

# Detection of in situ cleaved p115 with the cut specific antibodies in rapid protein inactivation system by tobacco etch viral protease cleavage

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**Abstract:** Gene perturbation methods are commonly used in the study of gene and protein function. The authors of this paper recently developed a rapid protein inactivation technique utilizing tobacco etch virus (TEV)-derived protease. TEV protease recognizes the ENLYFQG (Glu-Asn-Leu-Tyr-Phe-Gln-Gly) amino acid sequence and specifically cleaves between Q and G. The authors developed antibodies that recognize the cleaved TEV (ENLYFQ) sequence, both in vitro and in vivo, but do not bind to uncleaved TEV (ENLYFQG). Using these antibodies, in situ protein cleavage was successfully detected. These antibodies used in combination with the TEV protease may be a useful complement to other perturbation methods.

**Keywords:** TEV protease, Golgi, golgins, microinjection, recombinant proteins

## Introduction

The characterization of gene product function is an important challenge in the field of cell biology, especially since the completion of the Human Genome Project. Additionally, research into the causes of human disorders has revealed that defects in intracellular membrane trafficking can cause serious diseases.<sup>1,2</sup> Therefore, understanding how gene products are transported via membrane trafficking and how trafficking affects their function is crucial.

Ribonucleic acid (RNA) interference (RNAi) is a powerful technique used to disrupt a given messenger RNA using complementary double-stranded RNA, thereby reducing corresponding protein levels.<sup>3</sup> This technique is applicable for cell-based assays and therefore has been widely used by researchers in the field of cell biology, including membrane trafficking. Although RNAi is useful, there are certain limitations associated with its use. For example, the depletion of the Golgi matrix protein GM130 by RNAi has been described by three different groups and has resulted in contradictory phenotypes.<sup>4-6</sup>

According to published data and our experience, the depletion of Golgi-related proteins by RNAi takes 72–96 hours,<sup>6-8</sup> during which time the cells either die or compensate by adjusting the expression levels of other genes. Therefore, any phenotype observed may not reflect the primary loss of function phenotype for the disrupted gene. To address these concerns, we developed a technique for the rapid inactivation of proteins that allows accurate observation of the primary effects of gene product perturbation.<sup>9</sup>

For rapid inactivation, we chose the tobacco etch virus (TEV)-derived protease (TEV protease). TEV protease is unique in its extremely precise cleavage specificity: it recognizes the ENLYFQG (Glu-Asn-Leu-Tyr-Phe-Gln-Gly) consensus sequence and

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cleaves between Q and G. Using nested polymerase chain reaction, a TEV protease recognition site was introduced into a cDNA (complementary deoxyribonucleic acid) expression construct. After transfection, the protein was treated with the TEV protease by microinjection, both *in vitro* and *in situ*. Since the TEV recognition sequence is not found in the human gene database, this method can be used to rapidly remove any protein from the cell.

Using this method, we had previously demonstrated that *in situ* cleavage of p115, a coat protein I vesicle-tethering protein, caused reduced cargo trafficking.<sup>9</sup> However, we were unable to demonstrate direct protein cleavage in cells because of a lack of visualization tools. Here we raised anti-TEV (which recognizes only C-terminal ENLYFQ, not ENLYFQG or internal ENLYFQG) antibodies to detect the cleaved TEV sequence and were able to specifically detect *in situ* protein cleavage. Therefore, the use of these antibodies in combination with TEV protease can be a useful complement to other protein/gene perturbation methods.

## Materials and methods

### Antibodies

The antibodies used in this study are as follows: monoclonal anti-influenza hemagglutinin (anti-HA) (16B12, BAbCO, Richmond, CA), polyclonal anti-giantin,<sup>10</sup> Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA), and horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL). Anti-HA agarose was obtained from Sigma (St Louis, MO).

### Production of anti-TEV antibodies

Polyclonal rabbit anti-TEV antibodies were raised using TEV-cleaved hexapeptide (ENLYFQ) as an antigen (Figure 1A). A cysteine residue was added at its N-terminus for conjugation to keyhole limpet hemocyanin (KLH), and 6-aminohexanoic acid (Ahx) was used as a spacer (Figure 1A). Peptide synthesis by Fmoc (fluorenylmethoxycarbonyl) solid phase synthesis, immunization using the KLH-conjugated peptide and Freund's complete adjuvant, and a titer check by enzyme-linked immunosorbent assay (ELISA) were performed by Operon Biotechnologies (Tokyo, Japan). The first and second bleeds were collected a week after the first and second immunizations, respectively. Immediately after the third immunization and bleed, exsanguination was performed. Pooled anti-sera were used in this study.

### Plasmids and stable cell lines

The C-terminal TEV-cleaved sequence (ENLYFQ)-bearing p115 (Figure 1C) was generated by site-directed mutagenesis by introducing a stop codon between Q and G of the internal TEV-modified p115 (Figure 1B). The truncated p115 (1–886) and HeLa cells stably expressing the internal TEV-modified p115 were described previously.<sup>9</sup> We modified green fluorescence protein (GFP)-tagged golgin84 used in the study by Satoh et al<sup>11</sup> to insert the TEV cleavage site at the 300th amino acid of golgin-84 (Figure 4A). The internal TEV-modified golgin 84 was expressed transiently by transfection in HeLa cells.<sup>9</sup> The transient transfection was performed with Lipofectamine™ LTX (Invitrogen) following the manufacturer's protocol.

### Cell culture and microinjection

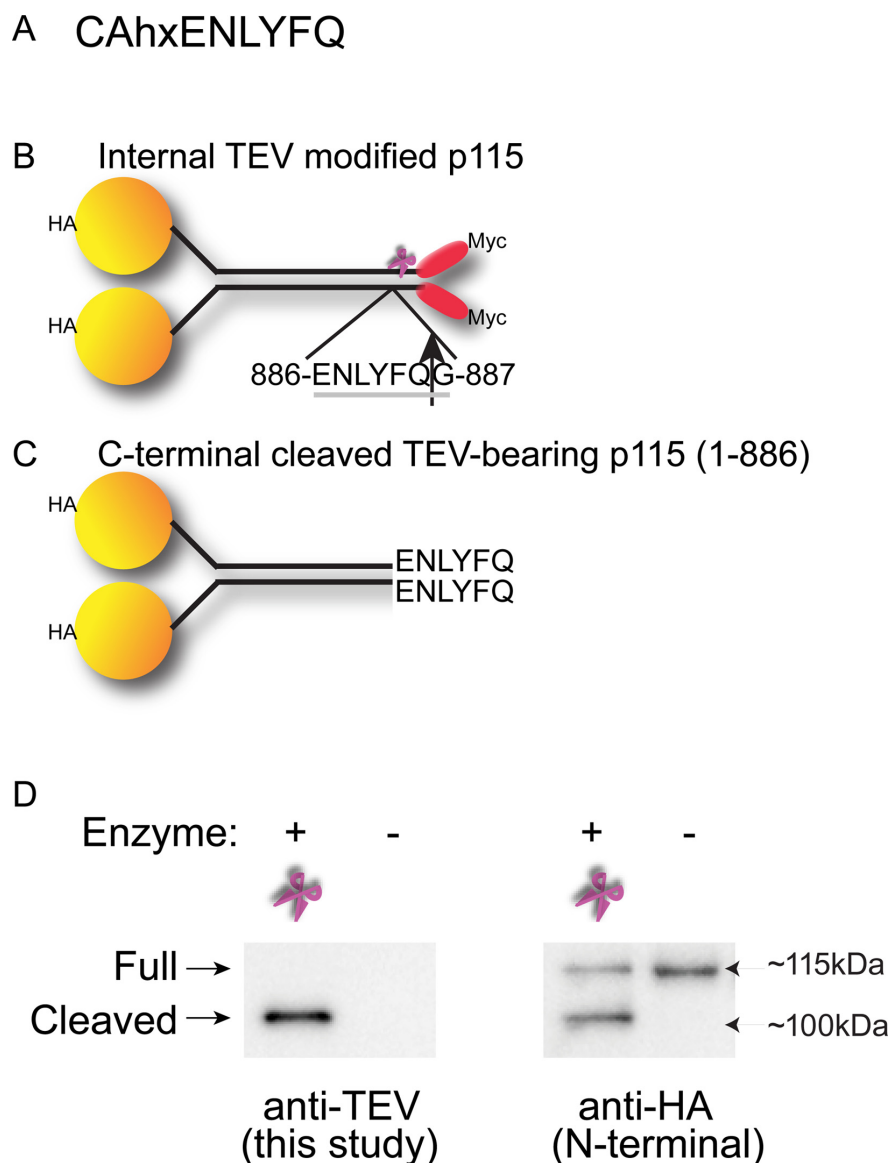
Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum on 12-mm glass coverslips. Desalted TEV protease (~4 U/μL, Invitrogen) together with Alexa-conjugated dextran (1 μg/μL) as an injection marker was injected into the cytoplasm. After the microinjection, the cells were incubated for 1.5–3.0 hours at 37°C and then processed for immunofluorescence. In typical experiments, 40–100 cells were injected.

### Immunoprecipitation (IP) and Western blotting<sup>9,11</sup>

Cell lysates were prepared with IP buffer (10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-KOH, pH 7.4, 100 mM KCl, 0.1 mM dithiothreitol [DTT], 2.5 mM MgCl<sub>2</sub>, 1% Triton® X-100 and the protease inhibitor cocktail from Roche, Penzberg, Upper Bavaria, Germany). After incubation for 10 minutes on ice, the extract was clarified by centrifugation at 14,000 g for 20 minutes. For IP, the supernatants (~100 μg protein) were incubated with anti-HA agarose (Sigma) or anti-GFP agarose (MBL, Woburn, MA) for 30 minutes at 4°C. After washing three times with IP buffer, the beads were washed three times with TEV buffer (50 mM Tris-HCl [pH 8.0], 0.5 mM ethylenediaminetetraacetic acid, 1 mM DTT). Proteins on the beads were then incubated with/without TEV protease (5 U, Invitrogen) for 1 hour at 30°C. The samples were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blotting, with antibodies indicated in the figures.

### Immunofluorescence microscopy<sup>9,11</sup>

Cells on coverslips were fixed with 3.7% formalin in phosphate buffered saline (PBS) for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at



**Figure 1** (A) The antigen peptide. Cysteine was added at the N-terminus for KLH conjugation. Ahx was used as a spacer. (B, C) Schematic models of modified p115 constructs used in this study. The TEV protease recognizes the heptapeptide sequence ENLYFQG and cleaves between Q and G with high specificity. This heptapeptide was inserted between amino acids 886 and 887 of bovine p115 (internal TEV-modified p115) (B). As a cleaved control, truncated p115 (1–886) followed by TEV-cleaved ENLYFQ sequence was constructed (C-terminal-cleaved TEV-bearing p115 (1–886)) (C). Both constructs were tagged with HA at their N-termini for detection. (D) Detection of in-vitro cleavage of internal TEV-modified p115. HeLa cells stably expressing the TEV-modified p115 with N-terminal HA tag were used. HA-tagged modified p115 was immunoprecipitated with anti-HA-agarose from cell lysates, and immunoreactive complexes were treated with or without the TEV protease for 30 minutes at 30°C and analyzed by Western blotting with anti-TEV (left panel) and anti-HA (right panel, to detect the N-termini) antibodies. The anti-TEV antibodies specifically recognized the cleaved protein but not the full-length protein. **Abbreviations:** Ahx, 6-aminohexanoic acid; ENLYFQG, Glu-Asn-Leu-Tyr-Phe-Gln-Gly; HA, hemagglutinin; KLH, keyhole limpet hemocyanin; TEV, tobacco etch virus.

room temperature. The cells were blocked with 4% bovine serum albumin (BSA) in PBS for 15 minutes, and then incubated for 15 minutes with primary antibodies diluted in 4% BSA in PBS. The cells were washed three times with PBS, and incubated for 15 minutes with secondary antibodies conjugated to Alexa Fluor® Dyes (Invitrogen). After washing the cells, the coverslips were mounted on microscope slides and imaged using an FV1000 confocal microscope equipped with a 60 × oil objective (Olympus, Tokyo, Japan).

## Results and discussion

### Production of cleaved TEV (ENLYFQ)-specific antibodies

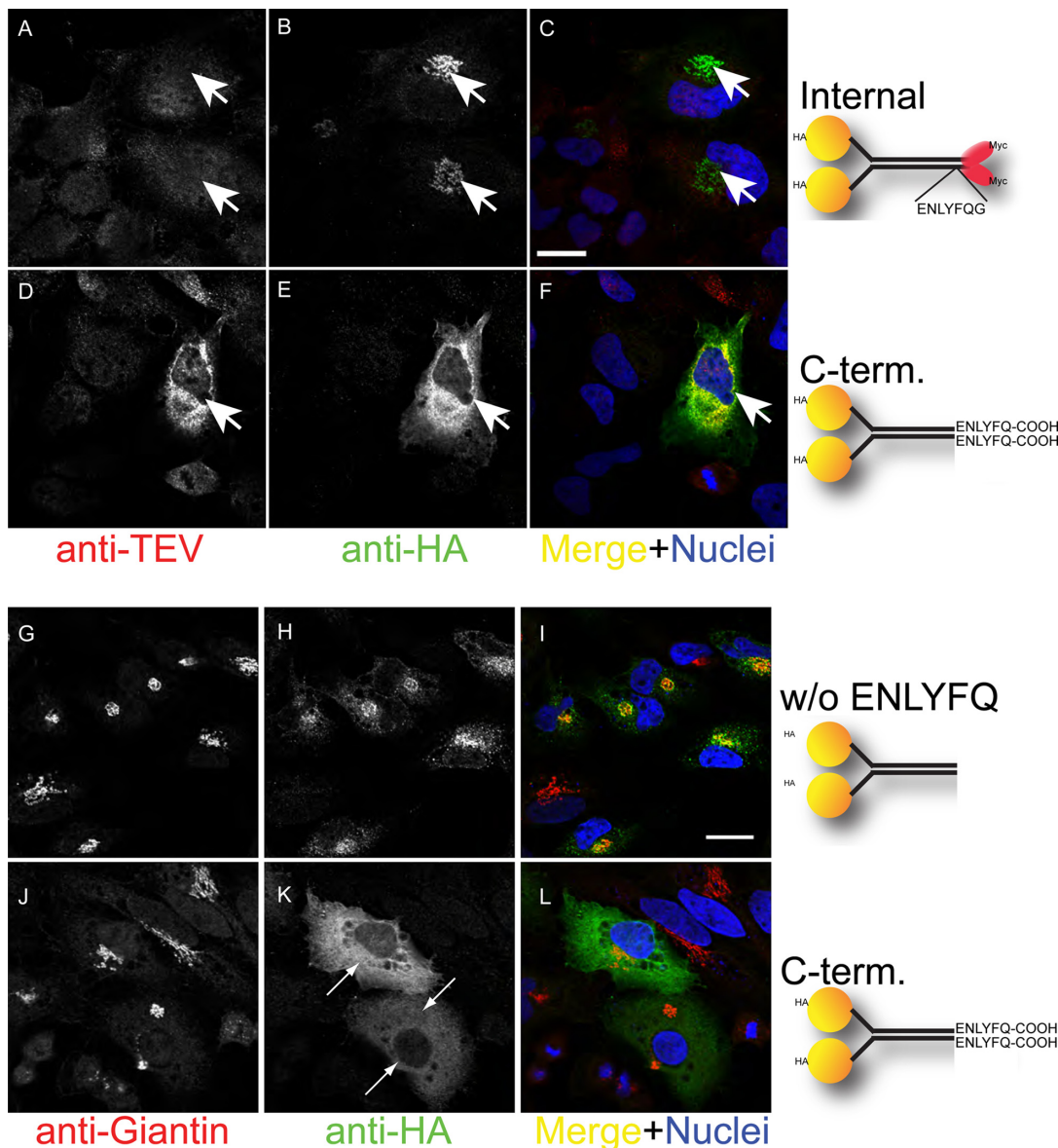
In general, sequence epitopes in proteins consist of 6–12 amino acids.<sup>12–14</sup> Since the cleaved TEV (ENLYFQ) was a hexapeptide, a non-antigenic Ahx was used as a spacer (Figure 1A) to maximize the presentation of the antigen peptide. The titers were determined by ELISA (not shown). The first and second bleeds were collected a week after the first and second

immunizations, respectively. Immediately after the third immunization and bleed, exsanguination was performed. Pooled anti-sera were used in this study.

## Detection of in vitro protein cleavage by anti-TEV antibodies

To test the specificity of the anti-TEV antibody, in vitro cleavage of the modified p115 was performed. The TEV-modified p115 was immunisolated from HeLa cells stably expressing

the modified p115<sup>9</sup> via its N-terminal HA-tag by IP with anti-HA-agarose. Immunoreactive complexes were treated with or without the TEV protease, and analyzed by Western blotting with anti-TEV (Figure 1D, left panel) and anti-HA (Figure 1D, right panel) antibodies. HA staining revealed that the cleavage was incomplete, with approximately 35% of the modified p115 uncleaved in the condition tested. The anti-TEV antibodies specifically recognized the cleaved protein but not full-length p115 (Figure 1D, left panel).



**Figure 2** Specificity of anti-TEV antibodies in immunofluorescence applications. HeLa cells transiently expressing the TEV-modified p115 with N-terminal HA tag (**A–C**) and C-terminal cleaved TEV-bearing p115 (**D–F**) were fixed and immunostained with anti-TEV (left panels, red) and anti-HA (middle panels, green) antibodies. The merged images with nuclear staining (blue) are shown on the right. Cells expressing HA-constructs are indicated with white arrows. The anti-TEV antibodies did not recognize uncleaved proteins in immunofluorescence applications. HeLa cells transiently expressing truncated p115 (1–886, **G–I**) and the C-terminal TEV-cleaved p115 (1–886, **J–L**) were immunostained with anti-giantin (**G, J**; red, Golgi marker) and anti-HA (**H, K**; green) antibodies. The merged images with nuclear staining (blue) are shown on the right. The addition of ENLYFQ to the truncated p115 (1–886) interfered with its localization to the Golgi apparatus (white arrows). **Abbreviations:** ENLYFQG, Glu-Asn-Leu-Tyr-Phe-Gln-Gly; HA, hemagglutinin; TEV, tobacco etch virus; w/o, without.

## Detection of in situ protein cleavage by anti-TEV antibodies

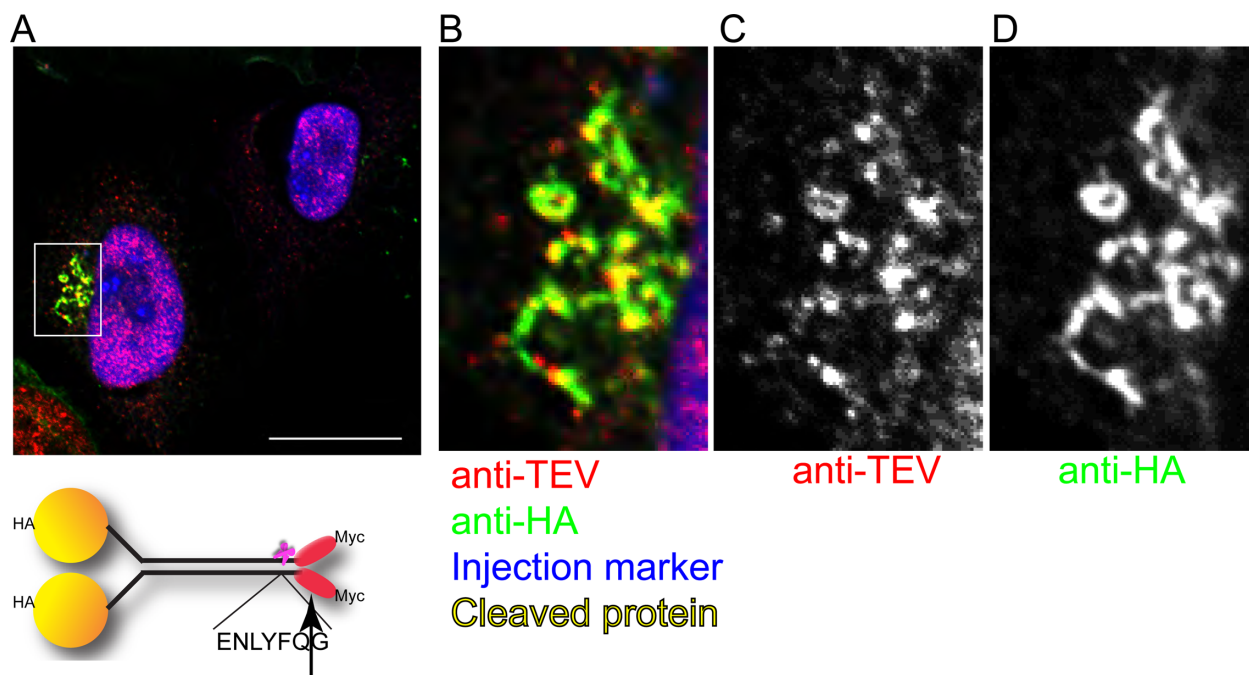
To test the specificity of the anti-TEV antibody in immunofluorescence applications, the internal TEV-modified p115 (Figure 1B) and C-terminal TEV-cleaved ENLYFQ-bearing truncated p115 (1–886) (Figure 1C) were expressed transiently, and cells were immunostained using anti-TEV and anti-HA antibodies. The latter was used to detect N-terminal HA-tags. The anti-TEV antibodies did not recognize the internal TEV-modified p115 (Figures 2A–2C), whereas it did recognize the C-terminal TEV-cleaved ENLYFQ-bearing p115 (1–886) (Figures 2D–2F).

Since the authors of this paper noticed that the C-terminal TEV-cleaved ENLYFQ-bearing p115 (1–886) localized throughout the cells, they compared the subcellular localization of this protein to that of p115 (1–886) without ENLYFQ. Both constructs were expressed transiently, and stained for giantin, a Golgi marker (Figures 2G and 2J), and HA to mark their N-termini (Figures 2H and 2K). The majority of the truncated p115 (1–886, without ENLYFQ) localized to the Golgi area marked by giantin (Figures 2G–2I), whereas the C-terminal TEV-cleaved ENLYFQ-bearing p115 did not (Figures 2J–2L). These data suggest that the addition of ENLYFQ may mask the binding site(s) in p115 (1–886) to the Golgi apparatus. These binding sites could be located in

the head (1–651 for GBF1, Cog2, and Rab1 bindings), the first coiled-coil (652–701 for SNARE and Rab1 bindings), and/or the second coiled-coil (704–776 for Rab1 binding) domains of p115.<sup>15–19</sup>

Furthermore, in situ protein cleavage was performed by microinjecting the TEV protease into HeLa cells stably expressing the internal TEV-modified p115. To mark the protease-injected cells, Alexa-labeled dextran was co-injected. Two hours after microinjection, cells were fixed and immunostained with anti-TEV and anti-HA antibodies. As shown in Figure 3C, the anti-TEV antibodies marked certain structures and primarily colocalized with HA, suggesting that the anti-TEV antibodies recognized in situ cleaved proteins. Protein cleavage could not be observed in shorter time points (20 minutes incubation). This could be because of the lower concentration of anti-TEV antibodies in the antisera used. Therefore, affinity purification of the anti-TEV antibodies may be needed to achieve the high concentrations required for the detection of cleaved proteins at earlier time points. Importantly, microinjection of the TEV protease and dextran did not affect anti-TEV reactivity.

We noticed that in situ cleaved p115 localized to the Golgi within the time observed (Figure 3), unlike C-terminal TEV-cleaved ENLYFQ-bearing p115 (Figure 2). The difference in the localization of these two proteins may be because

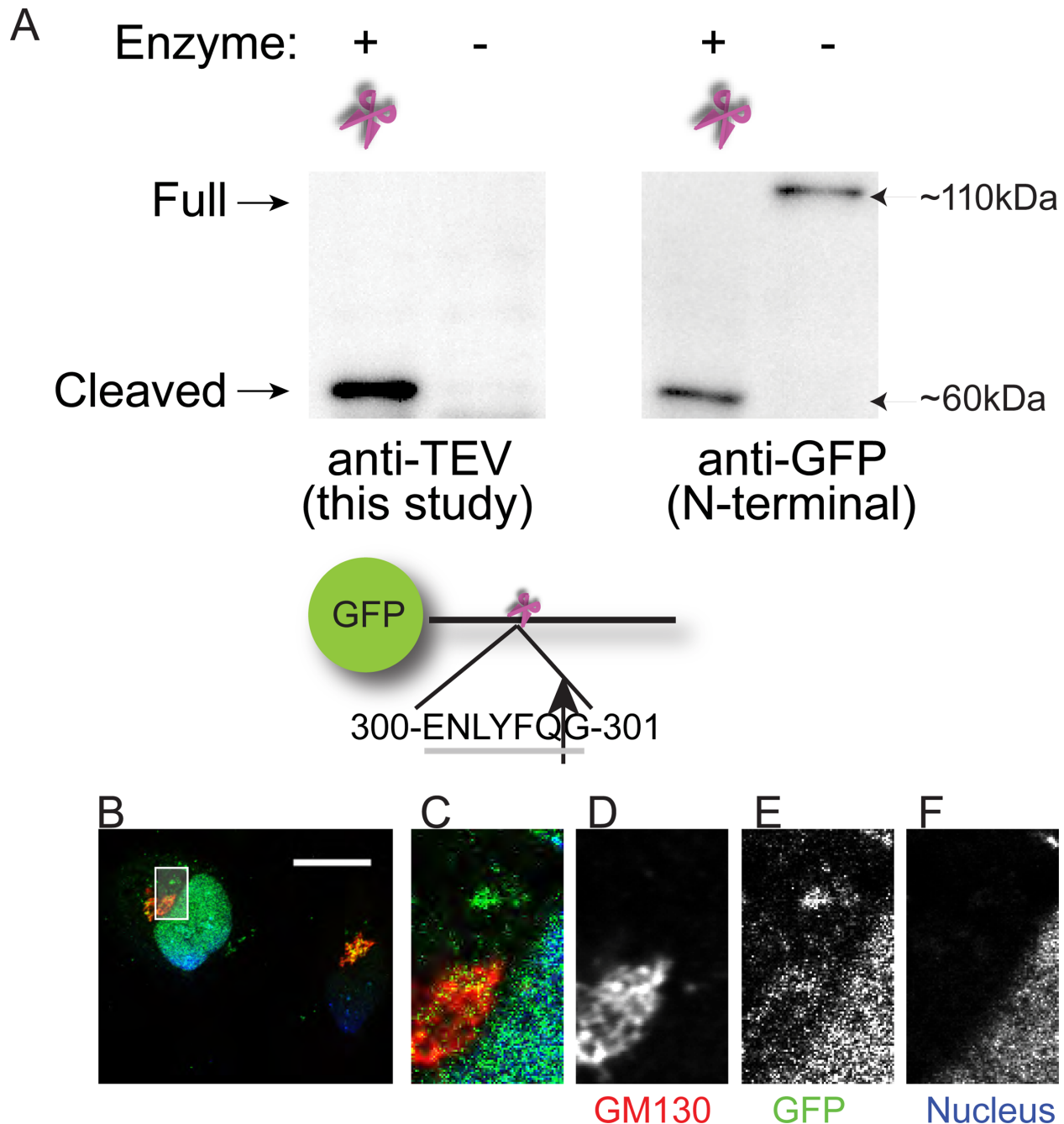


**Figure 3** Detection of in-situ protein cleavage by anti-TEV antibodies. HeLa cells stably expressing the internal TEV-modified p115 with N-terminal HA tag were microinjected with the TEV protease and injection marker (blue). After 2 hours incubation, cells were fixed and immunostained with anti-TEV (panel C, red in panels A and B) and anti-HA (panel D, green in panels A and B) antibodies. The merged images are shown in A and B. Images B–D represent a 3.5-fold magnification of the boxed area in A. Bar, 20  $\mu$ m. **Abbreviations:** ENLYFQG, Glu-Asn-Leu-Tyr-Phe-Gln-Gly; HA, hemagglutinin; TEV, tobacco etch virus.

the in situ cleaved p115 had bound to the Golgi apparatus before the cleavage, and ENLYFQ generated by the cuts may not have interfered with the pre-existing interactions.

Finally, to demonstrate the broad applications of this method, in vitro and in situ protein cleavage were performed using HeLa cells transiently expressing the internal TEV-

modified golgin 84, another Golgi-related protein. The TEV-modified golgin 84 was immunoprecipitated via its N-terminal GFP-tag by IP with anti-GFP-agarose as described above. Immunoreactive complexes were treated with or without the TEV protease, and analyzed by Western blotting with anti-TEV (Figure 4A, left panel) and anti-GFP (Figure 4A,



**Figure 4 (A)** Detection of in vitro cleavage of internal TEV-modified golgin 84. HeLa cells transiently expressing the TEV-modified golgin 84 with N-terminal GFP tag were used. GFP-tagged modified golgin 84 was immunoprecipitated with anti-GFP-agarose from cell lysates, and immunoreactive complexes were treated with or without the TEV protease and analyzed by Western blotting with anti-TEV (left panel) and anti-GFP (right panel), to detect the N-terminal antibodies. The anti-TEV antibodies specifically recognized the cleaved protein but not the full-length protein. **(B–F)** Detection of in-situ protein cleavage by anti-TEV antibodies. HeLa cells transiently expressing the internal TEV-modified golgin with N-terminal GFP tag were microinjected with the TEV protease and injection marker. After 2 hours incubation, cells were fixed and stained for anti-GM130 (panel D, red in panels B and C), anti-TEV (not shown) antibodies, and injection marker (blue, nuclei). Images C–F represent a 5-fold magnification of the boxed area in B. Bar, 20  $\mu$ m. **Abbreviations:** ENLYFQG, Glu-Asn-Leu-Tyr-Phe-Gln-Gly; GFP, green fluorescent protein; TEV, tobacco etch virus.

right panel) antibodies. The anti-TEV antibodies specifically recognized the cleaved protein but not the full-length golgin (Figure 4A, left panel). Moreover, in situ protein cleavage was performed as described above. As shown in Figures 4B–4F, the majority of GFP signal in the protease-injected cells appeared to localize to the nuclei, and the rest appeared to be very slightly, if any, colocalized with GM130, a Golgi marker. Any specific labeling of anti-TEV antibodies was not observed, suggesting that the in situ cleaved golgin-84 may have defused and been degraded. Golgin-84 has a Golgi localization signal at its C-terminus.<sup>20</sup> Cleaving the N-terminal part off this domain can cause a change of localization and degradation. For some reason, GFP signals in enzyme-injected cells appeared in their nuclei.

The TEV antibodies could be used for the kinetic analyses of receptor-mediated endocytosis or signaling. This can be achieved by introducing a TEV cleavage site in a receptor of interest, subsequent to which this TEV-receptor construct could be used to transfect cells, allowing for the inactivation of the receptor at the cell surface via the TEV protease cleavage. The inactivated/cleaved receptor could then be detected using fluorescently labeled anti-TEV antibodies. Furthermore, if both the TEV protease and labeled anti-TEV antibodies are added to a cell-culture media, the fluorescence of the surface-bound antibodies could be monitored by live imaging.

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## Disclosure

The authors report no conflicts of interest in this work.

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