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Studies on the epidemiology of severe fever with thrombocytopenia syndrome virus infection  
and the role of glycoproteins in the intracellular transportation of viral structural proteins

(重症熱性血小板減少症候群ウイルス感染の疫学およびウイルス構造タンパク質の細胞内輸送にお  
ける糖蛋白質の役割に関する研究)

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# Contents

|                                                                                                                                   |     |
|-----------------------------------------------------------------------------------------------------------------------------------|-----|
| Abbreviations                                                                                                                     | iii |
| Preface                                                                                                                           | v   |
| Chapter 1: Serological survey of severe fever with thrombocytopenia syndrome virus<br>infection in Sika deer and rodents of Japan | 1   |
| 1. Introduction.....                                                                                                              | 1   |
| 2. Materials and methods .....                                                                                                    | 3   |
| Study animals and sample collection.....                                                                                          | 3   |
| Antigen preparation for the detection of anti-SFTSV antibodies .....                                                              | 5   |
| Enzyme-linked immunosorbent assay (ELISA) .....                                                                                   | 5   |
| Indirect immunofluorescence assay (IFA).....                                                                                      | 6   |
| Focus reduction neutralization test (FRNT) .....                                                                                  | 7   |
| Statistical analysis.....                                                                                                         | 7   |
| 3. Results.....                                                                                                                   | 8   |
| Deer survey.....                                                                                                                  | 8   |
| Rodent survey .....                                                                                                               | 8   |
| 4. Discussion.....                                                                                                                | 15  |
| 5. Summary.....                                                                                                                   | 17  |
| Chapter 2: Targeting of SFTSV structural proteins to the ERGIC and Golgi complex                                                  | 18  |
| 6. Introduction.....                                                                                                              | 18  |
| 7. Materials and methods .....                                                                                                    | 20  |
| Cells .....                                                                                                                       | 20  |

|                                                                                                                                  |    |
|----------------------------------------------------------------------------------------------------------------------------------|----|
| Viral infection .....                                                                                                            | 20 |
| Plasmid construction.....                                                                                                        | 21 |
| Expression of recombinant proteins .....                                                                                         | 22 |
| Western blot (WB) analysis.....                                                                                                  | 22 |
| IFA .....                                                                                                                        | 23 |
| Immunoprecipitation.....                                                                                                         | 23 |
| 8. Results.....                                                                                                                  | 24 |
| Detection system of SFTSV structural proteins .....                                                                              | 24 |
| The subcellular localization of structural proteins in SFTSV-infected cells .....                                                | 27 |
| The subcellular localization of SFTSV structural proteins in cells singly transfected with<br>GP, NP or L encoding plasmids..... | 30 |
| GP interacts with L and NP .....                                                                                                 | 33 |
| GP is required for the recruitment of L to the ERGIC and Golgi complex.....                                                      | 35 |
| Localization of NP to the ERGIC and Golgi complex .....                                                                          | 38 |
| 9. Discussion.....                                                                                                               | 40 |
| 10. Summary.....                                                                                                                 | 44 |
| 11. Conclusion .....                                                                                                             | 45 |
| Acknowledgements .....                                                                                                           | 47 |
| References .....                                                                                                                 | 50 |
| Summary in Japanese .....                                                                                                        | 58 |

## Abbreviations

|                      |                                                      |
|----------------------|------------------------------------------------------|
| BSA                  | bovine serum albumin                                 |
| BUNV                 | Bunyamwera virus                                     |
| cDNA                 | complementary DNA                                    |
| DIC                  | disseminated intravascular coagulation               |
| DMEM                 | Dulbecco's modified Eagle's minimum essential medium |
| ELISA                | enzyme-linked immunosorbent assay                    |
| ER                   | endoplasmic reticulum                                |
| ERGIC                | endoplasmic reticulum-Golgi intermediate compartment |
| FBS                  | fetal bovine serum                                   |
| FRNT                 | focus reduction neutralization test                  |
| Gc                   | C terminal glycoprotein                              |
| Gn                   | N terminal glycoprotein                              |
| GPC                  | glycoprotein precursor                               |
| GP                   | glycoprotein                                         |
| HA                   | hemagglutinin                                        |
| <i>H.longicornis</i> | <i>Haemaphysalis longicornis</i>                     |
| HCl                  | hydrochloride                                        |
| hpi                  | hours post infection                                 |
| hpt                  | hours post transfection                              |
| Huh7                 | Hepatocellular carcinoma cells                       |
| IFA                  | indirect immunofluorescence assay                    |
| IFN                  | interferon                                           |
| L                    | large segment                                        |
| M                    | medium segment                                       |

|       |                                                         |
|-------|---------------------------------------------------------|
| MCS   | multi cloning site                                      |
| MEM   | Eagle's minimum essential medium                        |
| NaCl  | sodium chloride                                         |
| NP    | nucleocapsid protein                                    |
| NSs   | non-structural protein                                  |
| OD    | optical density                                         |
| ORF   | open reading frame                                      |
| PBS   | phosphate-buffered saline                               |
| PBST  | phosphate-buffered saline Tween-20                      |
| PCR   | polymerase chain reaction                               |
| PDI   | protein disulfide isomerase                             |
| RdRp  | RNA dependent RNA polymerase                            |
| rpm   | revolutions per minute                                  |
| RVFV  | Rift Valley fever virus                                 |
| S     | small segment                                           |
| SFTS  | severe fever with thrombocytopenia syndrome             |
| SFTSV | severe fever with thrombocytopenia syndrome phlebovirus |
| STAT  | signal transducer and activator of transcription        |
| UUKV  | Uukuniemi virus                                         |
| UV    | ultraviolet                                             |
| WB    | Western blotting                                        |
| WGA   | Wheat germ agglutinin                                   |
| YG    | Yamaguchi                                               |

## Preface

Severe fever with thrombocytopenia syndrome (SFTS) was first reported in China in 2011<sup>48)</sup>. The disease is caused by the SFTS virus (SFTSV) which belongs to the *Phenuiviridae* family in the order *Bunyavirales*<sup>1)</sup>. The order *Bunyavirales* was recently proposed to include 9 families of viruses and 13 genera. According to the International Committee on Taxonomy of Viruses, the genus phlebovirus is now classified into the *Phenuiviridae* family<sup>1)</sup>.

SFTS is a tickborne zoonotic infection that is transmitted by *Haemaphysalis longicornis* (*H. longicornis*) and *Amblyomma testudinarium* ticks<sup>48,52)</sup>. Ticks acquire SFTSV through blood meals from infected animals. Infected ticks transmit the virus to uninfected animals and humans. Within the tick population, SFTSV can be transmitted vertically from infected adult ticks to eggs<sup>19,48,52)</sup> (Figure 1). Though infection of humans through tick bite is the most common mode of transmission, human-to-human transmission of SFTSV is possible through contact with infected person's body fluids<sup>6)</sup>. Animal reservoirs have been suggested in the maintenance of SFTSV in nature. In Japan, SFTSV sero positive deer, wild boar, raccoon, goats, sheep and cattle have been reported<sup>8)</sup>. Since its discovery in Japan, SFTSV has continued to infect humans in western Japan, the affected prefectures are shown in the map in Figure 2. As of November 29, 2017, a total of 318 SFTS patients had been reported in Japan since 2013<sup>54)</sup>. The number of patients peaks around spring to autumn; probably as a result of increased tick activity<sup>54)</sup>. Infection in humans causes a syndrome that consists of fever, gastrointestinal symptoms, thrombocytopenia, leukocytopenia, hemorrhage disseminated intravascular coagulation (DIC) and multiple organ failure. The case fatality rates vary among the countries reporting cases, ranging from ~12% in China<sup>5,18)</sup>, to ~6.3–30% in Japan<sup>12,54)</sup>, to as high as 47% in South Korea<sup>38)</sup>. In Japan SFTSV was first isolated in 2013 from a patient of Yamaguchi (YG) prefecture who had developed symptoms such as fever, vomiting,

diarrhea and thrombocytopenia. The SFTSV strain that was isolated was designated as the YG1 strain<sup>40)</sup>. This is the SFTSV strain that was used in the experiments reported in this thesis.

The viruses in the family *Phenuiviridae* form spherical particles and have a tri-segmented negative stranded RNA genome which includes the large (L), medium (M) and small (S) segments. The viral particles have an envelope membrane that is studded by glycoproteins (Figure 3 A). The L, M and S segments encode RNA-dependent RNA polymerase (RdRp), envelope glycoprotein precursor (GPC) and the nucleocapsid protein (NP), respectively. The S segment of Phenuiviruses encodes a nonstructural protein (NSs) in an ambisense manner (Figure 3B).

The lifecycle of phenuiviruses begins with the attachment of glycoprotein to the cell membrane of host cells. The virus then enters the cell by receptor-mediated endocytosis followed by low pH-dependent fusion of the endosomal membrane and the envelope of the virus to release the nucleocapsid complex (a complex of the viral genome, NP and RdRp) to the cytoplasm. The released viral genome is replicated and transcribed by RdRp. GPC is translated by ribosomes on the endoplasmic reticulum (ER) and then modified to glycoproteins (GP) by cleavage into Gn at the N-terminus and Gc at the C-terminus. The synthesized GP traffics and accumulates in the ER - Golgi Intermediate Compartment (ERGIC) and the Golgi complex. NP and RdRp are synthesized on free ribosomes in the cytoplasm. The synthesized NP and RdRp form a nucleocapsid complex with the replicated viral RNA. The nucleocapsid associates with GP and buds into the Golgi complex to form progeny virus particles. Viral particles formed in the Golgi body are released from the cells by exocytosis (Figure 4).

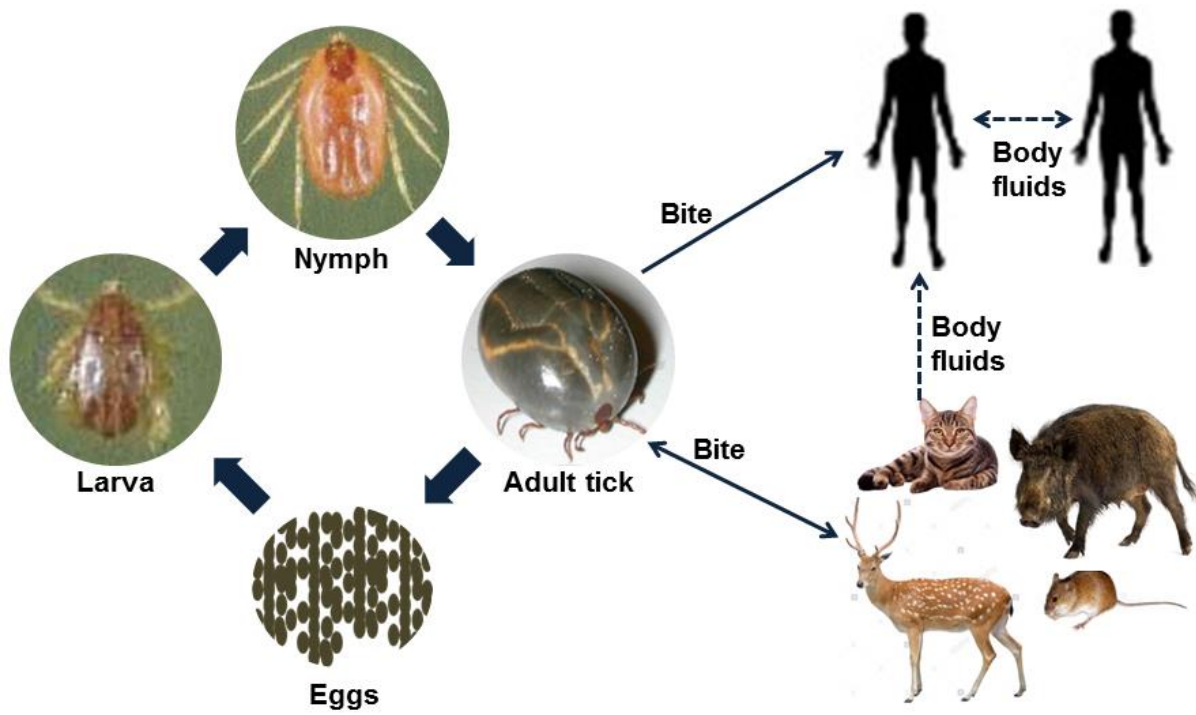


Figure 1 The transmission cycle of SFTSV. The maintenance of SFTSV in the tick population is shown as well as the role that ticks play in transmission of SFTSV to humans and animals. The solid arrows ( $\rightarrow$ ) represent the most common mode of transmission of SFTSV while the broken arrows ( $\dashrightarrow$ ) represent less common but possible transmission routes.

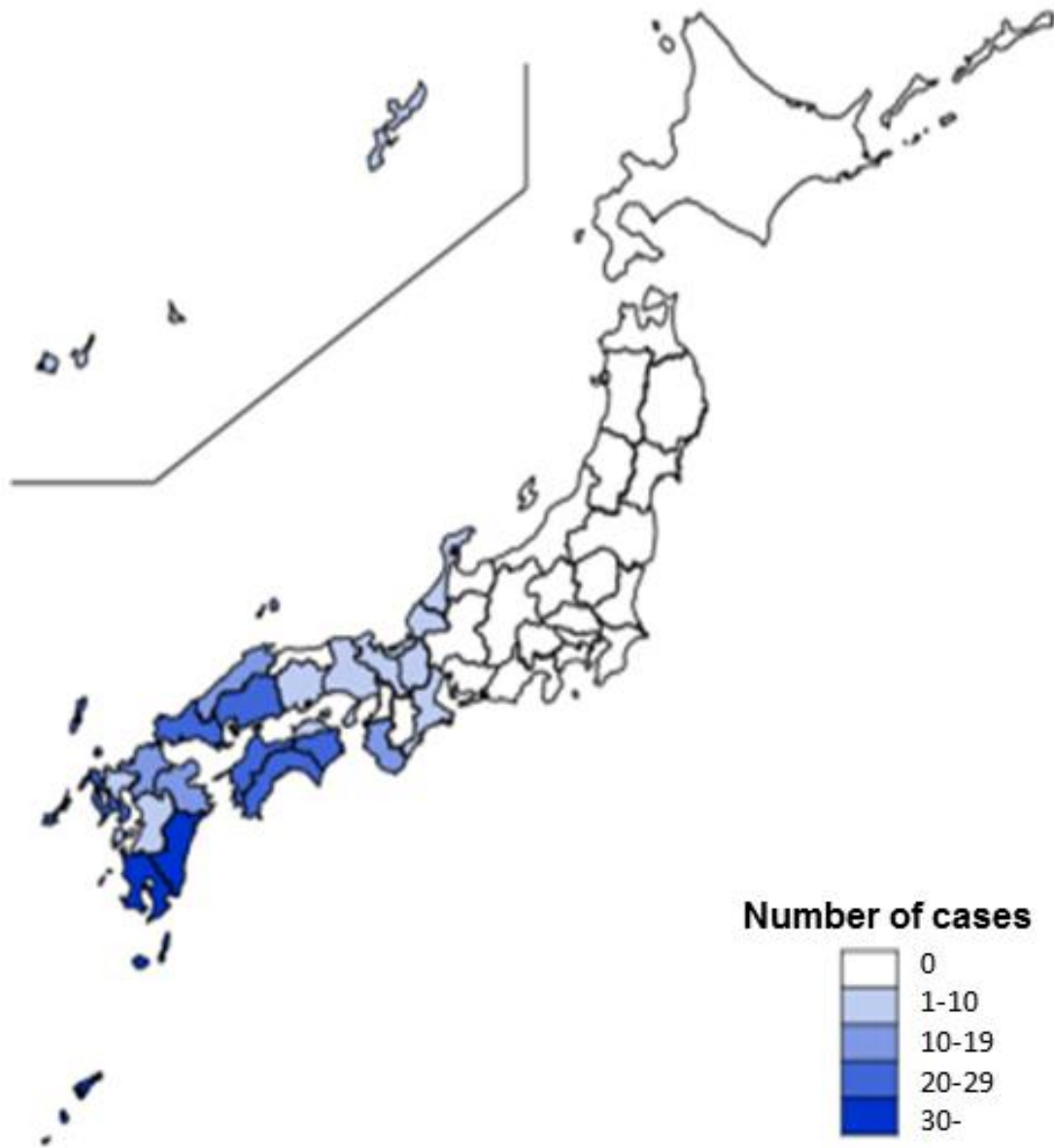


Figure 2 Map showing the prefectures where SFTSV infection occurred. The shades of blue represent the number of SFTS patients reported by each affected prefecture in Japan since 2013 as of 29 November 2017. Source: <https://www.niid.go.jp/niid/ja/id/2238-disease-based/sa/sfts/3143-sfts.html>

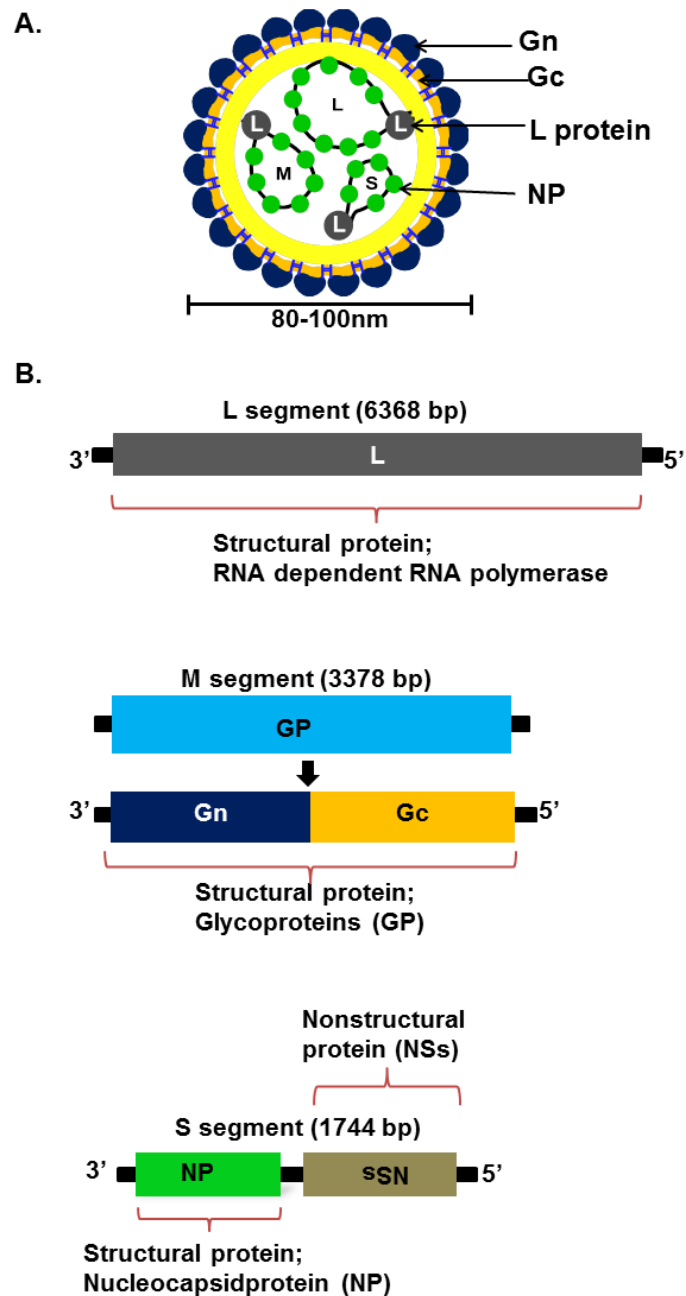


Figure 3 Schematic representation of (A) the morphology of SFTSV and (B) the organization of the genome showing the proteins that are encoded by the respective segments

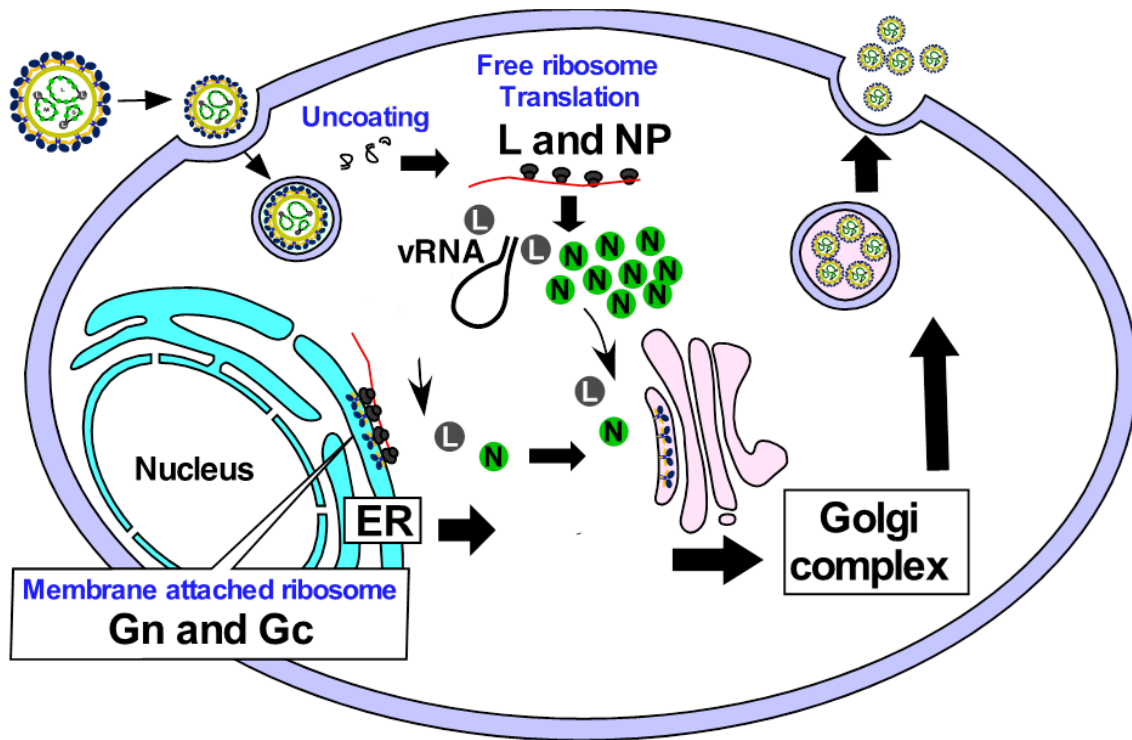


Figure 4 Schematic representation of the replication cycle of viruses in the family *phenuiviridae*

The structural proteins of SFTSV serve different functions; GP facilitates the entry of SFTSV into and fusion with membranes of host cells. NPs of phenuiviruses such as Rift valley fever phlebovirus encapsidate vRNA to form the nucleocapsid complex. The vRNA binding ability of NP is important for viral replication because loss of this function reduces the ability of vRNA to transcribe and replicate<sup>10)</sup>. The only non-structural protein encoded by SFTSV functions as an interferon antagonist and therefore suppresses host cell immune responses to facilitate invasion of the virus into host cells<sup>45,50)</sup>. SFTSV suppresses interferon (IFN) production by inhibiting retinoic acid-inducible gene I, TANK binding kinase 1, nuclear factor  $\kappa$ B and other molecules involved in type I IFN production by NSs<sup>32,35)</sup>. NSs also inhibits the intracellular signaling system by sequestering the signal transducer and activator of transcription (STAT) 2 and STAT 1<sup>23)</sup>.

Despite studies showing that ribavirin and T-705 (favipiravir) exhibit inhibitory effects on SFTSV production, there are still no specific antiviral drugs available to treat SFTS<sup>37,41)</sup>. The pathogenicity of SFTSV, the high case fatality rate caused by SFTS and the lack of antiviral treatment for the virus, make SFTSV an important human pathogen. Surveys conducted in wild and domestic animals to estimate the prevalence of anti SFTSV antibodies, have been useful for describing the epidemiology of the virus.

In chapter 1 of this thesis, the prevalence of anti-SFTSV antibodies in deer was assessed. Deer from Miyazaki, an area endemic to SFTSV and deer from Hokkaido, a non-endemic area, were screened.

Chapter 2 of this thesis is based on a study that was aimed at revealing the steps in the lifecycle of SFTSV in host cells. The intracellular localization of SFTSV structural proteins was assessed. The role of GP in targeting L protein and NP to the budding compartments in the ERGIC and Golgi complex was also analysed.

# Chapter 1: Serological survey of severe fever with thrombocytopenia syndrome virus infection in Sika deer and rodents of Japan

## 1. Introduction

SFTS recently emerged as a public health threat in China,<sup>47,48)</sup> Japan,<sup>40)</sup> and South Korea.<sup>13)</sup> SFTS is caused by the SFTSV, which belongs to the family *Phenuiviridae*. Until recently, SFTSV was considered a member of the *Bunyaviridae* family, genus *Phlebovirus*<sup>1,47,51)</sup>. Following a recent proposal to the International Committee on the Taxonomy of Viruses, the *Bunyaviridae* family was reclassified as the *Bunyavirales* order, and SFTSV now belongs to the *Phenuiviridae* family<sup>1)</sup>. SFTSV was first isolated in 2009 from the blood of a Chinese patient with SFTS<sup>48)</sup>. The majority of patients with SFTS have been identified in rural regions of China; however, SFTS cases and deaths due to the disease have also been reported in Japan and South Korea. SFTS is characterized by fever with gastrointestinal manifestations such as diarrhea, vomiting and nausea; drastic losses of leukocytes and platelets and hemorrhage. Severe cases progress to DIC and multiple organ failure, which usually results in death.<sup>48)</sup> Case fatality rates due to SFTS vary among the three countries with reported cases. The reported case fatality rates range from ~12% in China<sup>5,18)</sup>, to ~12–30% in Japan<sup>12)</sup>, to as high as 47% in South Korea<sup>38)</sup>. In Japan, SFTSV was first identified in 2013 and was traced back to as early as 2005 in a retrospective study of serum from patients who had had symptoms similar to those listed above.<sup>40)</sup> Following the discovery of SFTSV in Japan, the virus was found to be endemic to western Japan, where SFTS patients continue to be reported annually.<sup>40,53)</sup> SFTSV is transmitted by the ticks *H. longicornis*<sup>19,46)</sup> and *Rhipicephalus microplus*, from which the viral RNA was isolated in China and South Korea.<sup>52)</sup> In Japan, the ticks *H. longicornis* and *Amblyomma testudinarium* were identified as

SFTSV vectors,<sup>56)</sup> although other tick species may also carry the virus. The recent discoveries of pathogenic tick-borne phleboviruses<sup>9)</sup>, like Heartland virus in the USA<sup>10)</sup> and SFTSV in East Asia<sup>1,16)</sup>, have raised serious public health concerns. Elucidating the precise distribution of the pathogen within the regions and countries where patients are reported will provide useful information for disease risk assessment. By conducting serological surveys to estimate the prevalence of SFTSV antibodies in reservoir animals, the distribution of SFTSV in Japan can be determined. Serological studies of wild boar, deer, hedgehogs, wild rodents, hunting dogs, goats, sheep, cattle, chickens, and pigs suggest that a variety of animal species are infected with SFTSV.<sup>8,17,25,46)</sup> In Japan, the SFTSV tick–vertebrate–tick cycle seems to be well established, as observed by the high SFTSV seroprevalence in deer, wild boar, dogs, and raccoon dogs in the areas where SFTS occurs.<sup>53)</sup> Therefore, wild animals are useful sentinels for the sero-surveillance of SFTSV in Japan. Consequently, this study determined the geographic distribution of SFTSV activity in Japan. The data collected from this study will be useful for determining the risk of SFTSV transmission in endemic and non-endemic areas.

Deer from Miyazaki and Hokkaido and rodents from six areas in Japan were screened using enzyme-linked immunosorbent assays (ELISAs) and indirect immunofluorescence assays (IFA) for the presence of SFTSV antibodies in serum. By comparing the SFTSV antibody sero-prevalence in deer from Miyazaki with deer from Hokkaido, this study shows that the distribution of SFTSV activity was confined to the western parts of Japan during the sampling period, since antibody-positive animals were detected only in western Japan.

## 2. Materials and methods

### Study animals and sample collection

The sero-survey targeted animals in SFTSV endemic areas in western Japan and non-endemic areas in northern Japan. A total of 356 sika deer (*Cervus Nippon*) including 315 individuals of the subspecies of sika deer inhabiting Hokkaido (*Cervus nippon yesoensis*) and 910 rodents from Miyazaki, Toyama, Shimane, Gifu, Osaka and Yamagata Prefectures in western Japan were screened (Figure 5). The serum samples from the sika deer used in this survey were obtained by hunting and nuisance control culling from 2013 to 2014. The deer samples comprised 41 samples from Miyazaki Prefecture in western Japan and 315 from Hokkaido in northern Japan. The sample sizes and the inclusion of seven prefectures in this study were determined by the availability of samples. Banked rodent serum samples collected between 1997 and 2009 were included in this survey to assess whether SFTSV exposure could be traced back to before the virus was first identified. The rodent species that were sampled included 582 *Apodemus speciosus*, 51 *Apodemus argenteus*, 188 *Rattus norvegicus*, 1 *Rattus rattus*, 30 *Eothenomys smithii*, 19 *Eothenomys andersoni*, and 33 *Microtus montebelli*. The methods of rodent capture and serum preparation are described elsewhere.<sup>11)</sup>



Figure 5. Map showing the survey sites in Japan and animal species collected from each site. Rodents were collected from six prefectures (closed circles), while deer were collected from two sampling sites in north (closed blue triangle) and west Japan (closed red triangle). The grey areas are the prefectures where sika deer and rodents were captured.

## **Antigen preparation for the detection of anti-SFTSV antibodies**

Hepatocellular carcinoma (Huh7) cells grown in a 75-mL flask were infected with SFTSV YG-1 strain (an isolate from an SFTS patient in Yamaguchi Prefecture, Japan<sup>13</sup>) at a multiplicity of infection of 0.1 and cultured for 3 days in Dulbecco's modified Eagle's minimum essential medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). The cells were rinsed four times with phosphate-buffered saline (PBS). The cells were lysed with 1 mL of 1% NP-40 in PBS and incubated at room temperature for 10 min. The cell lysate was irradiated with ultraviolet (UV) light on a UV transilluminator for 10 min. The lysate was centrifuged at 12,000 rpm for 5 min in a microcentrifuge. The supernatant was collected and stored at  $-80^{\circ}\text{C}$  until use. The lysate of mock-infected cells was prepared similarly.

## **Enzyme-linked immunosorbent assay (ELISA)**

The serum samples were analyzed using an ELISA developed by the National Institute of Infectious Diseases in Japan. The SFTSV-infected Huh7 cell lysate was used as the antigen for the ELISA. First, 96-well EIA/RIA plates (Corning) were coated with 50  $\mu\text{L}$  of SFTSV-infected Huh7 cell lysate antigen diluted to 1:800 in PBS and incubated overnight at  $4^{\circ}\text{C}$ . Mock-infected Huh7 cells were used as the control antigen and diluted similarly before applying to the plates. After incubation, the wells were washed four times with washing buffer composed of PBS and 0.05% Tween 20 (PBST). This was followed by blocking with 3% bovine serum albumin (BSA) and incubation at  $37^{\circ}\text{C}$  for 1 h. The washing was repeated as above. Control and test sera diluted in 3% BSA-PBST were applied to the plates and incubated at  $37^{\circ}\text{C}$  for 1 h. After washing, the plates were coated with horseradish peroxidase-

conjugated chimeric protein A/G (Thermo Fisher Scientific) diluted in 3% BSA-PBST and incubated for 1 h at 37°C. Washing was repeated and 100 µl/well of o-phenylenediamine dihydrochloride substrate (Wako) was used for detection. The plates were incubated at room temperature for 15 min and the absorbance was measured using a spectrophotometer at 450 nm. The results are expressed as the difference between the optical density (OD) value of the control antigen and that of the SFTSV antigen. The cut-off for positivity was determined as the mean of the negative control OD value  $\pm$  2SD. Based on this criteria, samples were considered positive if the OD value was  $> 0.1$ .

### **Indirect immunofluorescence assay (IFA)**

Vero E6 cells grown in a 75-cm<sup>2</sup> flask were infected with SFTSV YG-1 strain and cultured for 3 days in MEM supplemented with 10% FBS. The cells were trypsinized, dispersed, and collected in a tube. The cells were seeded onto 24-well slides and cultured for 5 h in a CO<sub>2</sub> incubator. The slides were washed with PBS, fixed with acetone for 20 min, and used as IFA antigen slides. The fixed slides were stored at -80°C until use. Acetone-fixed SFTSV-infected and non-infected (control) Vero E6 cells were incubated with serum diluted in PBS for 1 h, washed three times in PBS for 5 min each, and stained with Alexa Fluor 488-labelled protein G (Thermo Fisher Scientific). Finally, the slides were observed under a fluorescence microscope. A positive reaction was indicated by granular fluorescence in the cytoplasm of the Vero E6 cells. The antibody titer was determined as the reciprocal of the highest serum dilution that produced granular fluorescence in the cytoplasm. Samples were considered positive if the antibody titer was  $>1:16$ .

### **Focus reduction neutralization test (FRNT)**

Two-fold serial dilutions of serum were prepared in 96-well plates with an initial dilution of 1/10. The SFTSV was then diluted in MEM supplemented with 2% fetal calf serum, penicillin G, and streptomycin. The virus was diluted in MEM to a concentration of 100 focus-forming units per 50- $\mu$ L volume. Next, 50  $\mu$ L of virus was added to diluted serum in each well and the virus-serum mixture was incubated for 1 h at 37°C in a CO<sub>2</sub> incubator. The inoculum was then applied to the Vero E6 cells and incubated for 1 h. The inoculum was removed and the cells were incubated for 3 days with an overlay of 1.5% carboxymethyl cellulose. To count the foci formed by each serum dilution, the plates were analyzed by IFA, as described above. The neutralizing antibody titer was determined as the reciprocal of the highest serum dilution that reduced the viral foci counts by 50% when compared with the negative control serum. Anti-SFTSV antibodies were confirmed in samples that tested positive on all three tests, *i.e.*, ELISA, IFA, and FRNT.

### **Statistical analysis**

A one-tailed Fisher's exact test was used to compare the antibody prevalence rate between deer in Hokkaido (proportions of positive and negative sera) and deer in Miyazaki. A  $p$ -value < 0.05 indicated statistical significance.

### 3. Results

#### Deer survey

This study surveyed 356 sika deer. Of the 41 deer from Miyazaki, 2 (4.9%) were sero-positive for SFTSV antibodies (Table 1). The ELISA OD values for the positive sera ranged from 0.1 to 0.3 (Figure 6A). In the IFA, scattered cytoplasmic fluorescence was observed in SFTSV-infected Vero E6 cells (Figure 7A and B) that were reacted with serum #268 and #326 respectively, but not with control serum (Figure 7C). The two sera showed IFA titers > 16 (#268: titer=64 and #326; titer=128). OD values ranging between 0 and < 0.1 were obtained by ELISA for the serum from Hokkaido deer, with the majority lying between 0 and 0.05 (Figure 6B). When compared with deer from Miyazaki, the OD values for Hokkaido deer were significantly lower ( $p = 0.02$ ). The FRNT<sub>50</sub> detected SFTSV neutralizing antibodies in deer serum from Miyazaki (Table 2), but not serum from Hokkaido (Hokkaido data not shown).

#### Rodent survey

A total of 910 rodent serum samples were screened for antibodies against SFTSV (Table 3). The ELISA OD values ranged from 0 to 0.2, with the majority lying between 0 and 0.1 (Figure 6C). Of the 910 rodent samples, 14 samples with OD values  $\geq 0.1$  were then tested by IFA and all 14 samples tested negative for anti-SFTSV antibodies. Serum was considered positive only if it tested positive by both ELISA and IFA. Therefore, it was concluded that all of the rodent serum samples were negative for anti-SFTSV antibodies. False positive samples may be encountered by ELISA in sera of wild animals, especially in rodents. On the other

hand, ELISA has the advantage of testing a large number of samples. Therefore, the two-step strategy to detect anti-SFTSV antibodies in wild animals was adopted. ELISA was used for screening then IFA or FRNT for confirmation. Hayasaka et al. detected anti-SFTSV antibodies in wild boars by ELISA and FRNT in a similar way<sup>2)</sup>.

Table 1 Sika deer screened for antibodies against SFTSV in northern and western Japan

| Prefecture | Collection Year | Number screened | Confirmed positive, no. (%) |
|------------|-----------------|-----------------|-----------------------------|
| Hokkaido   | 2013            | 315             | 0 (0)                       |
| Miyazaki   | 2015            | 41              | 2 (4.9)*                    |
| Total      |                 | 356             | 2 (0.6)                     |

\* $p < 0.05$

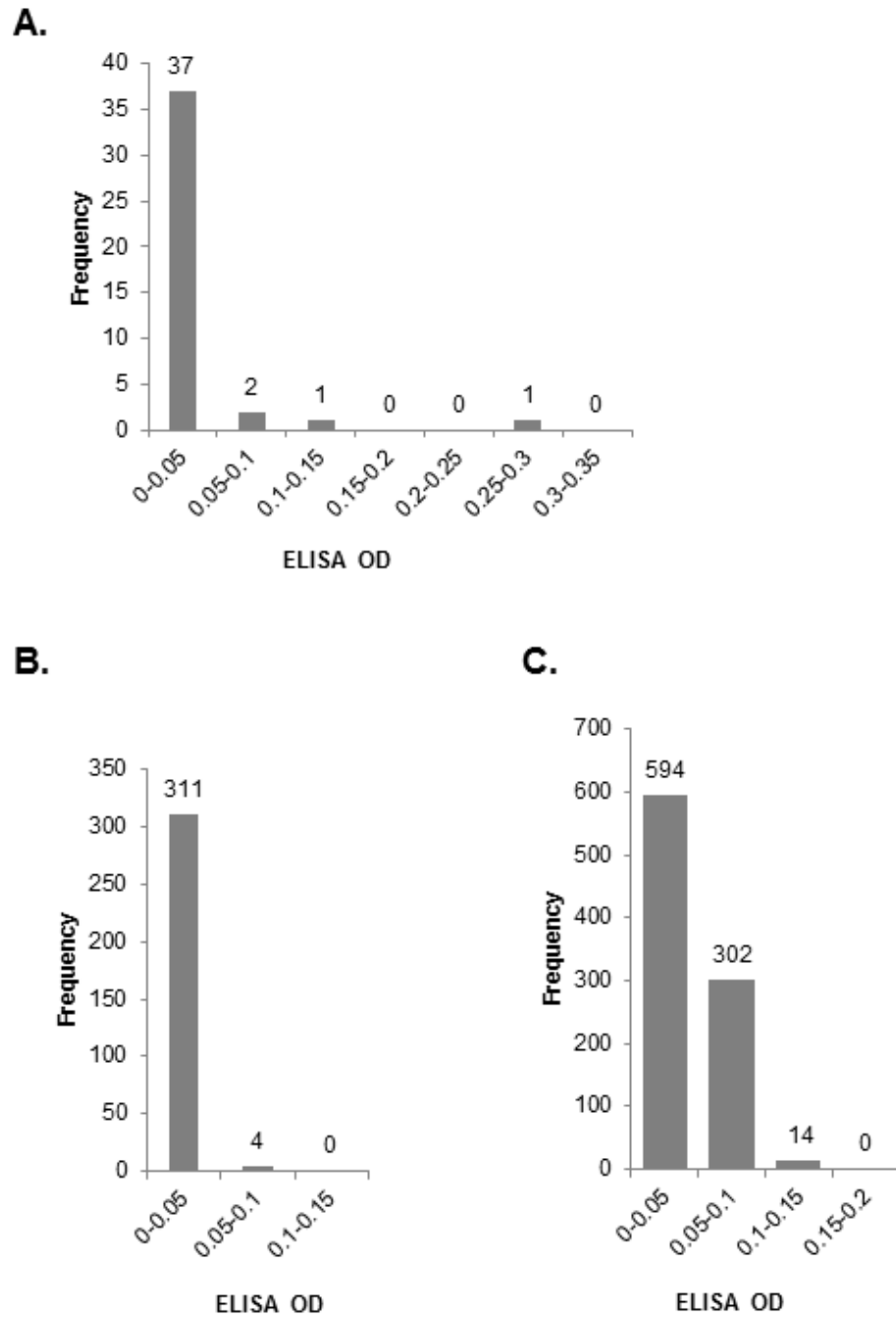


Figure 6. Detection of severe fever with thrombocytopenia syndrome (SFTS) phlebovirus (SFTSV) antibodies by enzyme linked immunosorbent assay (ELISA). The frequency distribution of ELISA optical density values for deer from Miyazaki (A) and Hokkaido (B) and rodent serum (C).

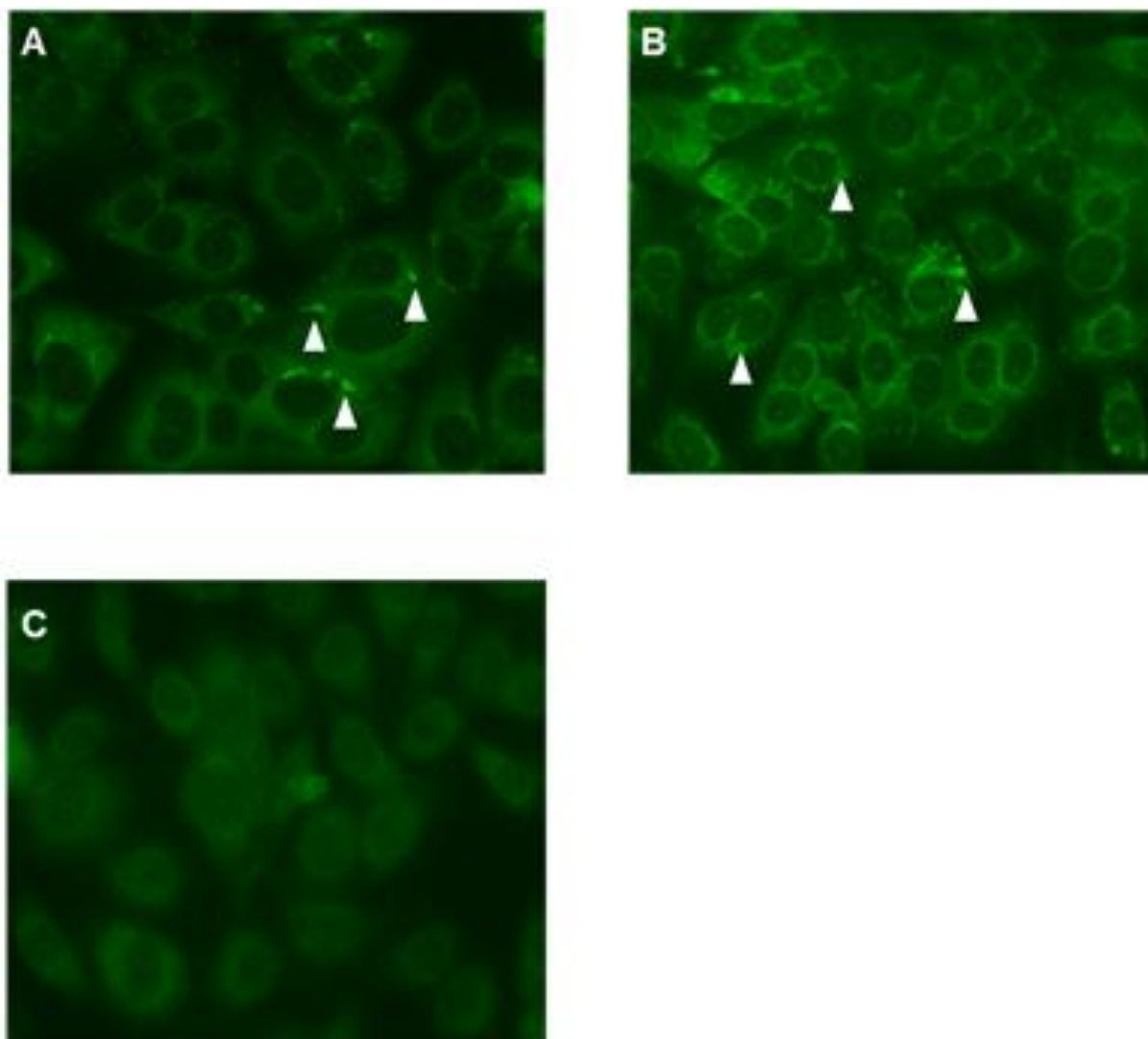


Figure 7 Detection of SFTSV antibodies with the immunofluorescence assay. A and B; SFTSV-infected Vero E6 cells that reacted with Sika deer serum (#268 and #362) showed cytoplasmic fluorescence in infected cells. The positive signal is indicated by the areas of fluorescence within the cytoplasm of SFTSV infected cells shown by the white arrow heads. (C) There was no fluorescence when the cells were stained with control serum.

Table 2 Reactivity of Sika deer serum on ELISA, IFA and focus reduction neutralization test

| Sample Number | ELISA | IFA titer | 50% focus reduction |
|---------------|-------|-----------|---------------------|
| 268           | +     | 64        | 40                  |
| 326           | +     | 128       | 320                 |

Table 3 Rodents screened for antibodies against SFTSV

| Prefecture | Collection Year | Number screened | Positive, no. (%) |
|------------|-----------------|-----------------|-------------------|
| Shimane    | 1997-2009       | 118             | 0 (0)             |
| Toyama     | 2000 & 2005     | 437             | 0 (0)             |
| Yamagata   | 2008            | 65              | 0 (0)             |
| Gifu       | 1997            | 176             | 0 (0)             |
| Osaka      | 2000            | 28              | 0 (0)             |
| Miyazaki   | 2001            | 86              | 0 (0)             |
| Total      |                 | 910             | 0 (0)             |

#### 4. Discussion

Using a combination of serological methods, the prevalence of SFTSV antibodies in Sika deer from Miyazaki in western Japan and Hokkaido in northern Japan was examined. Sika deer in Miyazaki had anti-SFTSV antibodies, whereas deer from Hokkaido had no antibodies to SFTSV, implying that SFTSV infections in deer continue to occur in western Japan. SFTSV is reported to be prevalent in both wild and domestic animals.<sup>25,40,46)</sup> In Japan, wild animals such as Sika deer<sup>53)</sup> and wild boar<sup>8)</sup> have tested positive for antibodies to SFTSV and serve as sentinels for estimating the seroprevalence of SFTSV antibodies, as well as for determining the geographic distribution of SFTSV. Seroprevalence studies in wild animals show that SFTSV antibodies are present in about 43.2% of the sika deer that were tested. In comparison, the percentage of seropositive wild boar ranges from 1.4 to 51%.<sup>8,53)</sup> Kurihara *et al.* reported that the risk of SFTSV infection in Nagasaki was low due to the absence of seropositive humans, even among populations that were considered to be at a high risk of SFTSV infection.<sup>15)</sup> Combined, these reports show that SFTSV infections in wild animals and humans are not widespread, even in areas where SFTSV is endemic. This concurs with the present survey, which found a low SFTSV prevalence of 4.9% in deer from Miyazaki, a known SFTSV endemic area. The serological survey was extended to Hokkaido, an area that is not endemic to SFTSV, to assess the possible geographical expansion of SFTSV activity. Comparing the SFTSV seroprevalence in deer from Miyazaki and Hokkaido, it was shown that SFTSV infections in deer have not spread to or are quite limited in Hokkaido. This concurs with the current status of SFTS patients being reported only in western Japan.<sup>53)</sup>

In this survey, only serum samples were available, therefore only serological testing could be conducted. With this limitation, a combination of serological tests is useful for screening for SFTSV activity. Interestingly, neutralizing antibodies were detected only in

deer from Miyazaki and not deer from Hokkaido. Therefore, leading to the conclusion that deer from Miyazaki had been exposed to SFTSV or a closely related virus. A report on SFTSV seroprevalence in Nagasaki also found SFTSV neutralizing antibodies in wild boar serum and the presence of a closely related virus that cross-neutralizes SFTSV antibodies was suggested.<sup>8)</sup> The cross reactivity of SFTSV and Heartland virus has been reported in animal serum in the USA.<sup>22)</sup> Going forward, it will be necessary to collect organs from animals for virus isolation and detection of the SFTSV genome.

The role of rodents in the transmission of SFTSV is not clear. Seropositive rodents have been reported in China and viral RNA detected in rodent species,<sup>16,17,25)</sup> suggesting that rodents are reservoir hosts for SFTSV. In the present survey, no rodent serum samples collected between 1997 and 2009 in Japan showed any evidence of SFTSV antibodies. At this point, it is unclear whether the SFTSV seroprevalence was generally low during this sampling period in the regions that were surveyed or the rodents were captured from areas of low endemicity. Further serological surveys of rodents in Japan will be useful for monitoring the expansion of SFTSV endemic areas and assessing the current risk for SFTSV infection in Japan. The results obtained in this survey will be useful for assessing the risk of SFTSV infection in Japan.

## 5. Summary

Severe fever with thrombocytopenia syndrome (SFTS) is a newly recognized zoonosis that occurs in China, Japan, and South Korea and is caused by the SFTS phlebovirus (SFTSV), which is in the genus *Phlebovirus*, family *Phenuiviridae*. Since its discovery in Japan in 2013, SFTS has been reported in the western parts of the country. To elucidate the distribution of SFTSV, a serological survey of Sika deer and rodents was conducted. Serum was screened using enzyme-linked immunosorbent assay (ELISA) and suspected cases were further tested with an indirect immunofluorescence antibody assay (IFA). Serum samples from 315 Sika deer from Hokkaido (non-endemic area), 41 Sika deer from Miyazaki (endemic area), and 910 rodents from six locations in Japan were tested. Of the 41 deer from Miyazaki, 2 (4.9%) had high ELISA optical density (OD) values ( $0.1 < OD < 0.3$ ) and a positive IFA result. All of the Sika deer samples from Hokkaido were negative by ELISA ( $OD < 0.1$ ). No SFTSV-positive rodents were found. These results indicate that Sika deer in Miyazaki were exposed to SFTSV, unlike deer from Hokkaido ( $p < 0.05$ ).

## Chapter 2: Targeting of SFTSV structural proteins to the ERGIC and Golgi complex

### 6. Introduction

SFTS is a febrile disease of humans characterized by severe fever, diarrhea, vomiting, nausea, leukocytopenia, thrombocytopenia, elevated liver enzymes and multiple organ failure<sup>12)</sup>. The syndrome caused by SFTSV is similar to the syndrome caused by Heartland virus another recently discovered pathogenic tick-borne phlebovirus, reported in the USA that causes severe illness in humans<sup>20)</sup>. Although ribavirin and T-705 (favipiravir) exhibit inhibitory effects on SFTSV production, there are still no specific antiviral drugs available to treat SFTS<sup>37,41)</sup>. Owing to the lack of specific antiviral treatments and to the severity of the disease caused by SFTSV and other recently discovered tick-borne phleboviruses such as Heartland virus, it is imperative to understand the lifecycle of SFTSV. Such knowledge will be useful for developing antivirals against SFTSV and other pathogenic phleboviruses.

The SFTSV genome consists of three single stranded negative-sense RNA segments. The large, medium, and small segments encode L, Gn/Gc and NP and NSs proteins, respectively. Viruses of the *Phenuiviridae* family replicate their genome in the cytoplasm of infected cells, and assemble and bud into the Golgi complex<sup>26,34)</sup>. After entry into cells, the viral components are released into the cytoplasm where transcription and replication of the viral genome occur. Similar to strategies of other phleboviruses, SFTSV entry into host cells is mediated by Gn. Previous studies have shown that SFTSV Gn is responsible for trafficking Gc to the Golgi complex for virion assembly via a signal peptide present in Gn<sup>31)</sup>. The NP and L proteins of Rift Valley fever virus (RVFV), Uukuniemi virus (UUKV), and presumably SFTSV lack signal peptides, and are translated on cytoplasmic ribosomes<sup>9,30,31)</sup>.

Phlebovirus GP is synthesized in the ER and then trafficked through the secretory pathway. Trafficking of viral components through the secretory pathway is necessary for the formation of progeny virus. It is generally known that the *Phenuiviridae* family viruses bud at the membranes of the Golgi complex. Recently, however, the ERGIC has also been proposed to be a site of virion assembly for other viruses in the *Bunyavirales* order such as UUKV<sup>9)</sup>, hantaviruses<sup>33)</sup>, and RVFV<sup>4)</sup>. For virus assembly to occur, viral structural components, as well as the genome, should be targeted to the same intracellular compartment. In the *Phenuiviridae* family, GP plays an important role in targeting other structural components to the sites of virus assembly and maturation. Immunocytochemical and electron microscopy studies have shown that UUKV, Bunyamwera virus (BUNV), RVFV, and hantavirus GP accumulate in the pre-Golgi and Golgi complex<sup>3,7,9,26,28,30,33,36)</sup>. However, it remains to be determined which cellular compartments are involved in the maturation of SFTSV. The accumulation of GP in the ERGIC and Golgi complex plays a central role in driving the formation of progeny virus. The NP and L proteins of RVFV<sup>4,30)</sup> and UUKV<sup>27)</sup> traffic through the secretory pathway via interactions with GP. It remains unknown how the NP and L proteins of SFTSV are recruited to the ERGIC and Golgi complex for virus assembly. Revealing the basic steps in the lifecycle of SFTSV could provide the necessary information needed to develop specific inhibitors for SFTSV. The high biosafety containment requirement for handling SFTSV has delayed progress in this area of research. Therefore, the use of plasmid vectors for the expression of SFTSV proteins is a convenient way to circumvent the requirement for conducting experiments in high biosafety containment facilities.

In this study, fluorescence microscopy was used to examine the subcellular localization of SFTSV structural proteins in cells infected with SFTSV and in cells expressing recombinant proteins. These results suggest that GP is required for targeting L to the ERGIC

and Golgi complex. However, the targeting of NP requires interaction with other factors besides GP.

## **7. Materials and methods**

### **Cells**

Vero-E6 cells were maintained in minimum essential medium (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (Biowest, Riverside, MO, USA) and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Human embryonic kidney 293T cells and Hepatocellular carcinoma (Huh7) cells were grown in high-glucose Dulbecco's modified eagle medium (Gibco, Thermo Fisher Scientific Inc.) supplemented with 10% FBS and penicillin/streptomycin. Cells were cultured at 37°C in 5% CO<sub>2</sub>.

### **Viral infection**

The SFTSV YG1 strain used for all virus infection studies was a generous gift from Dr. Ken Maeda, Yamaguchi University<sup>24,40</sup>. For IFA, Vero-E6 cells were grown on 24-well 4-mm HTC glass slides (Thermo Fisher Scientific Inc., MA, USA). The cells were inoculated with virus at a multiplicity of infection of 0.02. After incubation for 72 h, cells were fixed with 10% neutral buffered formalin (Nacalai, Kyoto, Japan) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. All virus work was performed in biosafety level 3 conditions following governmental and institutional guidelines.

## Plasmid construction

The full open reading frames (ORFs) of SFTSV NP, GP, and L were cloned into the pCAGGS-MCS mammalian expression vector. Cloning primers for NP, GP and L were designed based on the Genbank sequence with accession numbers AB817995, AB817987 and AB817979, respectively. The plasmids were generated using standard molecular cloning techniques and confirmed by sequencing. Briefly, complementary DNA (cDNA) was synthesized by polymerase chain reaction (PCR) from RNA extracted from cells infected with the SFTSV YG1 strain. The ORFs of NP and GP from the cDNA were amplified with primers flanked by *EcoRI* and *BglII* sites and cloned into the pCAGGS-MCS plasmid vector. The resultant plasmids were designated pCSFTSV-GP<sup>43</sup> and pCSFTSV-NP, containing the ORFs of GP and NP, respectively. Epitope-tagged NP was similarly constructed using primers containing the hemagglutinin (HA) tag sequence. Three fragments of the ORF coding for L protein—SFTSV L-I (17–1749), SFTSV L-II (1591–5261), and SFTSV L-III (5135–6271)—were amplified from cDNA with primers flanked by *ClaI/BamHI*, *BamHI/SpeI*, and *SpeI/KpnI* endonuclease recognition sites, respectively, and cloned into the aforementioned sites of the pATX vector. Each fragment was then digested with the appropriate enzymes and ligated to generate the full coding sequence of L. The entire coding sequence of L was then inserted into the *ClaI* and *KpnI* sites of the pCAGGS-MCS vector to generate the L expression plasmid pCSFTSV-L. The plasmid sequence was confirmed by Sanger sequencing prior to use.

## **Expression of recombinant proteins**

Vero-E6 cells were transfected with either single or combinations of expression plasmids; pCSFTSV-GP, pCSFTSV-NP, or pCSFTSV-L by lipofection using 3  $\mu$ L TransIT LT1/ $\mu$ g DNA (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's protocol. The cells were incubated at 37°C for 24 h and fixed for immunofluorescence assays as described above.

## **Western blot (WB) analysis**

Vero-E6 cells infected with SFTSV and 293T cells transfected with pCSFTSV-GP, pCSFTSV-NP or pCSFTSV-L were rinsed once with PBS followed by lysis in lysis buffer containing 25 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM Ethylenediaminetetraacetic acid; 1% Nonidet P-40; 1 mM Dithiothreitol, and 5% glycerol. Samples were subjected to WB analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed, followed by transfer of proteins to a 0.45- $\mu$ m pore immunoblot polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked for 1 h at room temperature with Blockace (Dai Nippon, Osaka, Japan) followed by incubation with viral protein specific primary antibodies; the mouse anti-Gn monoclonal antibody (#6G6) was a gift from Dr. K. Yamada (Oita University, Japan), rabbit anti-NP polyclonal antibody raised against a 15-amino acid synthetic peptide (40-54) region of NP (Sigma Aldrich Japan, Custom products, Japan), or rabbit anti-L peptide polyclonal antibody raised against a 15-amino acid synthetic peptide (386–400) region of L (Frontier Institute Co., ltd, Japan). Horseradish peroxidase-conjugated anti-mouse/ anti-rabbit IgG were used as secondary antibody and incubated with the membrane for 1 h. After each incubation step, membranes

were washed three times with an appropriate volume of PBS for 5 min. The signals were developed by ECL reagents (Amersham, GE Healthcare Life Sciences, Piscataway, NJ, USA).

## **IFA**

Cells were labeled with rabbit anti-Gn or Gc polyclonal antibody (#6647 or #6653; ProSci Inc., CA, USA) as described by Tsuda et al.<sup>43)</sup>, anti-NP, or anti-L antibody. The ER marker protein disulfide isomerase, PDI mouse monoclonal antibody (Enzo Life Sciences, Farmingdale, NY, USA), anti ERGIC-53 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Golgi marker Alexa Fluor 594-conjugated wheat germ agglutinin (WGA; Thermo Fisher Scientific Inc.) were used to label the organelles. Alexa Fluor 488/594 anti-rabbit/anti-mouse antibodies (Thermo Fisher Scientific Inc.) were used as secondary antibodies, respectively. The slides were mounted with ProLong<sup>TM</sup> Gold antifade reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. Fluorescence images were acquired using a Zeiss LSM 700 confocal laser scanning microscope (Zeiss, Urbana, IL, USA) with a 63x oil lens.

## **Immunoprecipitation**

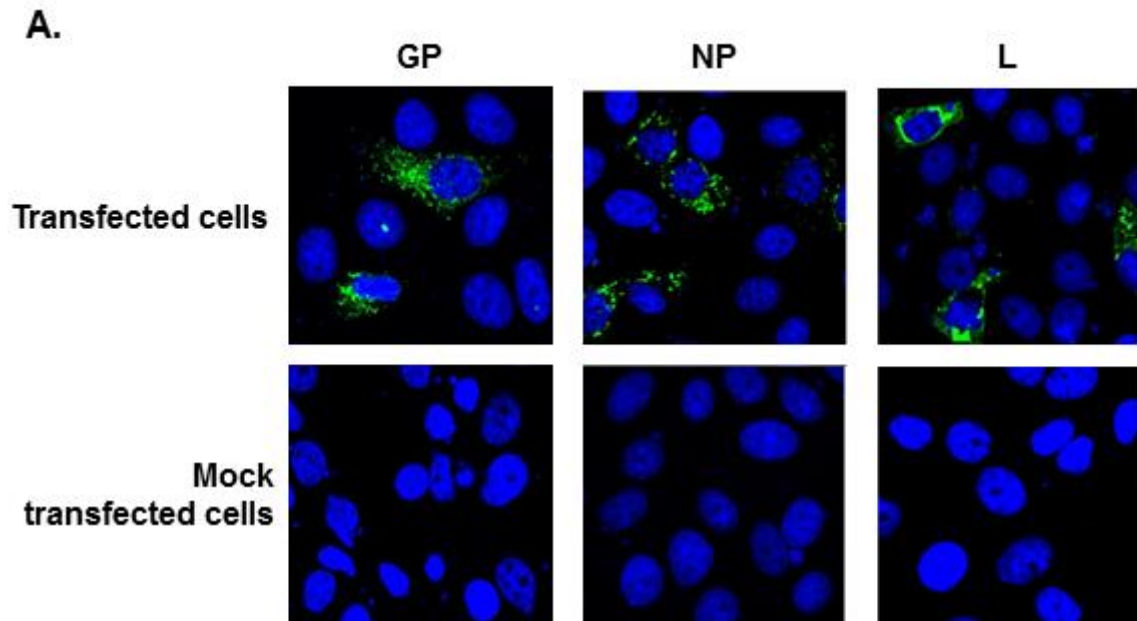
Huh7 cells infected with SFTSV were rinsed once with PBS followed by lysis in buffer containing 25 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM Ethylenediaminetetraacetic acid; 1% Nonidet P-40; 1 mM Dithiothreitol, and 5% glycerol. The cell lysates were incubated overnight at 4°C with protein A Sepharose beads (Pierce, Thermo Fisher Scientific Inc.) together with either rabbit anti-Gn IgG (#6647), rabbit anti-NP polyclonal, or rabbit anti-L

peptide antibodies. After incubation, the beads were washed with lysis buffer followed by elution of proteins in Laemli sample buffer (Bio-Rad, Hercules, CA, USA). Thereafter, samples were subjected to WB analysis.

## **8. Results**

### **Detection system of SFTSV structural proteins**

Viruses of the *Phenuiviridae* family use host cell intracellular compartments for the production of progeny virions. To understand the basic steps in the lifecycle of SFTSV, this work focused on the intracellular kinetics of virus structural proteins. Mammalian expression plasmids, pCSFTSV-GP, pCSFTSV-NP, or pCSFTSV-L, encoding GP, NP or L respectively were constructed. The ORF of each structural protein was inserted into the multi-cloning site of the mammalian expression vector (pCAGGS), and protein expression was confirmed by IFA and WB analysis. Using specific antibodies against the viral proteins the expressed proteins; GP, NP and L were specifically detected in the cytoplasm (Figure 8A). Rabbit anti-Gn and anti-Gc polyclonal antibodies specifically detected Gn and Gc in cells expressing GP. Both Gn and Gc showed similar expression pattern in GP expressing cells (data not shown), therefore, throughout this thesis only the term GP will be used to denote both Gn and Gc. The correct molecular weights of expressed proteins were confirmed by WB analysis (Figure 8B). The expression of L protein was much lower in infected cells compared to the plasmid expressing cells. Each protein expressed by the plasmids was the same size as viral protein detected in SFTSV-infected cells. These results indicate that the protein expression and detection system in this study are useful tools for analysis of viral protein dynamics in cells.



**B.**

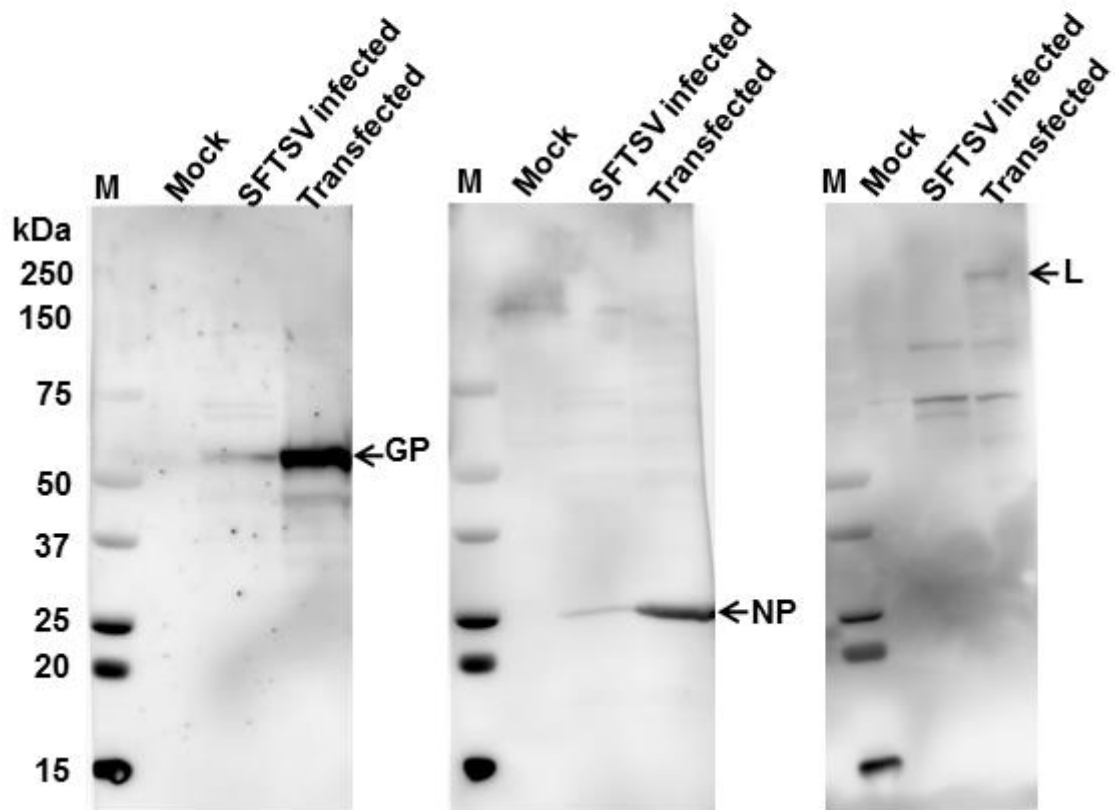


Figure 8. Expression of SFTSV structural proteins. (A) Vero-E6 cells were transfected with either expression plasmid, pCSFTSV-GP, pCSFTSV-NP or pCSFTSV-L and fixed at 24

hours post transfection (hpt). Cells were stained with anti- Gn, NP or L rabbit polyclonal antibodies for indirect immunofluorescence assay. 4',6-diamidino-2-phenylindole (DAPI) - stained nuclei (blue) and expression of the proteins (green) were analyzed by confocal microscopy. (B) Vero-E6 cells were infected with SFTSV YG1 and lysed at 72 hours post infection (hpi). 293T cells were transfected with expression plasmid, pCSFTSV-GP, pCSFTSV-NP or pCSFTSV-L and lysed at 24 hpt. Cell lysates were used for WB analysis and expressed proteins were determined by specific primary antibodies against each viral protein; mouse anti-Gn monoclonal antibody (#6G6), rabbit anti-NP polyclonal antibody or rabbit anti-L peptide polyclonal antibody. Arrows indicate the bands for the expressed viral proteins.

## **The subcellular localization of structural proteins in SFTSV-infected cells**

To determine the sites of formation of SFTSV particles, this study first investigated whether SFTSV structural proteins could be detected in pre-Golgi—namely the ER and ERGIC—as well as Golgi complex. Detailed analysis of the colocalization of SFTSV structural proteins with specific compartment markers was performed. Vero-E6 cells infected with SFTSV were fixed at 72 hpi and stained with organelle markers and antibodies to viral structural proteins (Figure 9). Colocalization experiments revealed that GP and L protein fluorescent signals colocalized with those of the ER, ERGIC and Golgi markers (Figure 9A and C). In contrast, a low degree of colocalization was observed between NP and the ER marker; NP mainly localized to the ERGIC and the Golgi complex (Figure 9B). These results suggested that SFTSV structural proteins start to assemble in the ERGIC as previously shown in Hantavirus virion assembly<sup>33)</sup>.

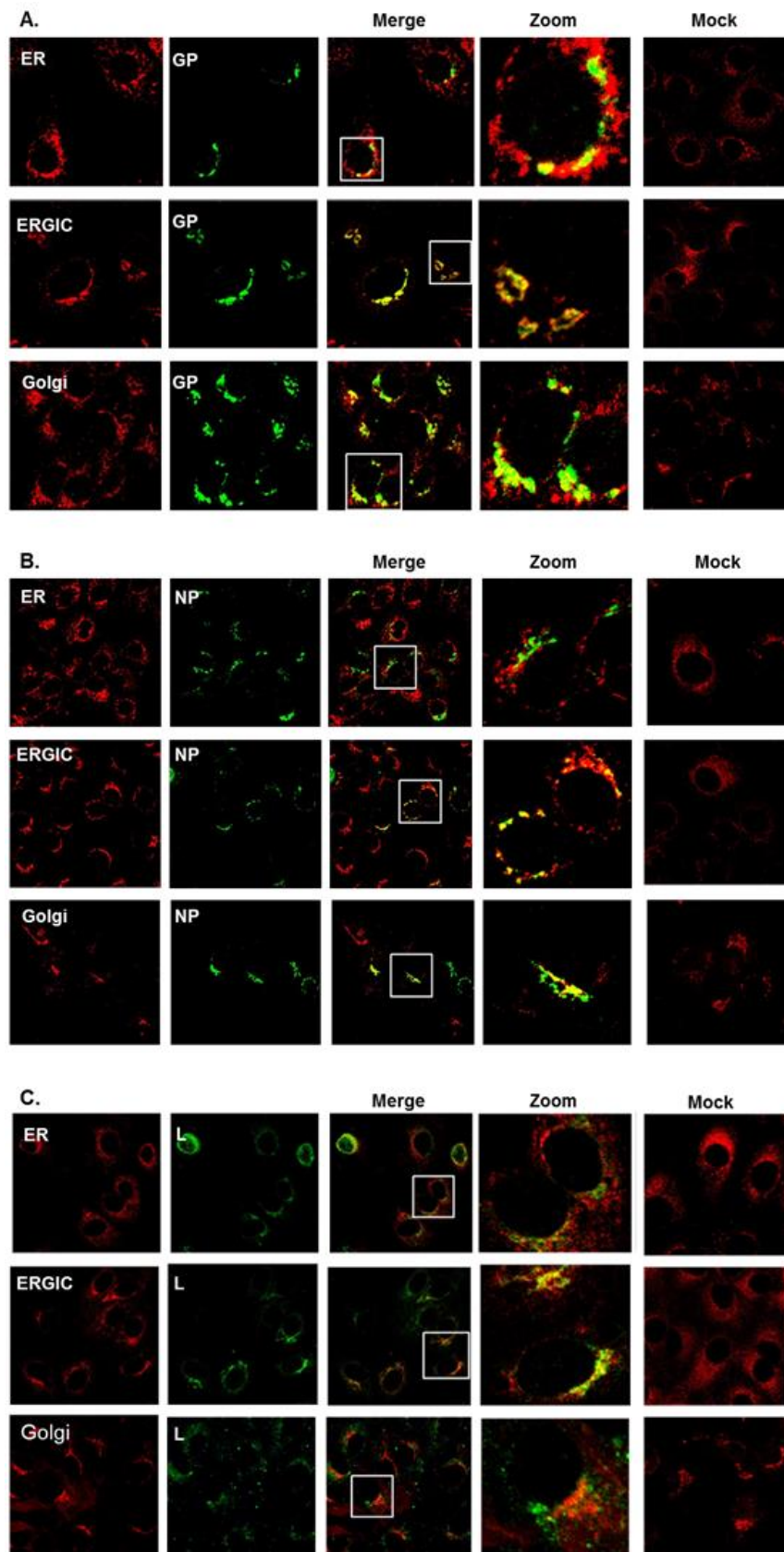


Figure 9. The subcellular localization of structural proteins in Vero-E6 cells infected with

SFTSV. Vero-E6 cells were infected with SFTSV YG1 and fixed at 72 hpi. Cells were stained with organelle markers along with antibodies against (A) GP, (B) NP or (C) L protein. Organelle markers were detected by Alexa Fluor 594 (red) secondary antibodies; the SFTSV proteins were detected by Alexa Fluor 488 secondary antibodies (green). The localization of each viral protein to the ER, ERGIC and Golgi was analyzed. The yellow areas in the merged image show colocalization of proteins with organelle markers. Magnification of the boxed areas is shown to the right of the merged image. Shown as mock, to the far right, are merged images of mock-infected cells.

## **The subcellular localization of SFTSV structural proteins in cells singly transfected with GP, NP or L encoding plasmids**

Vero E6 cells were transfected with either pCSFTSV-GP, pCSFTSV-NP, or pCSFTSV-L to assess whether GP, NP, and L could localize to the same intracellular compartments when the proteins were expressed singly. The subcellular localization of the SFTSV structural proteins was investigated in Vero-E6 cells at 24 hpt. Singly-expressed GP colocalized with the ER, ERGIC, and Golgi markers (Figure 10A). The subcellular localization of NP and L differed from that observed in infected cells shown in Figure 9; NP and L did not localize to the ER, ERGIC, and Golgi complex (Figure 10B and C). These results indicate that of the three SFTSV structural proteins, only GP localizes to the ER, ERGIC, and Golgi complex in the absence of other SFTSV components. In contrast, NP and L appeared to require other viral components for their ER, ERGIC, and Golgi complex localization.

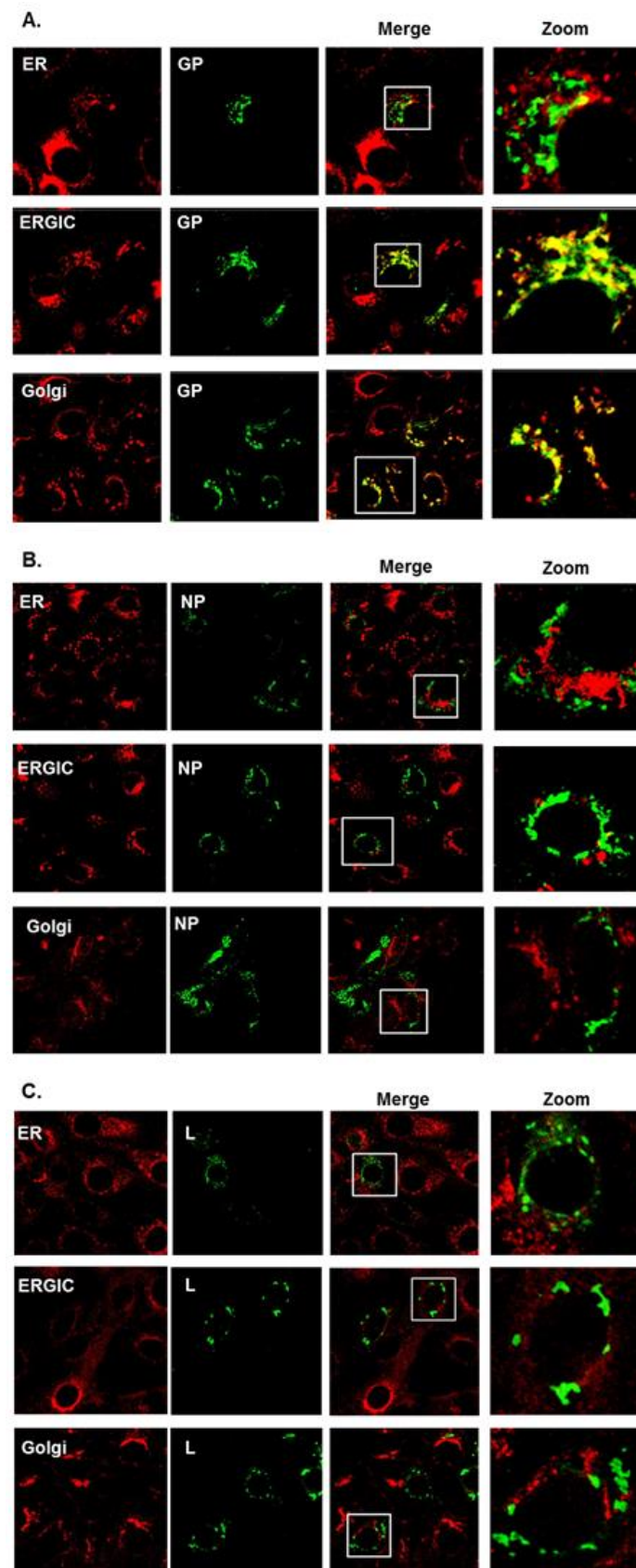


Figure 10. The subcellular localization of structural proteins in transfected cells. Vero-E6

cells were transfected with expression plasmid, pCSFTSV-GP, pCSFTSV-NP or pCSFTSV-L and fixed at 24 hpt. Cells were stained with organelle markers (red) along with antibodies against (A) GP, (B) NP or (C) L proteins (green). The localization of GP, NP and L to the ER, ERGIC and Golgi in cells singly transfected with plasmids was analyzed. The yellow areas in the merged image show colocalization of proteins with organelle markers. Magnification of the boxed areas is shown to the right of the merged image. Shown as mock, to the far right, are merged images of mock-transfected cells.

## **GP interacts with L and NP**

In phenuiviruses GP is important for targeting other viral structural proteins to the sites of assembly. To characterize the relationship of GP with L and NP, Huh7 cells were infected with SFTSV, and immunoprecipitation assays were performed using antibodies specific for Gn, L, and NP; the interacting proteins were detected by WB. GP was precipitated by anti-Gn and anti- NP antibodies (Figure 11A). NP was precipitated by anti-NP and anti-Gn antibodies (Figure 11B). These results suggest that GP and NP could form a complex in SFTSV-infected cells. The expression of L in the input was lower than that of GP and NP. L was precipitated by anti-L and anti-Gn antibodies. Weak interactions of L with GP may occur in SFTSV infected as observed by the thin bands of L detected in the SFTSV infected lane (Figure 11C). Taken together these results show that GP interacts with itself, L, and NP in SFTSV-infected cells.

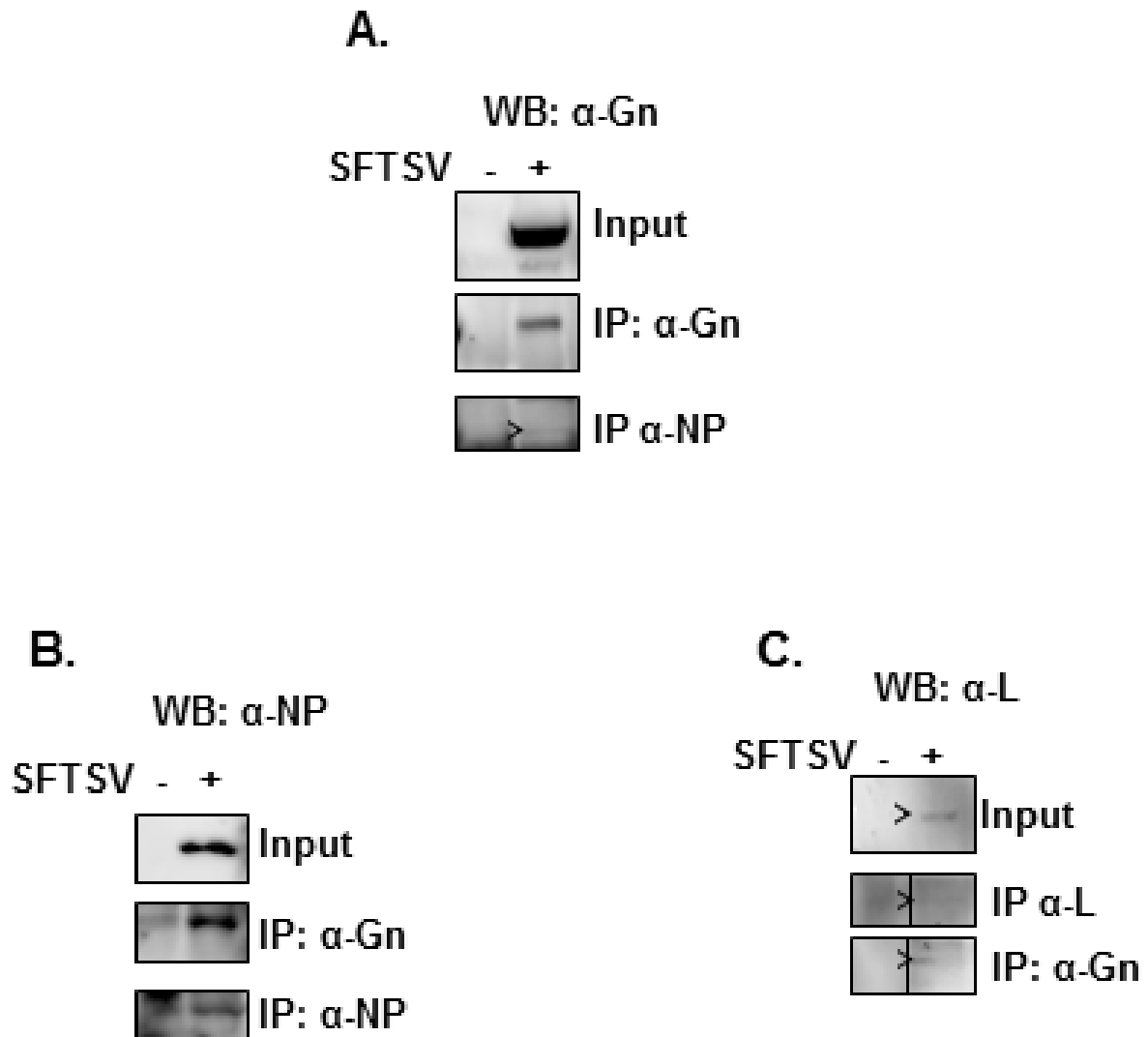


Figure 11 Interaction of GP with NP and L protein in infected cells. Cell lysates of Huh7 cells infected with SFTSV were incubated with antibodies against Gn, L, or NP and protein A Sepharose beads. The input and interacting proteins were then detected by western blot analysis with (A) anti-Gn (B) anti NP and (C) anti L. The arrowheads show the location of the bands of the precipitated proteins.

## **GP is required for the recruitment of L to the ERGIC and Golgi complex**

The GP of phenuiviruses is important for targeting other viral structural proteins to the sites of assembly. The intracellular localization of GP with NP or L in infected Vero E6 cells was evaluated (Figure 12A). GP colocalized with NP and L in perinuclear compartments in infected cells. To investigate the role of GP in the subcellular localization of L, Vero E6 cells were cotransfected with pCSFTSV-L and pCSFTSV-GP expressing L and GP, respectively. L partially colocalized with GP in transfected cells (Figure 12C). To further characterize the relationship between GP and L, the localization of L to the ERGIC and Golgi complex in cells that coexpressed GP and L, was assessed. In the presence of GP, L was detected in the ERGIC and Golgi complex (Figure 12C). These results showed that GP facilitates the localization of L to the budding compartments. Conversely, in cells that co-expressed NP and GP, NP did not colocalize with GP (Figure 12B). NP accumulated in the cytoplasm and was not detected in the ERGIC and Golgi complex. We further investigated whether the localization of NP could be altered in the presence of GP with L or NSs. In cells co-expressing NP and GP with L or NSs, NP did not colocalize with GP (data not shown). In addition, NP and NSs did not colocalize in SFTSV infected and in plasmid transfected cells (Figure 14). Furthermore, NP did not localize to the budding compartments in cells coexpressing NP and GP with either L or NSs. These results suggest that another virus factor except the known viral proteins are required for the ERGIC and Golgi complex localization of NP.

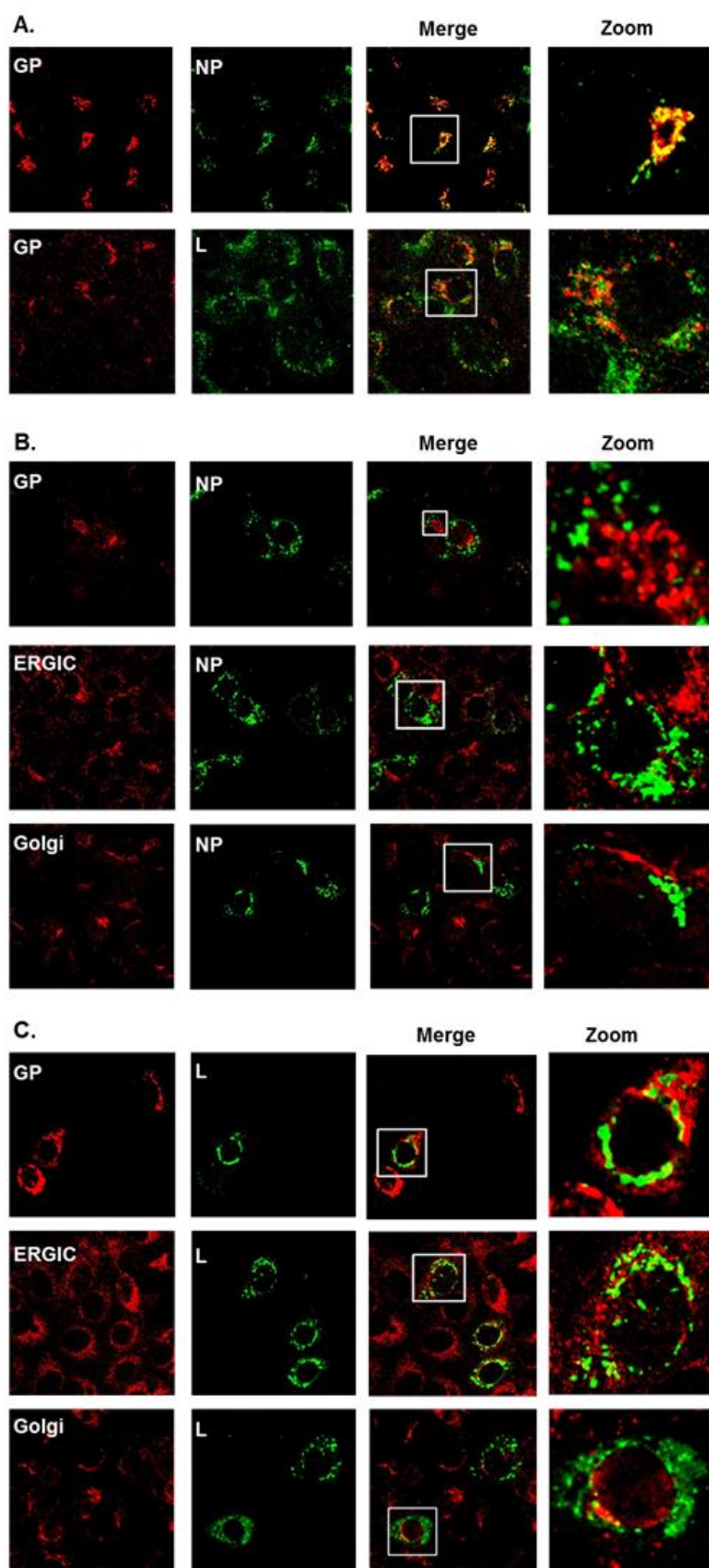


Figure 12. Colocalization of GP with NP and L protein. (A) Vero E6 cells were infected with

SFTSV, fixed at 72 hpi and stained with antibodies against NP or L (green) and GP (red). (B)

Vero-E6 cells were transfected with pCSFTSV-GP and pCSFTSV-NP and fixed at 24 hpt.

Cells were stained with antibodies against NP (green) and GP or organelle makers (red). (C)

Vero E6 cells were transfected with pCSFTSV-GP and pCSFTSV-L and fixed at 24 hpt.

Cells were stained with antibodies against L (green) and GP or organelle makers (red).

Magnification of the boxed areas is shown to the right of the merged image.

### **Localization of NP to the ERGIC and Golgi complex**

To determine whether other components of SFTSV are associated with localizing NP to the ERGIC and Golgi, Vero-E6 cells were transfected with plasmids containing NP-fused with HA tag (pCSFTSV-HA-NP). The cells were then infected or mock-infected with SFTSV and analyzed by IFA to assess whether the epitope-tagged NP could be detected in the ERGIC and Golgi complex. In SFTSV infected cells, HA-NP colocalized with GP (Figure 13). Furthermore, HA-NP was detected in the ERGIC and Golgi complex in SFTSV-infected cells. HA-NP was not detected in the ERGIC and Golgi complex in mock-infected cells, similar to results shown in Figure 10B when NP lacking the HA tag was expressed. These results indicated that HA-NP fusion protein expressed using the pCAGGS vector could correctly localize to the subcellular compartments in a manner comparable to the viral proteins in SFTSV infected cells. This result also indicates that other viral components may play a role in targeting NP to the ERGIC and Golgi complex.

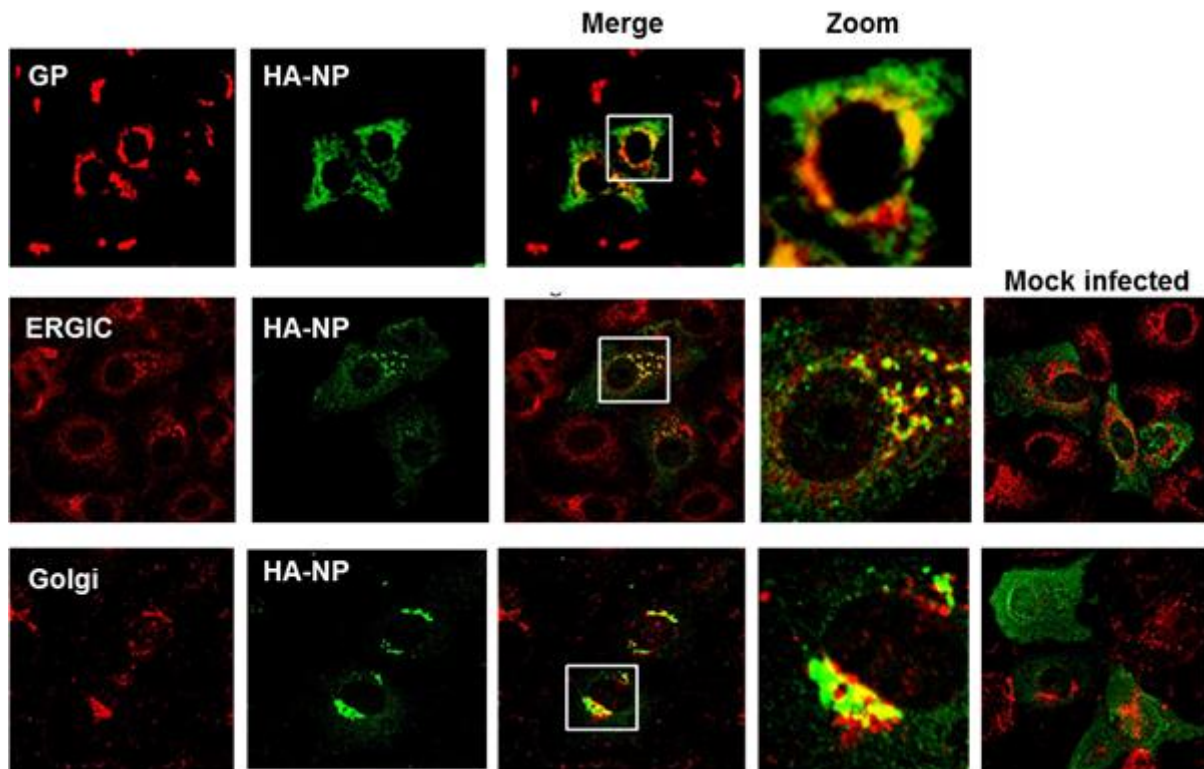


Figure 13. The effect of SFTSV replication on the subcellular localization of hemagglutinin (HA)-tagged NP. Vero-E6 cells transfected with HA-tagged NP were mock-infected or infected with SFTSV. At 72 hpi, the cells were examined for expression and colocalization between GP or organelle marker (red) and anti-HA antibody (green). Magnification of the boxed areas is shown to the right of the merged image.

## 9. Discussion

Negative strand RNA viruses use the host cell secretory pathway for the production of infectious virus particles<sup>29,31</sup>). Panda *et al.* used RNA interference screening of host proteins to show that lymphocytic choriomeningitis virus, human parainfluenza virus type 3, and vesicular stomatitis virus require the host cell secretory pathway for infection<sup>29</sup>). It is generally accepted that viruses of the *Bunyavirales* order assemble and bud into the Golgi complex<sup>26,34</sup>). Recently, not only the Golgi complex but the ERGIC has also been proposed as a site for virion assembly. The localization of viral structural proteins to the ERGIC and Golgi complex is involved in the production of infectious viral particles<sup>2,9,26,30,31,33,36</sup>). Herein, it was shown that SFTSV structural proteins, like those of other representative members of the *Bunyavirales* order, localized to the ERGIC and Golgi complex in infected cells (Figure 9). This result suggested that virion assembly of SFTSV starts in compartments such as the ERGIC that lie before the Golgi complex. This data supports previous reports on the targeting of NP and L to the ERGIC prior to movement to the Golgi complex<sup>9,33</sup>).

Elucidating the subcellular localization of SFTSV structural proteins is important for understanding the steps in the lifecycle of SFTSV. Using recombinant proteins to express the SFTSV structural proteins in cells singly transfected with plasmids the subcellular distribution of GP, NP, and L proteins was elucidated. GP was detected mainly in the ERGIC, and Golgi complex in cells that singly expressed GP in a manner similar to GP localization in SFTSV-infected cells (Figure 10). These findings support previous studies that showed SFTSV GP accumulation in intracellular compartments such as the ER and Golgi complex<sup>31,42</sup>). In contrast, NP and L proteins did not independently localize to the ERGIC and Golgi complex, indicating that additional viral factors are required for L and NP to localize to the ERGIC and Golgi complex.

Phlebovirus GP accumulates in the Golgi complex prior to virus assembly, and therefore, may serve to direct other structural proteins to the Golgi complex for maturation<sup>9,30,34</sup>). In RVFV and UUKV, targeting of the structural proteins to the ER, ERGIC or Golgi complex is mediated by GP<sup>27,30</sup>). Golgi complex accumulation of phlebovirus GP is due to the presence of a Golgi retention signal in the cytoplasmic tail of Gn<sup>7,28,36</sup>). Viruses employ different mechanisms, including protein–protein<sup>28,39</sup>) and protein–RNA interactions<sup>27</sup>), to deliver the viral components to the sites of assembly. In this study, the association of SFTSV structural proteins with one another to facilitate their localization to the budding compartments was investigated. GP is required for the localization of L protein to the ERGIC and Golgi complex. In SFTSV-infected cells and in cells expressing recombinant GP and L, GP colocalized with L protein. In addition, cotransfection of GP with L-expressing plasmids showed that GP could recruit L to the ERGIC and Golgi complex. This observation suggests that GP forms a complex with L, thereby recruiting L to the budding compartments. This result supports a previous study that showed the interaction of GP and L in RVFV<sup>30</sup>).

On the other hand, NP did not colocalize with GP in cells expressing recombinant NP and GP Figure 12B, although GP colocalized with NP in SFTSV-infected cells Figure 12A. A previous study by Wu *et al.* reported that SFTSV NP interacts with NSs, and that this interaction is important for virus replication<sup>45</sup>). Therefore, it was hypothesized that NSs might be required for the interaction of NP with GP. To test this hypothesis, pCAGGS-expressing NP and NSs were cotransfected to Vero-E6 and HeLa cells, the latter were used in the previous study. Contrary to expectation, in the present study NP did not colocalize with NSs in SFTSV-infected and in plasmid transfected cells (Figure 14). One possible explanation could be the difference in expression systems; Wu *et al.* used the pRK5 plasmid vector for protein expression.

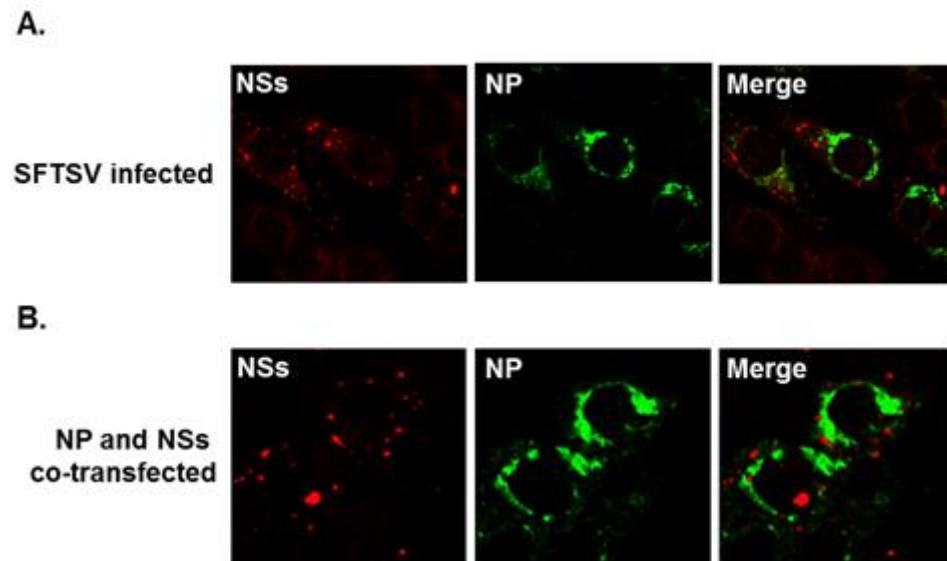


Figure 14. The relationship between NP and NSs in Vero E6 cells. (A) Vero E6 cells were infected with SFTSV or (B) transfected with plasmids encoding SFTSV NP and NSs and examined for colocalization between NP and NSs.

To confirm that the pCAGGS plasmid expression system for NP was functionally competent and to determine whether another component of SFTSV may be required for NP to colocalize with GP, epitope-tagged NP was used to analyze whether HA-NP could colocalize with GP from SFTSV-infected cells. HA-NP colocalized with GP in the ERGIC and Golgi complex only in SFTSV-infected cells and not in the mock-infected cells (Figure 13). This result confirmed that the inability of NP to localize to the ERGIC and Golgi complex when recombinant protein was expressed was not due to loss of function in the pCAGGS plasmid expression system; but that NP colocalization with GP likely requires other viral factors besides GP, L or NSs. Piper *et al.* reported that genomic RNA is required for RVFV packaging into virions <sup>30)</sup>. Whether this is the case with SFTSV requires further investigation. Currently experiments are underway to determine the other viral factors required for NP localization to the budding compartments. Budding of virions of RVFV <sup>4)</sup> and BUNV <sup>34)</sup> takes place at the Golgi complex. Pre-Golgi compartments are also sites for virion budding for UUKV <sup>9)</sup>. In hantavirus, <sup>33)</sup> budding of virions may start at the ERGIC and continue to the Golgi complex. The ERGIC-53 protein binds GP of several viruses including hantaviruses, arenaviruses, and corona viruses <sup>14,21,44)</sup>. In addition, binding of virus to ERGIC-53 is required for the production of infectious virus particles of filovirus, arenavirus, and corona virus <sup>14)</sup>. The Golgi complex is known as a site for virus budding in the *Bunyavirales* order. Plegge *et al.* reported that trafficking of SFTSV GP to the Golgi complex is required for the production of infectious virus particles <sup>31)</sup>. Further studies are required to clarify the role of the ERGIC and Golgi complex in virion assembly and maturation during the production of SFTSV particles. The present study showed that SFTSV structural proteins are targeted to the ERGIC and Golgi complex during the lifecycle of the virus. GP of SFTSV play an important role in the localization of structural proteins to the budding site, ERGIC and Golgi complex.

In addition to GP, NP may require other viral factors for trafficking through the secretory pathway.

## **10. Summary**

SFTSV is a newly emerged phlebovirus identified in China, Japan, and South Korea. Phlebovirus GP plays a key role in targeting viral structural components to the budding compartments in the ERGIC and Golgi complex. However, the role of SFTSV GP in targeting structural proteins to the ERGIC and Golgi complex remains unresolved. In this study, it was shown that SFTSV GP plays a significant role in targeting L and NP to the budding sites. Confocal microscopy was used to investigate the subcellular localization of SFTSV structural proteins. In SFTSV-infected cells, GP and L localized to the ER, ERGIC and Golgi complex, whereas NP localized to the ERGIC and Golgi complex. In addition, GP colocalized with L and NP in SFTSV infected cells. In cells singly transfected with GP, L or NP, GP localized to the same subcellular compartments as in infected cells. However, L or NP alone did not localize to the ER, ERGIC, or Golgi complex. Co-transfection experiments showed that GP altered the localization of L to the ERGIC and Golgi complex but not that of NP. Interestingly, plasmid-expressed NP fused with HA tag localized to the ERGIC and Golgi complex when expressed in SFTSV-infected cells and colocalised with GP, suggesting that GP plays a role in the subcellular localization of L and NP in infected cells. Thus, the SFTSV structural components start to assemble at the ERGIC to Golgi complex. GP is required for transporting L and NP to the ERGIC and Golgi complex. In addition, targeting of NP requires interaction with other factors besides GP.

## 11. Conclusion

SFTSV is a tick borne *Phlebovirus* in the family *Phenuiviridae* that emerged as an important human pathogen in China, Japan and South Korea. In Japan, SFTS is endemic to the western parts of the country and ticks including *Haemaphysalis longicornis* and *Amblyomma testudinarium* are the vectors. The SFTSV genome has been detected in ticks in geographic areas that are not in the endemic regions of SFTS. Thus, it is important to find out if there has been a geographic expansion of SFTSV infection in wild animals in Japan.

There are two chapters in this thesis. Chapter 1 is based on a serological survey that was conducted in deer and rodents from various locations in Japan. The deer sera used were from Hokkaido, a non SFTS-endemic area and Miyazaki, an SFTS-endemic area. The rodent sera were banked serum samples that had been collected between 1997 and 2009. The sero-survey showed that deer in Hokkaido did not harbor SFTSV antibodies (0%, 0/315). On the other hand, deer in Miyazaki had anti-SFTSV antibodies (4.9%, 2/41). This difference in occurrence of anti-SFTSV antibodies is in line with the reported distribution of SFTS patients. To the best of my knowledge, there are no SFTS patients in Hokkaido so far. The prevalence of 4.9% in Miyazaki shows that SFTSV is not widely distributed even in endemic areas. The information obtained in this survey is useful for defining the SFTSV endemic areas which is necessary for monitoring the spread of SFTSV and assessing the risk of human infection with SFTSV in Hokkaido and Miyazaki. In this study, anti-SFTSV antibodies were not detected in the rodent sera that were screened (0%, 0/910). The role of rodents in the transmission of SFTSV is not clear even though sero-positive rodents have been reported before in China. Further work is required for monitoring the circulation of SFTSV in Japan.

In chapter 2, the role of the SFTSV GP in targeting L protein and the nucleocapsid protein to the secretory pathway was studied. Progeny virions of representative viruses in the *Bunyavirales* order bud at the membranes of the Golgi complex. The ERGIC has also

recently been shown to be an important site for virus budding in the *Hantaviridae* family. Targeting the structural proteins to intracellular compartments where virus assembly occurs is critical for formation of new progeny virions. The localization of SFTSV structural proteins to intracellular compartments has not been described. This study thus was aimed at revealing the subcellular localization of SFTSV structural proteins in cells infected with SFTSV and in cells expressing recombinant proteins. The role of GP in targeting NP and L protein to the subcellular compartments was also studied.

The localization of GP, NP and L protein to the ER, ERGIC and Golgi was analysed by immunofluorescence assay in cells infected with SFTSV. This study revealed that in infected cells, GP and L protein localized to the ER, ERGIC and Golgi. NP localized to the ERGIC and Golgi. When the proteins were transfected singly, only GP was able to traffic through the secretory pathway independent of other SFTSV structural proteins. The effect of co-expressing GP with L protein or NP was assessed. In the presence of GP, L protein localized to the ERGIC and Golgi and NP did not localize to the ERGIC and Golgi when co-expressed with GP, however when NP fused with HA tag was expressed in the presence of SFTSV, HA-NP was detected in the ERGIC and Golgi, suggesting that another viral component is required for NP to localize to the budding sites. More studies are required to determine the viral factors that are required for NP to localize to the ERGIC and Golgi. The role of the ERGIC and Golgi in the formation of SFTSV virions needs to be clarified.

The information gathered in this study is useful for understanding the steps in the replication of SFTSV and could provide a basis for the development of virus inhibitors.

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## Summary in Japanese

重症熱性血小板減少症候群ウイルス（SFTSV）は中国、日本、および韓国における人の重要な新興感染症の原因ウイルスで、ダニ媒介性のフェニウウイルス科に属している。日本において、SFTSVは西日本において常在しており、フタトゲチマダニ、タカサゴキララマダニなどのマダニがベクターとなっている。SFTSVゲノムはSFTSの発生のない地域でも検出されている。したがって、SFTSVが日本の野生動物の間で流行域が拡大しているかどうかを調べることは重要である。

本論文は2つの章によって構成されている。第1章では、日本の様々な地域のシカやげっ歯類などの血清学的調査を実施した。SFTSの非流行地である北海道とSFTSの流行地である宮崎県から得られたシカの血清および、1997年から2009年の間に集められたげっ歯類の血清を検体に用いた。北海道のシカは一例も抗体を保有していなかったが（0%, 0/315）、宮崎では2例のシカが抗体を保有していた（4.9%, 2/41）。この抗体保有率の違いはSFTS患者の発生状況と一致していた。中国においてはげっ歯類から抗SFTS抗体が検出されているが、本研究においてはげっ歯類から抗体は検出されなかった（0%, 0/910）。したがって、SFTSVの伝播におけるげっ歯類の役割については未解明のままである。今後も日本においてSFTSVの流行状況を調べるために疫学調査が必要である。

第2章では、SFTSVのL蛋白質（L）と核蛋白質（NP）の細胞内分泌経路への輸送に係る糖タンパク質（GP）の役割について解析を行った。ブニヤウイルス目のウイルスではウイルスの出芽がゴルジ装置の膜において起こることが知られている。最近ハンタウイルス科のウイルスにおいて小胞体 - ゴルジ中間領域（endoplasmic reticulum Golgi intermediate compartment: ERGIC）がウイルスの出芽において重要な

部位であることが明らかになった。ウイルス粒子の産生にとって、ウイルスの構築が起こる細胞内器官に構造タンパク質が集積することは非常に重要である。しかし、これまでSFTSV構造タンパク質の各種細胞内器官における局在については明らかにされていなかった。本論文ではSFTSVの構造蛋白質の細胞内局在について、感染細胞と構造蛋白質を発現させた細胞において解析を行った。また、NPとLの各種細胞内器官への輸送におけるGPの役割についても解析を行った。

GP、NP、およびLの小胞体（endoplasmic reticulum: ER）、ERGIC、およびゴルジ装置における局在について感染細胞をIFAにより解析を行ったところ、GPとLはER、ERGIC、およびゴルジ装置のいずれにも局在することが明らかになった。NPはERには局在が見られず、ERGIC、およびゴルジ装置に局在していた。構造蛋白質を単独で細胞内に発現させた場合、GPのみが細胞の分泌経路に輸送された。LはGPと共発現した場合にはERGICおよびゴルジ装置に輸送されたが、NPはGPと共発現させてもERGICおよびゴルジ装置に輸送されなかった。NPにヘマグルチニン（HA）タグを付加させて感染細胞で発現させたところ、HA付加NPはERGICおよびゴルジ装置に局在した。したがって、NPの出芽部位への輸送にはさらに別の因子が必要であることが示唆された。NPのERGICやゴルジ装置への輸送に関与するウイルスの因子についてさらなる解析が必要である。ERGICやゴルジ装置のSFTSVのウイルス粒子産生における役割についても今後明らかにすることが重要である。

本研究で得られた知見はSFTSVの複製を理解する上で有用であるとともに、ウイルスの阻害剤を開発する上での基礎的情報を提供するものである。

## 正誤表

学位論文提出者：Tapiwa Lundu

学位論文題名：Studies on the epidemiology of severe fever with thrombocytopenia syndrome virus infection and the role of glycoproteins in the intracellular transportation of viral structural proteins

（重症熱性血小板減少症候群ウイルス感染の疫学およびウイルス構造タンパク質の細胞内輸送における糖蛋白質の役割に関する研究）

### <誤>

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### <正>

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