

**Virology**

## **Roles of the three L-domains in $\beta$ -retrovirus budding**

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## **Abbreviations**

M-PMV: Mason-Pfizer monkey virus

VLP: virus-like particle

ESCRT: endosomal sorting complexes required for transport

HTLV-1: human T-cell leukemia virus type 1

WT: wild-type

## **Abstract**

Retroviral Gag protein plays a critical role during the late stage of virus budding and possesses a so-called L-domain containing PT/SAP, PPxY, YxxL or FPIV motifs that are critical for efficient budding. Mason-Pfizer monkey virus (M-PMV) contains PSAP, PPPY, and YADL sequences in Gag. This study was performed to investigate the roles of these three L-domain-like sequences in virus replication using three different cell lines, 293T, COS-7, and HeLa cells. It was found that the PPxY motif plays an essential role in progeny virus production as a major L-domain in all three cell lines. The PSAP sequence was shown to function as an additional L-domain in HeLa cells and to promote efficient release of M-PMV; however, this sequence was dispensable for M-PMV production in 293T and COS-7 cells, suggesting that the role of the PSAP motif as an L-domain in M-PMV budding is cell type-dependent. Viruses possessing multiple L-domains appear to change the L-domain usage to replicate in various cells. On the other hand, the YADL motif was required for M-PMV production as a transport signal of Gag to the plasma membrane, but not as an L-domain.

**Keywords:** Budding, L-domain, M-PMV, Retrovirus

## **Introduction**

Retroviral Gag proteins play critical roles during the late stage of virus budding and, when expressed alone in cells, they are released in the form of virus-like particles. The Gag protein possesses a so-called L-domain containing PT/SAP, PPxY, YPxL or FPIV, which are motifs critical for efficient viral budding (1-8). Human immunodeficiency virus type 1 (HIV-1) possesses the PTAP motif as the L-domain, Whereas the core element of the L-domain of Rous sarcoma virus (RSV) is the PPxY sequence. A third L-domain motif, YxxL (or YPxL), has been identified in equine infectious anemia virus (EIAV) (6). FPIV and FPVI are the L-domains identified in the matrix (M) protein of human parainfluenza virus type 5 (hPIV-5) and mumps virus, respectively (9, 10). Some retroviruses, including Mason-Pfizer monkey virus (M-PMV), human T-cell leukemia virus type 1 (HTLV-1), and murine leukemia virus (MuLV), contain both the PPxY and the PT/SAP sequences within their Gag polyproteins. The PT/STAP motif reportedly interacts with the Tsg101 subunit of ESCRT-I, a ubiquitin-conjugating E2 enzyme variant (UEV), whereas the PPxY motif interacts with Nedd4-like ubiquitin ligases (11-15). The cellular factor that interacts with the YxxL motif has been shown to be AIP1/Alix (16-20). It has been reported that hPIV-5 and mumps virus, which contain the FPIV or FPVI sequence as the L-domain, respectively, utilize the ESCRT machinery in

virus budding, however, the cellular factors that interact with these L-domains have not been identified (9, 10).

M-PMV belongs to the genus  $\beta$ -retrovirus and has type-D morphology. Previously, we showed that the PPPY sequence within the pp24/16 region of M-PMV Gag plays a critical role in virus budding as a viral L-domain and that Nedd4-like ubiquitin ligase BUL1 interacts with this PPPY sequence and regulates virus budding (7, 14). In addition, Gottwein et al. reported that the PSAP sequence in the pp24/16 region also functions as an additional L-domain in HeLaP4 cells, and promotes the efficient release of M-PMV (21). Here, we found the YxxL sequence, which has been identified as the third L-domain motif in the Gag proteins of EIAV and HIV-1, in p10 (Matrix) protein of M-PMV (6, 20). No viruses possessing three L-domains have been reported previously.

Therefore, to examine whether this YxxL sequence functions as an L-domain and how these multiple L-domains regulate M-PMV budding, we investigated the roles of these putative L-domains of M-PMV in three different cell lines.

## **Materials and Methods**

### **Cell lines**

293T cells, COS-7 cells, and HeLa cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin.

### **Plasmids**

Plasmids expressing the intact M-PMV genome of wild-type (WT) or d3PY mutant, pSHRM15 or pd3PY, have been described previously (7, 22). To generate the expression plasmid for M-PMV mutants, pdPSAP, with deletion of the PSAP sequence within pp24/16, pAADL, pYAAL, and pYADA, with substitution of the YADL sequence with alanine within the p10 matrix were introduced into the wild-type M-PMV expression vector pSHRM15 by site-directed mutagenesis using a QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA).

### **Detection of viral proteins in cell lysates and virions**

293T, COS-7, and HeLa cells were transfected with pSHRM15, pd3PY, pdPSAP, pAADL, pYAAL, or pYADA (1 or 2 µg) using TransIT<sup>®</sup>-LT1 (Mirus Bio Corp., Madison, WI). For HeLa cells, to establish lines stably expressing WT or mutant

M-PMV, cells were incubated in the presence of hygromycin B (life technologies, Carlsbad, California). At 48 hours after spreading for HeLa cells and at 48 hours post-transfection for 293T and COS-7 cells, the culture supernatants were separated from cell debris by centrifugation ( $14200 \times g$ ; 3 min at  $4^{\circ}\text{C}$ ) and which virions were pelleted through a 16.5% sucrose cushion by ultracentrifugation ( $348000 \times g$ ; 45 min at  $4^{\circ}\text{C}$ ). Pelleted virions were resuspended with PBS (-). whereas cells were lysed with lysis A buffer (7). Cell lysates and virions were separated by SDS-PAGE (12% and 15%) and then transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). The blots were probed with anti-M-PMV Gag antibody and horseradish peroxidase-labeled secondary antibodies, and then detected using ECL prime (GE Healthcare) according to the manufacturer's instructions. Bands were visualized using an image analyzer (LAS-3000; GE Healthcare).

### **Production of anti-M-PMV Gag antibody**

M-PMV Gag precursor, Pr78, was expressed in *Escherichia coli* BL21 (DE3) as a GST fusion protein (from pGEX6P-2; GE Healthcare), and purified from the bacterial extracts according to the manufacturer's instructions. Because the GST fusion proteins attached to the glutathione-Sepharose 4B beads were digested with PreScission protease

(GE Healthcare), the purified Gag protein did not contain GST. The purified Gag protein was used to immunize rabbits.

### **Detection of intracytoplasmic procapsid assembly**

To analyze procapsid formation in mutant-expressing cells, the cells were washed with ice-cold PBS and lysed with Triton buffer (0.5% Triton X-100, 0.25 M sucrose, 1 mM EDTA, 0.14 M NaCl, 10 mM Tris-HCL, pH7.5) for 1 hour at room temperature. The cell lysates were clarified of cell debris by centrifugation ( $14000 \times g$ ; 2 min). The supernatants were fractionated into free and capsid-associated Gag polyproteins by ultracentrifugation ( $48500 \times g$ ; 30 min at  $4^{\circ}\text{C}$ ) through a 30% sucrose cushion. Gag polyproteins in supernatant and pellet fractions were analyzed by SDS-PAGE and Western blotting assay using anti-M-PMV Gag antibody.

### **Immunofluorescence microscopy**

293T and COS-7 cells were spread onto Millicell<sup>®</sup> EZ SLIDES (Merck Millipore, Darmstadt, Germany). After 12-hour incubation, cells were transfected with pSHRM15, pd3PY, pdPSAP, pAADL, pYAAL, or pYADA using TransIT-LT1. At 48 hours after transfection, cells were washed and then fixed with 4% paraformaldehyde for 15 minutes at room temperature. HeLa cell lines stably expressing WT or mutant M-PMV,

were incubated onto Millicell<sup>®</sup> EZ SLIDES for 48 hours and then fixed with 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 for 10 minutes and treated with blocking buffer (2.5% FCS and 0.2% Tween-20 in PBS) for 20 minutes. After blocking, the cells were incubated with anti-M-PMV Gag antibody for 1 hour, after which plasma membranes were incubated with Cholera Toxin Subunit B (recombinant), Alexa Fluor<sup>®</sup> 594 Conjugates (Molecular Probes Eugene, OR) for 2 hours and then FITC-conjugated Goat F(ab')<sub>2</sub> anti-Rabbit IgG–H&L polyclonal secondary antibody (Abcam, Cambridge, UK) was also used for detection. Primary and secondary antibodies were diluted in blocking buffer. Fluorescence was visualized with a confocal laser scanning microscopy LSM710/780 (Carl Zeiss, Oberkochen, Germany).

## Results

### Roles of putative three L-domains of M-PMV Gag

To examine the roles of the three putative L-domains of M-PMV Gag, the YxxL sequence within the p10<sup>MA</sup> region and the PPPY and PSAP sequences within the pp24/16 region, mutant M-PMV infectious clones expressing Gag with mutations in these L-domains were generated (Fig. 1). The mutants with deletion of the PPPY or PSAP sequence within pp24/pp16 were designated as d3PY and dPSAP, respectively. AADL, YAAL, and YADA are mutants with substitution of each amino acid within the YADL sequence to alanine. These plasmids expressing the L-domain mutants were introduced into three different cell lines: 293T, COS-7, and HeLa cells. At 48 hours transfection, Gag expression in 293T and COS-7 cells and virion production were analyzed by Western blotting. HeLa cells, cell lines stably expressing WT or mutant M-PMV were incubated on 6-well plates for 48 hours followed by analysis of Gag expression in cells and virion production by Western blotting.

The expression of Gag precursor (Pr78<sup>Gag</sup>) was observed in 293T, COS-7, and HeLa cells expressing either WT or mutants genomes (Fig. 2). WT and dPSAP virion production were also observed in 293T, COS-7, and HeLa cells; HeLa cells showed much less dPSAP virion production than WT (Fig. 2). d3PY and YADA were completely deficient in virion production in all three cell lines. Small amounts of AADL

mutant virions were produced from 293T cells, but not from COS-7 or HeLa cells. Virion production levels of YAAL was similar to that of WT in HeLa cells and less than that of WT in 293T cells, and faint in COS-7 cells. Gag precursor expressions of AADL and YAAL were much higher in COS-7 cells than in WT. Nevertheless, virion production from COS-7 cells expressing these mutants was not observed.

These results suggest that the PPPY motif and the tyrosine (Y) and leucine (L) residues within the YADL motif play essential roles in M-PMV virion production. Additionally, the PSAP motif was dispensable for virion production, at least in 293T and COS-7 cells; it was also required for efficient virion production in HeLa cells. The necessity of the aspartic acid (D) residue within the YADL motif for virion production differed between the cell lines.

### **Intracytoplasmic capsid assembly of L-domain mutants**

M-PMV is a type-D retrovirus, forms a viral procapsid in the cytoplasm of the host cell prior to virion release. To examine whether the mutant Gag polyproteins showed normal intracytoplasmic procapsid assembly, cells expressing WT or mutant Gag polyproteins were treated with Triton buffer after which intracytoplasmic procapsids were pelleted through a 30% sucrose cushion by ultracentrifugation as described in the Methods section. After ultracentrifugation, free Gag polyproteins remained in the

soluble fraction (S) whereas those incorporated into procapsids were recovered in the pellet fraction (P). The Gag polyproteins in each fraction were analyzed by SDS-PAGE and Western blotting. In this experiment, R55W mutant showing type-C morphogenesis, which assembles capsids at the plasma membrane but not in the cytoplasm, was used as a control (23). As shown in Fig. 3, WT and all mutant Gag polyproteins were found in the pellet fractions (P) of all three cell lines, whereas the Gag polyproteins of R55W mutant were found primarily in the soluble fraction (S), as reported previously (24). These results suggest that the Gag polyproteins of all L-domain mutants assemble intracytoplasmic procapsids normally in all three cell lines, as well as WT.

### **Subcellular localization of the L-domain mutant Gag polyproteins**

Next, to examine whether the intracytoplasmic procapsids assembled with the mutant Gag polyproteins are transported normally to the plasma membrane where the intact virions are formed and released from cells, the subcellular localization of the mutant Gag polyproteins was investigated by laser confocal microscopy. In this experiment, we used the transport-defective A18V mutant, which accumulates procapsids as cytoplasmic aggregates, was used as a control (25).

As shown in Fig. 4, WT, d3PY, dPSAP, YAAL, and R55W Gag polyproteins (Green)

were colocalized with the plasma membrane marker (Red) in 293T cells and HeLa cells, whereas A18V, AADL, and YADA mutants were observed only in the cytoplasm, and not in the plasma membrane. WT, d3PY, dPSAP, R55W, A18V, AADL, and YADA Gag polyproteins also showed similar subcellular localization in COS-7 cells as in 293T cells and HeLa cells. On the other hand, the YAAL mutant Gag, which showed membrane localization in 293T cells and HeLa cells, was observed only in the cytoplasm of COS-7 cells (Fig. 4, panel N).

These results indicated that the YxxL motif within p10<sup>MA</sup> plays an important role in the intracellular transport of Gag to the plasma membrane, but not in virus budding.

As found in virion production, the necessity of the aspartic acid (D) residue within the YADL sequence for membrane transport was different between cell lines.

## **Discussion**

To investigate the roles of three putative L-domains of M-PMV Gag in virus replication, we analyzed mutants with mutations within each L-domain motif in three cell lines. The results are summarized in Tables 1 – 3.

We have reported previously that the PPPY sequence within the pp24/pp16 region plays a critical role in M-PMV budding as an L-domain (7). Similar results have been reported for HTLV-1, Ebola virus, vesicular stomatitis virus (VSV), and Lassa virus, which contain both PPxY and PT/SAP sequences within their Gag or matrix proteins (26-30). Here, it was also shown that the PPxY motif plays an essential role in progeny virus production as a major L-domain in all three cell lines examined in this study (Fig. 2, Tables 1 – 3).

Gottwein et al. reported that the PSAP sequence in the pp24/16 region also functions as an additional L-domain in HeLaP4 cells and promotes the efficient release of M-PMV (21). In this study, we confirmed this observation in HeLa cells (Fig. 2, Tables 1 – 3). However, we found that PSAP is dispensable for M-PMV production in 293T and COS-7 cells, suggesting that the role of the PSAP motif as an L-domain in M-PMV budding is cell type-dependent. The PSAP motif appears to have no or only an additive role in M-PMV budding in the presence of the PPxY motif. Alternative cell

type-dependent usage of L-domains has also been reported in HTLV-1 (31). The cellular factors or systems that do not require the PSAP motif for virus budding should be present in COS-7 and 293T cells, but deficient or present at lower levels in HeLa cells. Viruses that possess multiple L-domains may change the L-domain usage to replicate in various cells.

In this study, we identified the third L-domain-like sequence, YADL, in the p10<sup>MA</sup> region of M-PMV Gag and analyzed the role of this YADL sequence in M-PMV replication. It has been reported that the YADL motif mediates endocytosis and plays a critical role in glycoprotein incorporation into virions as an intracellular trafficking signal (32). As shown in Fig. 2 and Tables 1 – 3, the AADL and YADA mutants are defective in transport to the plasma membrane, but not viral capsid assembly, indicating that the YADL motif is required for M-PMV production as a transport signal to the plasma membrane, but not as an L-domain.

## **Acknowledgments**

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## **Disclosure**

There are no conflicts of interest to declare.

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## Figure legends

Fig. 1. Schematic representations of wild-type and L-domain mutants of M-PMV. M-PMV Gag has a YxxL motif within the p10<sup>MA</sup> region and PPxY and PSAP motifs within the pp24/pp16 region.

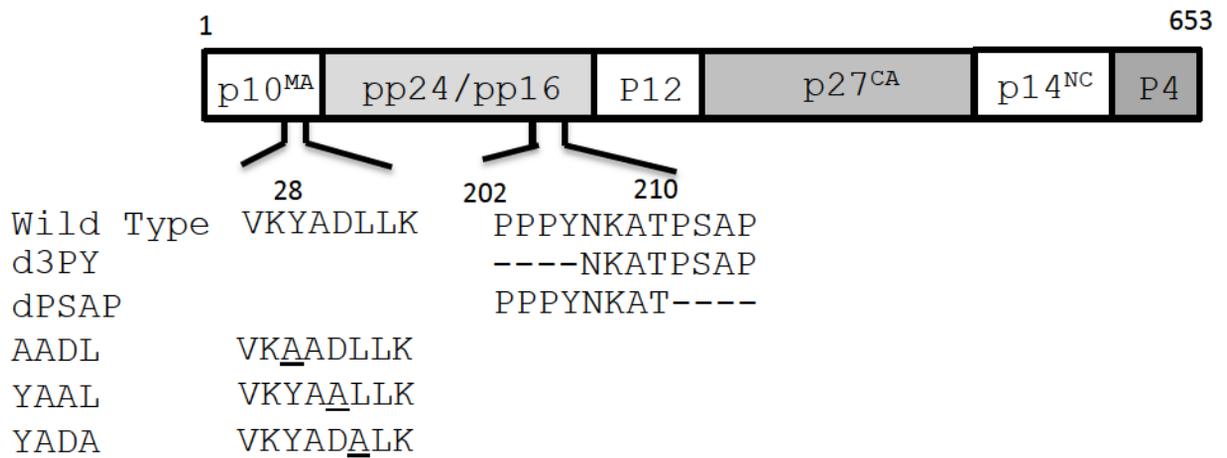
Fig. 2. Intracellular Gag protein expression and virus production of L-domain mutants in 293T, COS-7 and HeLa cells. In 293T cells (A), COS-7 cells (B), and HeLa cells (C), intracellular Gag polyprotein Pr78 expression (upper) and Gag-derived proteins (pp24 and p10<sup>MA</sup>) in virions produced from cells (lower) were detected by Western blotting. The efficiency of virus production in cells transfected with pSHRM15 (WT) was set to 1. The data are shown as averages and standard deviations of three independent experiments.

Fig. 3. Intracytoplasmic procapsid formation of L-domain mutants. 293T (A), COS-7 (B), and HeLa cells (C) expressing either WT or mutant M-PMV were lysed and assembled intracytoplasmic procapsids pelleted by ultracentrifugation. Free Gag polyproteins remained in the soluble fraction (S) and those incorporated into procapsids were recovered in the pellet fraction (P). The Gag polyproteins in each fraction were

analyzed by SDS-PAGE and Western blotting analysis.

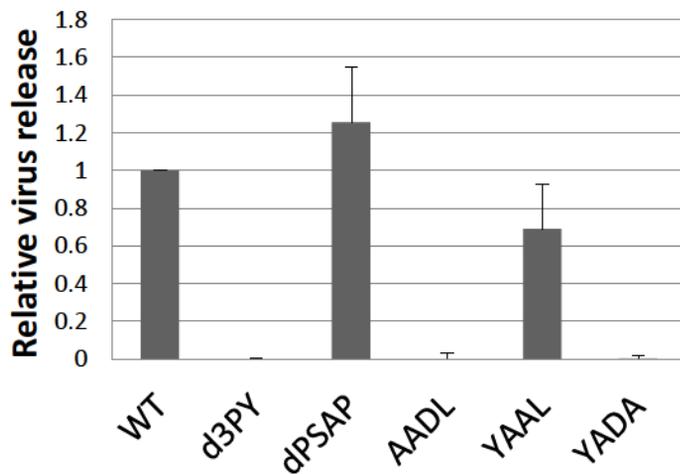
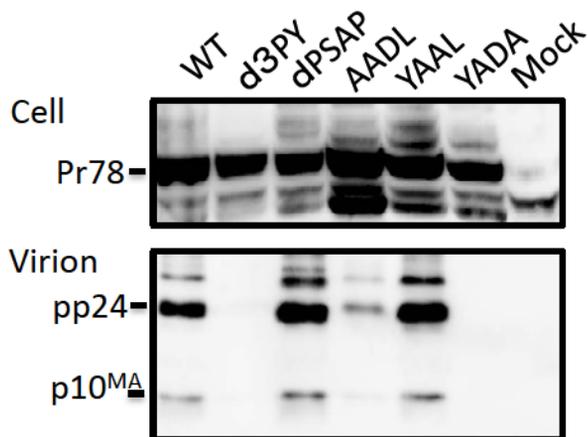
Fig. 4. Subcellular localization of WT or L-mutants of M-PMV Gag. In 293T, COS-7, or HeLa cells expressing WT or L-domain mutants of M-PMV, M-PMV Gag (Green) and the plasma membrane (Red) were stained as described in Materials and Methods. Nuclei (Blue) were stained with DAPI. The white arrowhead indicates co-localization of Gag and plasma membrane marker. Scale bar: 10  $\mu$ m.

M-PMV Gag Pr78

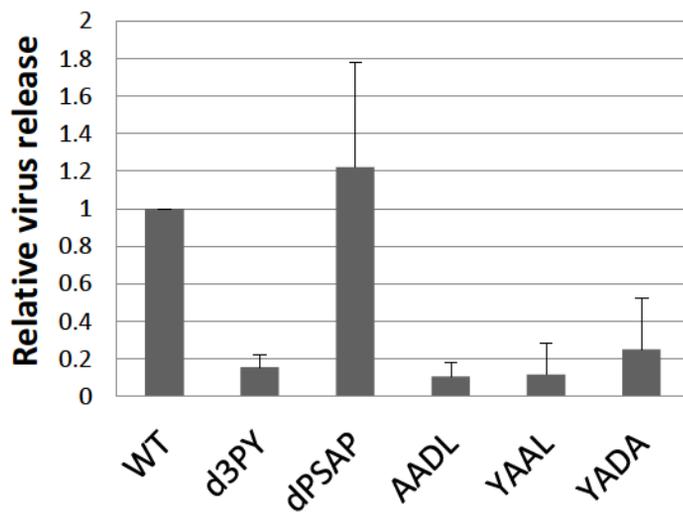
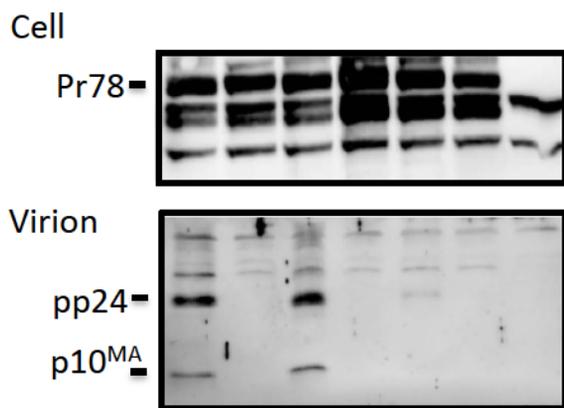


**Fig.1. Narahara & Yasuda**

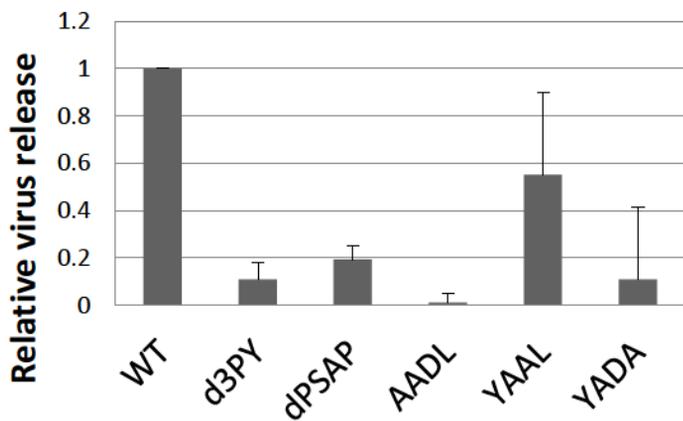
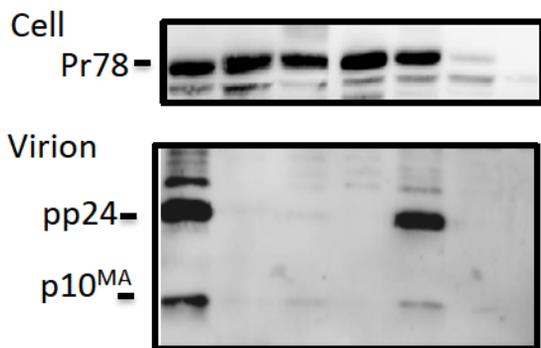
A. 293T cells



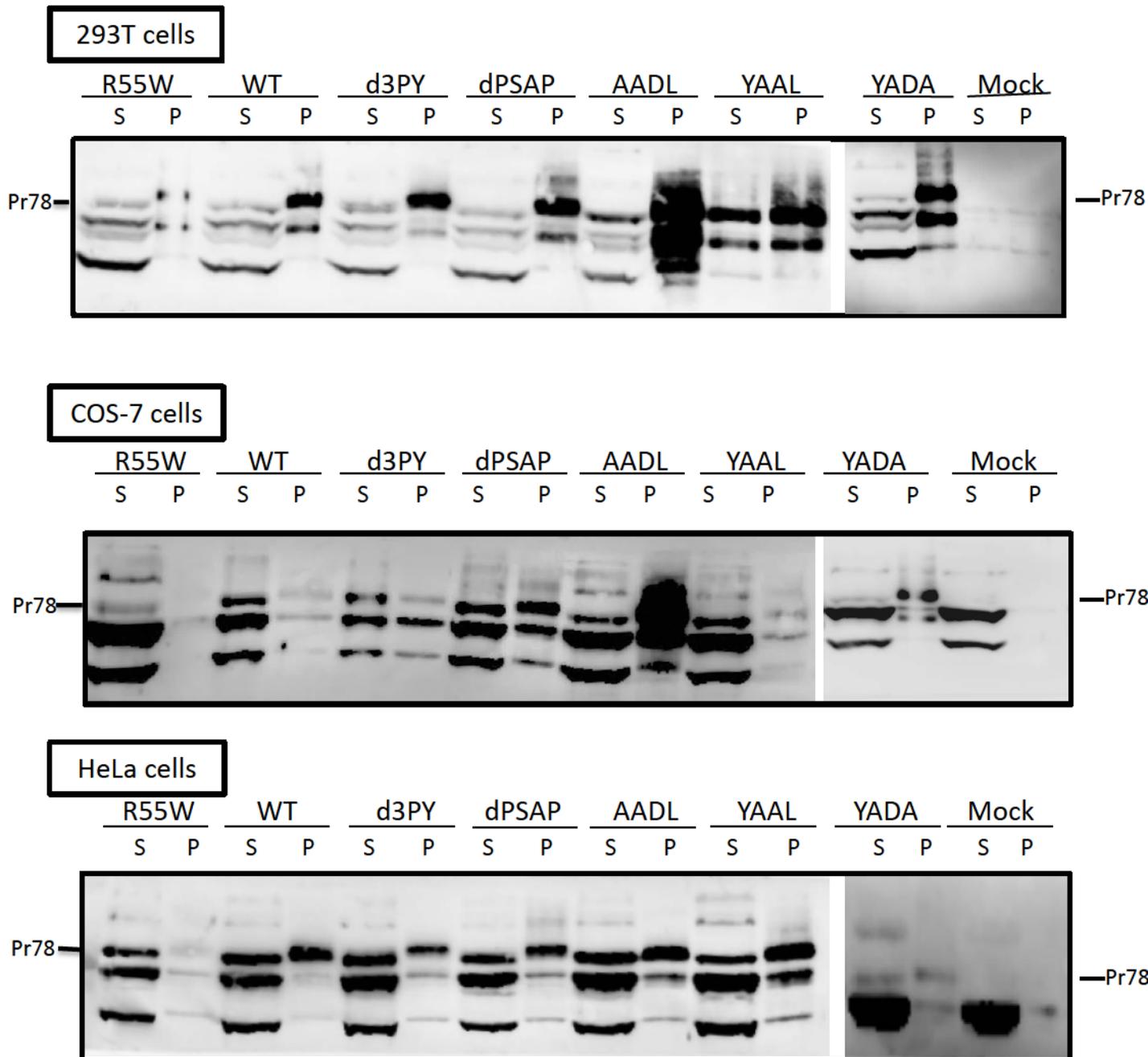
B. COS-7 cells



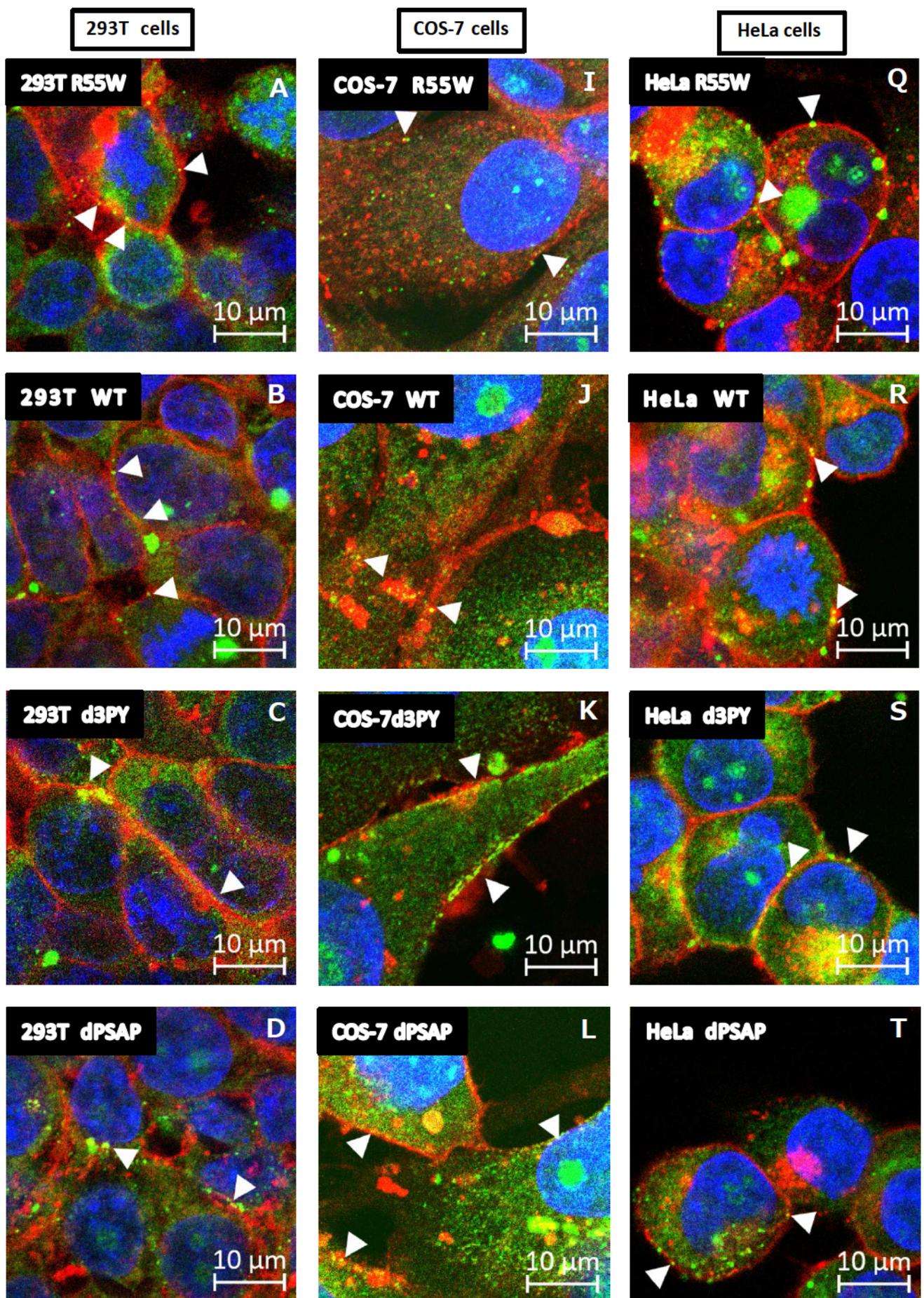
C. HeLa cells



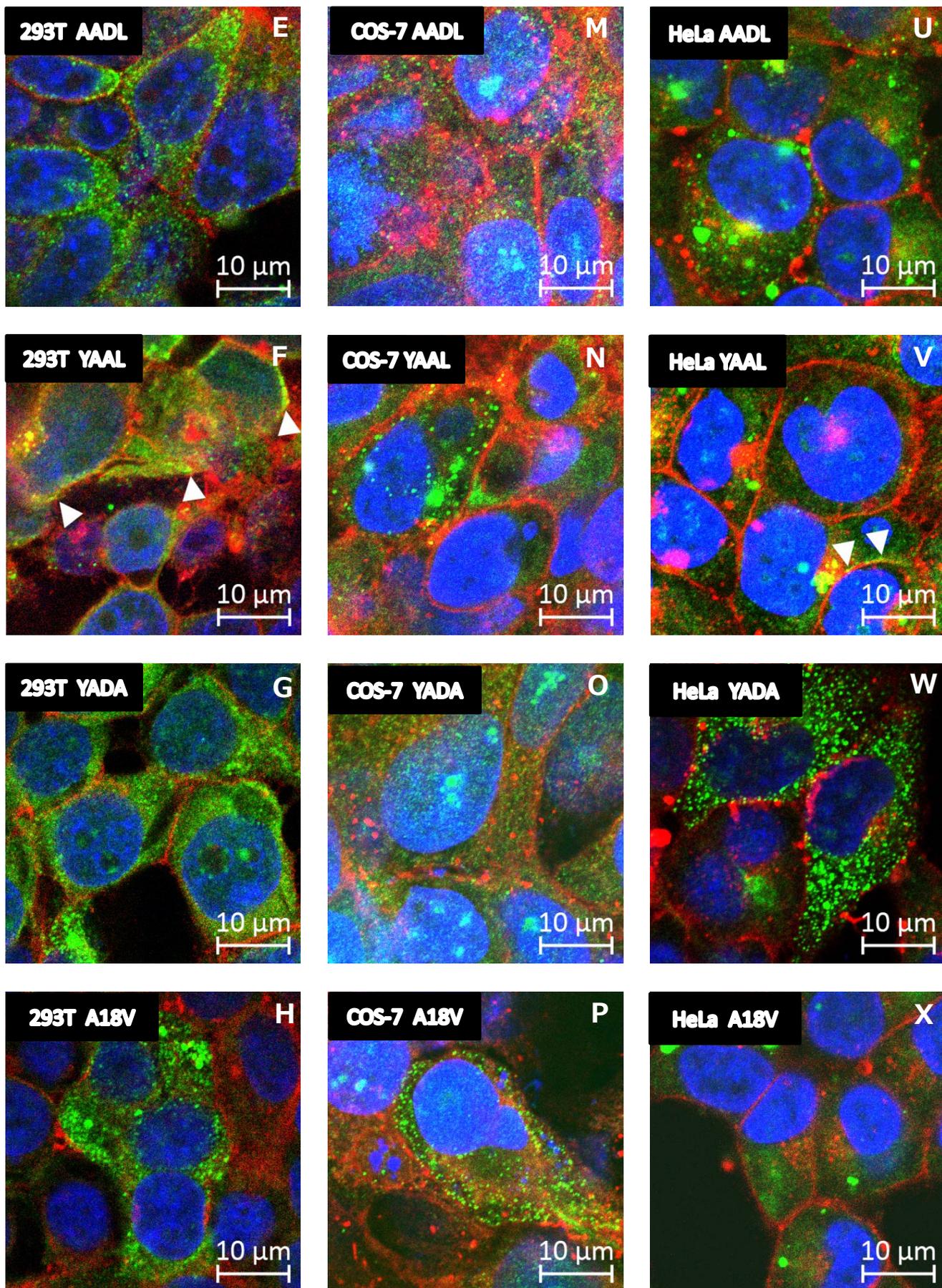
**Fig.2. Narahara & Yasuda**



**Fig.3. Narahara & Yasuda**



**Fig.4. Narahara & Yasuda**



**Fig.4. (Continued )** Narahara & Yasuda

**Table 1. Summary of the phenotypes of L-domain mutants of M-PMV in 293T cells**

Mutants	Mutations	Precursor stability	Capsid assembly	Virus release	Localization
Wild -Type		+++	+++	+++	Plasma membrane
Membrane direct transport mutant					
R55W	R(55)→W	+++	+		Plasma membrane
Transport-defective mutants					
A18V	A(18)→V	+++		-	Cytoplasm
Late domain mutants					
d3PY	PPPY→deletion	+++	+++	-	Plasma membrane
dPSAP	PSAP→deletion	+++	+++	+++	Plasma membrane
AADL	Y(28)→A	+++	++	-	Cytoplasm
YAAL	D(30)→A	+++	+++	++	Plasma membrane
YADA	L(31)→A	+++	+++	-	Cytoplasm

The efficiencies of Gag precursor stability, capsid assembly and virus release in each mutant are indicated as follows (those in WT set to 1.0).

+++; > 0.8; ++, > 0.4; + > 0.2; - ≤ 0.2; ND, not determined.

**Table 2. Summary of the phenotypes of L-domain mutants of M-PMV in COS-7 cells**

Mutants	Mutations	Precursor stability	Capsid assembly	Virus release	Localization
Wild -Type		+++	+++	+++	Plasma membrane
Membrane direct transport mutant					
R55W	R(55)→W	+++	+		Plasma membrane
Transport-defective mutants					
A18V	A(18)→V	+++		-	Cytoplasm
Late domain mutants					
d3PY	PPPY→deletion	+++	+++	-	Plasma membrane
dPSAP	PSAP→deletion	+++	+++	+++	Plasma membrane
AADL	Y(28)→A	+++	+++	-	Cytoplasm
YAAL	D(30)→A	+++	+++	-	Plasma membrane
YADA	L(31)→A	+++	+++	-	Cytoplasm

The efficiencies of Gag precursor stability, capsid assembly and virus release in each mutant are indicated as follows (those in WT set to 1.0).

+++ , > 0.8; ++ , > 0.4; + > 0.2; - ≤ 0.2; ND, not determined.

**Table 3. Summary of the phenotypes of L-domain mutants of M-PMV in HeLa cells**

Mutants	Mutations	Precursor stability	Capsid assembly	Virus release	Localization
Wild -Type		+++	+++	+++	Plasma membrane
Membrane direct transport mutant					
R55W	R(55)→W	+++	+	+++	Plasma membrane
Transport-defective mutants					
A18V	A(18)→V	+++		-	Cytoplasm
Late domain mutants					
d3PY	PPPY→deletion	+++	+++	-	Plasma membrane
dPSAP	PSAP→deletion	+++	+++	+	Plasma membrane
AADL	Y(28)→A	+++	++	-	Cytoplasm
YAAL	D(30)→A	+++	+++	+++	Plasma membrane
YADA	L(31)→A	+++	++	-	Cytoplasm

The efficiencies of Gag precursor stability, capsid assembly and virus release in each mutant are indicated as follows (those in WT set to 1.0).

+++ , > 0.8; ++ , > 0.4; + > 0.2; - ≤ 0.2; ND, not determined.