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Detection of a secreted metalloprotease within the nuclei of liver cells

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Abstract

ADAMTS13 is a secreted zinc metalloprotease expressed by various cell types. Here, we investigate its cellular pathway in endogenously expressing liver cell lines and after transient transfection with ADAMTS13. Besides compartmentalizations of the cellular secretory system, we detected an appreciable level of endogenous ADAMTS13 within the nucleus. A positively charged amino acid cluster (R-Q-R-Q-R-Q-R) present in the ADAMTS13 propeptide may act as a nuclear localization signal (NLS). Fusing this NLS-containing region to eGFP greatly potentiated its nuclear localization. Bioinformatics analysis suggests that the ADAMTS13 CUB-2 domain has a double-stranded beta helix (DSBH) structural architecture characteristic of various protein-protein interaction modules like nucleoplasmins, class I collagenase, tumor necrosis factor ligand superfamily, supernatant protein factor (SPF) and the B1 domain of neuropilin-2. Based on this contextual evidence and that largely conserved polar residues could be mapped on to a

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Competing Interests

The authors declare that they have no competing interests

Authors' Contribution

CK-S: conceived the study and helped prepare the manuscript

CA: performed the confocal microscopy studies

RH: construction of plasmids, transient transfections and subcellular fractionations

KH: performed the western blotting

SF: supplied cells and scientific discussion

SG: confocal microscopy and scientific discussion

RF: confocal microscopy

KS: provided the antibodies used in the study

PK: assisted in construction of plasmids

ES,GS & BS: conducted bioinformatic analysis

template CUB domain homolog, we hypothesize that a region in the ADAMTS13 CUB-2 domain with conserved polar residues might be involved in protein-protein interaction within the nucleus.

Introduction

ADAMTS proteases are a family of multi-domain secreted enzymes involved in an array of processes, including development, angiogenesis, arthritis and coagulation¹. One member of this family, ADAMTS13, is responsible for von Willebrand Factor (VWF) cleavage within circulating blood². Its synthesis occurs most abundantly in the liver,^{2,3} specifically within hepatic stellate cells^{4,5}. The ADAMTS13 protein has the basic structure of all ADAMTS proteases, but differs from other members by bearing two C-terminal CUB (complement C1r/C1s, Uegf (EGF-related sea urchin protein) and BMP-1 (bone morphogenic protein-1)) domains. Recent work suggests the CUB domains are important for activity under shear conditions⁶, in secretion⁷ and in binding of ADAMTS13 to its substrate, VWF⁸. The CUB domains may also function in apical sorting of ADAMTS13 so that it may be secreted properly⁹. Given its multi-domain architecture, biological function beyond that seen in an extracellular context is a tenable possibility, but this subject remains largely unexplored. We sought to explore novel roles of ADAMTS13 within the intracellular environment.

Results

Finding ADAMTS13 in nuclei of liver cells

The cellular localization of endogenous ADAMTS13 was tracked in a panel of liver cell lines, including LX2, Huh-7 and 7404 cells. One polyclonal and two monoclonal ADAMTS13 antibodies were used to follow the secretory pathway of ADAMTS13. These antibodies recognize epitopes in different domains along the ADAMTS13 polypeptide (Table 1). All antibodies localized ADAMTS13 to the cytoplasm of the liver cells studied, yet we also saw significant colocalization with the nuclear stain, DAPI (Figure 1A)—an unforeseen cellular localization for a protein understood for its function within circulating blood. We also examined the localization seen in transiently transfected HEK293 cells overexpressing ADAMTS13. It is worth noting that in this higher-expressing setting, the protein accumulated predominantly within the cytosol. We could trace ADAMTS13 to the endoplasmic reticulum and Golgi apparatus at various time points (Figure 2). To further survey this novel cellular localization, LoVo (human colorectal adenocarcinoma) cells were transiently transfected with WT ADAMTS13, harvested and separated into nuclear and cytoplasmic fractions. The two fractions were subsequently probed for the C-terminal V5 tag on recombinant ADAMTS13 and for calnexin, to exclude the possibility that the nuclear fraction was contaminated with ER membrane proteins (Figure 1B). The resulting Western blot supports the observations from confocal imaging: ADAMTS13 signal is seen in both cytoplasmic and nuclear fractions. Thus, although the exact proportion of protein accumulating within the nucleus may differ, ADAMTS13 can be detected in the nucleus both endogenously and following transient transfection.

Bioinformatic screening and identification of a potential nuclear localization signal

Clusters of surface-exposed, positively charged residues can direct proteins to the nucleus and are termed nuclear localization signals (NLSs). Such clusters have been shown to play a critical role in interacting with the heterodimeric import receptor complex, which ultimately facilitates nuclear translocation^{10,11}. An automated search of the ADAMTS13 peptide for the presence of a nuclear localization signal using the PSORT/PSORT II suite of programs¹² did not reveal any hits. Manually searching the ADAMTS13 sequence did reveal a cluster of positively charged amino acids located in the C-terminal region of the propeptide,

specifically 67-RQRQRQR-74, which could potentially act as a NLS seeing that such sequences have been shown to target nascent proteins to the cell nucleus¹³. A subset of this sequence (RQRR) in ADAMTS13 is acted upon by the propeptide convertase furin, although cleavage of the propeptide is not important for its activation or secretion¹⁴. A multiple sequence alignment of the propeptide region in ADAMTS13 homologs shows conservation of this positively charged region across a range of organisms (Figure S1). To examine the ability of these residues to function as a NLS, we fused the putative NLS and its flanking residues (Pro30-Asp99) to an exogenous protein, eGFP, which typically accumulates within the cytoplasm (Figure 1C, WT). We investigated the subcellular localization of this fusion construct in LoVo cells, which are recognized to be deficient in furin. Because part of the putative NLS is known to act as a furin-recognition site, active furin could attenuate nuclear inclusion by cleaving the fused NLS from eGFP. After transfecting the fusion construct alongside wild type eGFP, nuclear and cytoplasmic isolations were obtained via detergent purification. The fusion construct is sequestered preferentially within the nucleus relative to its WT counterpart, which remains predominantly within the cytosol (Figure 1C). Conducting a whole-cell lysis following transfection resulted in equivalent signal from cells expressing the fusion construct and those expressing WT eGFP, thus gross expression levels and antibody affinity are similar for both constructs. These results demonstrate that the presence of this putative NLS and its flanking residues are sufficient to elicit nuclear localization in an exogenous protein. We next studied an ADAMTS13 construct devoid of its prodomain¹⁴, comparing its nuclear inclusion to that of WT ADAMTS13 following transient transfection in LoVo cells. In the absence of the prodomain, the proportion of ADAMTS13 localized to the nucleus is just slightly less than that of WT ADAMTS13 (Figure 1D), and it is clear that the nuclear localization is not obliterated.

Exploring for functional roles of nuclear localized ADAMTS13

We next sought to predict what function(s) ADAMTS13 might carry out within the nucleus using a bioinformatics approach. We aimed to identify potential functional regions in ADAMTS13, giving particular attention to the distinguishing C-terminal, globular CUB domains. We mapped conserved polar residues of the CUB-2 domain in vertebrate homologs (Figure S2) onto a template crystal structure of the CUB domain from mammalian major seminal plasma glycoprotein PSP-II (PDB: 1spp chain B) and examined if they could potentially form a cluster in 3-D space (Figure 3A). We used the above crystal structure, as it was one of best PSI-BLAST hits with e-value $<10^{-5}$ in 2nd iteration. We identified a set of polar residues of the CUB-2 domain that mapped onto the exposed face of the overall structure (Figure S2). Therefore, we predict that this region could potentially constitute a binding region for the CUB-2 domain. This region may have a role in a nuclear context as it could interact with polar residues and main-chain/side-chain amino groups in other nuclear proteins.

Further, to understand potential broader functional themes of CUB domain-like proteins, we went on to identify similar 3-D structures. Using DaliLite software¹⁵, we performed sensitive structural searches with the experimentally determined CUB domain as a query against a locally constructed database of representative protein structures (approx. 8000 individual sub-units) from the PDB database¹⁶. We also confirmed the structural hits by querying the DALI database the world-wide web (<http://ekhidna.biocenter.helsinki.fi/dali/start>). We found a significant match in the core 8 β -strands (Figure 3B) of the CUB domain with double-stranded beta helix (DSBH) domains, which include nucleoplasmins, viral coat and capsid proteins of positive stranded ssRNA viruses and RmlC-like cupins superfamily proteins (Table S1). Figure S3 depicts the topology and shared fold of the CUB domain, B1 domain of neuropilin-2 and nucleoplasmin

domains. It is well known that DSBH proteins are part of protein complexes in various cellular localizations (e.g. nucleoplasmin and B1 domain of neuropilin-2), and protein-protein interactions are critical for the stability and functions of protein complexes. Hence, this might suggest that the DSBH fold could be major facilitator of protein-protein interactions, but the specificities are determined by local geometry and chemistry. Therefore, it is possible that the CUB-2 domain of ADAMTS13 could mediate protein-protein interactions in some nuclear protein complexes.

In summary, sequence and structural analyses of the CUB-2 domain in ADAMTS13 suggest that the CUB-2 domain might operate in chromatin-associated complexes, where it could possibly function as a protein-protein interaction mediating entity. This is also supported by observations that CUB domains can bind to various proteins and complexes in extracellular contexts^{6, 17–19}.

Discussion and Outlook

In this work, we show that the cluster of positively charged amino acid residues found at the distal end of the ADAMTS13 propeptide possesses NLS qualities. This sequence is highly similar to the K-K/R-X-K/R monopartite NLSs identified by Chelsky and colleagues²⁰. In the ADAMTS13 sequence, the requisite lysine is substituted by an arginine, which is then followed by the requisite R-X-R sequence. Such deviations from a consensus NLS sequence are frequent. In fact, others have documented significant variation in NLS sequence, even within an individual protein containing multiple NLS regions²¹. Although the 3-D structure of the ADAMTS13 protein is not fully known at high resolution, it is highly likely that the terminal part of the propeptide is readily accessible, as this region is susceptible to processing prior to maturation. These observations assign a possible function for the conserved, largely unaccounted for, propeptide region of ADAMTS13. The role prodomains can play in nuclear localization has been described previously in caspase 2²² and proprotein convertase 1 (PC1)²³. The prodomain of caspase 2 was found to contain a “classical” NLS, whereas a putative NLS (KHKNHPRRSRR) was discovered in PC1 that transiently facilitated nuclear localization. The RRSRR sequence in PC1 also serves as a furin cleavage site. Since removal of the putative NLS from ADAMTS13 does not fully mute its nuclear localization, we do not exclude the possibility of other NLSs or alternate mechanisms for nuclear localization. For example, the CUB domains, recognized for their ability to engage in protein-protein interaction, could associate with other proteins that have a strong NLS and thus facilitate entry into the nucleus.

Traditional thinking renders the finding of a secreted blood protein within the nuclei of liver cells as being highly dubious, but examples supporting this type of phenomenon do exist. Recent study of various matrix metalloproteinases (MMPs), which are regarded for their involvement in extracellular matrix remodeling, has established an intracellular role for these secreted proteins^{24–26}. MMP3, in particular, acts as a proteinase that degrades matrix components following its secretion, while behaving as transcription factor when present within the nucleus. These studies underscore the potential for intracellular functionality of secreted proteins; when localized to the nucleus, they may exert a function that is entirely distinct from that seen in the extracellular environment. Supporting this theme, the disintegrin metalloprotease ADAM-10 is recognized to be differentially localized in prostate cancer cells²⁷. While ADAM-10 remains at the cell membrane in benign prostatic hyperplasia, this metalloprotease is seen within the nucleus in prostate cancer, and this transposition is correlated to tumor growth and disease progression²⁸. These studies and the work presented here underscore the possibility that a single protein may take on a diverse set of functions. Members of the ADAM family in particular are increasingly recognized for their participation in an array of cellular tasks and pathological processes. This reality

should be accounted for if we hope to better understand the behavior of entire biological systems; carrying out biological modeling solely within the constraints of traditional understanding, or within one particular biological context, may be inadequate.

Although ADAMTS13 has been studied as a secreted metalloprotease, its nuclear inclusion renders additional biological function for ADAMTS13 plausible. The identification of nuclear sequestered ADAMTS13 and subsequent characterization a putative NLS, in sum, points to a broader realm of function for this protein. Whereas the metalloprotease domain is recognized as the functional domain in secreted ADAMTS13, the predicted conserved region of CUB-2 domains within vertebrate homologs and the significant structural similarity of the CUB-2 domain to DSBH-architecture proteins, points to the possibility that the C-terminal CUB-2 domain serves as a protein interacting mediator within the nucleus.

Materials and Methods

Cell lines and cell culture

Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) and LoVo cells, which have a point mutation in the endopeptidase furin (a gift from Dr. Wilson, CBER, FDA), were used in all transfection experiments. A panel of liver cells was tested for the localization and expression of ADAMTS13: Hep3B (ATCC), Huh7, Alexander (a gift from Sara Ladu, National Cancer Institute (NCI), NIH), 7404 cells (a gift from Michael M. Gottesman, NCI, NIH), and hepatic stellate cells (LX2)²⁹. All cells were grown in Dulbecco's Modified Eagle Medium with 1% glutamine, 1% penicillin- streptomycin and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C under humid conditions in 5% CO₂.

Plasmids

pcDNA4-ADAMTS13 (a gift from Evan Sadler, St. Louis, MO), which carries the full *ADAMTS13* cDNA (including the prodomain) conjugated to V5 and poly (His) tags was used for transfection as the WT construct. A plasmid expressing enhanced green fluorescence protein (Clontech peGFP-C1, referred here as eGFP) was used to create a C-terminal eGFP fusion construct that contains 70 amino acids (Pro30-Asp99) that follow the secretory signal peptide in ADAMTS13, thus including the putative NLS in ADAMTS13 and its flanking residues. The region of ADAMTS to be fused to eGFP was amplified with the following primers (5' EcoR1 and BamH1 sites underlined): F- 5' CCGGAATTCCGCCCTCCCATTTCAGCAGA R- 5' CGCGGATCCTGTGTCCTCCTGGTGAGCCT. Following amplification, products and plasmid were digested with EcoR1 and BamH1 and ligated to create a C-terminal fusion construct. We also used an rADAMTS13 construct that lacks its prodomain (a gift from Elaine M. Majerus) alongside pcDNA4-ADAMTS13 to assess changes in cellular localization with and without the influence of the putative NLS found within the prodomain.

Transfection

In preparation for confocal microscopy or for immunoblot, cells were plated in MatTeK dishes (MatTeK, Ashland, MA), or 6-well plates or T-75 flasks 24 hours before transfection at a confluency of 80–90%. Cells were transfected with 2–20 µg plasmid DNA (relative to the container size) using Lipofectamine Plus or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After transfection, the medium was replaced with OPTI-MEM medium (Invitrogen).

Confocal microscopy and immunostaining

Transfected cells were washed twice with phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA), and then permeabilized and fixed with 4% paraformaldehyde for 30 minutes and an optional step of 70% ethanol for 15 minutes. Antibody labeling was performed for one hour at room temperature (Table 1). Immediately before imaging the dish, 5 μ l of DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Molecular Probes) were added to stain the nucleus from a stock concentration of 1 μ g/ml, which was dissolved in water.

Confocal images were sequentially acquired with Zeiss AIM software on a Zeiss LSM 510 Confocal system (Carl Zeiss Inc, Thornwood, NY) with a Zeiss Axiovert 100M inverted microscope and 50 mW argon UV laser tuned to 364 nm, a 25 mW Argon visible laser tuned to 488 nm and a 1 mW HeNe laser tuned to 543 nm. A 63 $\times$ Plan-Neofluar 1.4 NA oil immersion objective was used at various digital zoom settings. Emission signals after sequential excitation of DAPI, Alexa Fluor 488 and Texas Red by the 364 nm, 488 nm or 543 nm laser lines were collected with a BP 435–485, BP 505–550 or LP 560 filter respectively, using individual photomultipliers. Fluorescence intensities were measured using regions of interest (ROI) for the entire cell and nucleus on 8-bit images. The intensity value for cytoplasm was calculated for each cell by subtracting the ROI value for the nucleus from the entire cell ROI value. Imaging transfected cells with secondary antibody only set the background conditions for time-course experiments.

Preparation of nuclear and cytoplasmic lysates

LoVo cells were scraped and washed twice with PBS 24 hours post-transfection. The cells were trypsinized, pelleted then resuspended in 200 μ L of buffer 1 (25 mM HEPES (pH 7.9), 5 mM KCl, 0.5 mM MgCl₂, 1 mM DTT, 1 mM PMSF) and 200 μ L of buffer 2 (1% IGEPAL) was added. The mixture was mixed gently at 4°C for 15 minutes and centrifuged at 2000 $\times$ g for one minute. The resulting supernatant contained the cytoplasmic fraction. The pellet was then washed with buffer 3 (1:1 mixture of buffer 1 and buffer 2) and pelleted as before with the supernatant being discarded afterwards. Finally, the pellet was mixed gently at 4°C for 1 hour in 500 μ L of buffer 4 (25 mM HEPES, 350 mM NaCl, 10% Sucrose, 0.05% NP-40, 1 mM DTT, 1 mM PMSF). The contents were centrifuged at 13,000 $\times$ g for 10 minutes. The supernatant contained the nuclear fraction. The cytoplasmic and nuclear fractions were stored at –80 °C.

Western blotting

Protein concentrations for the nuclear and whole cell lysates were determined using a BCA Protein Assay Reagent kit (Pierce, Rockford, IL) as per the manufacture's protocol. For electrophoresis, subcellular fractions were loaded at 20 μ g of total protein. The samples were mixed with SDS Sample Buffer (Invitrogen) and boiled for 5 minutes at 95 °C. The samples were electrophoresed on 7% Tris-Acetate SDS-PAGE gel, 12% Bis-Tris or 10% Tris-Glycine with corresponding running buffer (Invitrogen). Immunoblotting was performed using antibodies as detailed in Table 1 and secondary anti-mouse HRP antibody (Invitrogen).

Structural and sequence analysis of the CUB domain

Structure similarity searches were conducted using the standalone version of the DALI program called DaliLite with the query structures scanned against a local current version of Protein Data Bank (PDB) which has all chains as separate entries (construction detailed below). These hits were also confirmed by querying the DALI database. The structural hits for each query were collected and parsed for congruence of strand orientation with nucleoplasmin structures (e.g. 1 $\times$ e0). This was further confirmed by visual examination of

each structure. Protein structures were visualized using the Swiss-PDB viewer³⁰ and cartoon representations (figures) were constructed with the PyMOL program (<http://www.pymol.org>). Protein secondary structure predictions were made with the JPRED program (<http://www.compbio.dundee.ac.uk/~www-jpred>)³¹, using multiple alignments as queries. The residues of the CUB-2 domain that potentially interact with other proteins were deduced using alignment, which was generated by PCMA (Profile Consistency Multiple sequence Alignment)³² for the structural hits with the ADAMTS13 CUB-2 domain. Further alignment was adjusted manually based on secondary structure prediction by the JPRED program.

Construction of the DaliLite searchable local database

Over 30,000 atomic structures were downloaded from the PDB database, split into over 50,000 PDB chains and the corresponding amino acid sequences for each PDB chain were generated. The FASTA-formatted primary sequences were collated into a single file, and they were then clustered into unique, non-overlapping groups using BLASTCLUST with a sequence identity cut-off of 40% and a length threshold of 70%. A representative PDB chain from each group was identified, considering the following in order of preference: (1) resolution (2) R-factor (3) X-Ray > NMR (4) largest sequence length. The identified representative PDB chain from each group was used to build the local PDB database. Over 8,000 PDB chains were ultimately used to construct the local DaliLite searchable database.

Sequence analysis

The PSORT/PSORT II suite of programs was used to automatically search for an NLS via its web portal (<http://psort.nibb.ac.jp/form2.html>). The non-redundant (NR) database of protein sequences (National Center for Biotechnology Information, NIH, Bethesda, MD) was searched for sequence homologs of human ADAMTS13 protein with the BLASTP program³³. Profile searches were conducted using the PSI-BLAST program³⁴ with a profile inclusion expectation (e) value threshold of 0.01. Searches were iterated until convergence. Multiple alignments were constructed using the PCMA program³².

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tracking the cellular localization of endogenous ADAMTS13 and NLS-fusion constructs

(A) Human liver cell lines (7404, Huh-7, and LX2) were immunostained with the ADAMTS13-specific antibodies BL154G, Wh2-11-1 and Wh2-22-1A and probed with FITC or Alexa Fluor 488 secondary antibodies. The cells stained with secondary antibodies only served as a control (data not shown). The left column shows the green ADAMTS13 emission, the middle column contains cells stained with DAPI (nuclear specific) and the right column displays the merge. (B) LoVo cells were transfected with WT ADAMTS13 and harvested 24 hours later. The separated nuclear and cytoplasmic fractions were subsequently probed with anti-V5 antibody (top). The immunoblot of nuclear extract and the cytoplasmic extract were tested for contamination by ER membrane proteins using calnexin antibody (bottom). (C) LoVo cells were harvested 24 hours post-transfection with either prodomain-fusion eGFP or WT eGFP. Subcellular extracts were obtained via detergent lysis and analyzed by Western blot. Constructs used for transfection are identified as follows: WT (WT eGFP), FUS (prodomain-fusion eGFP), No T (no transfection). (D) LoVo cells were harvested 24 hours post-transfection with either WT ADAMTS13 (WT) or an ADAMTS13 construct devoid of its prodomain (DEL). Subcellular extracts were obtained via detergent lysis and analyzed by Western blot.

Figure 2. Cellular localization of ADAMTS13 in transfected HEK293 cells

Cells were transfected with the ADAMTS13-V5 tag construct and the subcellular localization of the recombinant protein was monitored using confocal microscopy at 4 and 24 hours post-transfection. Cells were immunostained with an anti-V5 antibody and one of two secondary antibodies: Rhodamine Red™ (red, left panel) or Alexa Fluor 488 (green, right panel) to detect recombinant ADAMTS13. The Golgi were immunostained with a GM-130 antibody followed by Alexa Fluor 488 (green, left panel). The endoplasmic reticulum was marked with a BiP/GRP78 antibody followed by Rhodamine Red™-X (red, right panel). The DAPI-stained nuclei are seen in bottom left quadrant of each image. The

overlay of the green, red and blue emissions (bottom right quadrant) demonstrates the colocalization of ADAMTS13 with the ER and Golgi apparatus.

Figure 3. Structural analysis on the CUB domain

(A) A potential binding region in the CUB-2 domain mapped on to a CUB domain of Major Seminal Plasma Glycoprotein PSP-II (PDB: 1spp chain B). The residue positions shown as blue sticks are polar residues and are largely conserved in the CUB-2 domains of ADAMTS13 across species (Figure S2). (B) Double-Stranded Beta Helix topology shared between CUB domains and protein families listed in Table S1 and Figure S3. This fold consists of core 8 anti-parallel β -strands. The strands colored similarly are approximately in a plane in 3-D.

Table 1

Antibodies employed in this study

Antibody specificity (Antigen)	Primary Antibody	Secondary Antibody
ADAMTS13 (Metalloprotease)	BL154G (Bethyl, Montgomery, TX)	FITC anti-goat (Bethyl, Montgomery, TX)
ADAMTS13 (TSP-3)	Wh2-11-1	Alexa 488 goat anti-mouse IgG (H+L) or Rhodamine Red TM -X anti-mouse (Invitrogen, Carlsbad, MA)
ADAMTS13 (Disintegrin domain)	Wh2-22-1A	Alexa 488 goat anti-mouse IgG (H+L) or Rhodamine Red TM -X anti-mouse (Invitrogen, Carlsbad, MA)
V5 tag	Anti- V5 (Invitrogen)	Alexa 488 goat anti-mouse IgG (H+L) or Rhodamine Red TM -X anti-mouse (Invitrogen); for Western blot anti-mouse HRP (Invitrogen)
Endoplasmic Reticulum (ER)	Calnexin (cell Signaling, Boston, MA)	Goat anti-Rabbit IgG HRP conjugate (Rockland)
GFP	Anti- GFP (Invitrogen)	Donkey Anti-Rabbit IgG HRP conjugate (Jackson ImmunoResearch)