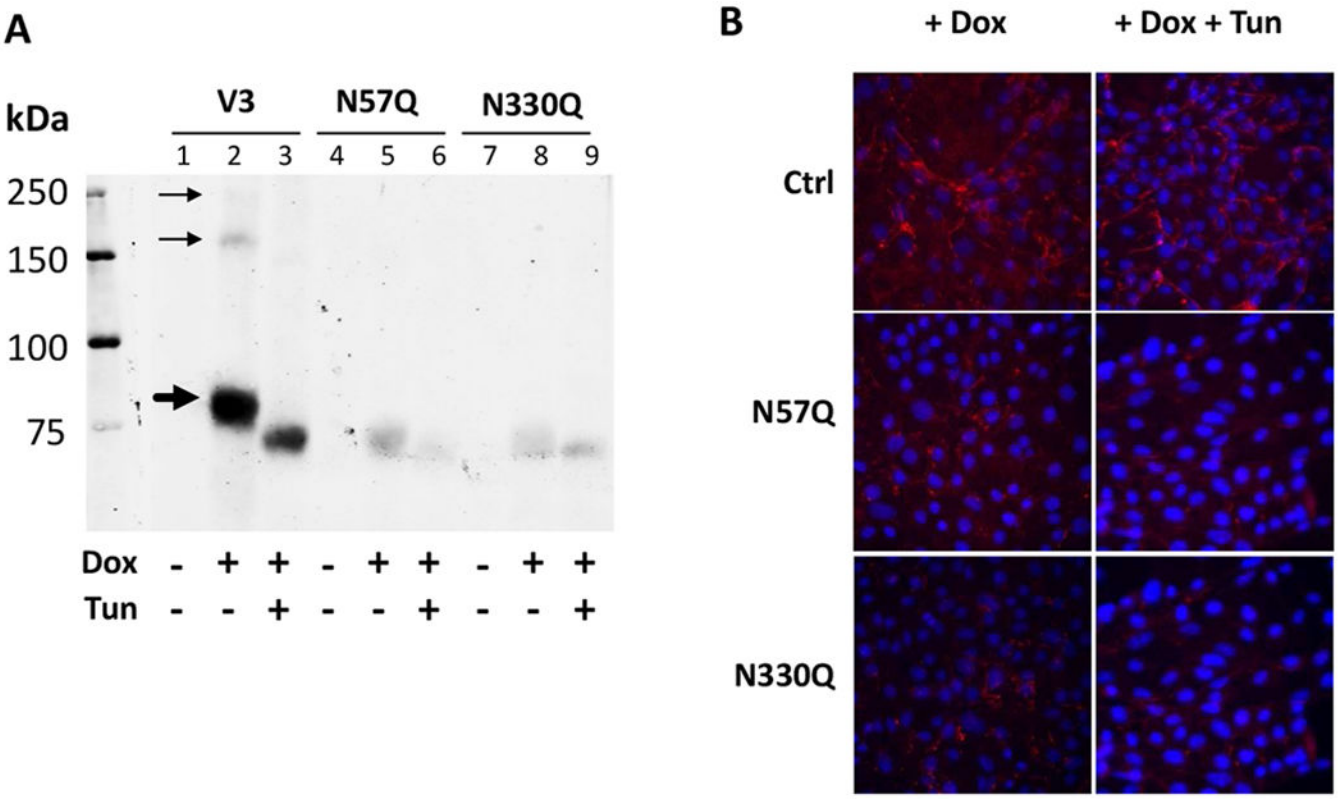

Figure 7. Loss of N-glycosylation at either site markedly reduces secretion of V3.

A) Western blot of conditioned media from control and mutant V3-expressing NIH 3T3 cultures treated ± Dox and ± Tun and probed for Hag-tag. Conditioned media volumes normalized to equal cellular protein were loaded in each lane. Heavy arrow indicates soluble monomeric V3, whereas thin arrows indicate high molecular weight, likely multimeric, V3. **B)** Immunohistochemistry of NIH 3T3 cells expressing native (Ctrl) and mutant (N57Q and N330Q) rat V3 (red). Nuclei are stained in blue.

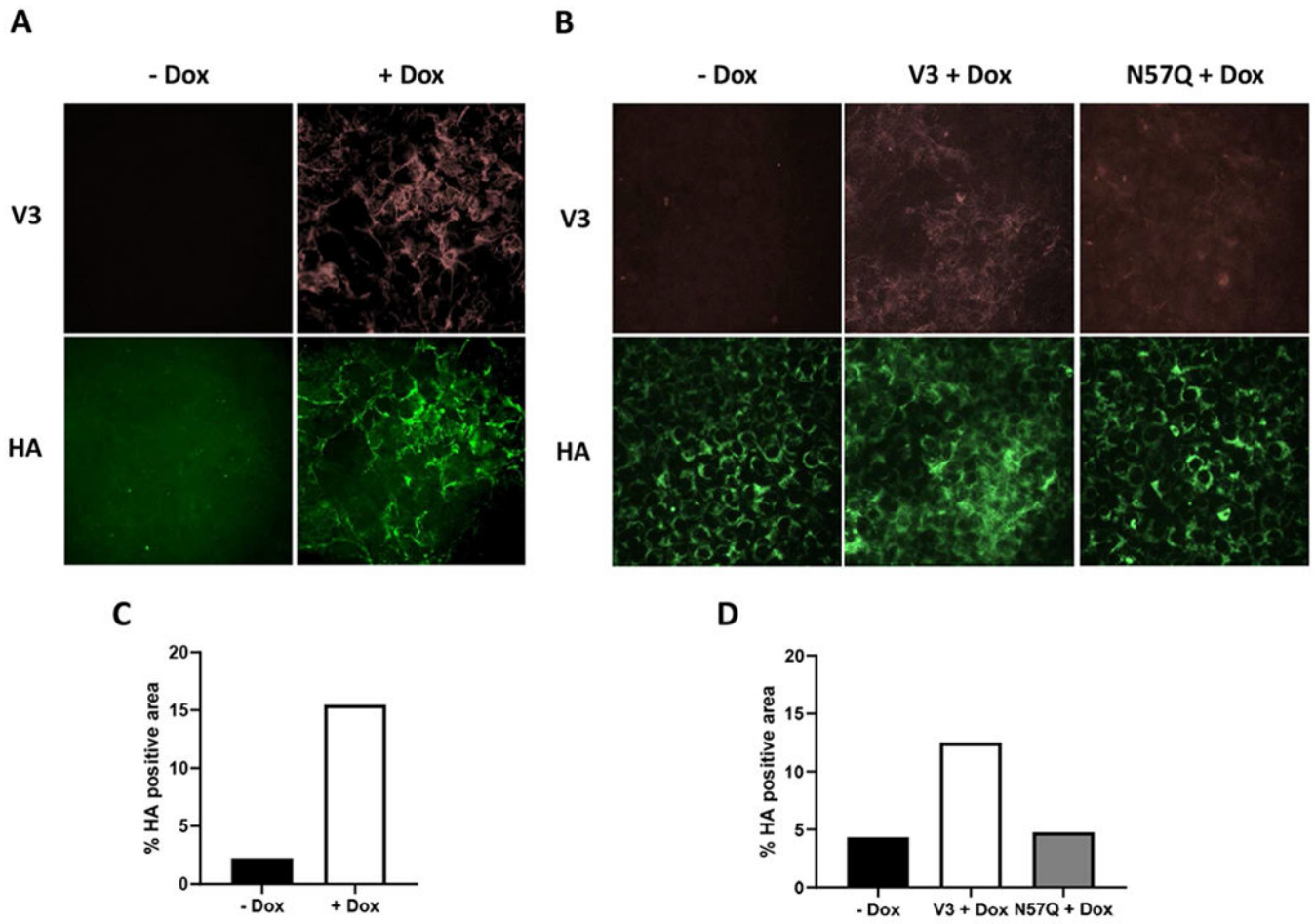

Figure 8. Overexpression of V3 increases the amount of exogenous HA bound at the cell surface.

A) NIH 3T3 cells overexpressing V3 were incubated with 1 μg/ml high molecular weight HA labeled with fluorescein (green) ± Dox for 24 h. After washing, the cells were fixed and counterstained for V3 (red). **B)** NIH 3T3 cells overexpressing native V3 or mutant N57Q V3 were treated identically as in **A**. **C** and **D)** Digital densitometric analyses of percent HA-positive areas in **A** and **B**, respectively.

Table 1.

Primer Sequences for V3 Construct Assembly

Primer	Sequence
rV3SpeIF	TTTTACTAGTGCCACCATGTTGATAAATATGAACGGCATCCTATGG
rV3XbaIR	TTTTTCTAGATCAAGCGTAATCTGGAACATCGTATGGGTAGCGCCTCGTTTCCTGCCACC

Table 2.

Primer Sequences for rat V3 QPCR

Primer	Sequence
rV3F	AGCAGATTTGATGCCTACTGCTTT
rV3R	GCACAGGTGCACACATAGGA