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Optimized Delivery of C3 Transferase by Lentiviral Vectors for CNS Injury Therapy

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Abstract

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Identifying targets for effective treatment of neurodegenerative diseases, brain and spinal cord injuries is one of the fundamental goals of modern neuroscience. These pose tremendous challenges and burdens on our society, especially as life expectancy increases.

In the past decades, through the elucidation of several inhibitory pathways governing the non-permissive nature of the central nervous system, RhoA GTPase has emerged as an important inhibitory signal convergence point, and therefore an attractive therapeutic target. Investigators in previous studies have successfully demonstrated axonal regeneration both *in vitro* and *in vivo* by RhoA inhibition using various forms of C3 transferase from *Clostridium botulinum*, a potent RhoA inhibitor. However, the poor permeability of this enzyme together with its short-term expression limited the success of such strategies.

In this study we have explored a novel axonal regeneration approach, based on the delivery of C3 transferase into the central nervous system utilizing FIV or HIV lentiviral vectors. Such strategies will potentially overcome limitations associated with direct C3 enzyme delivery, and lead to its constitutive, sustained cellular expression. Consequently, this will lead to continuous axonal regeneration necessary for complete neuronal recovery.

We were able to develop several C3-containing lentiviral plasmids that can be used for lentiviral vector production. Moreover, we assessed the C3 expression levels and functionality of these plasmids to determine their readiness for lentiviral production. Achieving constitutive, sustained production of C3 transferase through plasmid development and subsequent lentiviral generation is an important milestone in exploring the full regenerative and therapeutic potentials of RhoA inhibition.

Optimized Delivery of C3 Transferase by Lentiviral Vectors for CNS Injury Therapy

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Tables and Figures

Introduction

Traumatic brain or spinal cord injury, stroke, and neurodegenerative diseases such as Parkinson's disease (PD) are all major public health problems. Each can seriously debilitate a patient's quality of life, causing a variety of physical, emotional, and behavioral predicaments up to severe disability or death. The prevalence of these conditions and their detrimental impact on health demands the conception of effective neuronal rehabilitation therapies that can better patients' health and improve recovery. However, the developmental process associated with such therapies is full of challenging obstacles stemming from the unique characteristics of the human central nervous system (CNS), best characterized by its non-permissive nature.

More specifically, the CNS fails at regenerating the axons of injured neurons and does not allow uninjured neurons to restore lost connections which would otherwise compensate for the damaged synaptic contacts (1). There are two primary reasons for this non-permissiveness: the inhibitory environment created by a variety of extrinsic factors, and significantly reduced regenerative capacity of CNS in the adult stage (2).

Intrinsic factors of adult neurons as a barrier to neuronal regeneration

In contrast to the axons of both peripheral nervous system (PNS) or neonatal CNS which, upon injury, can regenerate and restore lost connections, adult mammalian CNS axons fail to do so (3). One of the reasons for this different response to injury is attributable to an altered expression profile of a variety of growth-associated factors. In fact, it has been shown that some of them are downregulated in adult CNS and upregulated in PNS or neonatal CNS (2). This evidence suggests that with maturation the intrinsic capacity for axonal growth decreases in adult CNS. Nevertheless, despite such

limitations, it has also been shown that neurons of adult CNS can, in fact, extend their processes over a significant length when provided with an alternative, more permissive environment, such as peripheral nerve grafts (4). Thus, the non-permissive environment of the CNS can be one of the driving factors in restricting axonal regeneration. Since the inhibitory processes of such an environment impede functional neuronal recovery, a better understanding of these processes on a molecular and cellular level can provide insight into the mechanisms underlying CNS regeneration failure. Subsequently, this can allow advancing CNS injury therapy to a new level towards increased effectiveness.

Extrinsic factors of CNS environment as main obstacles to CNS regeneration

The best characterized extracellular inhibitory molecules that significantly contribute to the establishment of a non-permissive environment in CNS and subsequently prevent neuronal regeneration are of three main types: Myelin associated inhibitors (MAIs), chondroitin sulfate proteoglycans (CSPGs) and axon guidance repulsive molecules.

MAIs and CNS injury

The three known myelin-derived inhibitory proteins: NogoA, myelin associated glycoprotein (MAG) and oligodendrocyte – myelin glycoprotein (OMgp) are all potent inhibitors of axonal regeneration (5). In addition, myelin contains other inhibitory factors such as Versican V2, a member of CSPGs (6). Immediately upon CNS injury, disruption of myelin leads to significant exposure to and subsequent interaction between these molecules and neuronal growth cones, ultimately resulting in growth cone collapse and inhibition of neurite outgrowth (7). Therefore, MAIs are considered to be the main inhibitory factors at the early stages of injury.

CSPGs and CNS injury

Approximately 24 hours after CNS injury, the processes underlying the formation of a glial scar start playing a prominent role in the inhibition of neurite outgrowth (8). Astrocytes, which are the main components of the glial scar, undergo reactive gliosis, a process characterized by an increase in cell division, hypertrophy, and the production of intermediate filaments (9). Apart from creating a physical barrier to axonal outgrowth through the scar tissue, astrocytes produce an excess of a variety of proteoglycans, including CSPGs such as Neurocan, Phosphacan, and Brevican (1, 10). This class of molecules has been shown to compromise neurite outgrowth significantly and to lead to the formation of axonal dystrophic endings (9, 11, 12). Moreover, their differential expression was found to last for up to six months following injury, underlining their prolonged inhibitory effect on neuronal regeneration (13).

Axon guidance repulsive molecules and CNS injury

As the scar reaches its mature stage, another class of inhibitory factors called axon guidance repulsive molecules begins to have a significant role in preventing neuronal regeneration. Certain members of this class, such as class III semaphorins, are secreted along with CSPGS by meningeal cells that have infiltrated the injury site due to the disruption of the blood-brain barrier and have been shown to serve as potent inhibitors of CNS regeneration (14, 15). Other types of axon guidance repulsive molecules, such as ephrins, have also been implicated in inhibiting axonal outgrowth, as it was shown that mice without functional EphA4 (ephrin receptor) displayed significant axonal regeneration upon spinal cord injury, leading to its improved functional recovery (16).

Thus, it seems clear that the establishment of a non-permissive environment in CNS is a complex process involving a variety of different inhibitory molecules that exert their influence in a cooperative manner and come into play at specific stages of neuronal injury response.

RhoA as a key mediator in the creation of an inhibitory environment of the CNS:

RhoA has long been considered to play a significant role in impeding the regrowth of axons following injury. RhoA belongs to a family of small GTPases that are active in GTP-bound conformation and inactive in GDP-bound conformation. It has been shown that treatment of neuronal cells with lysophosphatidic acid (LPA), an activator of RhoA, leads to axonal retraction, growth cone collapse, and cell body rounding (17). At the same time, inhibition of RhoA results in increased axonal regeneration, even when on non-permissive substrates, both *in vitro* and *in vivo* (10, 18, 19). Additionally, apart from being involved in cell division, growth, migration and neural development, RhoA also serves as a key mediator between extracellular signals and cytoskeleton rearrangement (20). This characteristic is especially important considering the fact that upon injury to CNS, neurons undergo extensive morphological changes characterized by axon retraction and cell rounding.

Over the past few decades, the molecular mechanisms by which RhoA participates in axonal growth inhibitory transduction cascades have been clarified and it was shown that activation of RhoA is required for mediating the activity of all types of inhibitory factors present in adult CNS: MAIs, CSPGs, and axon guidance repulsive molecules, despite the fact that they have diverse receptor and effector targets (5, 7, 10, 18, 21 - 28).

MAIs and RhoA

A variety of experiments have shown a strong association between MAIs and RhoA. It has been observed that myelin or MAIs lead to an increase in cellular content of active RhoA (22). At the same time, inactivation of RhoA results in counteraction of the inhibitory effects of MAIs (18, 23). Clarification of the molecular pathway utilized by MAIs to inhibit axonal regeneration allowed the pinpointing of the role of RhoA in this process. Despite structural differences between all three MAIs, all have been found to mediate their inhibitory effects by binding to the same GPI – linked cell surface receptor Nogo Receptor (NgR) (5). NgR belongs to a receptor complex that also includes Lingo-1 and $p75^{NTR}$, a member of the tumor necrosis factor receptor family. Activation of this complex subsequently leads to conversion of RhoA into its active state. Active RhoA, in turn, leads to the sequential activation of Rho kinase (ROCK), myosin light chain kinase (MLCK) and myosin light chain (MLC), ultimately resulting in actin filament depolymerisation and subsequent growth cone collapse (7, 10).

Thus, RhoA can be considered as an essential molecular switch in MAIs inhibitory pathway. Moreover, it has been shown that the N - terminus axon growth inhibitory domain of NogoA, NiG, also acts through RhoA via the NgR/$p75^{NTR}$ independent pathway (24).

CSPGs and RhoA

Similarly to NiG, Versican V2, a CSPG expressed by myelin, was found to exert its inhibitory effects via NgR/$p75^{NTR}$ independent pathway that converges on RhoA (24). Moreover, there is compelling evidence that the level of active RhoA is elevated when exposed to such CSPGs as Aggrecan or Versican and that the inhibition of RhoA or its

downstream effector RhoA kinase (ROCK) leads to neurite extension of various neuronal cells, including cortical neurons and retinal ganglion cells (RGCs), in the presence of CSPG substrates (10, 25). Thus, even though the specific mechanism by which RhoA is activated and mediates inhibitory effects of CSPGs have not been explained, it is clear that RhoA plays a significant role in this process.

Axon Guidance Repulsive Molecules and RhoA

In previous studies RhoA was shown to participate in the signal transduction pathways of axon guidance repulsive molecules such as ephrins and semaphorins. For instance, RhoA was implicated to serve as a key mediator in the ephrins transduction cascade through its interaction with Eph-interacting exchange protein (Ephexin) (26). Even though involvement of RhoA in semaphorin transduction cascades seems less significant (27), it still plays a considerable role for inhibitory effects of specific types of semaphorin molecules. For example, cellular inactivation of ROCK by Y27632 inhibitor or administration of dominant negative RhoA leads to a reduction of Sema-4D effects such as neural growth cone collapse (28). Thus, although there is much more to be discovered, it seems that RhoA is involved in mediating the effects of at least some of these types of inhibitory factors.

RhoA as an attractive target for CNS injury therapy

Overall, an accumulation of evidence suggests that RhoA functions as a common inhibitory signal convergence point for all three types of inhibitory molecules existing in the CNS. This represents a significant advantage in designing an effective therapeutic strategy for increasing neuronal regeneration.

In fact, previous attempts at targeting only specific types of inhibitory molecules or their unique downstream effectors were not as effective in significantly increasing neuronal regeneration as targeting RhoA itself. For example, *in vivo* studies on mice with null $p75^{NTR}$ did not result in neuronal regeneration following spinal cord injury, suggesting that blocking the NgR/$p75^{NTR}$ pathway utilized by MAIs is insufficient for allowing neurite outgrowth (29). Similarly, digestion of CSPGs by chondroitinase ABC (ChABC) did not lead to significant axonal outgrowth of retinal ganglion cells (RGCs (30). In contrast, inactivation of RhoA or ROCK led to stimulated axon regeneration both *in vitro* and *in vivo* (31). Thus, it seems that due to its central role in a variety of transduction cascades initiated by different types of inhibitory molecules, inactivation of RhoA results in counteraction of a majority of them and leads to a better functional recovery. Moreover, RhoA has also been shown to play a role in the regulation of apoptototic cell death upon CNS injury, further highlighting the level of its contribution in impeding neuronal regeneration (32). Thus, inhibiting RhoA represents a promising approach in CNS injury therapy.

C3 as a potent inhibitor of RhoA

Bacterial 27kDa exoenzyme C3 transferase from *Clostridium botulinum* has been implicated as a potent inhibitor of RhoA through ADP ribosylation of its effector domain (33). In fact, previous studies have indicated that administration of C3 had neuroprotective effects and promoted neurite outgrowth both *in vivo* and *in vitro* (18,19, 32). Nevertheless, while there is a great potential for C3 to be used in CNS regeneration therapy, several obstacles must first be overcome. First, this enzyme is not cell permeable, which makes its introduction into neuronal cells problematic (18, 34).

Second, while C3 delivery by direct injection results in active C3 for several days, it might be necessary to achieve a more sustained delivery of this enzyme where it would be able to suppress RhoA for a period of sufficient length as would be necessary for axon regeneration.

In order to introduce C3 into neuronal cells, investigators in previous studies have used scrape - loading or neuronal cell trituration (19, 23, 35). Nevertheless, even though both of these methods can serve as a conceivable C3 delivery method *in vitro*, they are not applicable for *in vivo* studies due to their disruptive effects on cells. Others have tried to circumvent the same issue by fusing C3 to various types of transport peptides (18). Even though such an introductory approach resulted in efficient C3-like protein translocation across the cell membranes without alteration of the enzyme's behavior, the inhibitory effects on RhoA lasted for up to 36 hours, an insufficiently short period of time for significant recovery.

Lentiviral delivery of C3 – an advantageous strategy for CNS regeneration

The limitations associated with the current methods of C3 delivery into the neuronal cells can potentially be circumvented with the employment of lentiviral vectors. In fact, these vectors might serve as a more efficient therapeutic approach for C3-mediated CNS regeneration due to several advantages associated with this method of C3 delivery: First, lentiviruses can efficiently transduce non-dividing cells (36). Second, they do not lead to generation of a significant immune response (37). Third, when pseudotyped by appropriate envelope glycoproteins with incorporation of neuron specific promoters, a high preference for the neuronal, rather than glial cells was displayed (38).

Forth, the lentiviral C3 delivery method allows incorporation of the C3 enzyme into the neuronal genomic DNA, leading to its constitutive C3 expression.

Thus, the lentiviral approach can lead to not only efficient delivery of C3 into neuronal cells, but also to its sustained cellular production and, consequently, inhibition of RhoA for a prolonged period of time. The outcome of this, in turn, can be increased CNS regeneration and consequent alleviation of the conditions associated with CNS injuries. Moreover, such therapy has a potential application in treatment of such neurological disorders as PD, which is characterized by progressive death of dopaminergic neurons in substantia nigra pars compacta. The use of C3 lentiviral therapy could promote axonal outgrowth of these neurons and thereby increase their natural regenerative capacity, subsequently leading to a delay of the progress of the disease.

Since the lentiviral C3 delivery method has never been approached before, we decided to explore it. For this purpose C3 was incorporated in a specific manner into different types of lentiviral backbones, following comparative assessment of the generated plasmids on C3 expression and C3 functionality. This *in vitro* comparative study led to identifying the most efficient and suitable constructs for lentiviral production and thus served as a necessary foundation step for not only lentiviral production but also for their use in future *in vitro* and *in vivo* studies.

Material and Methods

Lentiviral plasmids construction

For each plasmid constructed, the insert of interest was amplified through polymerase chain reaction (PCR) using primers containing specific restriction enzymes (Table 1). Following this, both the vector and the insert were cut with a particular set of enzymes and run on 1% agarose gel. Bands of a needed molecular weight corresponding to the vector and insert were cut out from the gel and purified with Geneclean Turbo kit (MP Biomedicals, Solon). Next, the vector and the insert were ligated using T4 ligase, dNTPs, and ligase buffer overnight at 4 ^{0}C. The next day the DH5 alpha bacterial strain was transformed with the ligation mixture in the following way: 50ul of cells placed on ice were incubated with 5ul of ligation mixture for half an hour, put for 45 seconds into 45 ^{0}C water bath, transferred back on ice for 2-3 minutes and diluted in 1.5 ml of Super Optimal broth with catabolite repression (SOC) medium. The mixture was transferred to a shaker at 37^{o}C for an hour and plated on ampicillin or kanamycin agarose plates. Following this, the agarose plates were put into a 37^{o}C incubator overnight.
The next day, single bacterial colonies were selected using a sterile toothpick and transferred into solution of Luria-Bertani (LB) medium containing the appropriate antibiotic. The solution was put into shaker overnight at 37^{o}C. The next day, the DNA was extracted from each sample using GeneJET plasmid miniprep kit (Fermentas, Glen Burnie). Briefly, the mixture of LB and amplified bacteria was centrifuged for 5 minutes to form a pellet. Next, the precipitate was resuspended, lysed, and neutralized. The

resulting mix of cellular debris and DNA was centrifuged for 10 minutes and the supernatant was carefully taken out and transferred into a DNA binding column. The column was washed and the DNA was eluted with 50 µl of Tris EDTA elution buffer.

Prior to executing any combinatorial manipulations with the lentiviral backbones FUGW (obtained from Dr. David Baltimore) or Fuginsin (obtained from Dr. Gary Bassell), we focused first on incorporating C3 into non-lentiviral plasmids pEGFPN2 and pEGFPC1 that are readily available and that possess a significant advantage over FUGW and Fuginsin in that they are smaller in size and have an abundance of restriction enzyme sites. The C3 sequence was amplified from the plasmid pRK5-C3, donated by Doctor Allan Hall's lab, and the 2A sequence was incorporated into the plasmids by means of PCR whereby one of the primers used for amplification of C3 contained this sequence within itself. Upon completion of this preliminary step, the pEGFPC1 (GFP2AC3) and pEGFPN2(C32AGFP) plasmids served as a convenient source for the template.

Confirmation of the successful generation of each of the constructs was accomplished through a restriction enzyme digestion procedure where each plasmid was cut with specific restriction enzymes and run on an agarose gel, upon which the size of the bands on the gel was compared to the theoretical prediction based on the map of the generated construct (Table 2, Figure 1, Figure 2). Upon such confirmation, the maxiprep corresponding to each plasmid was prepared using QlAfilter Plasmid Maxi Kit (QIAGEN, Valencia).

Transfection of 293T and PC12 cell lines

Either human embryonic kidney 293T cell line or PC12 cell line derived from rat adrenal pheochromocytoma was used for the comparative assessment of generated plasmids. These cell lines were plated on either poly-L-lysine (PLL) or PLL and CSPGs, and upon reaching 70-80 % confluency transfected with FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W, FU(GFP2AC3)W, Fuginsin, Fuginsin (C3), Fuginsin (C3GFP), Fuginsin (C3GFP) truncated (trunc.), Fuginsin (C32AGFP) or Fuginsin (GFP2AC3) using Lipofectamine™ 2000 Transfection Reagent as described by the manufacturer. The transfected cells were placed into a 5% CO_2 incubator at 37^0C and after 3 hours the Opti-MEM® Media was replaced with either nerve growth factor (NGF) containing media in the case of PC12 or DMEM media in the case of 293T cells. The cells were put back into the incubator until further use.

Immunocytochemistry (ICC)

Transfected 293T or PC12 cells, grown on laminin and PLL coverslips, with or without CSPGs, were immunostained as follows: The cells were fixed in 4% paraformaldehyde and 4% sucrose in phosphate buffer (PB) for 15 minutes at room temperature and washed with Phosphate Buffered Saline (PBS) three times for 10 minutes each. To increase their permeability, we incubated the cells in a PBS solution containing 0.25% Triton X-100 for 10 minutes at room temperature. The cells were then washed once with PBS and incubated in a blocking solution containing 1% BSA in PBS buffer for 30 minutes. Upon blocking, the cells were washed again with PBS and incubated overnight at 4^0C in PBS solution with the diluted primary antibody of interest. The next day, the cells were washed with PBS 3 times for 5 minutes each and incubated

with the secondary antibody diluted in PBS for 1 hour at room temperature. After an hour, the cells were washed with PBS for 10 minutes, incubated in 4',6-diamidino-2-phenylindole (DAPI) with PBS for 10 minutes, and rinsed again with PBS. Using Fluoro-Stain, the coverslips were mounted on slides and analyzed under a calibrated epifluorescence microscope.

Antibodies used

Primary antibodies: Monoclonal anti mouse C-myc antibodies (1:500; Cell Signaling Technologies) for C3 visualization, monoclonal anti rabbit GFP antibodies for enhancement of GFP visualization (1:500, Cell Signaling Technologies), and βIII-tubulin antibodies (1:5000, SIGMA) for cytoskeleton visualization.

Secondary antibodies: Secondary anti-mouse or anti-rabbit antibodies conjugated with Alexa 488 or 594 (1:5000, Invitrogen).

RhoA (Rhotekin) assay

The level of active, GTP - bound RhoA in the transfected 293T cells was determined using a RhoA activity assay (Cytoskeleton Inc.). Briefly, 4 days after transfection the 293T cells were lyzed, their cellular contents were collected, centrifuged at 4^0C for 5 minutes at 14000g, and analyzed by the Bradford assay to determine their protein concentration. 300 ug of each protein sample were incubated with 15ul of Rhotekin beads for 1hour at 4^0C. Beads were washed and resuspended in protein sample buffer. The amount of active RhoA bound to Rhotekin beads was determined by running each purified sample through a Western Blot, using a monoclonal anti-mouse RhoA

(1:500) antibody and infrared secondary goat antimouse antibody conjugated with Alexa 680 (1:2000). The Western blot was read using an Odyssey reader (Licor).The relative intensity of the visualized bands corresponding to active RhoA in each type of transfected cell was analyzed quantitatively by J Image Software.

Western Blot

Proteins were separated on 4-20% Tris-HCl acrylamide gels at a constant voltage of 110V. Proteins were then blotted onto PVDF immobilon membranes for one hour at a constant current of 0.15 A. The membrane was then incubated in Odyssey blocking buffer for half an hour, following incubation with a specific primary antibody diluted in 5ml of Tris buffered saline with Tween 20 (TTBS) overnight at 4^0C. The next day, the membrane was washed 3 times with TTBS for 10 minutes each, incubated with a secondary antibody diluted in TTBS for an hour at room temperature, washed again with TTBS 3 times, transferred to a methanol solution, and analyzed using Odyssey Western Blot Reader (Licor).

Antibodies used

Primary antibodies: Monoclonal anti mouse C-myc antibodies (1:500, Cell Signaling Technologies) for C3 detection, monoclonal anti rabbit GFP antibodies (1:500, Cell Signaling Technologies) for GFP detection, and monoclonal anti-mouse RhoA antibodies (1:500, SIGMA) for RhoA detection.

Secondary antibodies: Secondary infrared goat anti-mouse or anti rabbit antibodies conjugated with Alexa 680 or 800 (1:2000, Invitrogen).

Lentiviral production

The first lentiviruses based on Fuginsin (C3GFP) truncated plasmid were produced by the Emory lentiviral core at a titer of 10^6.

Results

Creating a generation of C3-containing plasmids that would serve as a core element of future lentiviruses, was the first objective of this study. In order to do so, we used two different lentiviral backbones FUGW and Fuginsin. FUGW is a Human Immunodeficiency Virus – 1 (HIV-1) based backbone while Fuginsin is a Feline Immunodeficiency Virus (FIV) based backbone. The decision to use both backbones for this study allowed us to expand the range of lentiviral vectors produced, and compare the advantages of different vectors within one type (FUGW or Fuginsin) as well as between types (FUGW versus Fuginsin). Using basic molecular cloning protocol, a variety of C3-containing plasmids were produced which can be classified into four main types: control plasmids expressing only GFP, C3-containing plasmids expressing only C3, C3-containing plasmids expressing C3 fused to GFP, and C3-containing plasmids expressing C3 and GFP as separate proteins (Table 3, Figure 3).

The last type of lentiviral plasmid where C3 and GFP were expressed as separate proteins was generated by incorporating between them a 2A sequence that is a short peptide consisting of 16 amino acids from the Foot and Mouth Disease Virus (FMDV) that cleaves itself at the C-terminus at the posttranslational stage both *in vitro* and *in vivo* (39). We chose to construct this type of plasmid because when C3 is fused to GFP, they are expressed as one protein and thus their function can be compromised. Consequently, the incorporation of the 2A sequence allows circumvention of this issue due to its cleaving capacity. Since 2A cleaves itself at the C terminus, the position of C3 and GFP in relation to 2A becomes important because if C3 is at the N terminus of 2A, it will

acquire the 2A sequence after its cleavage, and if it is at the C terminus it would not (Figure 3). In order to explore these possibilities, we generated both plasmids with the C32AGFP order and with a reversed order GFP2AC3. Thus, for both Fuginsin (C32AGFP) and FU(C32AGFP)W C3 will be present in the cells fused to the 2A peptide while for Fuginsin(GFP2AC3) and FU(GFP2AC3)W it will be present in the cells without any modifications.

Given that previous attempts to produce viruses using Fuginsin (C3GFP) as the core plasmid encountered complications including low titer viral production attributable to the large size of the plasmid, we decided to shorten it and create a truncated version of Fuginsin (C3GFP). This has been achieved by cutting Fuginsin (C3GFP) with ApaI and BplI restriction enzymes and excising a fragment about 1.3 kb, rendering the size of plasmid to about 8.7 kb. This issue was taken into consideration in designs of all the subsequent C3-containing Fuginsin plasmids. In fact, in each case the incorporation of the desired insert was achieved by choosing specific restriction enzymes which also excised an unnecessary fragment of the Fuginsin plasmid.

Acquisition of all the necessary FUGW and Fuginsin C3-containing plasmids served as an important step for this study and allowed us to progress to the second objective – an *in vitro* plasmid comparison study for the identification of the plasmids most advantageous for lentiviral production and for future testing *in vivo*. In consideration of the relative lack of time, we opted to pass down the lentiviral production based on Fuginsin constructs to Dr. Robert McKeon's lab and focus on FUGW constructs, even though we had already performed some preliminary analysis with Fuginsin plasmids.

The ideal lentiviral C3-containing plasmid had to meet several requirements in order to be successfully applied in CNS injury therapy. Most importantly, it had to express specific levels of C3 that would lead to significant axonal outgrowth. Thus, the focus was made on performing comparative assessment of *C3 expression* and *functionality* of these plasmids.

Assessment of C3 expression:

Immunocytochemistry and Western Blot were performed in order to assess C3 expression by the various plasmids.

*1) Immunocytochemistry (ICC***)**

ICC was performed to visualize C3 and/or GFP expression in 293 T cells transfected with the following lentiviral plasmids: FUGW (control), FU(C3GFP)W, FU(C32AGFP)W FU(GFP2AC3)W, Fuginsin (control), Fuginsin (C3), Fuginsin (C3GFP), Fuginsin (C3GFP) truncated, Fuginsin (C32AGFP) and Fuginsin(GFP2AC3). Apart from serving as a convenient tool for confirming expression of C3 and/or GFP in corresponding plasmids, this assay was also helpful in determining the localization of these proteins within cells. In order to detect C3, the 293T cells plated on PLL were fixed 48 hours post – transfection and stained with an anti - myc primary antibody corresponding to myc - an epitope that was incorporated at the N-terminus of C3 in all plasmids with the specific purpose of facilitating the protein's detection since antibodies to C3 are not available commercially. No antibodies were used for GFP since this protein fluoresces by itself. Additionally, cells were stained with DAPI to visualize their nuclei. Using this method, the presence of C3 and/or GFP was determined by light fluorescence micrsocopy using and FITC (for GFP) and a Rhodamine (for myc).

FUGW constructs:

As expected, FUGW treated cells expressed GFP only, FU(C3)W containing cells expressed C3 only and cells transfected with FU(C3GFP), FU(C32AGFP)W or FU(GFP2AC3)W expressed both GFP and C3. In all cases C3 and GFP appeared to be distributed uniformly within the cells (Figure 4).

Fuginsin constructs:

In a similar manner to FUGW constructs, cells transfected with Fuginsin C3 expressed C3 only while Fuginsin (C3GFP), Fuginsin (C3GFP) truncated, Fuginsin (C32AGFP) or Fuginsin (GFP2AC3) containing cells expressed both C3 and GFP (Figure 5). Localization of C3 and/or GFP within cells was also uniform. However, extremely low C3 expression was detected in Fuginsin (GFP2AC3) transfected cells, suggesting that some irregularities had occurred during the integration process of the insert into Fuginsin plasmid or during transfection. Because of this, Fuginsin (GFP2AC3) was not included in the subsequent assessment studies of Fuginsin C3 containing plasmids.

Surprisingly, both C3 and GFP expression were detected in Fuginsin-transfected cells. Later in the study, however, it was determined by digestion analysis of the plamid used with AgeI and KpnI restriction enzymes, that the plasmid we considered as a control and subsequently employed in all experiments was, in fact, Fuginsin(C3GFP) (Figure 6). As a consequence, the Fuginsin data were dismissed and FUGW served as a control plasmid for the assessment studies of the Fuginsin C3 containing constructs described below. This seemed reasonable since both FUGW and Fuginsin lentiviral backbones

contain only GFP and thus it is not likely that such a comparison compromised the assessment of Fuginsin C3 - containing plasmids.

2) Western Blot

Apart from allowing confirmation of C3 and/or GFP expression, Western Blot (WB) was also useful in determining the functionality of 2A. That is, whether the 2A peptide does indeed cleave itself in C32AGFP- or GFP2AC3-containing plasmids. Considering the fact that both C3 and GFP proteins weigh about 27 kDA, the appearance of a band around 60 kDa would indicate failure of 2A to cleave itself, while appearance of a 27 kDA band only would confirm that 2A does indeed function in an expected manner.

In order to do so, the WB was performed on 50ug of cell lysate obtained from 293T cells transfected with either no vector, FUGW lentiviral plasmids (FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W, FU(GFP2AC3)W), or Fuginsin lentiviral plasmids (Fuginsin (C3GFP), Fuginsin (C3GFP) truncated, Fuginsin (C32AGFP)). The blot was immunostained with myc antibodies with the following results:

C3 expression in FUGW constructs:

As expected, C3 was not detected in cells transfected with no vector or FUGW (Figure 7). In contrast, a band of approximately 27 kDA corresponding to C3 appeared in the 3rd lane which contained samples from FU(C3)W transfected cells. Similarly, a band of a similar, slightly greater molecular weight appeared in the lane corresponding to FU(C32AGFP)W transfected cells. This observation suggests that 2A does indeed cleave itself at its C terminus and is attached to C3 thereby slightly increasing the molecular weight of C3. Nevertheless, no C3 was detected in FU(GFP2AC3)W nor FU(C3GFP)W

containing cells, which is in contrast to the microscopic examination of the cells prior to harvesting as well as the ICC results (Figure 3) and previous results from the lab (data not shown). At least with respect to FU(C3GFP)W, C3 was detected by WB in the previous attempts (Figure 8). Thus, it is most likely that the absence of C3 recognition in cells containing these plasmids occurred due to a technical errore during the WB procedure and it will have to be redone in the future for a definite assessment of C3 expression, especially in the case of FU(GFP2AC3)W.

C3 expression in Fuginsin constructs:

A band of approximately 60kDa corresponding to C3 (27 kDA) fused to a GFP (~30kDa) protein was detected in both Fuginsin(C3GFP) truncated and Fuginsin(C3GFP) containing cells (Figure 9). At the same time, a band around 27kDa was detected in Fuginsin(C32AGFP) containing cells. Thus, WB confirmed that in all these plasmids C3 is expressed in an expected manner: As a fused protein in the case of Fuginsin(C3GFP) and Fuginsin (C3GGFP) truncated, and in its original form in the case of Fuginsin(C32AGFP), suggesting that the 2A sequence does indeed function.

Assessment of plasmids' functionality:

The functionality of a variety of generated FUGW and Fuginsin lentiviral plasmids was assessed through 293T cell studies, immunocytochemistry with PC12 transfected cells, and RhoA activity (Rhotekin) assay.

1) Effect of C3 expression on process number and length in 293T cells

The microscopic analysis of 293T cells transfected with a variety of FUGW and Fuginsin generated constructs served as a convenient method for preliminary testing of

the functionality of these plasmids. For this purpose the 293T cell line was transfected with FUGW (control), FU(C3GFP)W, FU(C32AGFP)W FU(GFP2AC3)W, Fuginsin(control), Fuginsin (C3GFP), Fuginsin (C3GFP) truncated and Fuginsin (C32AGFP) constructs and analyzed under the microscope after 48 hours. Since the analysis was based on the detection of fluorescent cells due to GFP expression, the FU(C3)W and Fuginsin(C3) plasmids were not included in this preliminary testing. Visually, transfection of all FUGW constructs except FU(C3GFP)W and control vector resulted in an appearance of multitude of processes extending fluorescent cells (Figure 10). In contrast, cells treated with FU(C3GFP)W did not appear as distinctly bright and did not display as many processes, suggesting that fusion of GFP to C3 compromised the functionality of both proteins. Another possibility is that it is hard to see the processes because of the weak GFP expression levels. With regards to Fuginsin constructs, the administration of each lentiviral C3-containing plasmid led to augmentation of the processes extension (Figure 11).

Several pictures corresponding to each type of cell were taken and subsequently analyzed using the NIS – Elements software to determine quantitatively the processes outgrowth, the number of processes per cell, and the percentage of cells with processes compared to the total number of cells.

The FUGW constructs:

While cells treated with control plasmid FUGW or with FU(C3GFP)W displayed processes with similar length of about 15 μm, administration of FU(C32AGFP)W or FU(GFP2AC3)W led to the increased outgrowth of processes, with the average length of about 19 μm and 18 μm, respectively (Figure 12). Nevertheless, only administration of

FU(C32AGFP)W plasmid resulted in a statistically significant increase (t-test $p<0.05$) when compared with the control. While FUGW or FU(C3GFP)W containing cells on average had 0.6 processes per cell, cells with FU(C32AGFP)W and FU(GFP2AC3)W had significantly more processes (1 and 0.9 respectively) (Figure 13). Moreover, while only about 50% of the observed cells treated with FUGW or FU(C3GFP)W plasmids extended processes, 74% of FU(C32AGFP)W and 61% of FU(GFP2AC3)W treated cells did so (Figure 14). These observations have led to the conclusion that both FU(C32AGFP)W and FU(GFP2AC3)W plasmids are more effective than the FU(C3GFP) plasmid in promoting the outgrowth of processes.

The Fuginsin constructs:

According to the quantitative analysis, administration of Fuginsin(C3GFP) resulted in an increase of the outgrowth of processes compared to the control, with the average process length of 16.6 μm and 15 μm respectively (Figure 15). Similarly, treatment with Fuginsin (C3GFP) truncated and Fuginsin (C32AGFP) have also led to increased processes outgrowth (16 μm and 15.6 μm respectively). However, none of these results are statistically significant. At the same time, cells transfected with any of the C3-expressing constructs had, on average, a similar number of processes per cell (about 0.9) and a similar percentage of processes extending cells (about 71%) (Figure 16 and 17). Thus, even though treatment with Fuginsin(C3GFP), Fuginsin (C3GFP) truncated or Fuginsin(C32AGFP) resulted in a significant increase in the number of processes per cell compared to the control (0.9 neurites/cell and 0.6 neurites/cell), and percentage of processes displaying cells (71% compared to 50%), the particular identity of the Fuginsin C3-containing construct did not seem to play a significant role.

FUGW constructs vs, Fuginsin constructs:

According to our preliminary analysis, FU(C32AGFP)W and FU(GFP2AC3)W plasmids are both more effective at promoting outgrowth of processes than any of the Fuginsin C3 – containing plasmids. However, all the tested Fuginsin plasmids (except control) led to similar increase in percentage of cells displaying processes as FU(C32AGFP)W and were more effective at it than FU(GFP2AC3)W. Moreover, their administration also led to a comparable significant increase in the number of processes per cell as FU(C32AGFP)W and FU(GFP2AC3)W. Thus, it seems that both of these types of lentiviral plasmids, especially FU(C32AGFP)W, FU(GFP2AC3)W and any of the assessed Fuginsin C3 – containing plasmids might be good candidates for lentiviral construction. Nevertheless, only FUGW C3-containing plasmids were subjected to the subsequent morphological analysis using the PC12 cell line due to the lack of time.

2) Effect of C3 expression on neurite outgrowth revealed by tubulin staining.

In this assay the PC12 cell line plated on either PLL or PLL with CSPGs (typical inhibitory molecules at the site of injury) was transfected with FUGW (control), FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W, or FU(GFP2AC3)W plasmids, fixed after 96 hours, stained with βIII-tubulin antibodies and analyzed under microscope. A quantitative determination of the neurite outgrowth and the number of neurites per cell for each type of transfected cells was made in a manner identical to the preliminary 293T cells studies.

Visually, non-C3-containing PC12 cells plated on PLL appeared normal with multiple extending neurites, but in the presence of CSPGs they acquired a spherical shape and were unable to extend neurites (Figure 18 and 19). FU(C3)W- or FU(C3GFP)W-

containing cells looked similar to the control in the presence of inhibitory substrate, suggesting that administration of these plasmids did not lead to a counteraction of the inhibitory effects of CSPGs. In contrast, cells transfected with FU(C32AGFP)W and FU(GFP2AC3)W displayed visible neurite outgrowth on CSPGs. Due to these observations, only FU(C32AGFP)W and FU(GFP2AC3)W were subjected to further comparative assessment.

Neurite outgrowth: FU(C32AGFP)W vs. FU(GFP2AC3)W

As expected, the cells transfected with control plasmid showed a drastic (about 50%) drop in neurite outgrowth in the presence of CSPGs (Figure 20). At the same time, the cells transfected with FU(C32AGFP)W plasmid displayed not only significantly elevated neurite outgrowth on PLL (which was expected considering the results of the preliminary analysis) but also were resistant to the inhibitory effects of CSPGs. In fact, the length of their processes on CSPGs was more than 100% longer compared to the control on inhibitory substrate. Moreover, treatment with FU(C32AGFP)W resulted in restoration of neurite outgrowth to the same level as the control on PLL.

By comparison, treatment with FU(GFP2AC3)W resulted in insignificantly elevated neurite outgrowth on PLL compared to the control. Nevertheless, it also led to a counteraction of the inhibitory effects of CSPGs, resulting in significantly elevated neurite outgrowth by 69% compared to the control on CSPGs and 82% restoration of the neurite outgrowth compared to the control on PLL. According to this analysis, FU(C32AGFP)W seems more efficient in counteracting the inhibitory effects of CSPGs compared to FU(GFP2AC3)W and thus can be considered as a more advantageous plasmid, at least in this respect, for lentiviral production.

The number of neurites per cell: FU(C32AGFP)W vs. FU(GFP2AC3)W

Determination of the number of neurites per cell has led to the following results (Figure 21). In the absence of an inhibitory substrate, the cells transfected with FU(C32AGFP)W exhibited a significant increase in the number of neurites per cell, while cells treated with FU(GFP2AC3)W displayed a similar number of neurites per cell compared to the control (3.9, 3.2, 2.9 neurites per cell correspondingly). Surprisingly, in the presence of CSPGs, the number of neurites per cell was not only maintained but also significantly increased in cells containing FU(GFP2AC3)W plasmid while it was decreased in FU(C32AGFP)W transfected cells and even more in case of the control (3.2, 2, and 0.7 neurites per cell, correspondingly). Thus, it seems that the FU(GFP2AC3)W plasmid, though not as efficient in promoting neurite outgrowth in the presence of inhibitory substrate as FU(C32AGFP)W, nevertheless induces significantly better increase in the number of processes put out by each cell in the presence of CSPGs.

3) RhoA activity (Rhotekin) assay

A Rhotekin assay was employed to determine the relative inhibitory effects of a variety of C3-containing plasmids on RhoA. Since the Rhotekin assay measures the amount of cellular active RhoA, it was theorized that transfection with the most advantageous plasmid, at least in this respect, would result in the least amount of detected active RhoA. With this reasoning, 293T cells transfected with no vector, FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W, FU(GFP2AC3)W, Fuginsin (C3GFP), Fuginsin (C3GFP) truncated and Fuginsin (C32AGFP) was analyzed for the presence of active RhoA.

FUGW plasmids

Through a quantitative determination of the intensity of the resulting RhoA bands, it was concluded that FU(C3)W inhibits RhoA most efficiently, followed by FU(C32AGFP)W, FU(GFP2AC3)W, FU(C3GFP)W, No vector, and FUGW (Figure 22). However, none of the RhoA inhibitory effects exerted by the various C3 expressing plasmids reached statistical significance. All FUGW C3 - expressing plasmids rather showed a trend in decreasing RhoA activity with FU(C3)W approaching significance with a T-Test p value of 0.08 when compared to FUGW.

The RhoA activity inhibition displayed by FU(C3)W seems contradictory to the results of the previous PC12 cytoskeleton ICC study where treatment with this plasmid did not result in significant counteraction of CSPGs effects. One possible explanation is that the C3 enzyme expressed by this plasmid is simply too potent in its effects, inadvertently leading to cells death due to its cytotoxicity. Therefore, the observed PC12 cells treated with FU(C3)W in the previous study were most likely the ones that did not take up this plasmid and thus were unaffected by its cytotoxicity. In regards to FU(C32AGFP)W and FU(GFP2AC3)W, the Rhotekin assay results were agreeing with the previous observations in that the former plasmid is more efficient than the latter in counteracting effects of CSPGs through inhibition of RhoA.

Fuginsin plasmids:

Fuginsin (C3GFP) truncated plasmid turned out to be the most effective at deactivating RhoA, followed by Fuginsin(C32AGFP), control and Fuginsin(C3GFP) (Figure 23). However, similarly to FUGW C3 – containing plasmids, none of the obtained results was statistically significant, rather showing a trend in decreasing active cellular RhoA.

In consideration to preliminary 293 T cell studies, the low inhibition potential of Fuginsin(C3GFP) is unexpected. This discrepancy is accentuated by an additional fact that Fuginsin(C3GFP) truncated, the most efficient plasmid in inactivating RhoA, is in essence a shortened version of Fuginsin(C3GFP). In order to provide reasonable explanation for such an outcome, the Rhotekin study will have to be repeated since it is possible that some procedural mistake was, in fact, the underlying reason of this inconsistency. Alternatively, it is possible that the amount of total RhoA in the sample corresponding to Fuginsin(C3GFP) was greater than in the other samples. Thus, even though C3 expressed by this plasmid potentially might have led to efficient RhoA inhibition, it was not recognized due to the remaining active RhoA. To rule out this possibility, the total amount of RhoA will also be assessed in each sample by WB.

FUGW vs. Fuginsin plasmids:

It is clear that the Rhotekin assay needs to be repeated as no statistically significant results were obtained from it, possibly due to some procedural mistake. However, out of all the plasmids tested by this assay, FU(C3)W seemed to the most potent plasmid in inhibiting RhoA. Fuginsin(C3GFP) truncated and FU(C32AGFP)W were the second best displaying identical inhibitory activity. Thus, at least in this respect, all three of these plasmids were considered to be the most advantageous for lentiviral production.

Lentiviral production

Even though successful lentiviral production, based on constructed FUGW and Fuginsin C3-containing plasmids, is yet to be accomplished in our study, a lentivirus

based on the Fuginsin(C3GFP) truncated plasmid was generated in Dr. Robert McKeon's lab with the help of Emory's lentiviral Core facility. The ICC assay performed in our lab in PC12 cells infected with this lentivirus confirmed cellular expression of both C3 myc, red) and GFP (green) (Figure 24). Moreover, visually, transduced PC12 cells displayed striking alterations in their morphological structure compared to the control as most of them extended multiple long processes (Figure 25). Thus, such observations, albeit preliminary, confirm the functionality of this lentivirus. In the future we are planning to produce the rest of lentiviruses based on the generated FUGW and Fuginsin C3 containing plasmids and perform the same assessment analysis as with the plasmids in *vitro*, followed by studies in *vivo* in normal adult rats or rats with neuronal lesions.

Discussion

1. Assessment of produced FUGW and Fuginsin C3 - containing plasmids:

This study, for the first time, explored the possibility of incorporating C3 exoenzyme into lentiviral plasmids and confirmed that such a method of C3 delivery into cells results in prolonged C3 expression *in vitro* and leads to increased neurite outgrowth on inhibitory substrates. Moreover, the comparative assessment analysis performed on Fuginsin and FUGW C3-containing plasmids that were either expressing C3 only (type 1), C3 fused to GFP (type 2), or both C3 and GFP as separate proteins (type 3) provided important observations on the advantages and limitations associated with each type of produced plasmids, and, subsequently, led to the determination of the set of most appropriate plasmids for application in the future production of either HIV or FIV lentiviral vectors.

Type 1 – plasmids expressing C3 only:

Even though no functionality analysis was made with Fuginsin(C3) plasmid, FU(C3)W plasmid was shown to be inefficient at promoting neurite outgrowth on inhibitory substances, despite its high RhoA inactivation effect compared to the rest of all the analyzed plasmids. This suggests that this plasmid, although effective, might exert unfavorable cytotoxic effects on its carrier cells, subsequently leading to their death. Thus, even though production of lentiviruses based on a plasmid expressing only C3 would be practical for therapy applications and will constitute the final goal of this study, the current design of such a plasmid poses major limitations on the overall benefit of such lentiviruses. If toxicity is indeed the problem the use of this vector would necessitate the

incorporation of a regulatory system to reduce C3 levels of expression and the toxic effect.

Type 2 – plasmids expressing C3 fused to GFP

Fuginsin(C3GFP), Fuginsin(C3GFP) truncated and FU(C3GFP)W constructs belong to the second type of the lentiviral plasmids produced in this study and assessment of their functionality led to contradictory results.

Despite the fact that C3 expression was detected in cells transfected with FU(C3GFP)W by ICC and by WB in previous studies, administration of this plasmid did not lead to any significant morphological changes in either 293T or PC12 cells. This can be attributed to its inability to inhibit RhoA efficiently which was determined by Rhotekin assay. Thus, most likely fusion of C3 to GFP resulted in conformational alterations of the structure of the C3 enzyme and subsequent loss of its proper functionality. Therefore, it can be assumed that C3 delivery with lentivirus based on this plasmid will not lead to significant axonal regeneration and thus is disadvantageous for our study.

However, the functionality assessment of both Fuginsin(C3GFP) and Fuginsin(C3GFP) truncated plasmids led to completely contradictory results which suggested that using this type of plasmids in lentiviral vector production, in fact, might result in efficient therapeutical C3 delivery. First, both of them proved to be efficient in significantly increasing the number of processes per cell as well as the percentage of cells with processes when introduced into 293T cells. Second, Fuginsin(C3GFP) truncated showed a trend in decreasing the cellular content of active RhoA according to the Rhotekin assay. Surprisingly, Fuginsin(C3GFP) was found to be the least efficient at

inhibiting RhoA which was most likely attributed to a technical mistake. Overall, regardless of the source of the observed discrepancy in effects of Fuginsin(C3GFP), it seems unlikely that successful production of lentiviruses based on this plasmid is feasible due to its large size. Thus, Fuginsin(C32AGFP) truncated is a better choice.

Moreover, the first lentiviral vector produced in this study was, in fact, based on a Fuginsin(C3GFP) truncated plasmid, and administration of it in PC12 cells confirmed the successful expression of both C3 and GFP, resulting in visible neurite outgrowth compared to the control. Thus, it seems that out of all generated plasmids that express C3 fused to GFP Fuginsin(C3GFP) truncated is the most advantageous plasmid to be employed in lentiviral studies. However, it is important to acknowledge that the lentiviral vectors based on this type of plasmid will only be suitable for exploration of the benefits associated with lentiviral C3 delivery in preliminary studies, while in more advanced studies a separate expression of C3 might be desired.

Type 3 – plasmids expressing C3 and GFP as separate proteins:

The last type of plasmids, which includes FU(C32AGFP)W, Fuginsin(C32AGFP) , FU(GFP2AC3)W and Fuginsin(GFP2AC3), was generated in order to accomplish expression of C3 and GFP as two separate proteins, as opposed to the fused version expressed by the previous type of plasmids. The 2A sequence taken from FMDV served as a necessary tool to accomplish such expression, and its cleaving capability was confirmed for both FU(C32AGFP)W and Fuginsin(C32AGFP) plasmids but remains undetermined for the (GFP2AC3) expressing plasmids. Even though based on the ICC analysis Fuginsin(GFP2AC3) was considered unusable for future studies, the rest of this type of plasmids displayed not only strong C3 expression, but also proved to be efficient

in altering morphological structure of the 293T cells in preliminary microscopical study with FU(C32AGFP)W plasmid being the most efficient in all three analyzes performed.

Since no PC12 assessment study was performed on Fuginsin(C32AGFP) plasmid and results of Rhotekin assay indicated its mediocre RhoA inhibiting efficiency, it is hard to definitively determine the benefit of the application of this plasmid for future lentiviral production. However, with subsequent PC12 morphological analysis and repetition of Rhotekin study the advantages of Fuginsin(C33AGFP) construct will be assessed with greater certainty.

Data from the functionality assessment in PC12 cells suggest that the use of FU(C32AGFP)W plasmid results in significant PC12 cells neurite outgrowth on both permissive and inhibitory substances compared to the control. Moreover, this plasmid was second most efficient out of all C3-expressing plasmids in inhibiting RhoA after FU(C3)W. Together with results from the293T cell studies, it seems that FU(C32AGFP)W, in fact, might be the most advantageous plasmid for application in lentiviral therapy not only out of the third type of produced plasmids, but also out of all tested plasmids.

Lastly, with regards to FU(GFP2AC3)W, it is unreasonable to assess its applicability for lentiviral therapy without confirming C3 expression levels for this plasmid. However, PC12 cells treated with this plasmid displayed both significant neurite outgrowth and increase in the number of neurites per cell. Even though FU(GFP2AC3)W did not induce as impressive PC12 neurite outgrowth as FU(C32AGFP) it nevertheless was extremely effective in promoting cellular branching that was most evident in the presence of CSPGs. In contrast, the number of neurites per cell was decreased in cells

treated with FU(C32AGFP)W on CSPGs compared to PLL. Therefore once C3 expression is confirmed it might prove useful to generate lentiviral vectors using this plasmid and further explore its effects on process outgrowth.

Based on these studies we propose to move forward on lentiviral production and further *in vitro* and *in vivo* experiments using the following plasmids in the following order: FU(C32AGFP)W, FU(GFP2AC3)W, and Fuginsin(C3GFP) truncated. It is likely that the HIV or FIV lentiviruses based on these plasmids will lead to significant neurite outgrowth, increase in the number of neurites per cell, and increase in the percentage of cells with neurites. The other plasmids would benefit from further assessment before potentially moving forward with them.

2. C3 lentiviral delivery - future directions:

The development of lentiviruses based on the designed C3-expressing FUGW and Fuginsin plasmids, especially FU(C32AGFP) and Fuginsin(C3GFP) truncated, will be a major progress towards a more thorough understanding of the benefits of this new strategy with the expected outcome being prolonged RhoA inhibition and consequent axonal regeneration both *in vitro* and *in vivo*. The importance of developing such strategy for *in vivo* therapy was underscored a long time ago, as it was determined that administration of non permeable C3 at the crush site of injured optic nerves resulted in thriving but short lived axonal regeneration (19). Other studies have used C3-like chimeric proteins whereby it was fused to various transport peptides to achieve enhanced translocation across cellular plasma membranes. However, with this approach C3 administration into neuronal cell lines resulted in effective RhoA inactivation for only up

to 36 hours, suggesting that after this period of time C3 like proteins were no longer active or were all taken up (18). In contrast, C3 was detected by ICC in PC12 cells transfected with some of the produced plasmids up to 48 hours after transfection, and the functionality analysis revealed that at least 84 hours post transfection C3 was able to counteract the inhibitory effects of CSPGs, leading to increased neurite outgrowth. It is expected that with application of lentiviral vectors based on constructed FUGW and Fuginsin plasmids the duration of C3 expression in infected cells will be increased significantly more, leading to active cellular C3 for the period of time necessary for complete recovery.

However, it is also acknowledged that the future lentiviruses based on the currently produced plasmids hardly will be perfect vehicles for C3 introduction into the CNS, and there are still a lot of issues to be considered, ranging from the level of C3 expression to the targeting of particular neuronal cell types. Thus, several generations of lentiviruses will be needed for the creation of an efficient and safe therapeutic strategy for CNS regeneration.

One of our goals regarding lentiviral C3 delivery is to accomplish cell-type specific C3 expression only in neuronal cells of interest. Previous studies have shown that C3 introduction into astrocytes, the main components of the glial scar, leads to substantial increase in their neurite outgrowth and branching, which is extremely disadvantageous for CNS regeneration (40). Moreover, it was also observed that C3 leads to a microglial inflammatory response (41). Therefore achieving neuronal specifity in lentiviral C3 delivery is necessary for avoiding unwanted immune response and effect on or by glial cells.

There are several types of neuronal cells of interest for specific C3 lentiviral targeting. For example, in PD, one would want to protect or replace the dopaminergic (DA) neurons of substantia nigra (SN). In the case of spinal cord injury (SCI) or amyotrophic lateral sclerosis (ALS), motor neurons are relevant lentiviral targets. In the case of brain injury or stroke, only the transduction of damaged neurons is desired. It was decided that DA neurons will be the first type that will be transducted with the C3 expressing lentiviruses in this study.

Two main approaches can be employed for accomplishing targeted lentiviral delivery: promoter-based strategy and envelope-based strategy (38). Both can be incorporated into the enhancement of the design and application of the future generations of lentiviral vectors.

Promoter-based strategy

This strategy is based on the incorporation of a specific cell-type endogenous promoter into the lentiviral plasmid leading to the transgene expression only in the cells activating or using this promoter. The current FUGW and Fuginsin C3-containing lentiviral plasmids use the ubiquitin C (UbC) promoter that has been shown to induce transgene expression for a prolonged period of time in only neuronal cells (42). Such promoters might be advantageous for acute brain injury or stroke treatment where the specific type of affected neurons is unknown. However, the UbC promoter is not suitable for targeting DA neurons of the SN due to its lack of specificity. This issue can be circumvented in the future by replacing the existing UbC promoter with a 2.5 kb tyrosine hydroxylase (TH) promoter which has been shown to be specific for induction of

transgene expression in DA neurons alone (43). Thus, such modification will lead to a more precise targeting of this type of neuron without affecting surrounding cells.

Envelope-based strategy

This strategy leads to increased specificity of lentiviral transgene delivery by utilizing envelope proteins that can only bind to specific receptors located on the neuronal cells of interest. According to the current design, the C3 containing FUGW plasmids will be pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G) while Fuginsin plasmids with the glycoprotein of rabies virus. Even though several studies linked VSV-G pseudotyping with increased neuronal transduction (44), it seems that achievement of targeted expression was due mainly to a choice of promoter rather than utilization of VSV-G (38). Nevertheless, administration of VSV-G lentiviruses was shown to lead to a stable and long term transgene expression (45), and thus, together with utilization of a promoter specific to neuronal cells it can result in a feasible therapeutic strategy targeting only neuronal cells. Pseudotyping with Rabies - G glycoprotein is also advantageous, especially in regards to targeting motor neurons, as it was shown to induce efficient retrograde gene transfer from peripheral intramuscular injections to this poorly accessible type of neuron (46, 47). Thus, the use of both promoter-based and envelope-based strategies will be incorporated in the design of future lentiviral vector generation.

Another issue that is important to consider with regards to lentiviral delivery of C3, as was observed with FU(C3)W plasmid, is regulation of the C3 expression. Currently, no regulatory system is incorporated into FUGW or Fuginsin C3-expressing constructs. Due to this, lentiviral C3 expression will solely depend on the activity of UbC promoter, on the number of lentiviruses present in one cell, and on the nature of the

neuronal cell. Such lack of control is disadvantageous because C3 expression might be therapeutic only during a regenerative period, after which its effects might not be beneficial. Additionally, sustained strong C3 expression might be cytotoxic to the neuronal cells, and thus its expression needs to be under stringent control.

To overcome this potential drawback of our current strategy, several regulatory gene expression systems can be employed in future generations of C3 lentiviruses. For instance, C3 expression can be modulated through incorporation in the lentiviral FUGW or Fuginsin plasmids tetracycline (Tet) regulated system. This system is based on the tetracycline-resistance operon of *Escherichia Coli* where in the absence of tetracycline Tet repressor (TetR) protein binds to the Tet operator (tetO) and inhibits transcription of the genes downstream of tetO. The first regulatory gene expression system based on this *E. coli* operator was created by constructing tet-controlled transactivator (tTa) by means of fusing TetR to the herpes simplex virus VP16 transactivation domain and by placing several tandem copies of TetO upstream of the transgene promoter (48). This system produces transgene expression only in the absence of tetracycline when tTa is bound to the tetO sequences and abolishment of transgene expression with administration of antibiotic. Later, this Tet-Off system was modified into a Tet-On system whereby tTa acquired reverse phenotype (rtTA) and led to transgene expression upon antibiotic treatment (49).

In order for this system to work, both tetO-harbored transgene and rtTA must be present in the same cell. In the past this was achieved by either separating the necessary elements of this system on two different lentiviral vectors or, alternatively, placing them all on one lentivirus (38). Given the current design of lentiviral FUGW and Fuginsin C3

containing plasmids, the first approach is more attractive due to the limiting cloning capacity of these lentiviral plasmids. Overall, incorporation of this system might lead to the desired sustained C3 delivery only for a particular period of time controlled by antibiotic administration. However, there are certain drawbacks even with this approach due to the observed "leakiness" of the Tet system in the absence of an inducer (50). However, with the future improvement of the functionality of the elements of this system such as rtTA, this issue might be circumvented (51).

Alternatively, controlled C3 expression might be achieved through incorporation of the Rheo-switch mammalian inducible expression system composed of synthetic ligand receptor RSL1 and engineered nuclear receptor, the Rheoreceptor-1 protein. Implementation of this system leads to both specific induction and fine-tuning of the transgene expression (52) and compared to the Tet-On system, results in practically no background expression. Thus, control of C3 expression can be achieved through a variety of methods which will be explored in the future.

It must be understood that even with a stable and sustained C3 expression level in the neuronal cells of interest, which is going to take multiple generations of lentiviral vectors with a variety of modifications, the goal of CNS functional regeneration still remains unreached. That is, even with axonal outgrowth through the non-permissive environment of the adult CNS, it is unlikely that the lost synapses will be recreated and the damaged neuronal pathways will be restored.

During development, specific neuronal circuits are formed due to positive and negative cues exerted by a variety of guidance molecules, such as netrines, semaphorins, slits, and ephrins. Based on the balance of attractive and repulsive forces established by a

diversity of these signals, the axonal growth cone can either continue on its path, stop, collapse, turn, or retract (53, 54). Potentially, this guidance system can be manipulated for restoration of the lost neuronal connections due to the CNS injury. However, a variety of limitations exist for successful employment of this system. For instance, even though guidance factors and their correspondent receptors remain to exist in the adult CNS, their expression patterns are altered (55), and further changes in their gene expression occur after damage to the CNS (54).

The overall outcome of these changes leads to a creation of an environment characterized by a scarcity of a variety of positive cues and a predominance of negative cues. Moreover, upon injury the non-permissive environment of the CNS becomes even more hostile to axonal regeneration due to the release of a variety of reactive immune cells, oxygen species and astrocytes which can lead to an alteration of the integration response of the growth cones to the guidance cues (9).

Based on these observations, it can be concluded that the reformation of the damaged neuronal pathways, even with achieving axonal regeneration through lentiviral C3 delivery, constitutes an extremely complicated process. Nevertheless, the task of generating such a strategy is not impossible. For example, one study achieved sensory axon regeneration upon dorsal root injury throughout the dorsal horn with the reestablishment of lost neuronal connections by administration of adenoviruses containing either positive (nerve growth factor) or negative (Sema3A) cues in a particular spacial and temporal manner (56). Thus, it seems that exogenous administration of appropriate guidance molecules is possible and leads to successful reestablishment of the

lost synaptic connections, albeit a unique strategy is required depending on the nature of the injury and its location in the CNS.

It is clear that achieving functional CNS recovery will remain an elusive goal for the foreseeable future. Nevertheless, as more is learned about molecular transduction pathways obstructing CNS regeneration, and as we identify important molecular switches governing the decrease of the intrinsic neuronal regenerative capacity, potentially effective neuronal recovery strategies such as the lentiviral C3 therapy explored in this study will be created. Implementation of such therapeutical approaches together with strategies for controlling axonal guidance will more than likely result in the repair of damaged neuronal pathways and lead to unprecedented improvement in those patients suffering from CNS injuries and neurological disorders.

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Tables and Figures

Final construct	Vector	Template	Insert	Restriction enzymes
pEGFPN2(C32AGFP)	pEGFPN2	pRK5 - C3	C32AGFP	KpnI and EcoRI for both vector and insert
pEGFPC1(GFP2AC3)	pEGFPC1	pRK5 - C3	GFP2AC3	KpnI and EcorI for both vector and insert
FU(C3)W	FUGW	pEGFPN2 (C32AGFP)	C3	AgeI and EcorI for both vector and insert
FU(C3GFP)W	FUGW	pRK5-C3	C3GFP	NheI and EcorI for both vector and insert
FU(C32AGFP)W	FUGW	pEGFPN2 (C32AGFP)	C32AGFP	AgeI and EcorI for both vector and insert
FU(GFP2AC3)W	FUGW	pEGFPC1 (GFP2AC3)	GFP2AC3	Age I and SmaI for insert Ecor I/AgeI for vector (followed by blunting)
Fuginsin (C3)	Fuginsin (C3GFP)	N/A	N/A	AleI and BplI followed by blunting
Fuginsin (C3GFP)	Fuginsin	pRK5-C3	C3GFP	AgeI only for both vector and insert
Fuginsin(C3GFP) tr.	Fuginsin (C3GFP)	N/A	N/A	ApaI and BplI followed by blunting
Fuginsin (C32AGFP)	Fuginsin	pEGFPN2 (C32AGFP)	C32AGFP	BplI and AgeI for both vector and insert
Fuginsin (GFP2AC3)	Fuginsin	pEGFPC1 (GFP2AC3)	GFP2AC3	BplI/AgeI for both vector and insert

Table 1 Description of vectors, templates, and restriction enzymes employed in construction of each type of FUGW or Fuginsin plasmid.

Constructs	Enzymes	Expected bands with succesful ligation	Expected bands with unsuccesful ligation
FUGW (control)	AgeI/EcorI	700 bp	Not applicable
FU(C3)W	AgeI/EcorI	700 bp	3 kb
FU(C3GFP)W	EcorI/NheI	1.5 bp	0bp (linearization)
FU(C32AGFP)W	AgeI/EcorI	1.5 bp	700 bp
FU(GFP2AC3)W	AgeI/EcorV	800 bp, 1.5 kb	700 bp, 800 bp, 80 bp
Fuginsin (control)	AgeI/KpnI	1.3 kb	Not applicable
Fuginsin (C3)	EcorI/NheI	900 bp	865 kb, 1.5 kb
Fuginsin (C3GFP)	AgeI/KpnI	700 bp, 1.2 kb	1.2 kb only
Fuginsin(C3GFP)tr.	AgeI/NheI	700bp, 1.8 kb	700 bp, 3.1 kb
Fuginsin(C32AGFP)	KpnI/NheI	1.8 kb	0 bp (linearization)
Fuginsin(GFP2AC3)	AgeI/NheI	2.5 kb	1 kb

Table 2 Description of specific restriction enzymes employed for confirmation of succesful construction of each type of FUGW and Fuginsin plasmid which was determined through appearance of expected bands on an agarose gel upon digestion.

Type of FUGW or Fuginsin plasmid		Description
FUGW	**Fuginsin**	Control plasmids
FU(C3)W	**Fuginsin(C3)**	Plasmids expressing only C3
FU(C3GFP)W	**Fuginsin(C3GFP) and Fuginsin(C3GFP) truncated**	Plasmids expressing C3 fused to GFP
FU(C32AGFP)	**Fuginsin(C32AGFP)**	Plasmids expressing C32A and GFP
FU(GFP2AC3)	**Fuginsin(GFP2AC3)**	Plasmids expressing C3 and 2AGFP

Table 3 Various types of FUGW and Fuginsin plasmids produced for lentiviral construction.

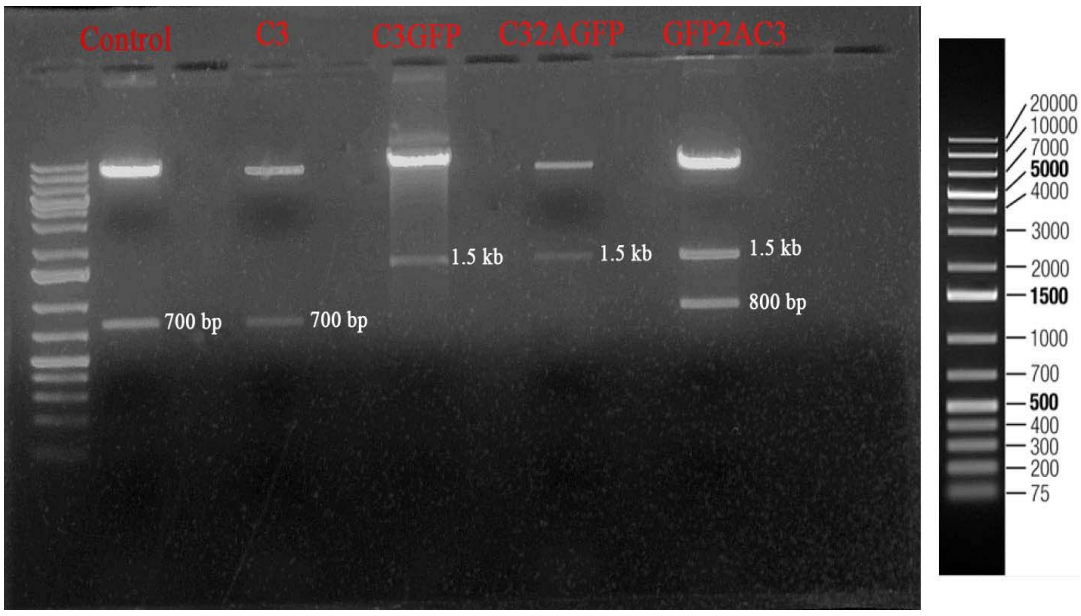

Figure 1 Restriction enzyme digestion of FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W, and FU(GFP2AC3)W plasmids as was described in *Table 2* in order to confirm their successful production.

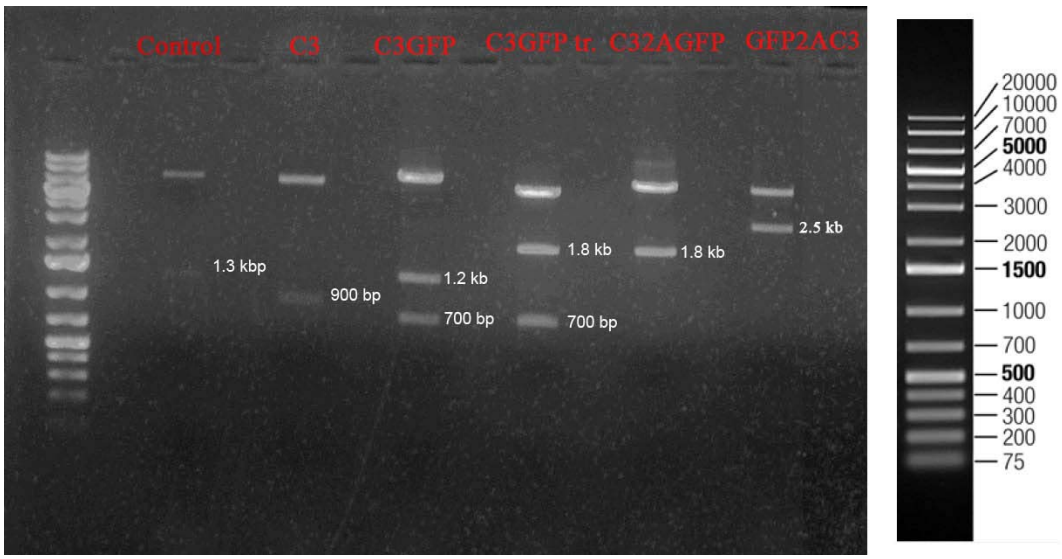

Figure 2 Restriction enzyme digestion of Fuginsin, Fuginsin(C3), Fuginsin(C3GFP), Fuginsin(C3GFP) truncated, Fuginsin(C32AGFP), and Fuginsin(GFP2AC3)W plasmids as was described in *Table 2* in order to confirm their successful production.

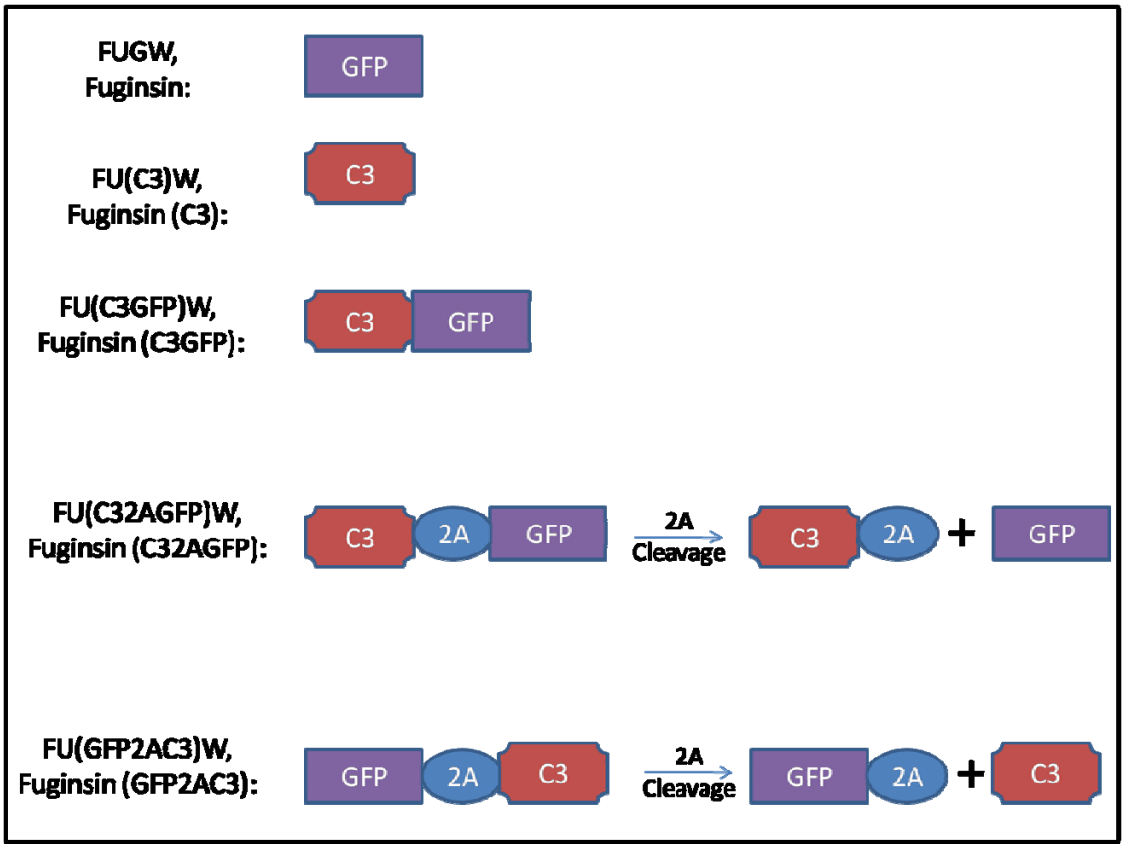

Figure 3 Schematic representation of the expected C3 and/or GFP cellular expression when transfected with correspondent plasmids.

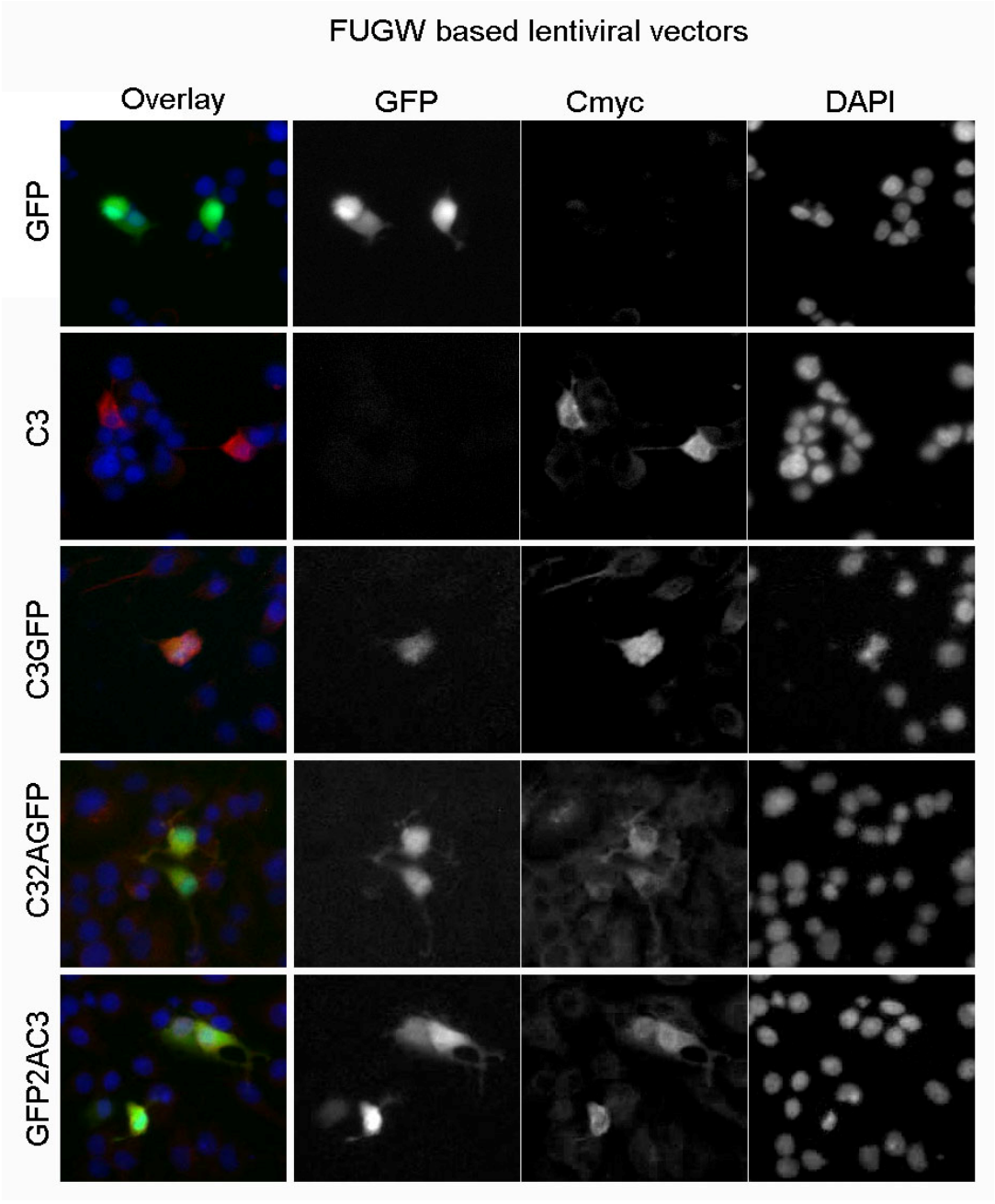

Figure 4 ICC panel representing FUGW (row 1), FU(C3)W (row 2), FU(C3GFP)W (row 3), FU(C32AGFP)W (row 4), FU(GFP2AC3)W (row 5) using anti - myc antibodies for visualization of C3 (red). Green pertains to endogenous GFP. DAPI was used to visualize nuclei (blue).

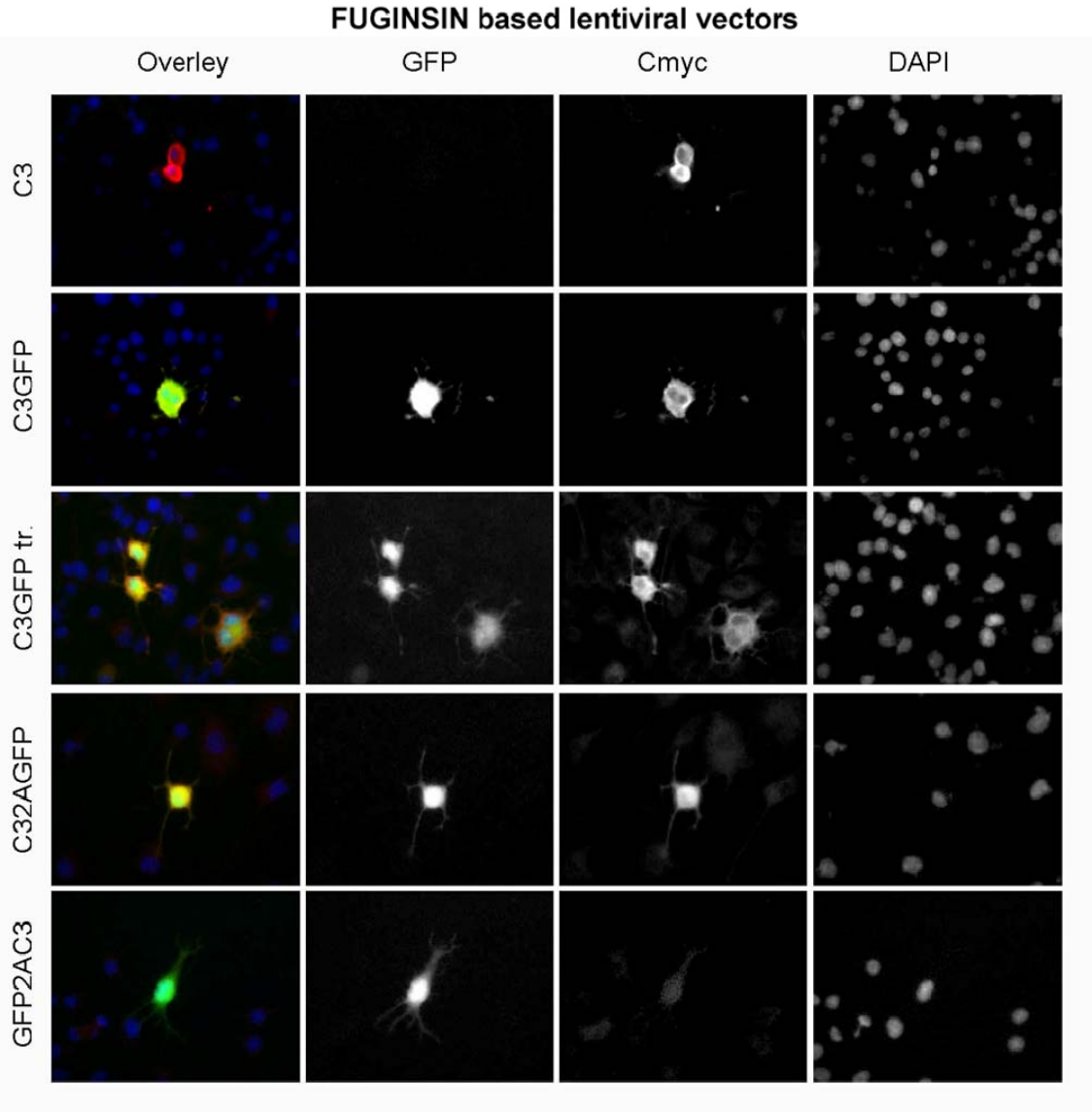

Figure 5 ICC panel representing Fuginsin(C3) (row 1), Fuginsin(C3GFP) (row 2), Fuginsin(C3GFP) truncated (row 3), Fuginsin(C32AGFP) (row 4), and Fuginsin(GFP2AC3) (row 5) using anti - myc antibodies for visualization of C3 (red). Green pertains to endogenous GFP. DAPI was used to visualize nuclei (blue).

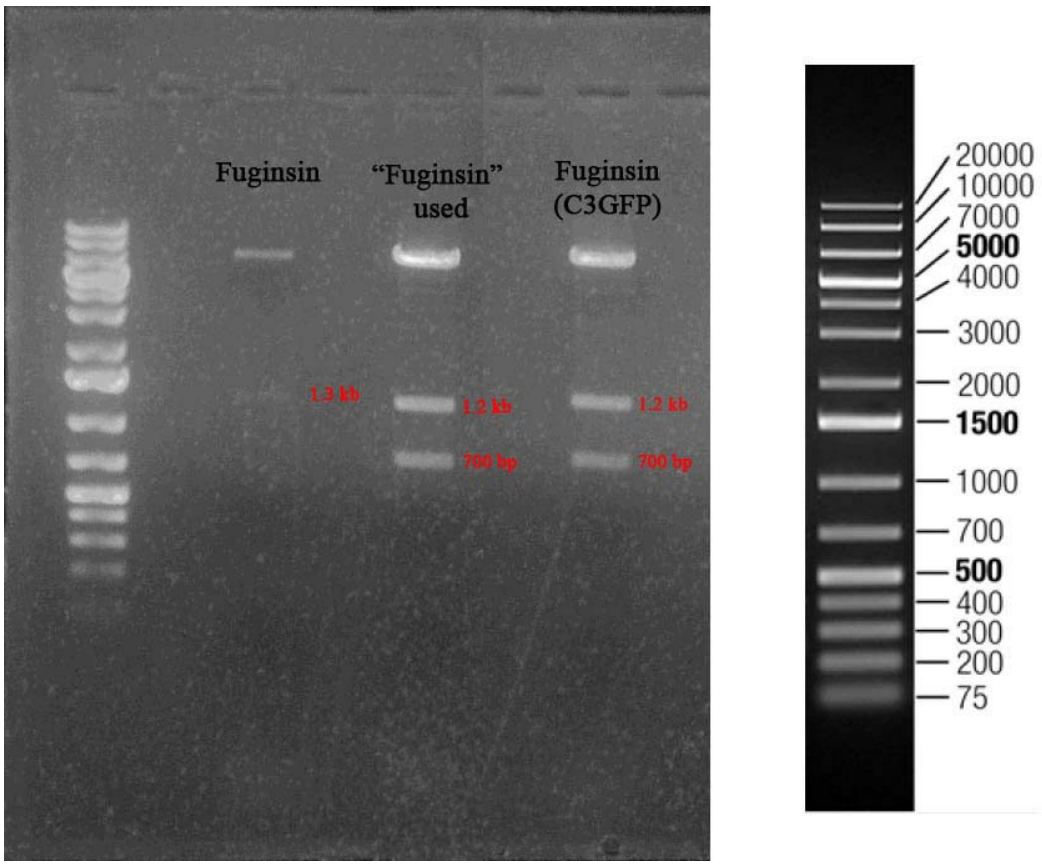

Figure 6 Restriction enzyme digestion of Fuginsin (lane 1), the plasmid used in all studies as control Fuginsin (lane 2), and Fuginsin (C3GFP) with AgeI and KpnI. The identity of the plasmid used as control was determined to be Fuginsin(C3GFP**).**

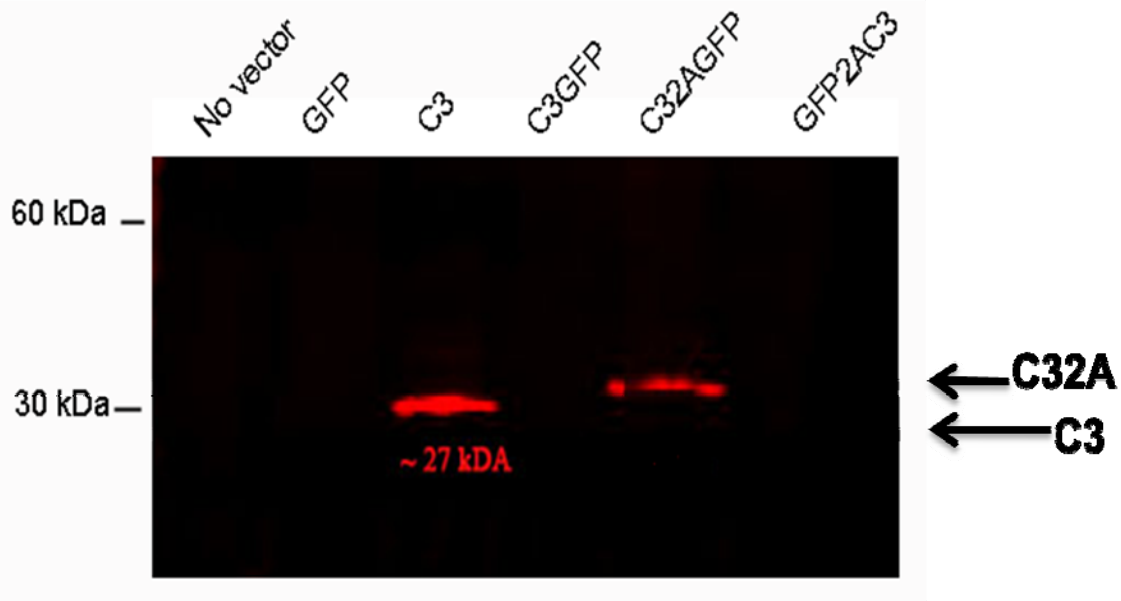

Figure 7 Western Blot of 293T cell lysates 24 hours after transfection with FUGW plasmids using anti-myc antibody for detection of C3. From left to right: No vector, FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W and FU(GFP2AC3)W.

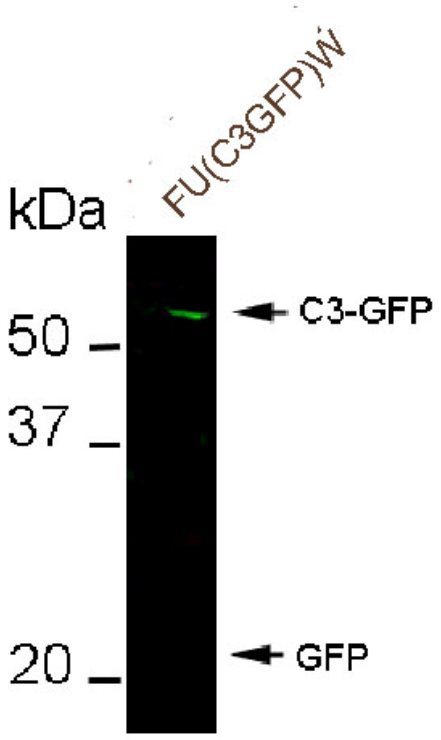

Figure 8 Western Blot of 293T cell lysate 24 hours after transfection with FU(C3GFP)W plasmid using anti-C3 antibody.

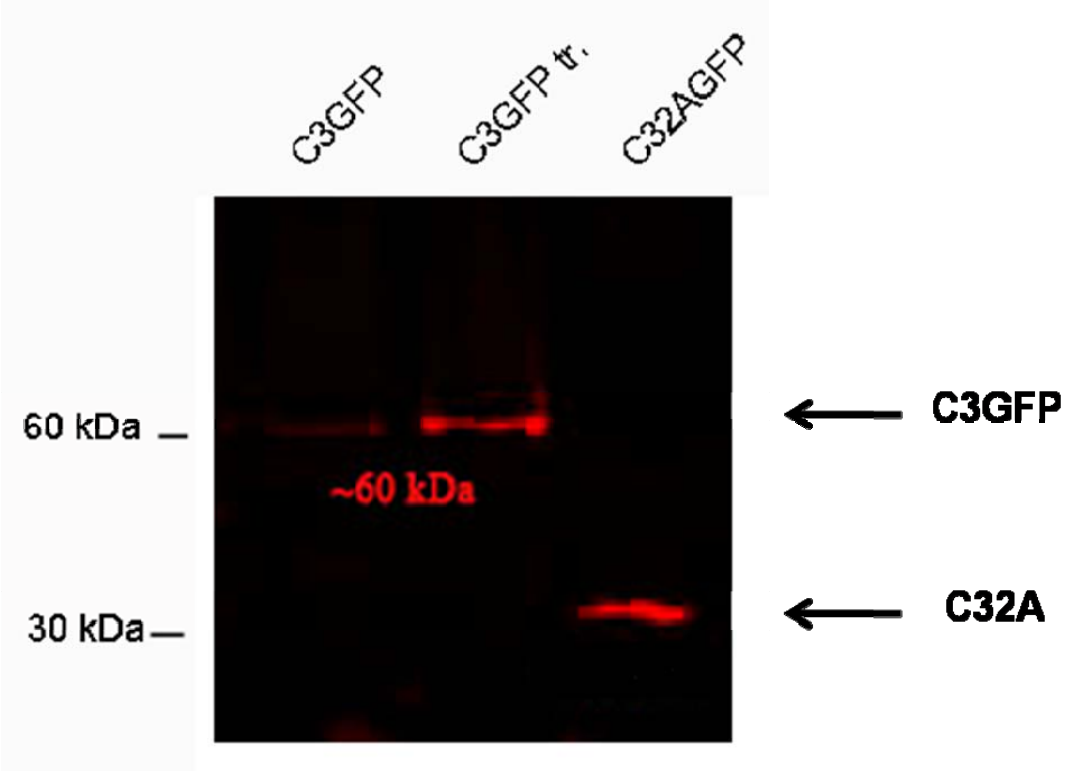

Figure 9 Western Blot of 293T cell lysates 24 hours after transfection with Fuginsin C3 -containing plasmids using anti-myc antibody. From left to right: Fuginsin(C3GFP), Fuginsin(C3GFP) truncated, and Fuginsin(C32AGFP).

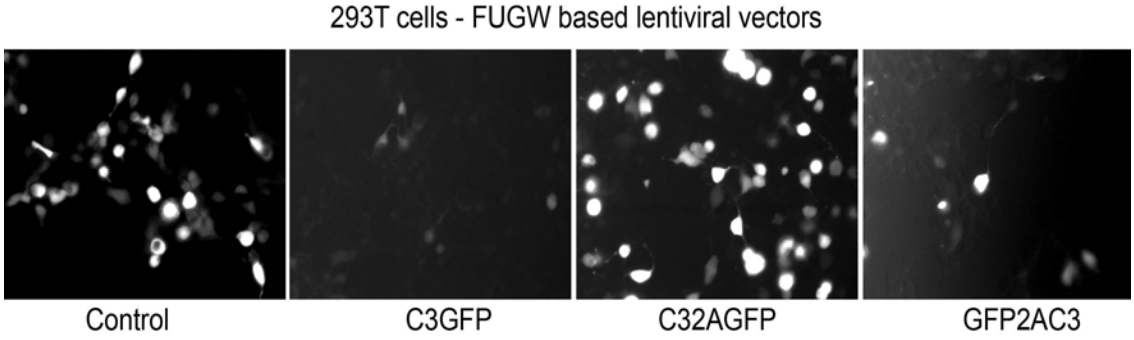

Figure 10 Visualization of GFP-expressing cells 48 hours after transfection with FUGW C3 - containing plasmids. From left to right: Control, FU(C3GFP)W, FU(C32AGFP)W, and FU(GFP2AC3)W.

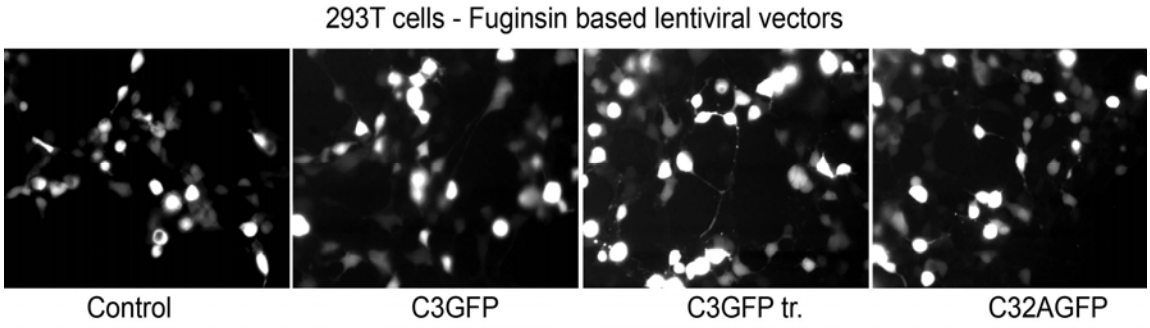

Figure 11 Visualization of GFP-expressing cells 48 hours after transfection with Fuginsin C3 – containing plasmids. From left to right: Control, Fuginsin(C3GFP), Fuginsin(C3GFP) truncated, and Fuginsin(C32AGFP).

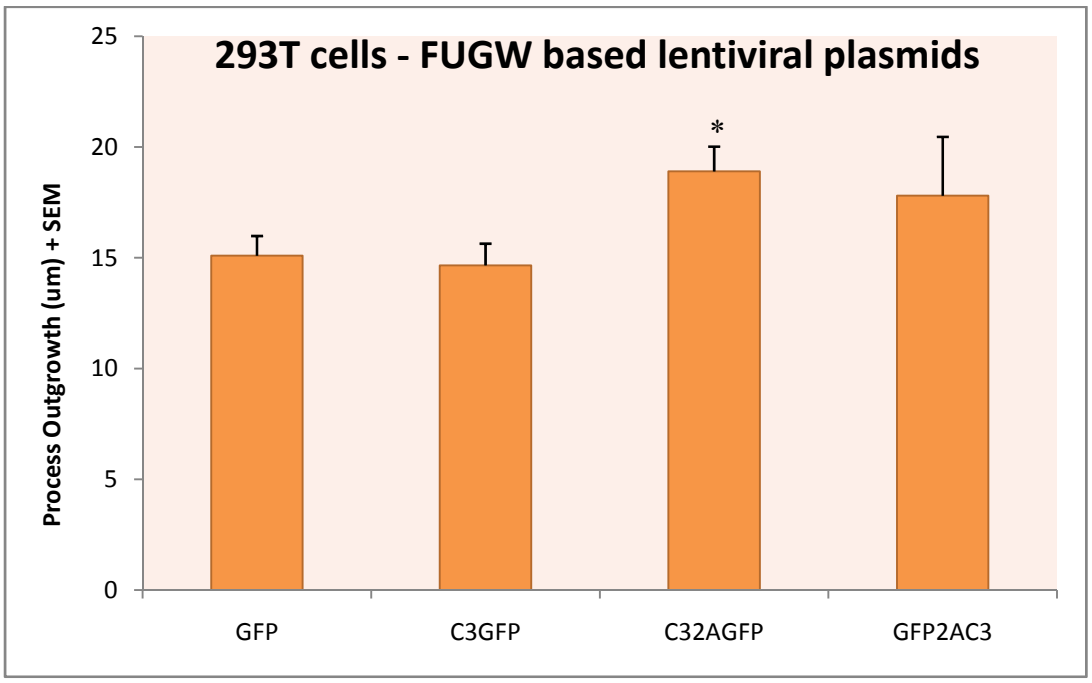

Figure 12 Process outgrowth (μm) of 293T cells plated on PLL. Processes were counted 48 hr after transfection with FUGW, FU(C3GFP)W, FU(C32AGFP)W or FU(GFP2AC3)W plasmids. * indicates a t-test $p<0.05$ as compared to control.

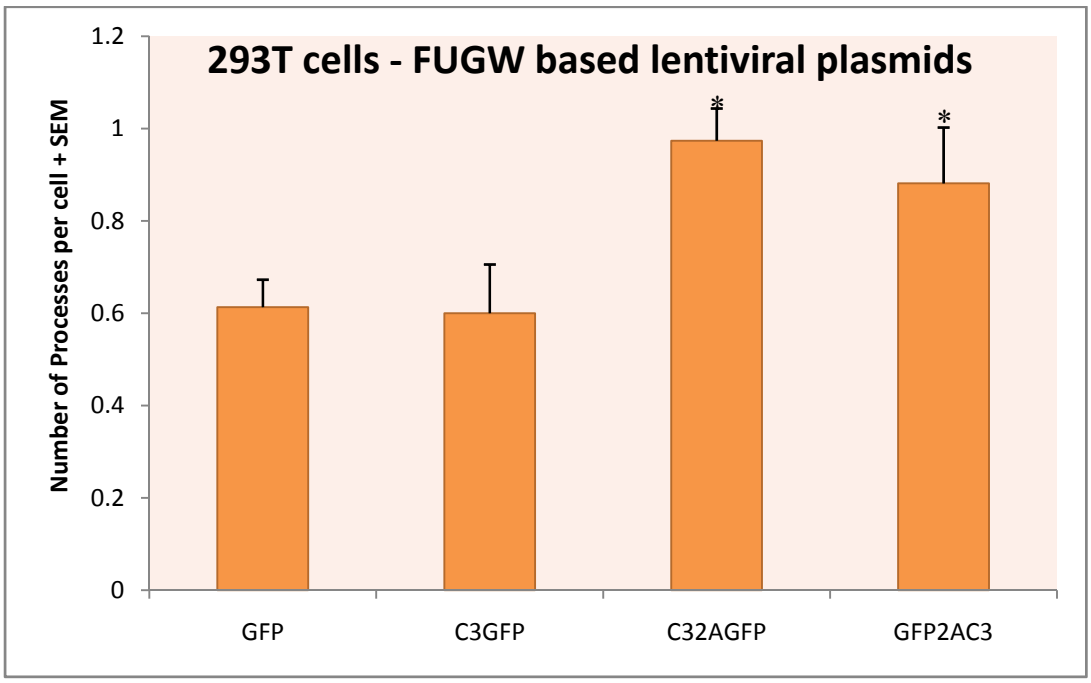

Figure 13 Number of processes per 293T cell plated on PLL. Processes were counted 48 hr after transfection with FUGW, FU(C3GFP)W, FU(C32AGFP)W or FU(GFP2AC3)W plasmids. * indicates a t-test $p<0.05$ as compared to control.

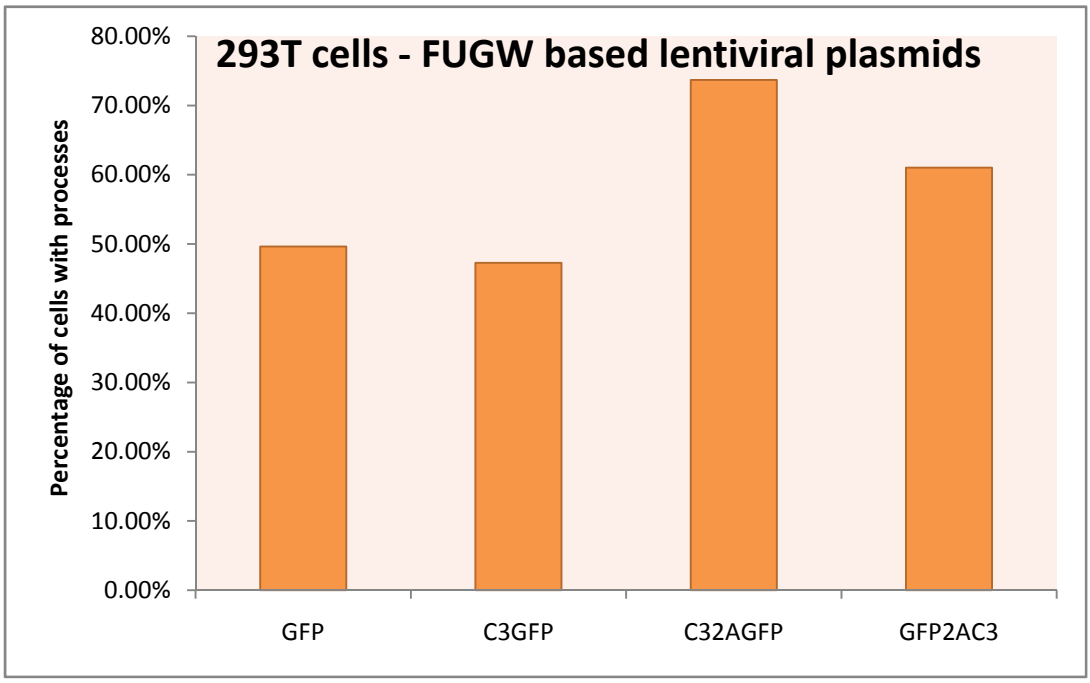

Figure 14 Percentage of 293T cells with processes plated on PLL. Processes were counted 48 hr after transfection with FUGW, FU(C3GFP)W, FU(C32AGFP)W or FU(GFP2AC3)W plasmids.

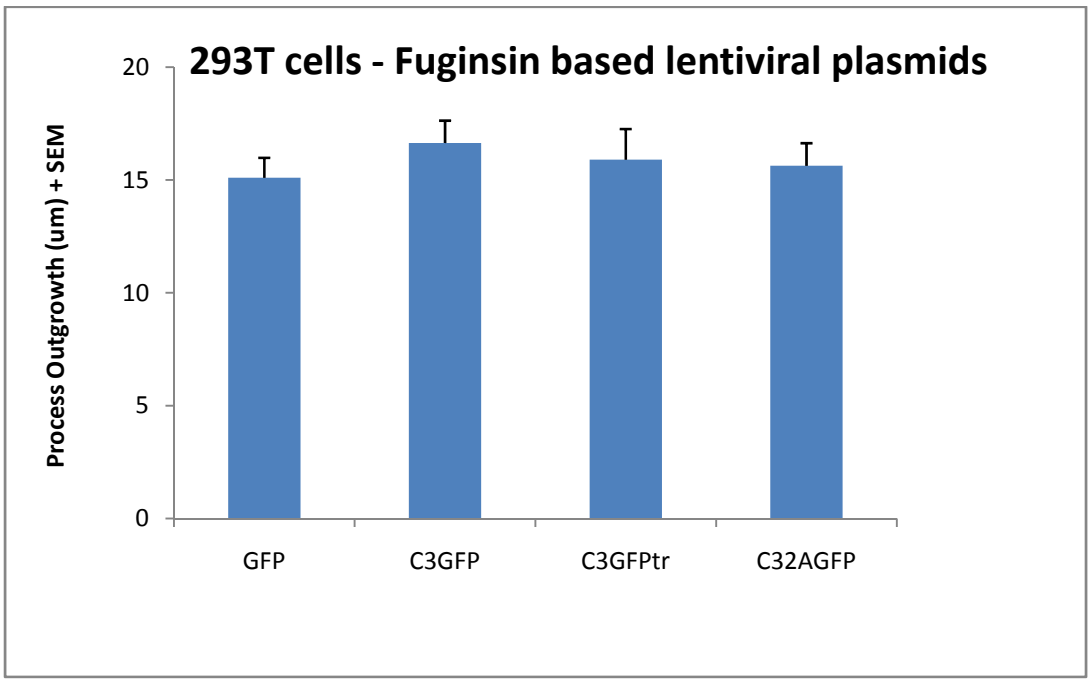

Figure 15 Process outgrowth (μm) of 293T cells plated on PLL. Processes were counted 48 hr after transfection with FUGW, Fuginsin(C3GFP), Fuginsin(C3GFP) truncated or Fuginsin(C32AGFP) plasmids. Only a trend in increased process outgrowth was seen compared to control vector.

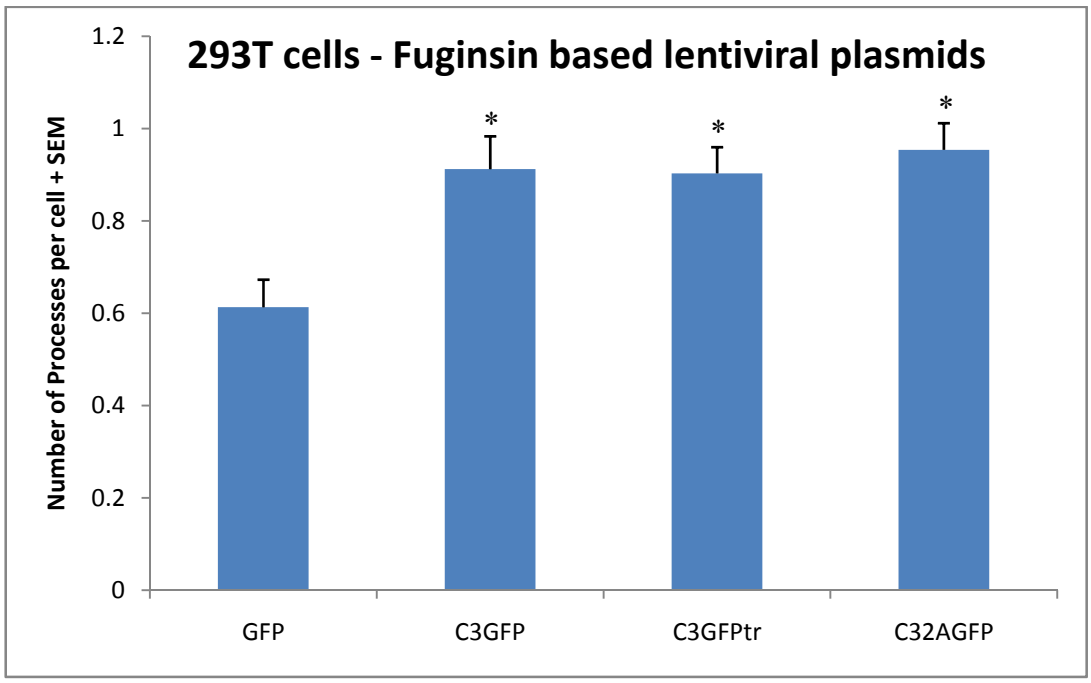

Figure 16 Number of processes per 293T cell plated on PLL. Processes were counted 48 hr after transfection with FUGW, Fuginsin(C3GFP), Fuginsin(C3GFP) truncated or Fuginsin(C32AGFP) plasmids. * indicates a t-test $p<0.05$ as compared to control.

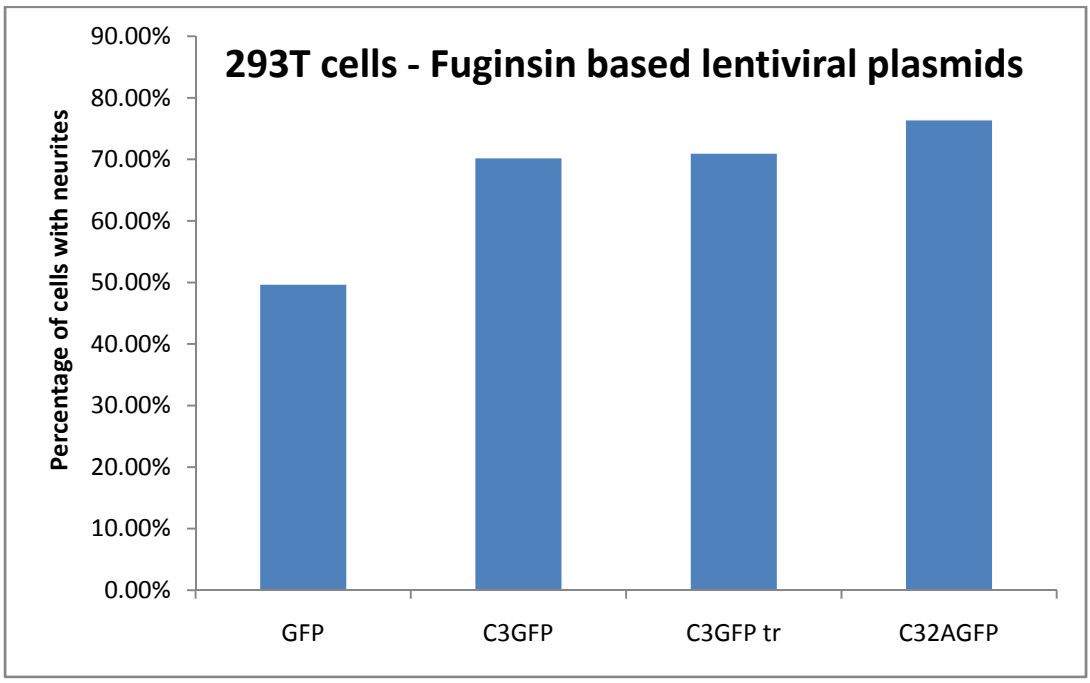

Figure 17 Percentage of 293T cells with processes plated on PLL. Processes were counted 48 hr after transfection with FUGW, FU(C3GFP)W, FU(C32AGFP)W or FU(GFP2AC3)W plasmids.

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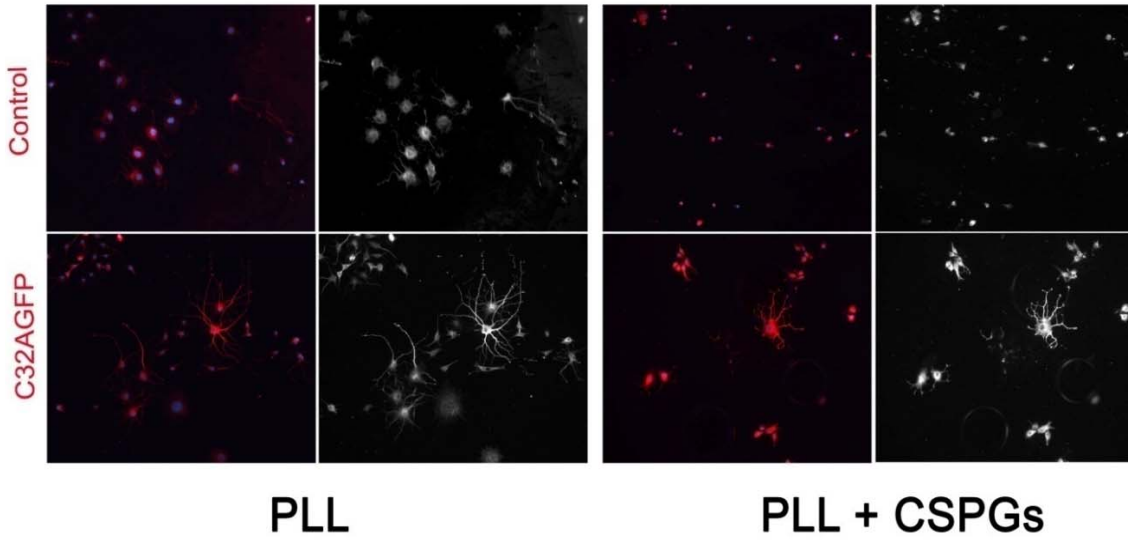

Figure 18 PC12 cells transfected with either no vector or FU(C32AGFP)W plasmids and immunostained with anti- βIII-tubulin antibody (red) for cytoskeletal staining. DAPI was used for nuclear visualization.

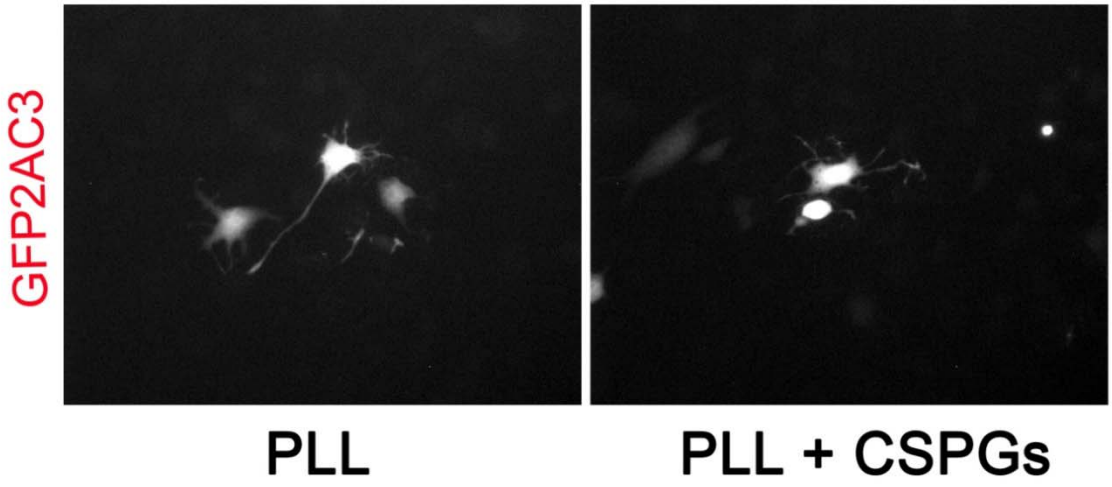

Figure 19 PC12 cells transfected with FU(GFP2AC3)W plasmid and immunostained with anti-βIII-tubulin antibody for cytoskeletal staining. Due to the antibody failure to stain cytoskeleton, the morphology of FU(GFP2AC3)W transfected cells was observed through GFP fluorescence.

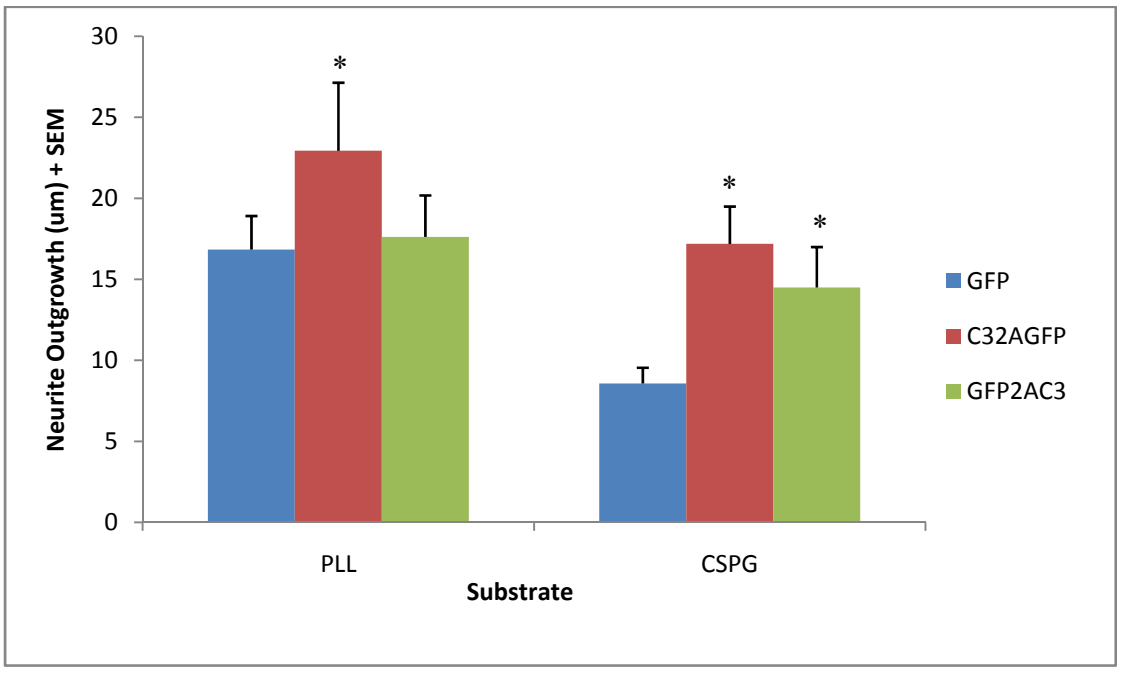

Figure 20 Neurite outgrowth (µm) of PC12 cells plated on PLL or PLL and CSPGs. Processes were counted 84 hr post transfection with either FUGW, FU(C32AGFP)W or FU(GFP2AC3)W plasmids. * indicates a t-test $p<0.05$ as compared to control.

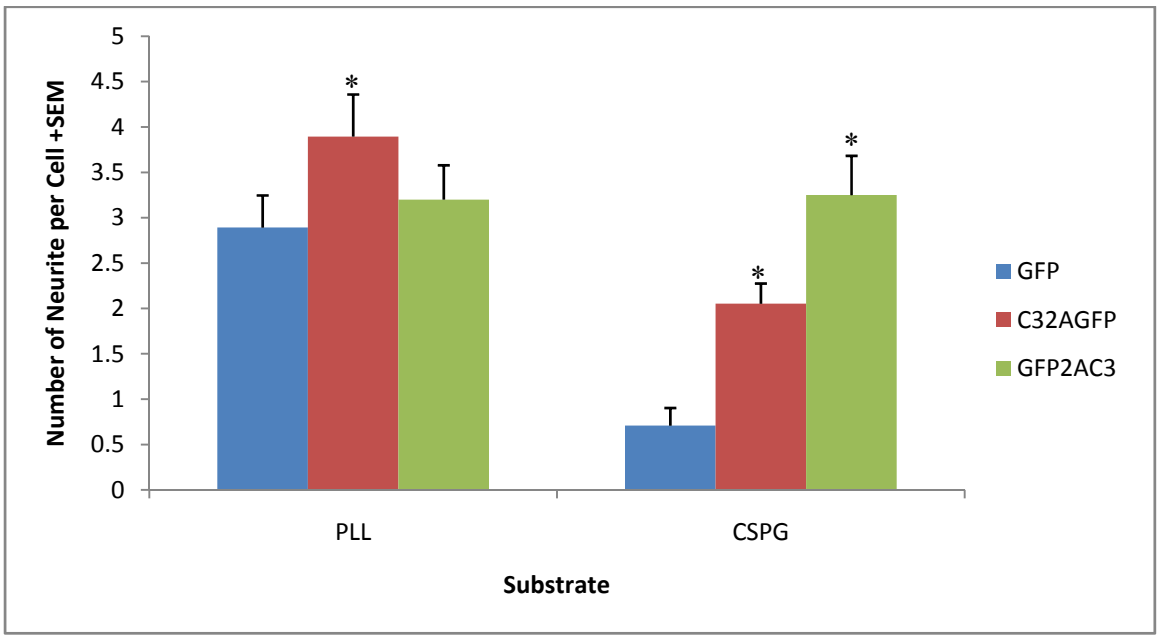

Figure 21 Number of neurites per cell of PC12 cells plated on PLL or PLL and CSPGs 84 hours after transfection with FUGW, FU(C32AGFP)W or FU(GFP2AC3)W plasmids. * indicates a t-test $p<0.05$ as compared to control.

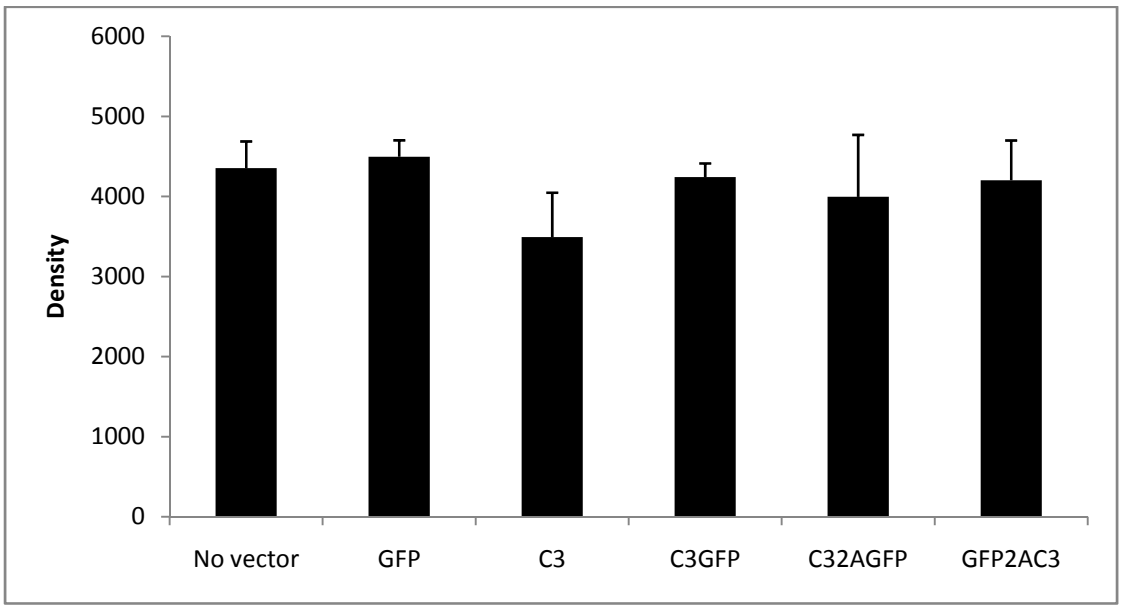

Figure 22 Effect of FUGW- based C3 expression on RhoA activation. Rhotekin assay was performed in triplicates on 293T cell lysates 24 hours post transfection. Density of RhoA immunostaining was measured using NIH Image software and graphed. From left to right: No vector, FUGW, FU(C3)W, FU(C3GFP)W, FU(C32AGFP)W and FU(GFP2AC3)W.

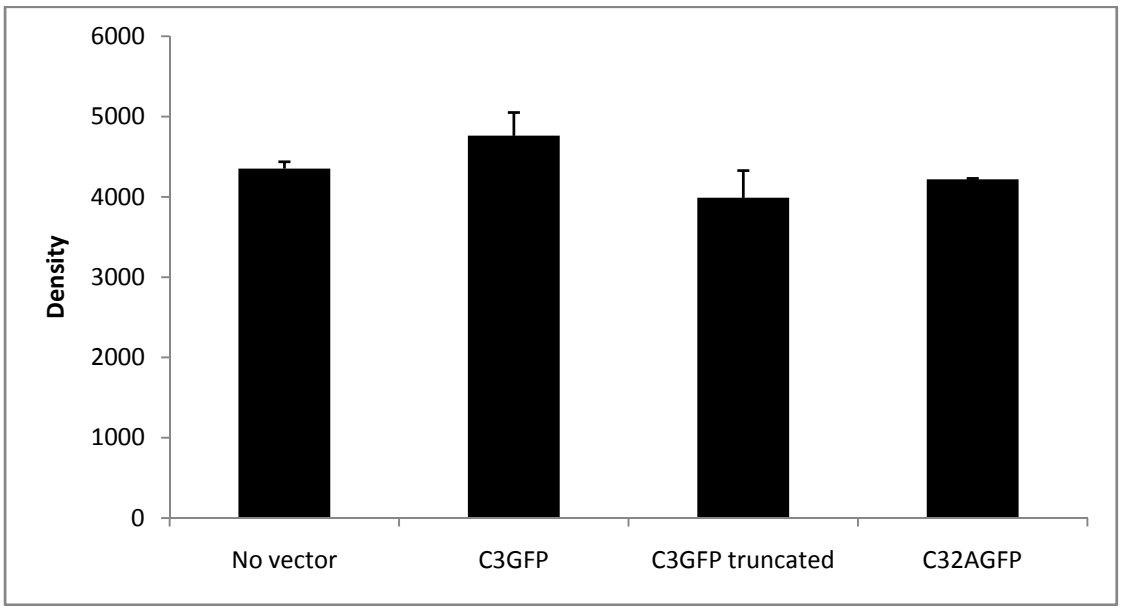

Figure 23 Effect of Fuginsin based C3 expression on RhoA activation Rhotekin assay was performed in triplicates on 293T cell lysates 24 hours post transfection. Density of RhoA immunostaining was measured using NIH Image software and graphed. From left to right: From left to right: No vector, Fuginsin(C3GFP), Fuginsin(C32AGFP), and Fuginsin(GFP2AC3).

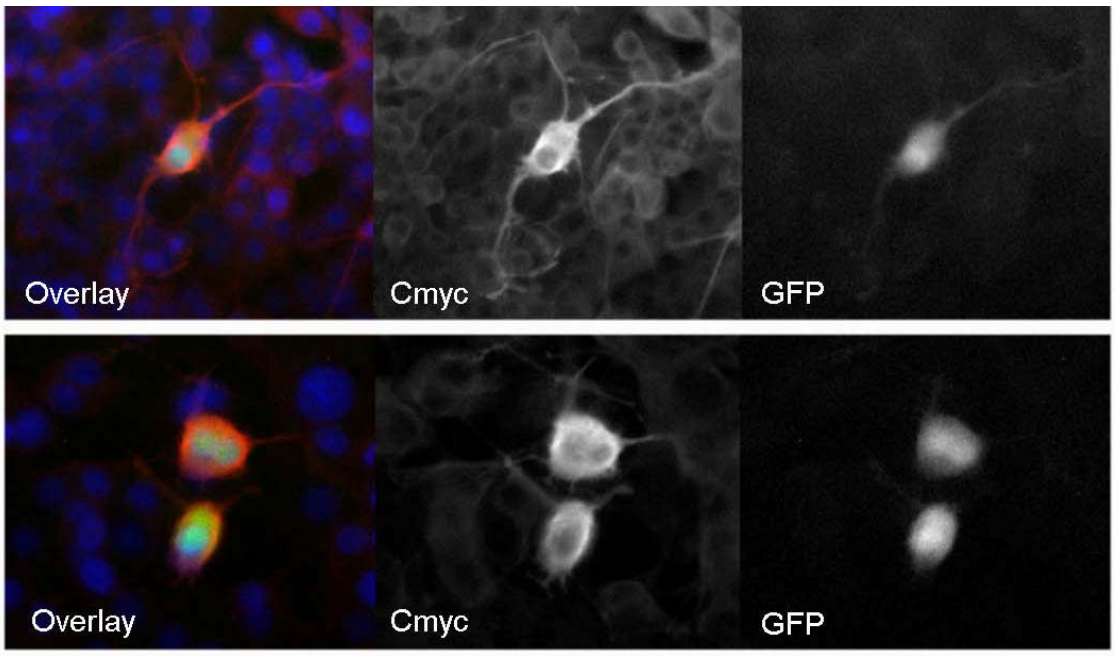

Figure 24 PC 12 cells were infected with 2ul of Fuginsin(C3GFP) truncated lentiviral vector an immunostained using an anti – myc antibody for visualization of C3 and anti – GFP antibody for visualization of GFP. Transduced cells show colocalization of GFP (green) and myc (red). DAPI was used to visualize nuclei (blue).

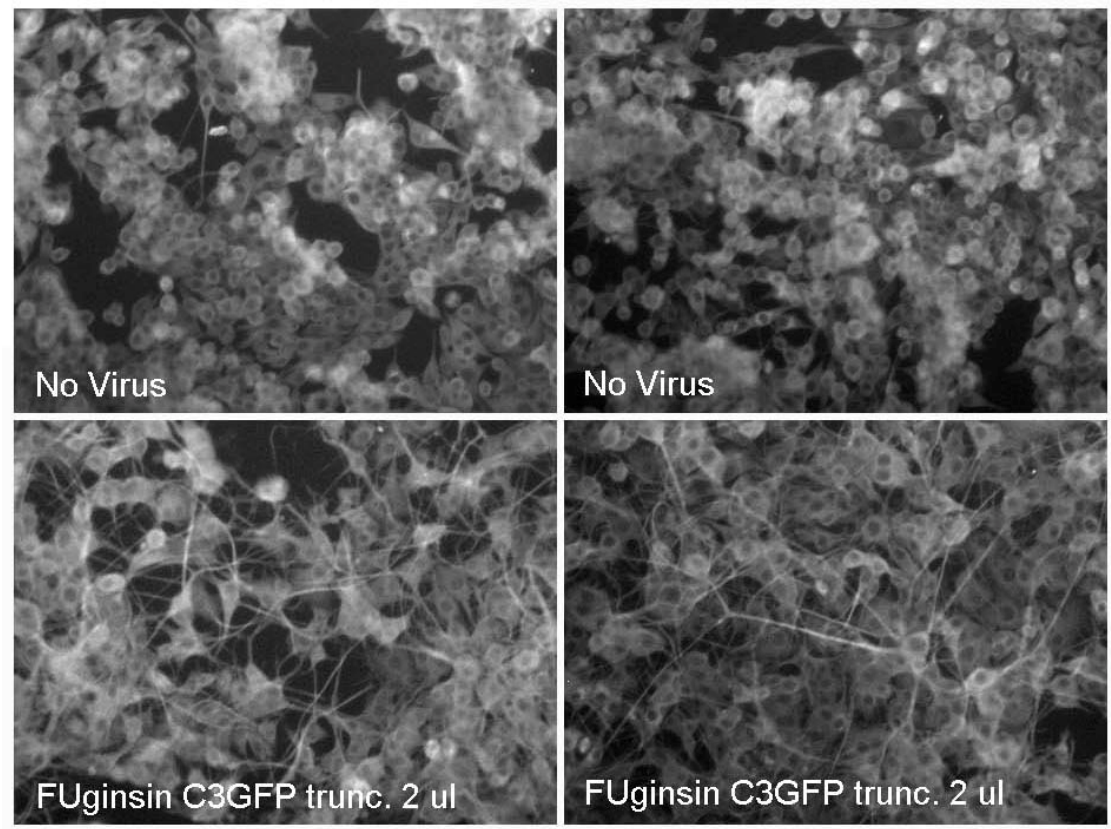

Figure 25 Morphological alterations of PC12 cells infected with 2ul of Fuginsin(C3GFP) truncated lentiviral vector compared to no virus control.