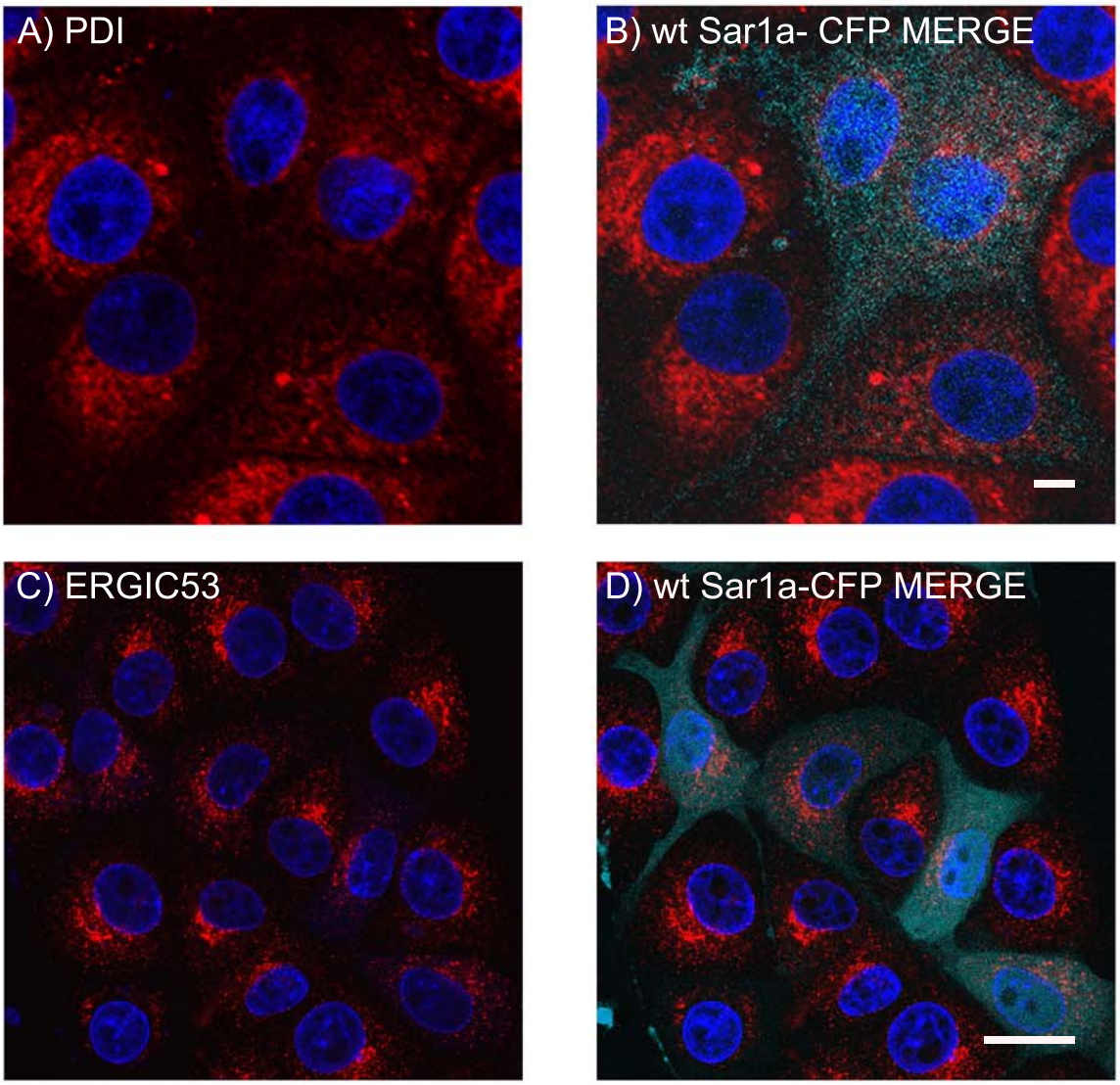


Figure 4.2 The effects of wt Sar1a expression on the ER and ERGIC
IBRS-2 cells were transfected to express wt Sar1a-CFP for 14 hours and labelled for the ER (PDI; panels A & B) or the ERGIC (ERGIC53; panels C & D). Cells expressing Sar1 are shown as cyan; the ER and ERGIC are shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

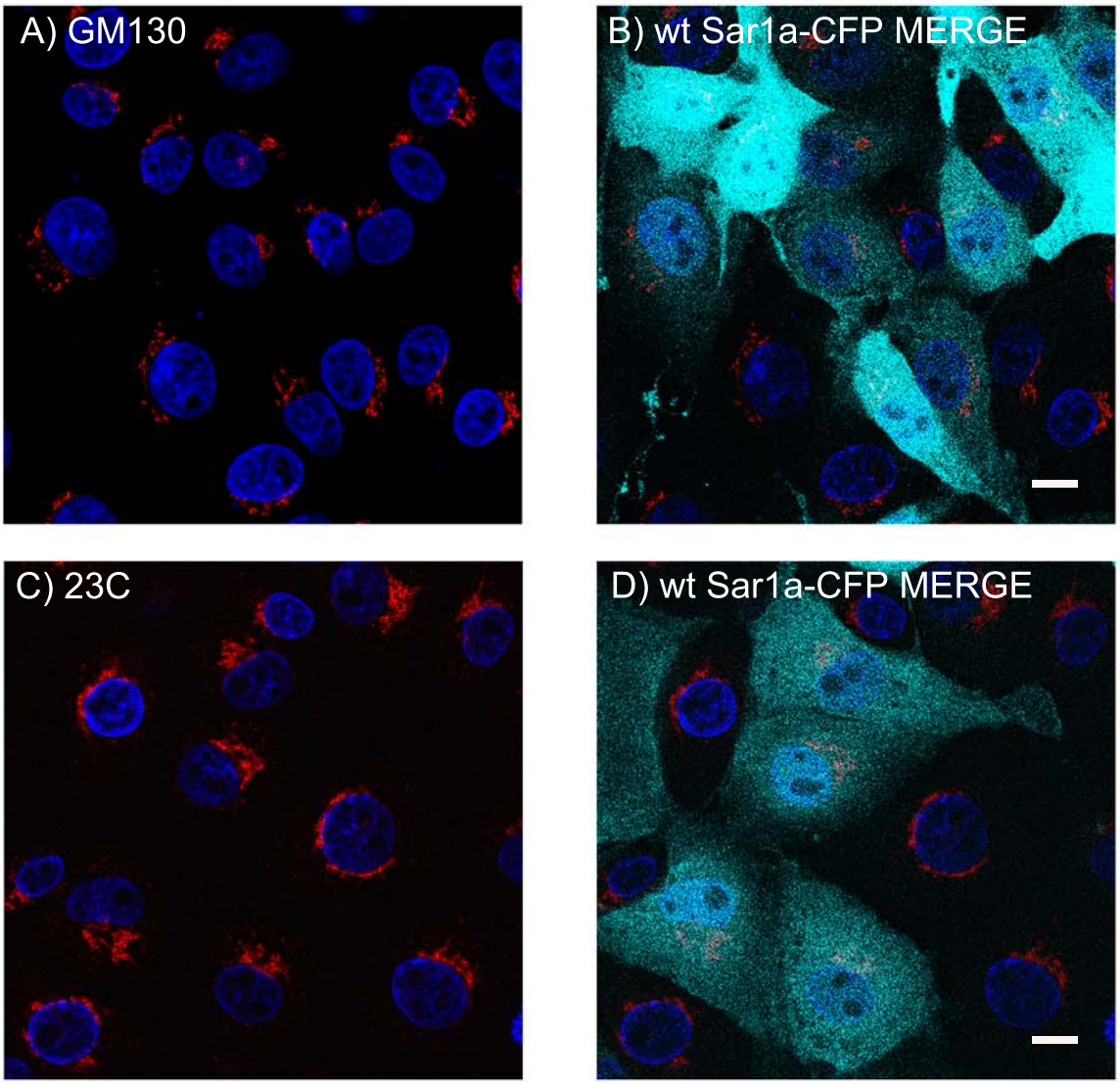


Figure 4.3 The effects of wt Sar1a expression on the Golgi
IBRS-2 cells were transfected to express wt Sar1a-CFP for 14 hours and labelled for the Golgi (GM130 panels A & B or 23C panels C & D). Cells expressing Sar1a are shown as cyan; the Golgi is shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

Expression of dn Sar1a T39N-CFP has been previously reported to result in Golgi disruption and a block to protein transport (Kuge et al., 1994; Zaal et al., 1999). Figure 4.4 shows cells expressing dn Sar1a T39N-CFP (shown as cyan, in panels B and D) labelled for the ER (shown as red, panels A-B) and the ERGIC (shown as red, panels C-D). The ER appeared severely disrupted in cells expressing dn Sar1a T39N-CFP compared to the surrounded non-expressing cells. The peri-nuclear labelling for the ERGIC was also dispersed in cells that expressed a high level of dn Sar1a T39N-CFP, while in cells expressing a low level of the dn protein the ERGIC remained largely intact (Fig 4.4, panel D). The effect of dn Sar1a T39N-CFP (cyan in panels B and D) on the Golgi is shown in Figure 4.5 (labelled with GM130 in red, panels A-D) and Figure 4.6 (labelled with 23C in red). In a similar manner to the ERGIC, variable effects on the Golgi were observed, but in the majority of cells expressing a high level of dn Sar1a T39N-CFP, the Golgi was dispersed (Fig 4.5 panels A-D and Fig 4.6 panels A-D). In general, over-expression of dn Sar1 T39N-CFP caused loss of ER and disruption to the ERGIC and Golgi. This would indicate that a block in protein transport occurs when dn Sar1 T39N-CFP is expressed at high levels. However, these observations also indicate that this dn form of Sar1a does not efficiently block membrane transport through the secretory pathway when expressed below a certain threshold.

Figure 4.7 shows cells expressing dn Sar1a H79G-CFP (shown as cyan, panels B and D) labelled for the ER (shown as red, panels A-B) and the ERGIC (red, panels C-D). ER labelling was clearly reduced in cells expressing this dn protein. The characteristic peri-nuclear labelling of the ERGIC was completely lost in dn expressing cells (Fig 4.7 shown as red, C-D). Figure 4.8 shows dn Sar1a H79G-CFP expressing cells (shown as cyan, panels B and D) labelled for

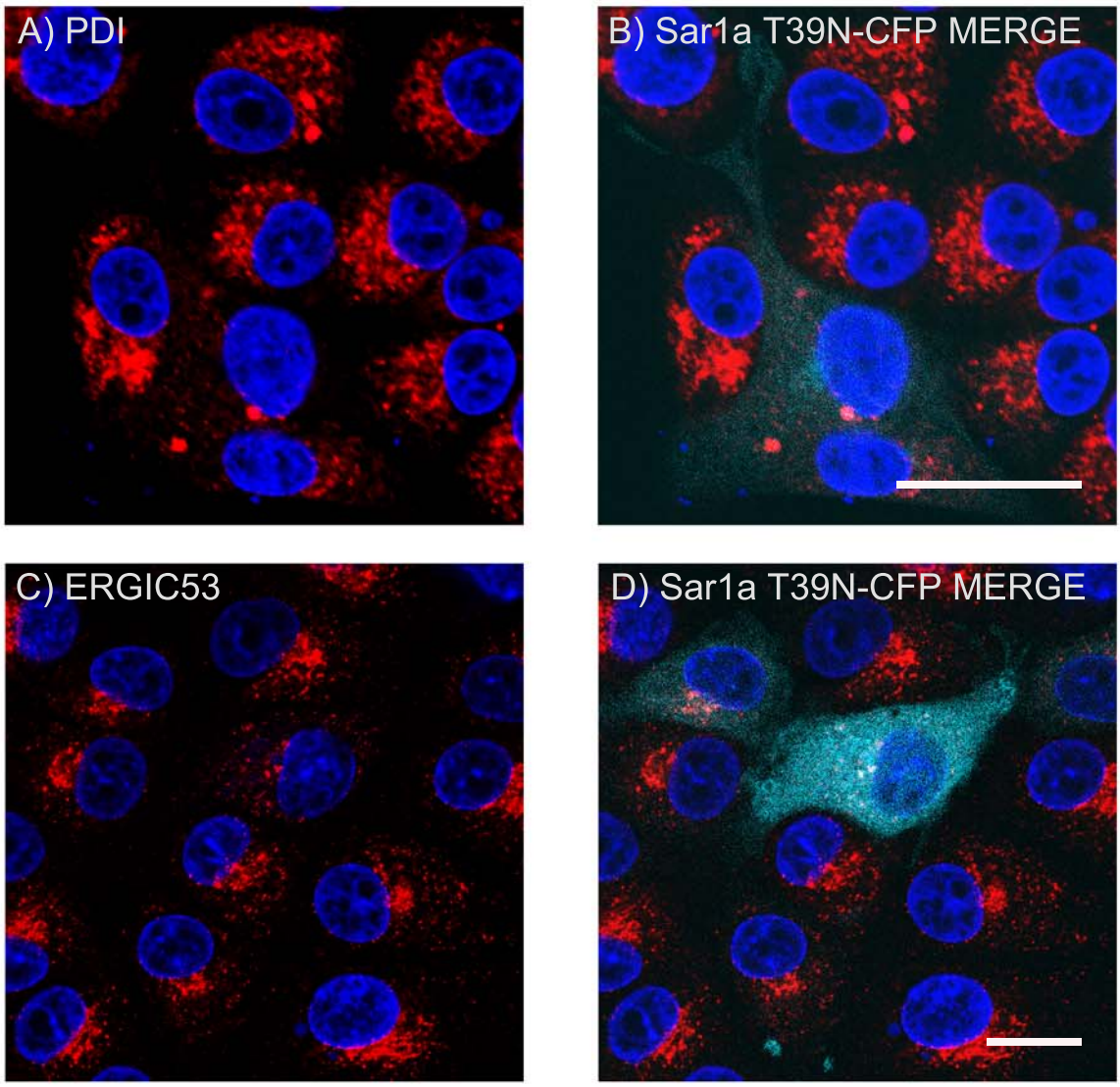


Figure 4.4 The effects of dn Sar1a (T39N) expression on the ER and ERGIC
IBRS-2 cells were transfected to express dn Sar1a T39N-CFP for 14 hours and labelled for the ER (PDI; panels A & B) or the ERGIC (ERGIC53; panels C & D). Cells expressing Sar1a T39N are shown as cyan; the ER and ERGIC are shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
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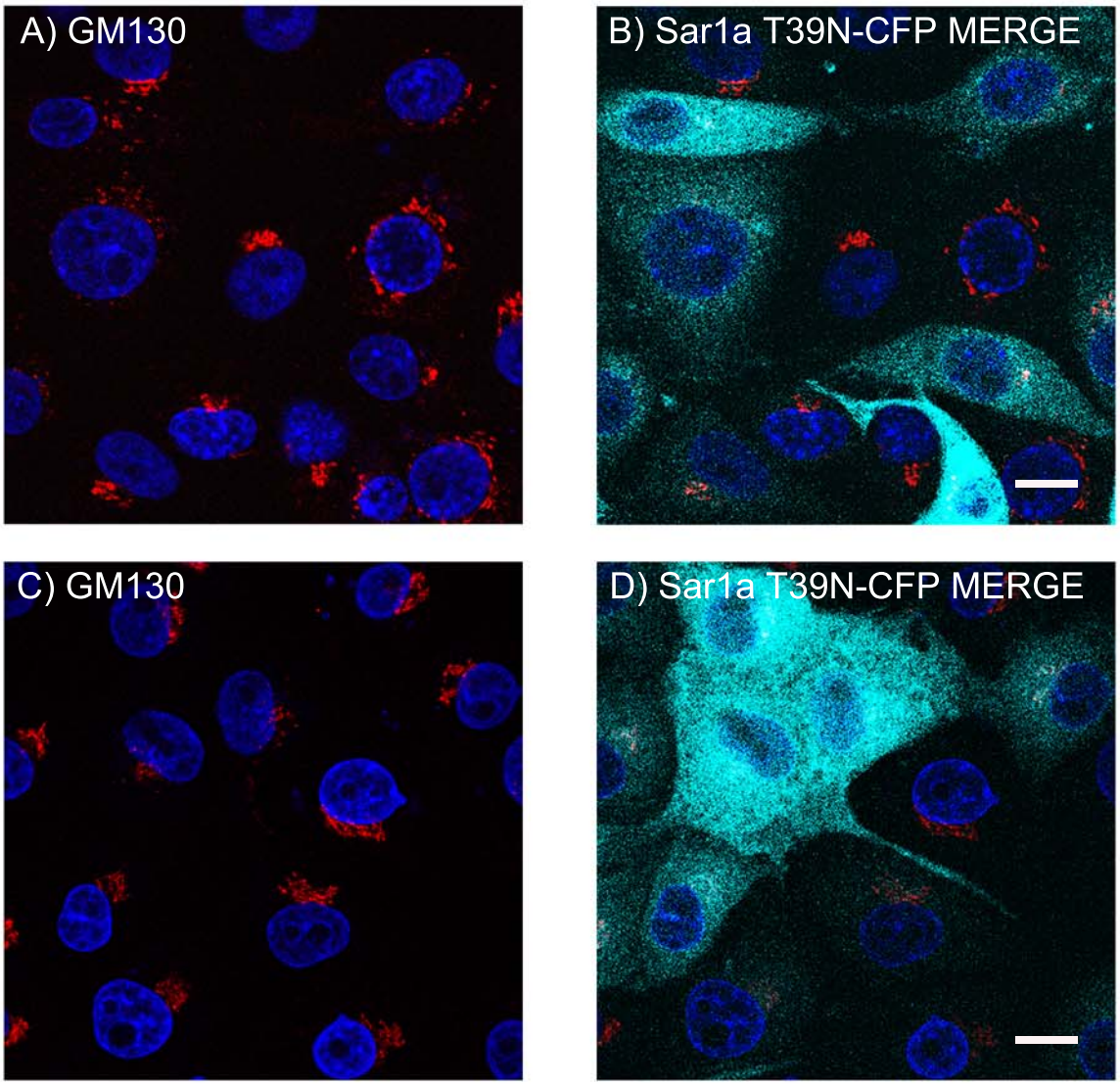


Figure 4.5 The effects of dn Sar1a (T39N) expression on the Golgi (GM130)
IBRS-2 cells were transfected to express dn Sar1a T39N-CFP for 14 hours and labelled for the Golgi (GM130 panels A-D). Cells expressing Sar1a T39N are shown as cyan; the Golgi is shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

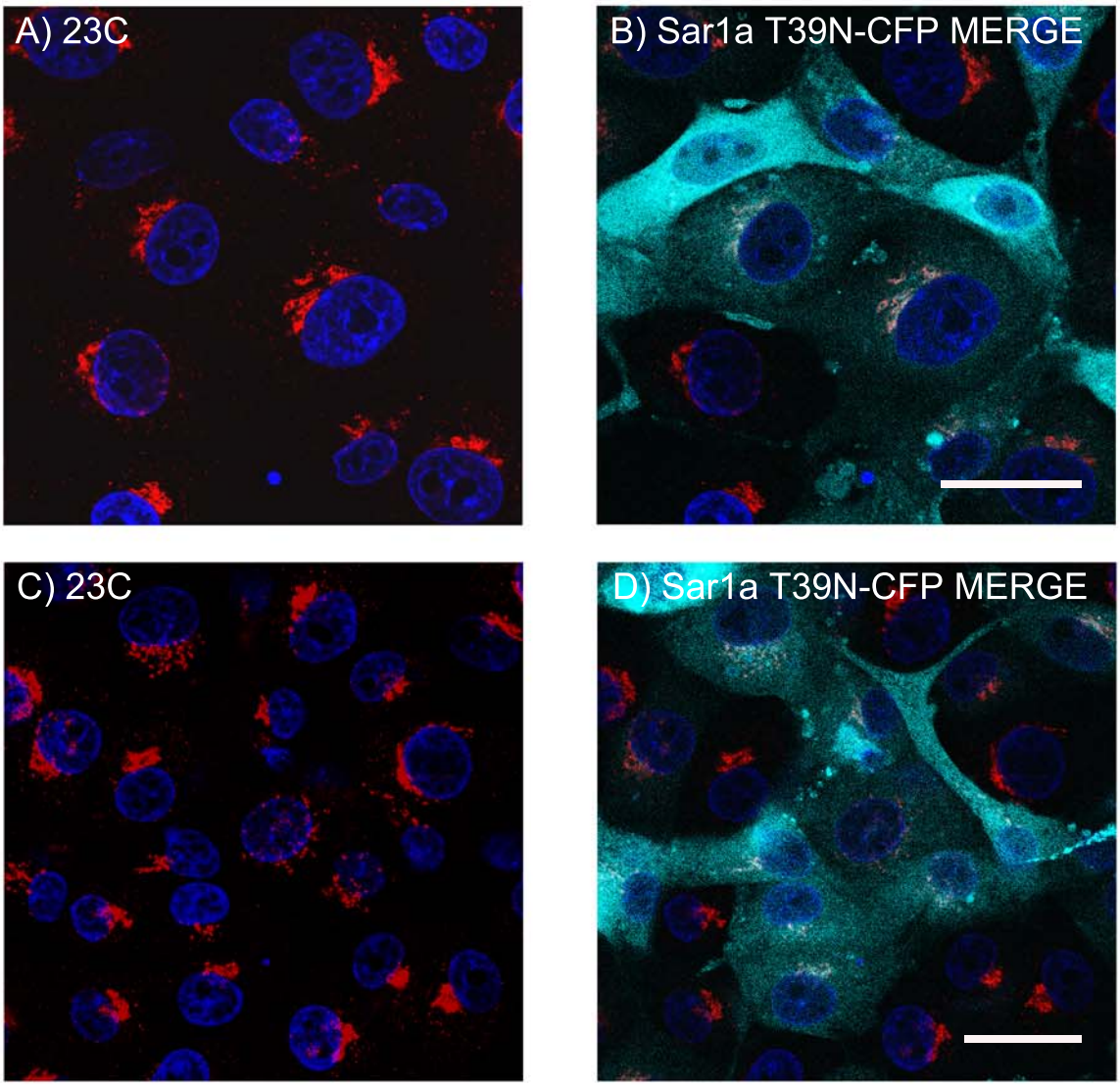


Figure 4.6 The effects of dn Sar1a (T39N) expression on the Golgi (23C)
IBRS-2 cells were transfected to express dn Sar1a T39N-CFP for 14 hours and labelled for the Golgi (23C panels A-D). Cells expressing Sar1a T39N are shown as cyan; the Golgi is shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

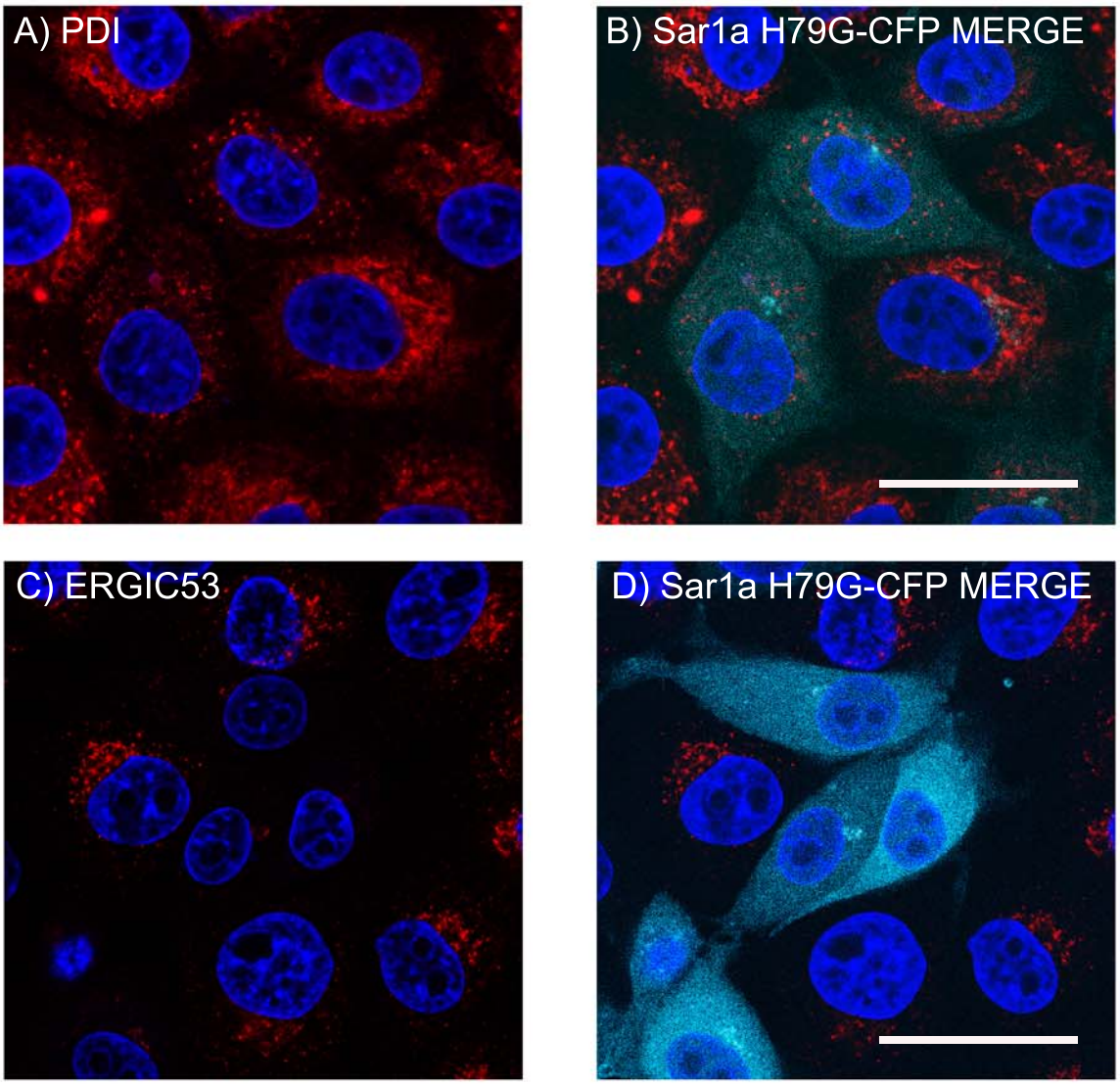


Figure 4.7 The effects of dn Sar1a (H79G) expression on the ER and ERGIC
IBRS-2 cells were transfected to express dn Sar1a H79G-CFP for 14 hours and labelled for the ER (PDI; panels A & B) or the ERGIC (ERGIC53; panels C & D). Cells expressing Sar1a H79G are shown as cyan; the ER and ERGIC are shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

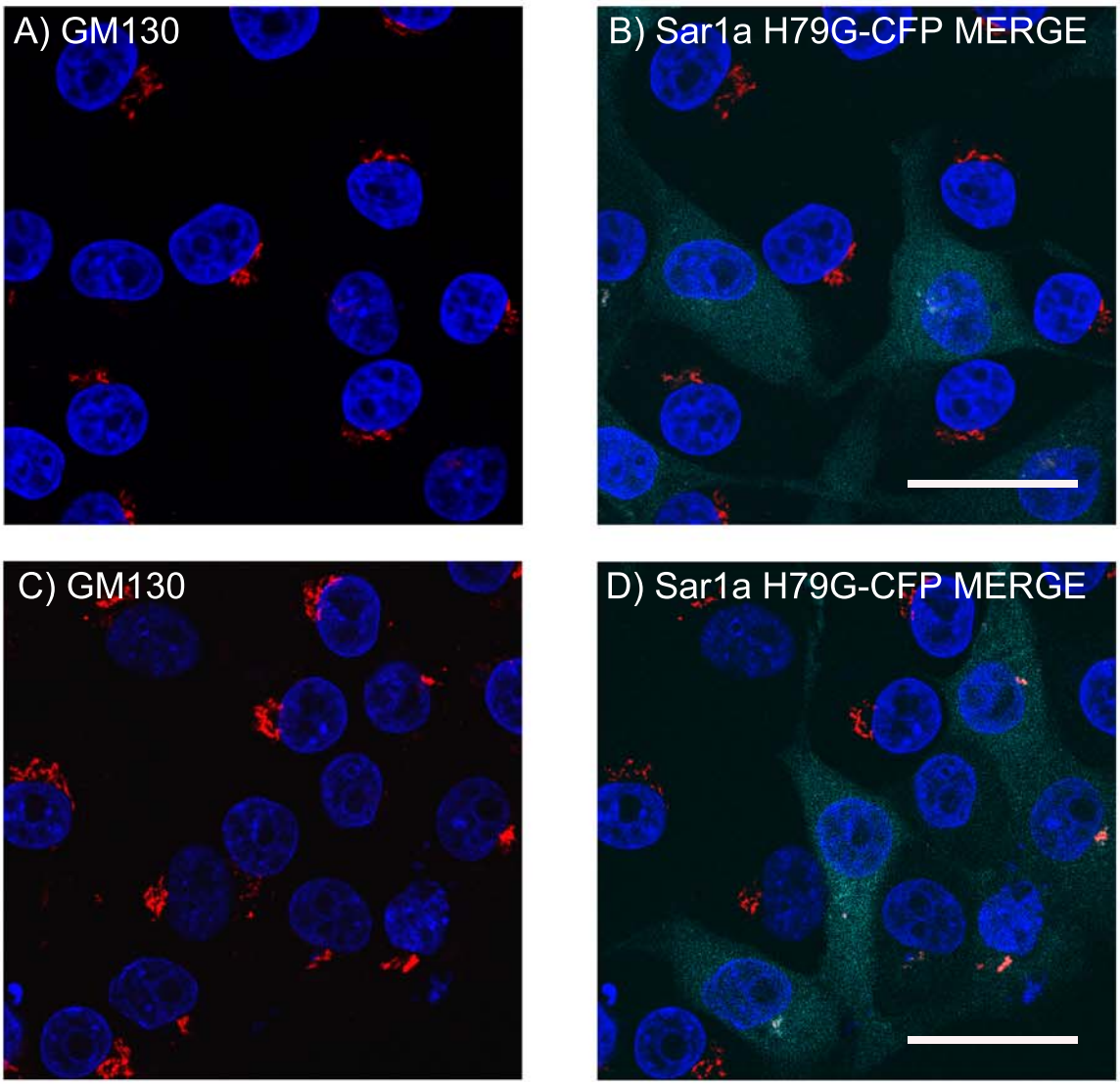


Figure 4.8 The effects of dn Sar1a (H79G) expression on the Golgi (GM130)
IBRS-2 cells were transfected to express dn Sar1a H79G-CFP for 14 hours and labelled for the Golgi (GM130 panels A-D). Cells expressing Sar1a H79G are shown as cyan; the Golgi is shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

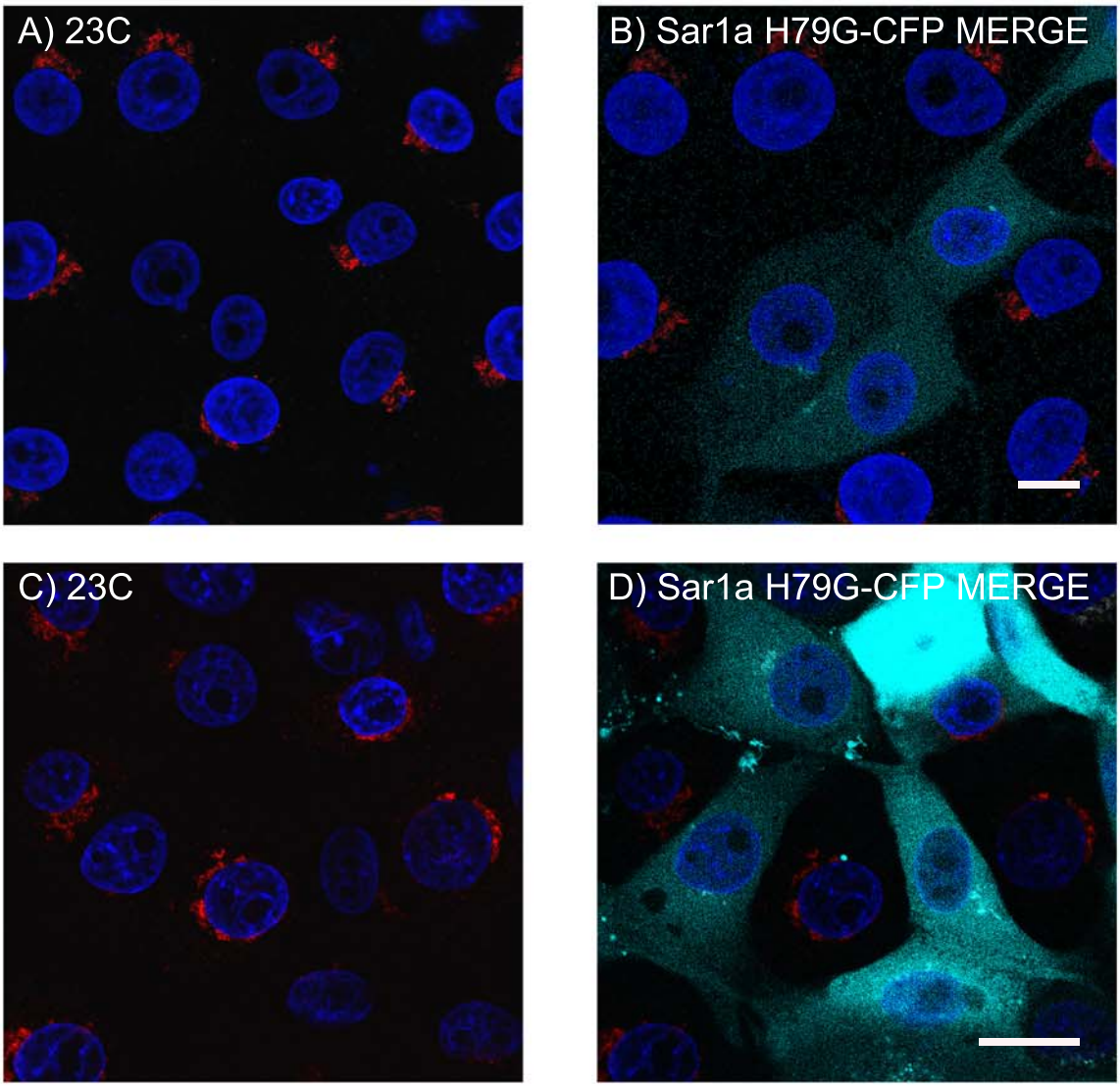


Figure 4.9 The effects of dn Sar1a (H79G) expression on the Golgi (23C)
IBRS-2 cells were transfected to express dn Sar1a H79G-CFP for 14 hours and labelled for the Golgi (23C panels A-D). Cells expressing Sar1a H79G are shown as cyan; the Golgi is shown in red. Panels B and D showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

the Golgi marker GM130 (shown as red, panels A-D). Again a loss of labelling is observed in cells expressing the dn protein indicating disruption of the Golgi (Fig 4.8, A-D). Similarly, figure 4.9 shows cells expressing Sar1 H79G-CFP (shown as cyan, panels B and D) labelled for the Golgi marker 23C (shown as red, panels A-D). Again labelling was lost as a result of dn Sar1 H79G expression. The above results indicate that expression of dn Sar1a H79G-CFP creates a strong block to membrane flow through the secretory pathway and most likely blocks protein secretion. The disruption of the ERGIC suggests that the protein blocks ER to ERGIC transport.

4.2.2 The effect of dominant-negative Sar1a on FMDV infection

Next, the effect of dn Sar1 on FMDV infection was investigated. IBRS-2 cells were transfected with either wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours. The transfected cells were then infected with FMDV O1Kcad_2 at a low MOI (MOI 0.5). Infection was allowed to proceed for 4 hours before the cells were fixed with 4% PFM, permeabilised and labelled for virus using a rabbit anti-FMDV antibody and visualised with a goat anti-rabbit Alexa-fluor 568 secondary antibody. Infected cells are shown as red on the figures.

Figure 4.10 shows IBRS-2 cells expressing wt Sar1a-CFP (shown as cyan, in panels B-C) or dn Sar1a T39N-CFP (shown as cyan, in panels E-F) and infected with FMDV (shown as red, in panels A, C, D, F). Cells expressing wt Sar1a-CFP (Fig 4.10, B-C) were infected with FMDV but at a lower frequency than that of cells of the non-expressing population (i.e. the cells not expressing CFP). Infection appeared to be restricted to cells expressing a low level of Sar1, while cells that appeared to express a high level of the protein showed a low frequency of infection. In cells expressing dn Sar1a T39N-CFP

similar observations were made, i.e. cells expressing dn Sar1 appeared to be infected at a lower frequency when compared to the cells of the non-expressing population. Infection also tended to be restricted to cells that expressed a lower level of the dn protein. Figure 4.11 shows cells expressing dn Sar1a H79G-CFP (cyan, panels B-C) and infected with FMDV (infected cells are shown as red in panels A and C). The frequency of infection appeared similar for both the expressing and non-expressing cell populations. In addition the level of expression of the dn protein did not appear to influence infection (Fig 4.11, A-C).

The above experiments were repeated four times and the effect of the dn Sar1 proteins on infection quantified. When quantifying the effect of a dn protein on a biological system the data is often normalised to the effect of the wt protein. However, the results above (section 4.2.1 and 4.2.2) indicate that when over-expressed, wt Sar1-CFP may function as a dn protein and inhibit the flow of membranes through the secretory pathway. To take into account this possibility, the level of infection of cells expressing a Sar1 transgene (wt Sar1, dn Sar1 T39N or dn Sar1 H79G) were normalised to the frequency of infection of cells of the non-expressing population i.e. for each transfection, the frequency of infection of the cells expressing wt Sar1-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP was normalised to the frequency of infection of cells of the non-expressing population. This analysis would reveal if over-expression of the wt or dn proteins results in an inhibitory effect on infection.

Figure 4.12 (panels A-E) shows data for four independent experiments. The first experiment (Figure 4.12 [A]) shows that cells expressing wt Sar1a-CFP were infected at a reduced frequency (~40% reduction) when compared to cells of the non-expressing population. Similarly, cells expressing dn Sar1a T39N-

CFP showed a reduced level of infection (~30%). In contrast, cells expressing the dn Sar1a H79G-CFP showed an increased frequency of infection (~25% increase). The second experiment (Figure 4.12 [B]) also showed a decrease in the frequency of infection in cells expressing either wt Sar1a-CFP or dn Sar1a T39N-CFP by 40-60%. However, in this experiment dn Sar1a H79G-CFP also reduced the frequency of infection by 25%. Figure 4.12 (C) shows the third experiment in which wt Sar1a-CFP reduced infection levels by ~50% and dn Sar1a T39N-CFP by ~60%. In this experiment dn Sar1a H79G-CFP had a small inhibitory effect on infection (~15% reduction). Figure 4.12 (D) shows the fourth experiment and a similar pattern to the previous experiments, as both wt Sar1a-CFP and the dn Sar1a T39N-CFP reduced infection levels (~50 and ~30% respectively), while dn Sar1a H79G-CFP expression showed little or no effect on infection.

The mean level of infection for the four experiments is shown in Figure 4.12 (E). Overall, it appears wt Sar1a-CFP reduced the frequency of FMDV infection by ~50%, and dn Sar1a T39N-CFP by ~40%. In contrast dn Sar1a H79G-CFP showed little effect on infection levels. The apparent reduction of FMDV infection caused by wt Sar1a-CFP may be due to the over-expression of the protein having a dominant-negative effect on endogenous Sar1. The inactive version of Sar1 (dn Sar1a T39N-CFP) prevents COPII vesicle formation and the inhibitory effect of this dn on FMDV infection may indicate a requirement for Sar1 activity and/or COPII vesicle formation for virus replication. In contrast the constitutively active mutant of Sar1, dn Sar1a H79G-CFP did not appear to inhibit FMDV infection which suggests that FMDV may target COPII vesicles for replication.

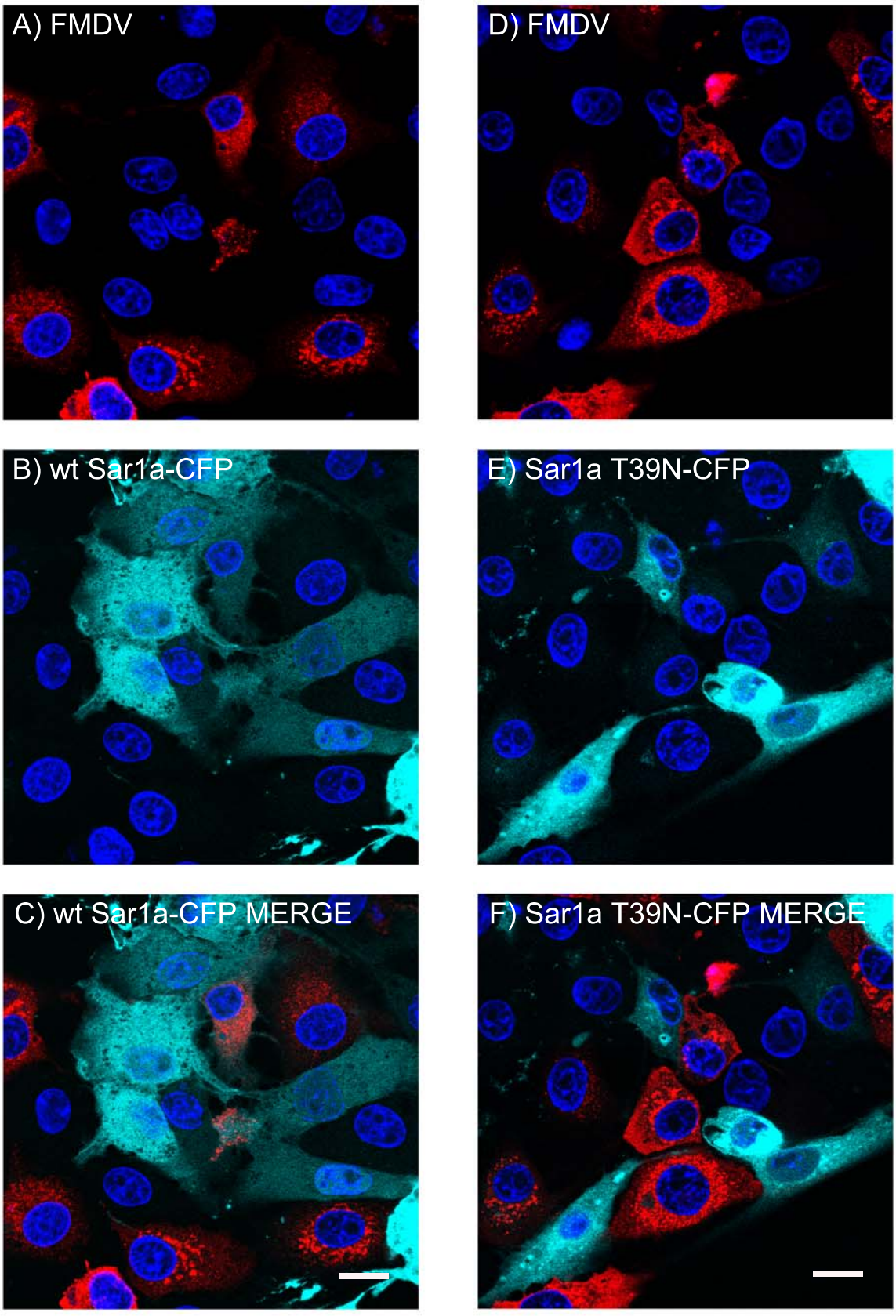


Figure 4.10 FMDV Infection of cells expressing wt or dn Sar1a (T39N)
IBRS-2 cells were transfected to express wt Sar1a-CFP (A-C) or dn Sar1a T39N-CFP (D-F) for 14 hours and then infected with FMDV. Cells expressing Sar1a are shown as cyan. Infected cells are shown as red. Panels A and D show labelling for FMDV. Panels B and E show the signal for Sar1-CFP. Panels C and F showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

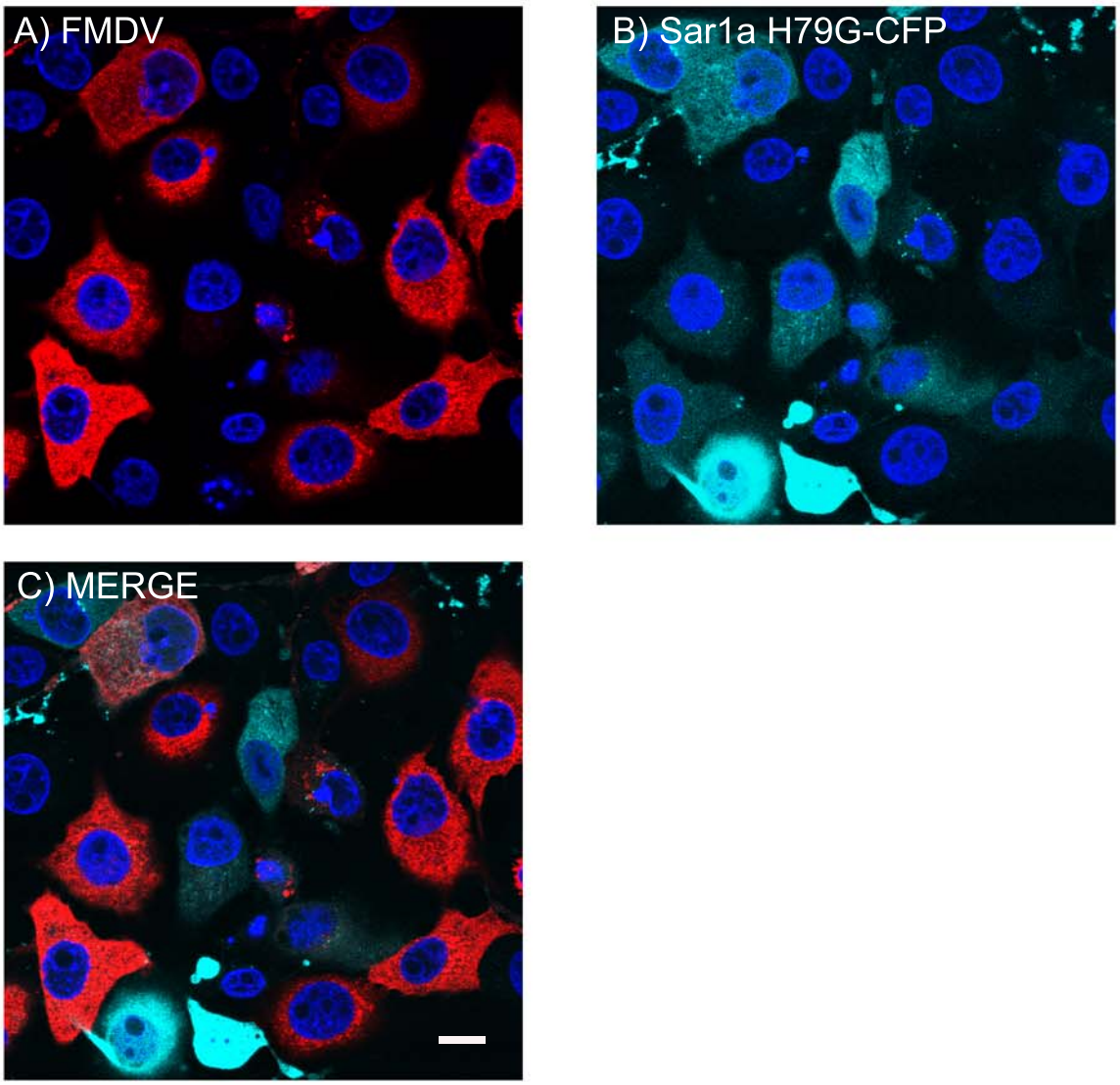


Figure 4.11 FMDV Infection of cells expressing dn Sar1a (H79G)
IBRS-2 cells were transfected to express dn Sar1a H79G-CFP (A-C) for 14 hours and then infected with FMDV. Cells expressing Sar1a H79G are shown as cyan. Infected cells are shown as red. Panel A shows labelling for FMDV. Panel B shows the signal for Sar1a H79G-CFP. Panel C shows merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**36**
SAR1a wildtype CFP	**Expressing**	49	25
	Non-expressing	51	36
SAR1a T39N CFP	**Expressing**	45	26
	Non-expressing	55	32
SAR1a H79G CFP	**Expressing**	49	50
	Non-expressing	51	37

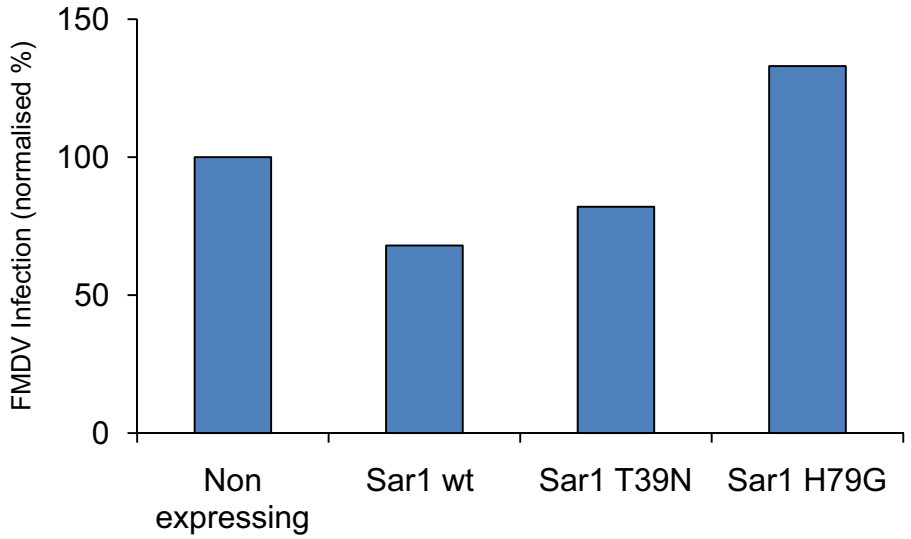


Figure 4.12(A): Experiment 1. The effects of dominant-negative Sar1a on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**54**
SAR1a wildtype CFP	**Expressing**	32	23
	Non-expressing	68	55
SAR1a T39N CFP	**Expressing**	32	28
	Non-expressing	68	47
SAR1a H79G CFP	**Expressing**	34	33
	Non-expressing	66	46

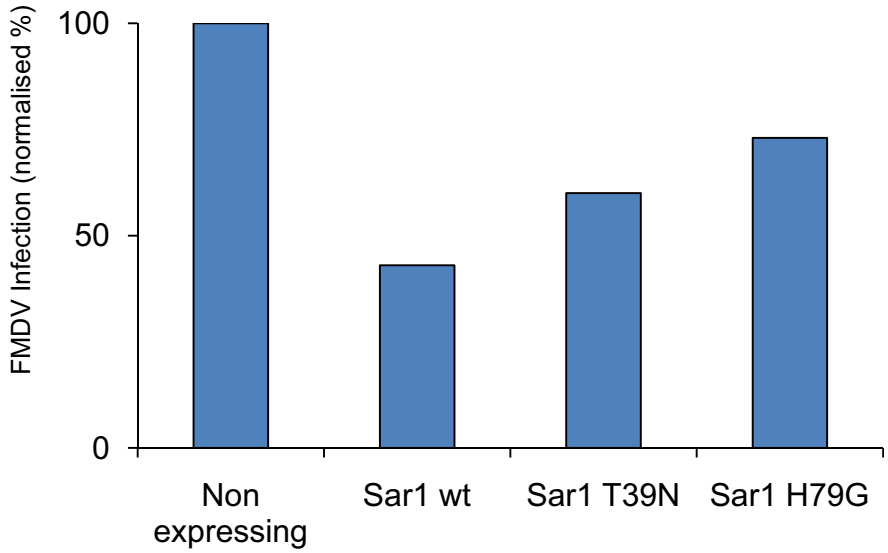


Figure 4.12 (B): Experiment 2. The effects of dominant-negative Sar1a on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**48**
SAR1a wildtype CFP	**Expressing**	25	26
	Non-expressing	75	54
SAR1a T39N CFP	**Expressing**	16	22
	Non-expressing	84	51
SAR1a H79G CFP	**Expressing**	37	49
	Non-expressing	63	56

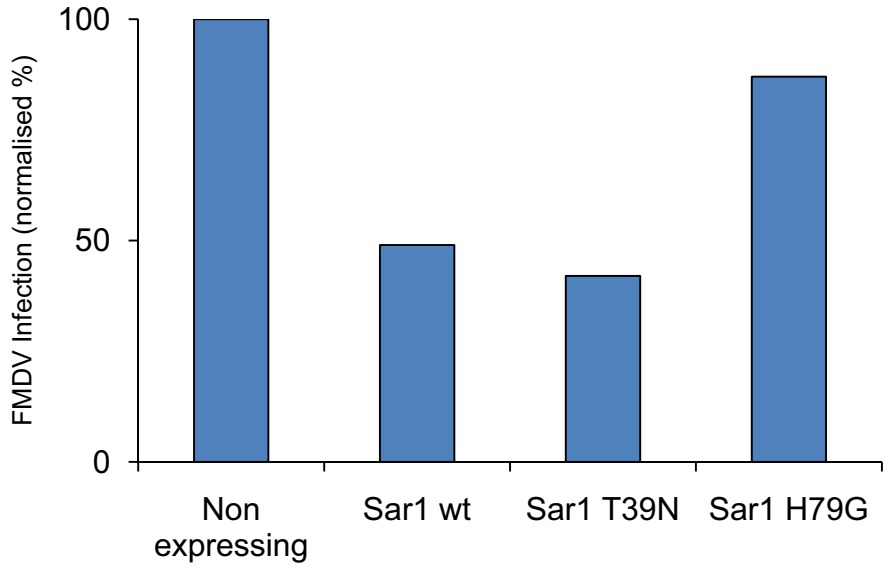


Figure 4.12(C): Experiment 3. The effects of dominant-negative Sar1a on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**26**
SAR1a wildtype CFP	**Expressing**	42	13
	Non-expressing	58	27
SAR1a T39N CFP	**Expressing**	39	17
	Non-expressing	61	24
SAR1a H79G CFP	**Expressing**	53	38
	Non-expressing	47	38

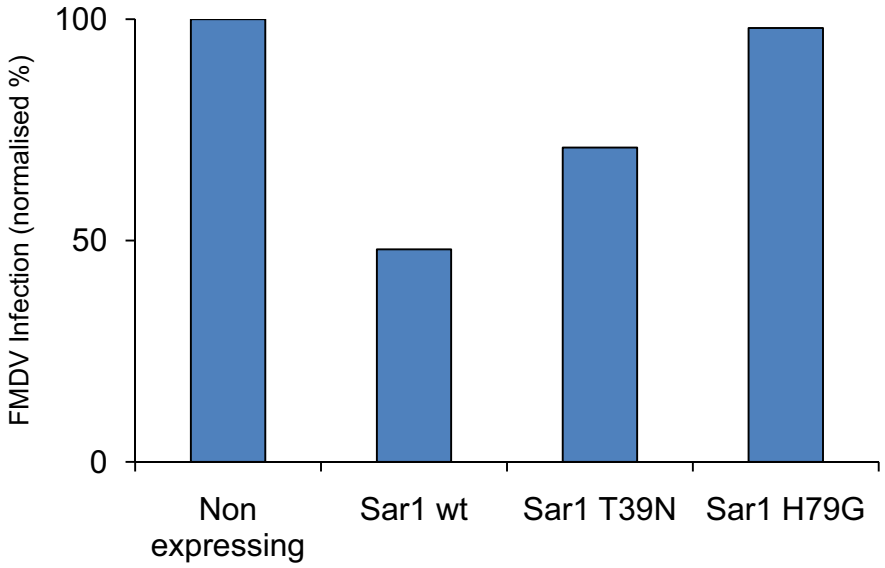


Figure 4.12(D): Experiment 4. The effects of dominant-negative Sar1a on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

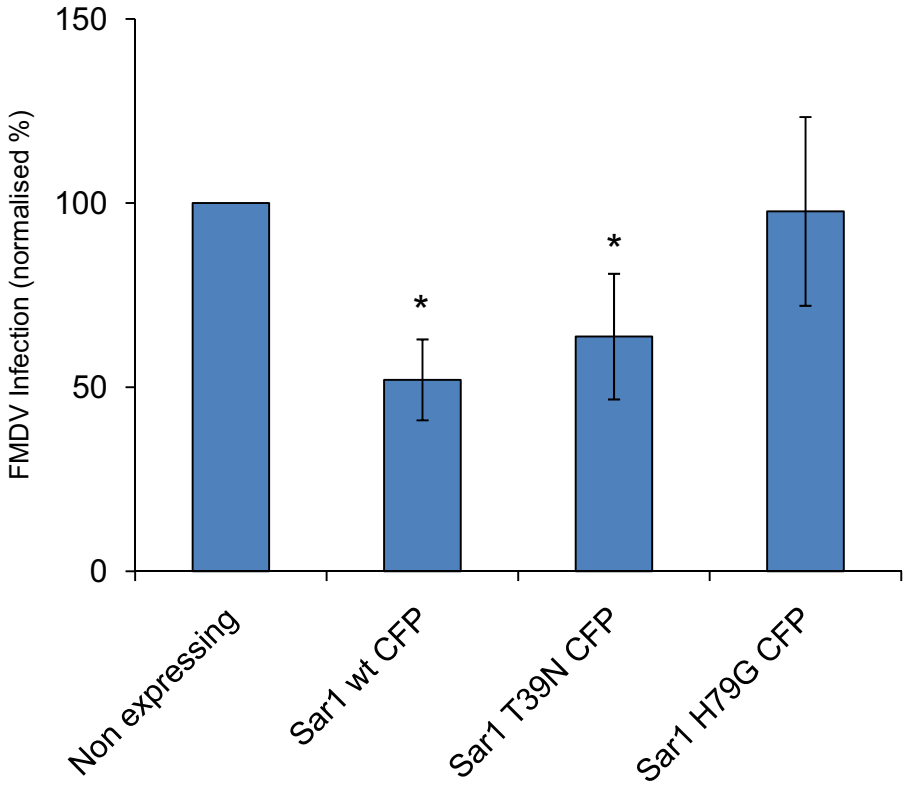


Figure 4.12(E): The effects of dominant-negative Sar1a on FMDV infection of IBRS-2 cells
Shown is the mean ± SD for the experiments shown in figure 4.12 (A-D). For each transgene (wt, dn T39N or dn H79G) the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population (*P* values: * <0.05).

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4.2.3 The effect of dominant-negative Sar1a on BEV-1 infection

The above experiments were repeated using BEV-1 in the place of FMDV. BEV-1 was included as an example of a BfA sensitive virus. Cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with BEV-1 at an MOI of 0.5. After 4 hours cells were fixed in 4% PFM and labelled for infection with an anti-BEV-1 primary antibody and an anti-Guinea pig Alexa 568 secondary antibody. BEV-1 infected cells are shown in red on the figures.

Figure 4.13 shows cells expressing wt Sar1a-CFP or dn Sar1a T39N-CFP and infected with BEV-1. Figure 4.14 shows cells expressing the dn Sar1a H79G-CFP and infected with BEV-1. Cells expressing a transgene are shown in cyan and infected cells are shown as red. As seen with FMDV, cells that appeared to express a high level of a Sar1 protein tended not to be infected. The above experiments were repeated three times and the effect of the dn Sar1 proteins on infection, quantified. The results of these experiments are shown in figure 4.15 (A-C) and the mean level of infection for the experiments in Figure 4.15(D). Overall infection was reduced by each Sar1 construct. Experiment 1 (Figure 4.15 [A]) showed a slight knockdown of BEV-1 infection of ~20% in cells expressing wt Sar1a-CFP. Expression of dn Sar1a T39N-CFP also reduces infection by ~40%, and in cells expressing dn Sar1a H79G infection was inhibited by ~10%. Figure 4.15 (B) shows the second experiment and a ~ 25-30% decrease in infection in cells expressing all three Sar1a proteins. Similar results were seen in the third experiment (Figure 4.15 [C]); all three Sar1a proteins reduced BEV-1 infection by ~25-30%.

The mean level of infection for the four experiments is shown in Figure 4.15 (D). Overall there was a ~25% inhibition of BEV-1 infection in cells

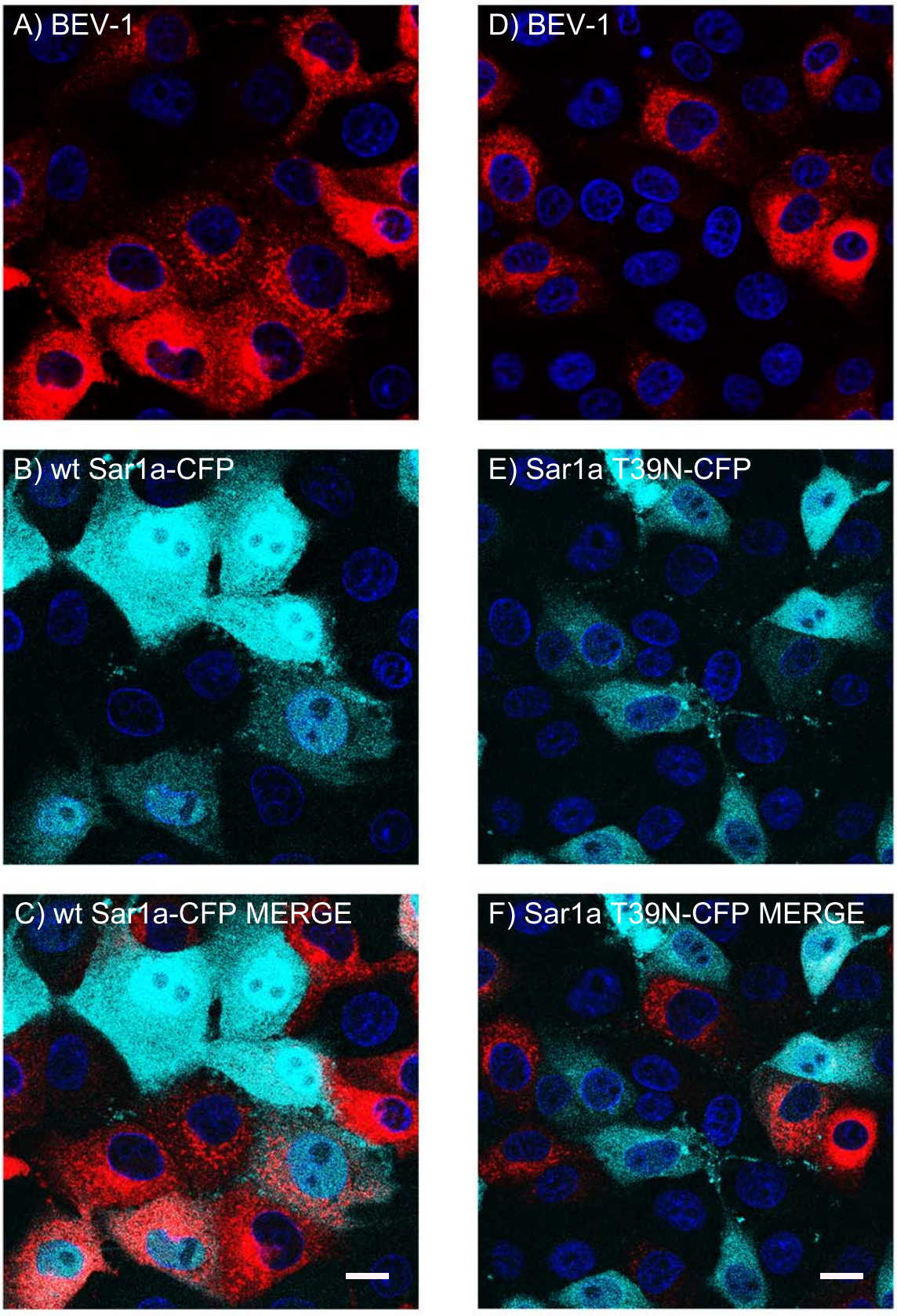


Figure 4.13 BEV-1 Infection of cells expressing wt or dn Sar1a (T39N)
IBRS-2 cells were transfected to express wt Sar1a-CFP (A-C) or dn Sar1a T39N-CFP (D-F) for 14 hours and then infected with BEV-1. Cells expressing Sar1a are shown as cyan. Infected cells are shown as red. Panels A and D show labelling for BEV. Panels B and E show the signal for Sar1a-CFP. Panels C and F showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

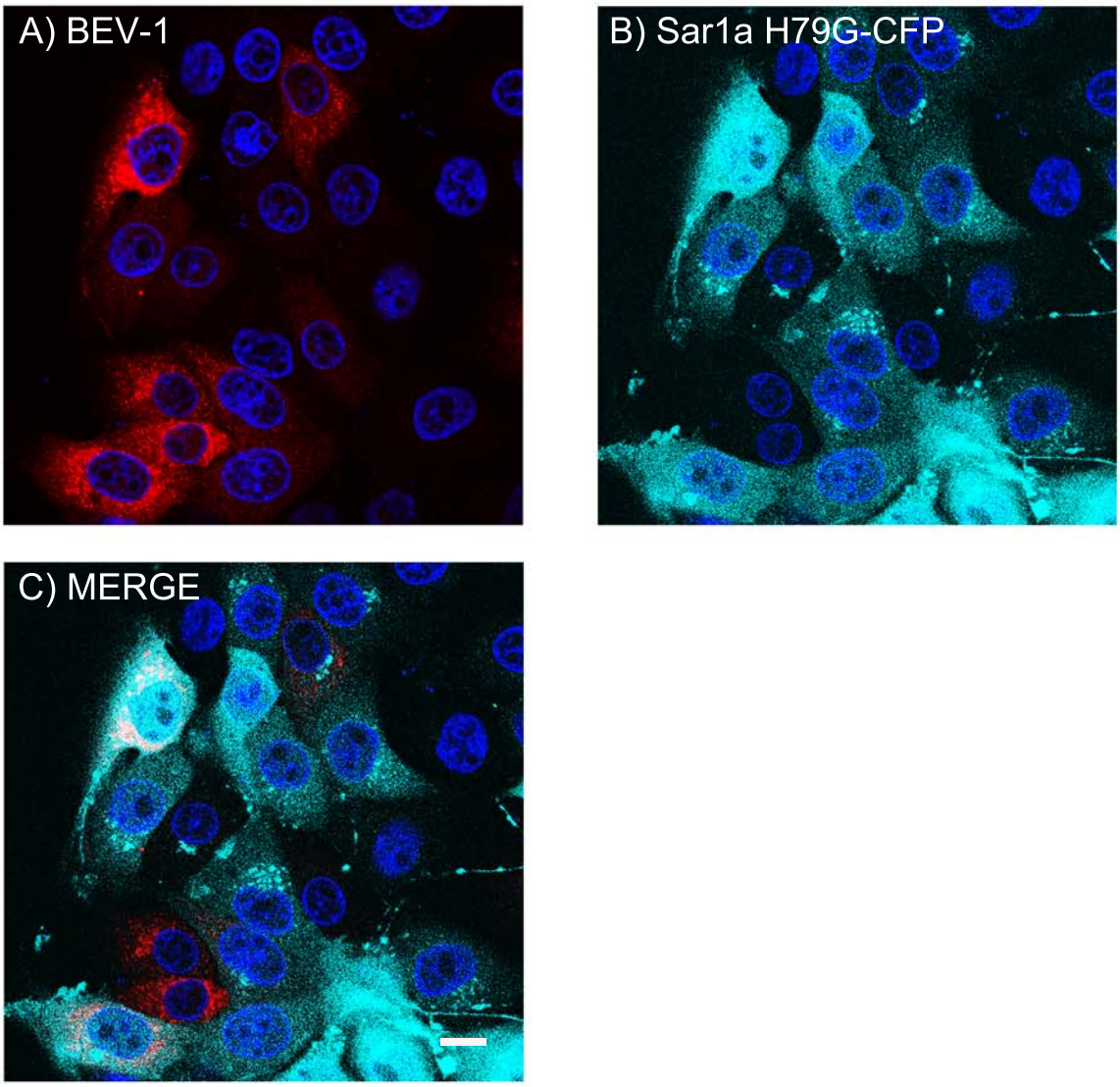


Figure 4.14 BEV-1 Infection of cells expressing dn Sar1a (H79G)
IBRS-2 cells were transfected to express dn Sar1a H79G-CFP (B-C) for 14 hours and then infected with BEV. Cells expressing Sar1a H79G are shown as cyan. Infected cells are shown as red. Panel A shows labelling for BEV. Panel B shows the signal for Sar1a-CFP. Panel C shows merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**48**
SAR1a wildtype CFP	**Expressing**	9	31
	Non-expressing	91	37
SAR1a T39N CFP	**Expressing**	31	21
	Non-expressing	69	37
SAR1a H79G CFP	**Expressing**	16	36
	Non-expressing	84	38

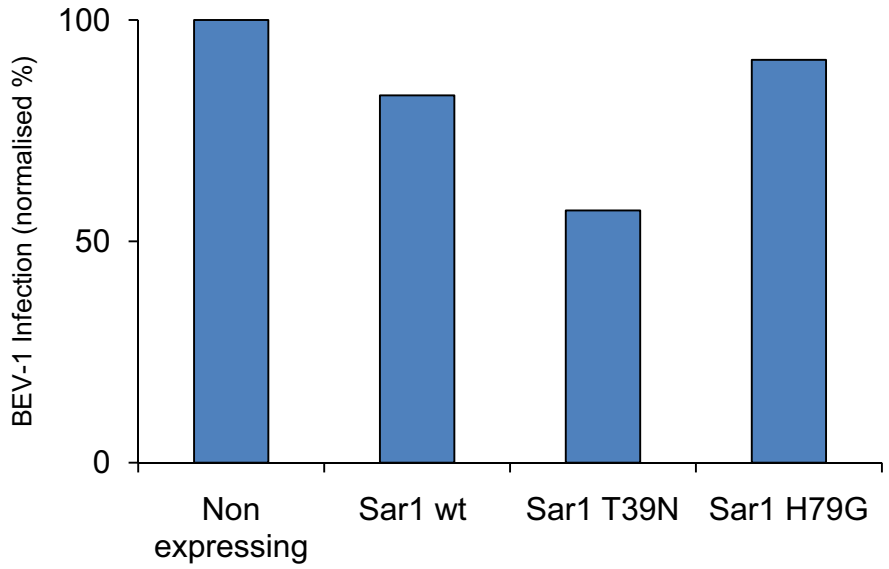


Figure 4.15(A): Experiment 1. The effects of dominant-negative Sar1a on BEV-1 infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sara1 T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**52**
SAR1a wildtype CFP	**Expressing**	27	40
	Non-expressing	73	58
SAR1a T39N CFP	**Expressing**	55	35
	Non-expressing	45	54
SAR1a H79G CFP	**Expressing**	62	33
	Non-expressing	38	45

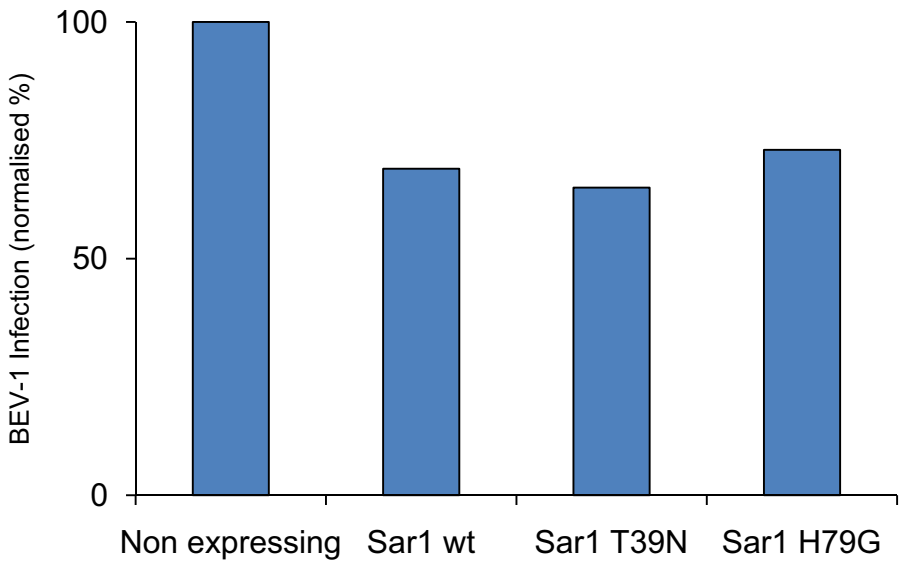


Figure 4.15(B): Experiment 2. The effects of dominant-negative Sar1a on BEV-1 infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**38**
SAR1a wildtype CFP	**Expressing**	34	24
	Non-expressing	66	35
SAR1a T39N CFP	**Expressing**	42	27
	Non-expressing	58	33
SAR1a H79G CFP	**Expressing**	53	25
	Non-expressing	47	38

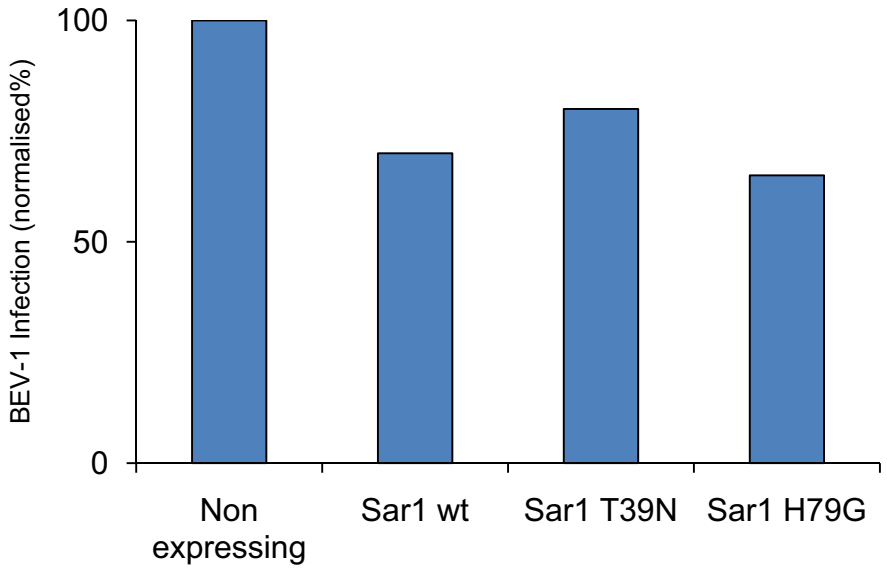


Figure 4.15 (C): Experiment 3. The effects of dominant-negative Sar1a on BEV-1 infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

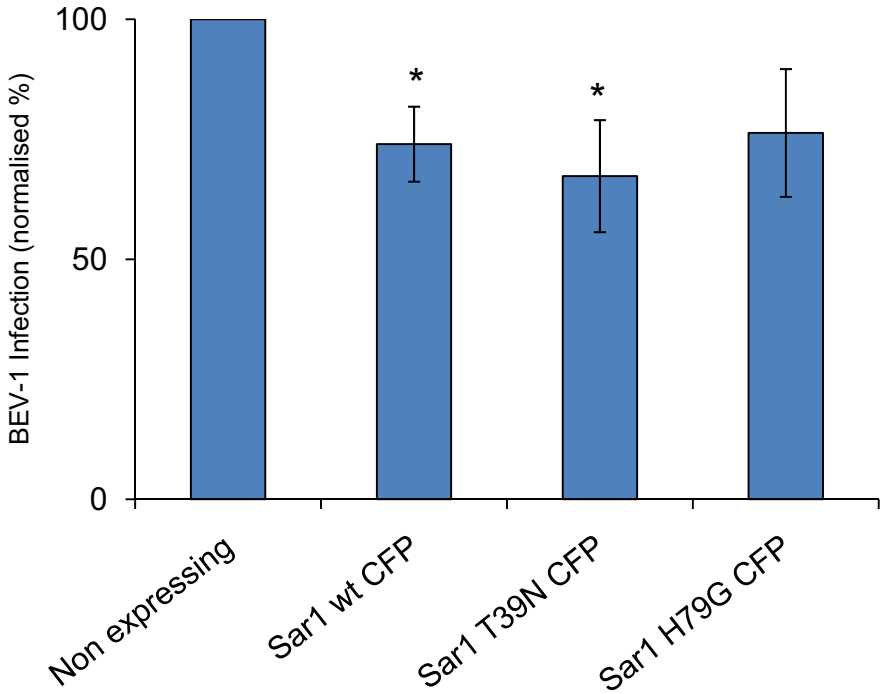


Figure 4.15(D): The effects of dominant-negative Sar1a on BEV-1 infection of IBRS-2 cells
Shown is the mean ± SD for the experiments shown in figure 4.15 (A-C). For each transgene (wt, dn T39N or dn H79G) the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population (*P* value: * <0.05)

expressing any of the Sar1 proteins. This suggests a potential involvement of Sar1 in BEV-1 infection.

4.2.4 The effect of dominant-negative Sar1a on FMDV infection in MAX cells

Next, the effects of dn Sar1 on FMDV infection were investigated using a second cell line (MAX cells). MAX cells, like IBRS-2 are derived from pig kidney. Max cells, transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP, were either (i) fixed using 4% PFM and labelled for the Golgi (for GM130) as described above or (ii) infected with FMDV at an MOI of 0.5 . At 4 hours post-infection the cells were fixed in 4% PFM and labelled for FMDV with a rabbit anti-FMDV type O antibody as described above. Infected cells are shown as red on the figures.

MAX cells expressing the Sar1a proteins were labelled for the Golgi to confirm if expression of the dn proteins disrupted the Golgi in a similar manner to that observed in IBRS-2 cells. Figure 4.16 shows untransfected MAX cells labelled for the Golgi (green) and that the Golgi locates to a peri-nuclear region. Figure 4.17 shows cells expressing wt Sar1a-CFP (cyan, panel B), dn Sar1a T39N-CFP (cyan, panel D) and dn Sar1a H79G-CFP (cyan, panel F) labelled for the Golgi with GM130 (shown in green [A-F]). As seen in IBRS-2 cells (see section 4.2.1), expression of wt Sar1a-CFP (Fig 4.17 cyan, panel B) did not appear to affect the Golgi. MAX cells expressing dn Sar1a T39N-CFP (Fig 4.17 cyan, panel D) showed a range of effects on GM130 labelling, from a complete loss of labelling in cells that express a high level of the dn protein, to fragmentation of Golgi labelling in cells that express a low level of the protein (Fig 4.17, C-D). Expression of dn Sar1a H79G-CFP resulted in severe fragmentation of the Golgi, however labelling for GM130 was not completely

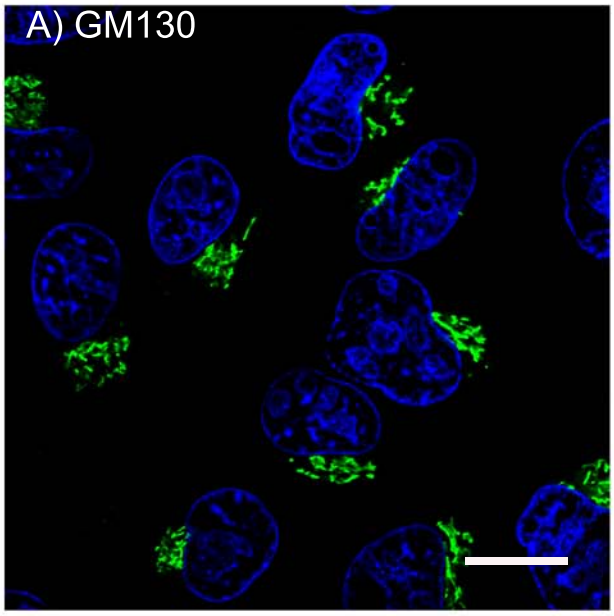


Figure 4.16 Labelling of the Golgi in MAX cells
MAX cells on coverslips were fixed, permeablized and labelled for the Golgi (GM130) and visualised with an anti-mouse Alexa-488 conjugated secondary antibody (shown as green on the figure). The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

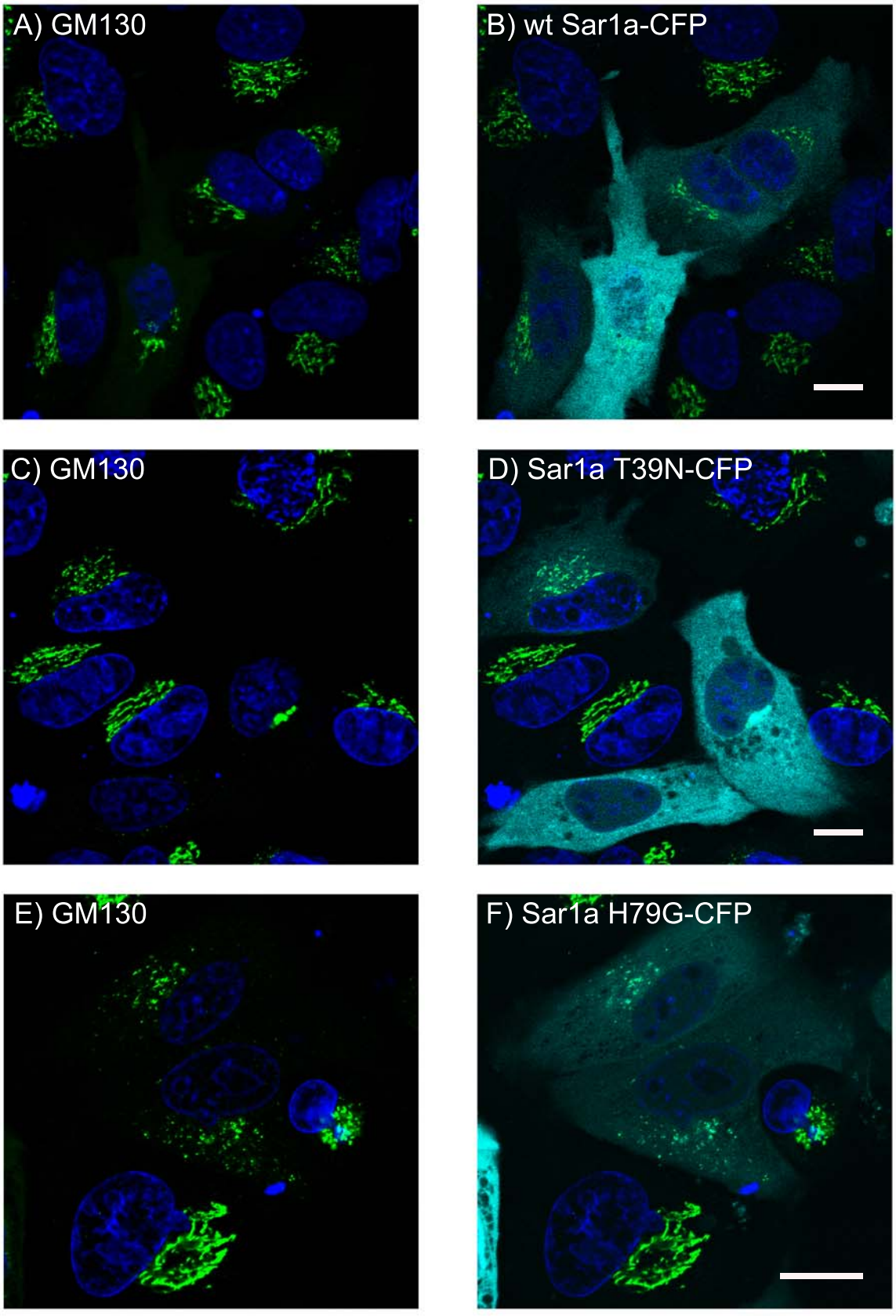


Figure 4.17 The effects of Sar1a expression on the Golgi in MAX cells
MAX cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G -CFP for 14 hours and labelled for the Golgi (GM130, panels A-F). Cells expressing Sar1a are shown as cyan; cells labelled for the Golgi are shown in green. Panels B, D and F show merged images for green and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

lost. Disruption of the Golgi suggests that membrane flow through the secretory pathway and protein transport is blocked by both dn Sar1a proteins, and that the extent of the block is dependent on the level of the dn protein.

Figure 4.18 shows MAX cells expressing wt Sar1-CFP (shown as cyan, panels B-C) or dn Sar1 T39N-CFP (shown as cyan, panels E-F) and infected with FMDV (shown as red). The frequency of infection of cells expressing wt Sar1-CFP was noticeably reduced in comparison to cells in the non-expressing population. The frequency of infection of cells expressing dn Sar1a T39N-CFP also appeared to be reduced in comparison to the non-expressing cell population. The cells expressing the wt or dn proteins that were infected appeared to be those expressing a lower level of the transgene. Hence, both wt Sar1a-CFP and dn Sar1a T39N-CFP expression appears to reduce FMDV infection levels. Figure 4.19 shows MAX cells expressing dn Sar1a H79G-CFP (cyan, panels B-C). There did appear to be a slight reduction in FMDV infection for cells expressing this dn, however as seen with IBRS-2 cells, the level of dn expression did not appear to influence infection (see Fig 4.17).

As described above for IBRS-2 cells, the results for each transfection were normalised to the frequency of infection of the non-expressing cell population. Figure 4.20 (graphs A-C) shows three independent experiments and the mean effect of the Sar1 proteins on infection is shown in figure 4.20 (D). The results of the first experiment is shown in Figure 4.20 (A); a ~50% reduction in FMDV infection was observed in cells expressing either wt Sar1a-CFP or dn Sar1a T39N-CFP, while dn Sar1a H79G-CFP expression reduced FMDV infection by ~30%. The second experiment is shown in Figure 4.20 (B); similar results were seen with wt Sar1a-CFP and dn Sar1a T39N-CFP as infection was reduced by ~55-60%. For dn Sar1a H79G-CFP, infection was reduced by 20%.

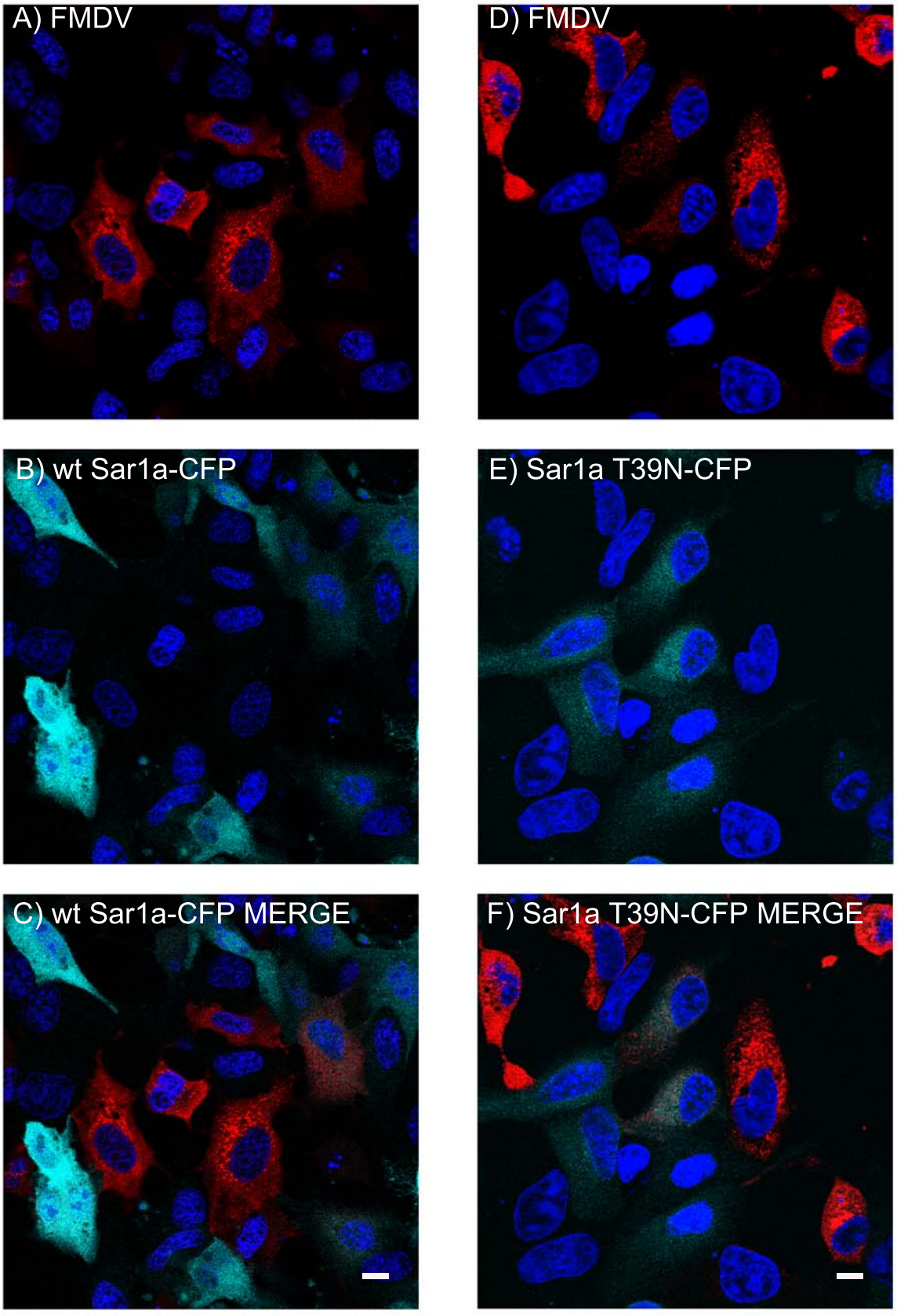


Figure 4.18 FMDV Infection of MAX cells expressing wt or dn Sar1a (T39N)
MAX cells were transfected to express wt Sar1a-CFP (A-C) or dn Sar1 T39N-CFP (D-F) for 14 hours and then infected with FMDV. Cells expressing Sar1a are shown as cyan. Infected cells are shown as red. Panels A and D show labelling for FMDV. Panels B and E show the signal for Sar1a-CFP. Panels C and F showed merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

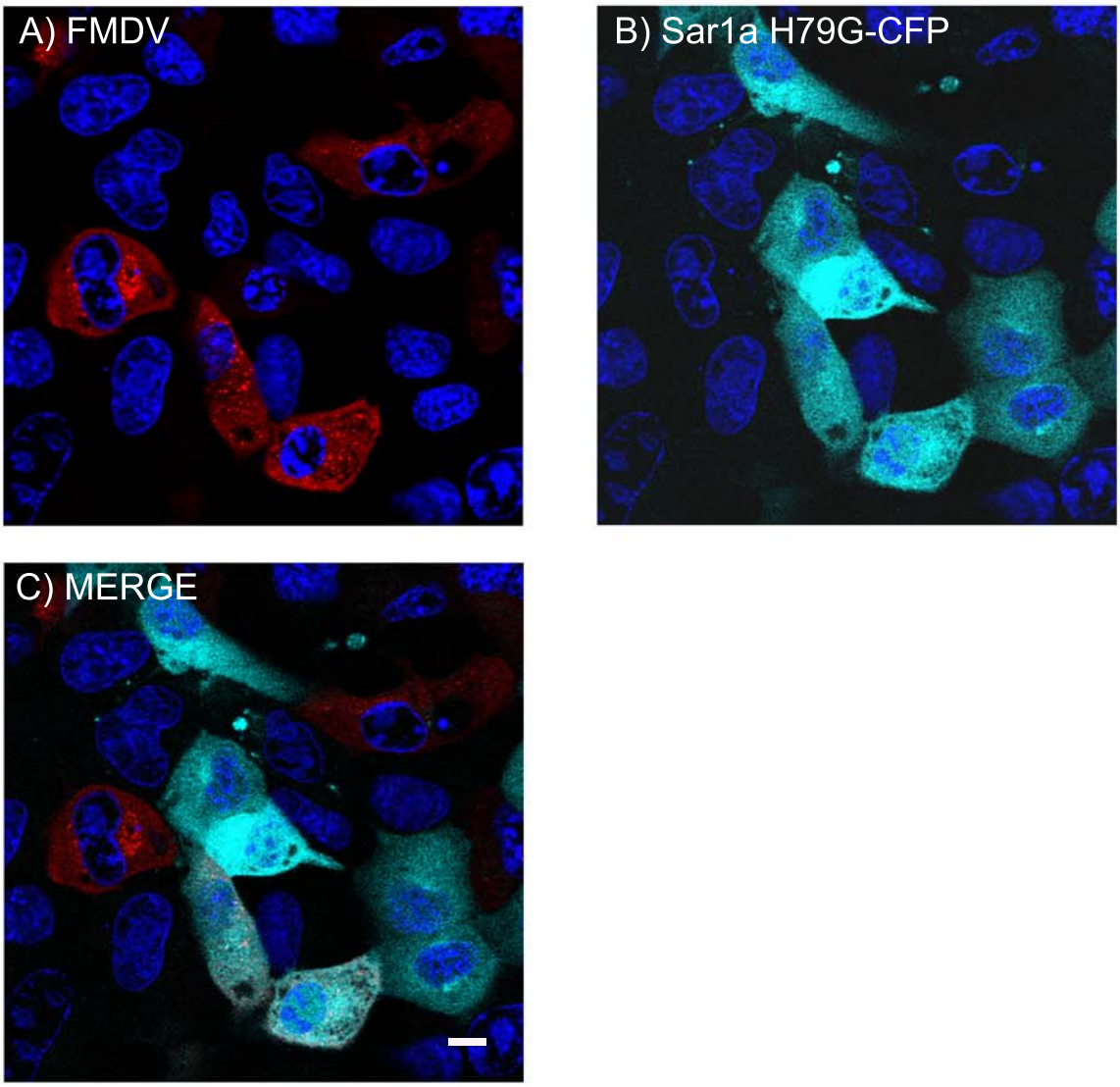


Figure 4.19 FMDV Infection of MAX cells expressing dn Sar1a (H79G)
MAX cells were transfected to express dn Sar1a H79G-CFP (B-C) for 14 hours and then infected with FMDV. Cells expressing Sar1a H79G are shown as cyan. Infected cells are shown as red. Panel A shows labelling for FMDV. Panel B shows the signal for Sar1a -CFP. Panel C shows merged images for red and cyan fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**41**
SAR1a wildtype CFP	**Expressing**	35	19
	Non-expressing	65	41
SAR1a T39N CFP	**Expressing**	34	18
	Non-expressing	66	38
SAR1a H79G CFP	**Expressing**	37	25
	Non-expressing	63	38

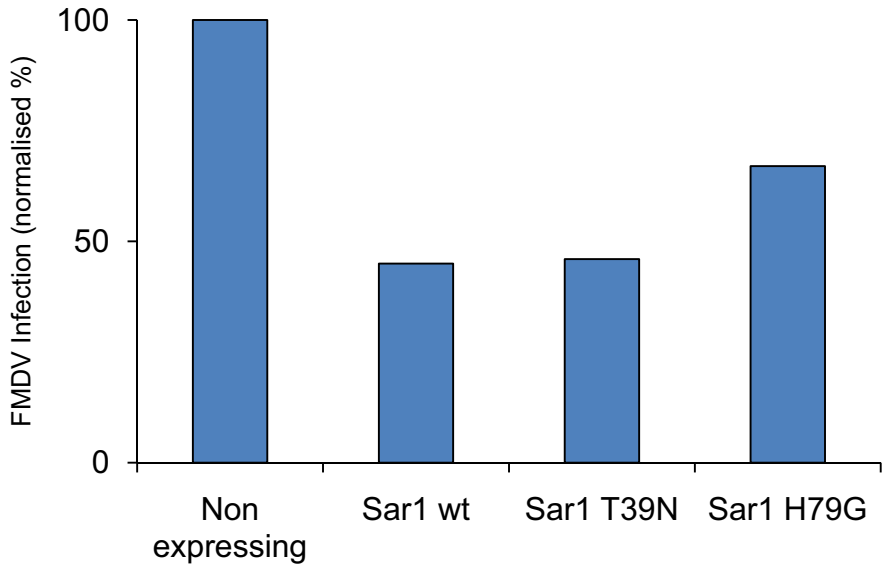


Figure 4.20(A): Experiment 1. The effects of dominant-negative Sar1a on FMDV infection of MAX cells
MAX cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**46**
SAR1a wildtype CFP	**Expressing**	35	19
	Non-expressing	65	41
SAR1a T39N CFP	**Expressing**	39	17
	Non-expressing	61	44
SAR1a H79G CFP	**Expressing**	37	30
	Non-expressing	63	39

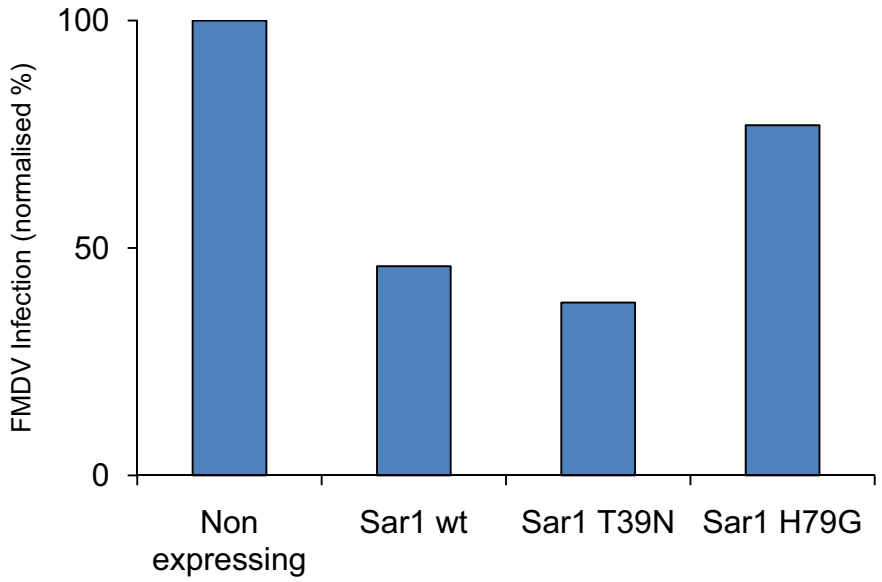


Figure 4.20(B): Experiment 2. The effects of dominant-negative Sar1a on FMDV infection of MAX cells
MAX cells were transfected to express wt Sar1a-CFP, dn Sar1a T39N-CFP or dn Sar1a H79G-CFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**38**
SAR1a wildtype CFP	**Expressing**	40	18
	Non-expressing	60	38
SAR1a T39N CFP	**Expressing**	47	25
	Non-expressing	53	44
SAR1a H79G CFP	**Expressing**	45	27
	Non-expressing	55	36

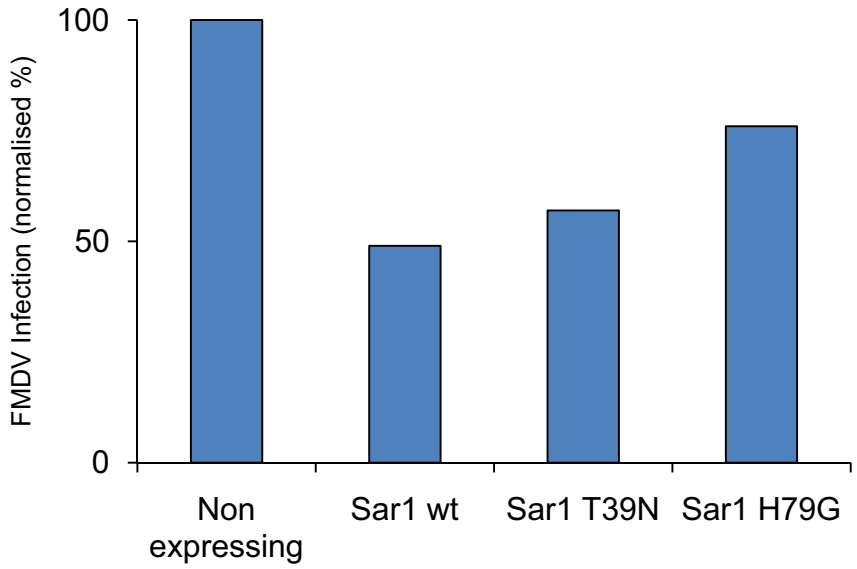


Figure 4.20(C): Experiment 3. The effects of dominant-negative Sar1 on FMDV infection of MAX cells
MAX cells were transfected to express wt Sar1a-CFP, dn Sar1 T39N-CFP or dn Sar1 H79G-CFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. cyan expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population.

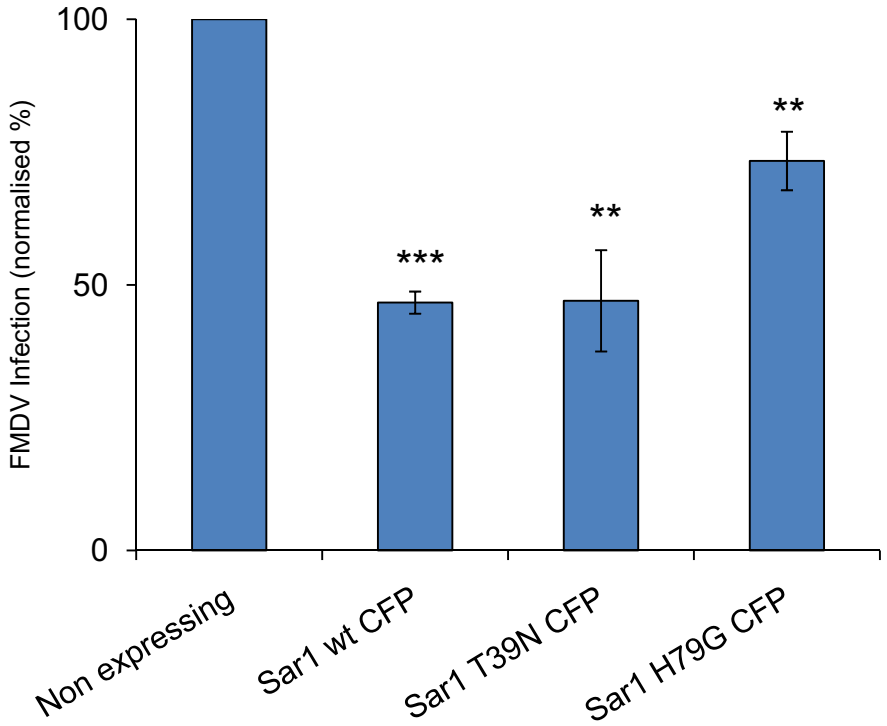


Figure 4.20(D): The effects of dominant-negative Sar1a on FMDV infection of MAX cells

Shown is the mean ± SD for the experiments shown in figure 4.20 (A-C). For each transgene (wt, dn T39N or dn H79G) the frequency of infection of cells expressing Sar1 was normalised to the results for the non-expressing cell population (P values: ** <0.01, *** <0.001).

The third experiment is shown in Figure 4.20 (C); expression of wt Sar1a-CFP, dn Sar1a T39N-CFP and dn Sar1a H79G-CFP reduced infection by 50%, 40% and 25 % respectively.

Figure 4.20 (D) shows the mean reduction for the three experiments. Both wt Sar1a-CFP and dn Sar1a T39N-CFP reduced FMDV infection by ~50-55%, while expression of dn Sar1a H79G-CFP inhibited infection by ~30%. The reduction to FMDV infection seen in wt Sar1a-CFP and dn Sar1a T39N-CFP expressing cells agrees with the IBRS-2 cells data, suggesting a role for Sar1 in FMDV infection. However, the inhibitory effect of dn Sar1a H79G-CFP on virus infection of MAX cells appeared greater than for IBRS2 cells (see discussion 4.5).

4.3 Investigating the role of Sec16 on FMDV infection using Dominant-negative proteins

The previous experiments indicate a possible requirement for Sar1 and COPII vesicle formation at the ERES for efficient FMDV infection. A dominant negative version of Sec16 was used to further investigate the requirement for the ERES and COPII vesicles in infection. Sec16 is thought to serve as a scaffold protein at the ERES as its C-terminal region is reported to interact with Sec23, Sec24, Sec31 and Sar1, all components of the COPII complex. Sec16 is reliant on Sar1a-GTP activity to form the ERES. The inhibition of Sec16 activity should prevent the formation of normal ERES organisation and block to ER-Golgi transport (Battacharyya and Glick 2007). The dominant-negative Sec16 protein used in these experiments has a mutation in the C-terminal of the protein in the residues that interact with the COPII complex. This dominant-negative should prevent efficient formation of the COPII coat at the ERES and therefore reduce protein transport in the early secretory pathway.

4.3.1 The effects of dominant-negative Sec16 on membranes in the early secretory pathway

In the first instance the effect of dn Sec16 C-term on membranes of the early secretory pathway was investigated by immunofluorescence confocal microscopy (see methods, section 2.7) using IBRS-2 cells. IBRS-2 cells were transfected to express Sec16 C-term fusion with GFP (which allows for detection of transfected cells) for 14 hours. The cells were processed for IFCM as described above and labelled the ER (PDI), the ERGIC (ERGIC-53) or the Golgi (GM130). Cells expressing a transgene (i.e. dn Sec16 protein) are shown as green on the figures.

Figure 4.21 shows cells expressing dn Sec16 C-term-GFP (shown as green in panels B, D and F) and labelled for the ER (shown as red on panels A-B), the ERGIC (shown as red on panels C-D) or the Golgi (shown in red on panels E-F). No obvious alteration in ER labelling was observed in cells expressing the dn Sec16 C-term-GFP (Fig 4.21 A-B), however the ERGIC peri-nuclear labelling did appear dispersed (Fig 4.21 C-D). In cells labelled for the Golgi, the labelling became fragmented in cells expressing dn Sec16 C-term-GFP (Fig 4.21 E-F). Overall, this data suggests that expression of dn Sec16 C-term-GFP is likely to block protein secretion from the ER to the ERGIC and Golgi.

4.3.2 The effect of dominant-negative Sec16 on FMDV infection

Next the effect of dn Sec16 C-term-GFP on FMDV infection was investigated. IBRS-2 cells were transfected with either full length Sec16L-GFP or dn Sec16 C-term-GFP for 14 hours. The transfected cells were then infected with FMDV O1Kcad_2 at a low MOI (MOI 0.5). Infection was allowed to proceed for 4 hours before the cells were fixed with 4% PFM, permeabilised and labelled

for virus using a rabbit anti-FMDV antibody and then visualised with a goat anti-rabbit Alexa-fluor 568 secondary antibody. Infected cells are shown as red on the figures.

Figure 4.22 shows IBRS-2 cells expressing dn Sec16 C-term-GFP (shown as green, in panels B-C) and infected with FMDV (shown as red, in panels A, C). In cells expressing dn Sec16 C-term-GFP a slight reduction in the frequency of FMDV infection was observed when compared to the cells of the non-expressing population.

The above experiment was repeated three times and the effect of the dn Sec16 C-term on infection quantified. The level of infection of cells expressing a transgene (Sec16) were normalised to the frequency of infection of cells of the non-expressing population. In addition cells were transfected to express Sec16L-GFP, the full length version of the Sec16 protein, as a control to compare the effects of the dn Sec16 C-term with.

Figure 4.23 (panels A-C) shows data for three independent experiments. The first experiment (Figure 4.23 [A]) shows that cells expressing Sec16L-GFP were infected at a reduced frequency (~25-30% reduction) when compared to cells of the non-expressing population. Similarly, cells expressing dn Sec16 C-term-GFP showed a reduced level of infection (~35%). The second experiment (Figure 4.23 [B]) showed a very slight reduction of ~3% in the frequency of infection in cells expressing Sec16L-GFP and ~20% reduction in cells expressing Sec16 C-term-GFP. Figure 4.23 (C) shows the third experiment in which Sec16L-GFP increased infection levels by ~25% and dn Sec16 C-term-GFP also increased infection slightly by ~5-10%.

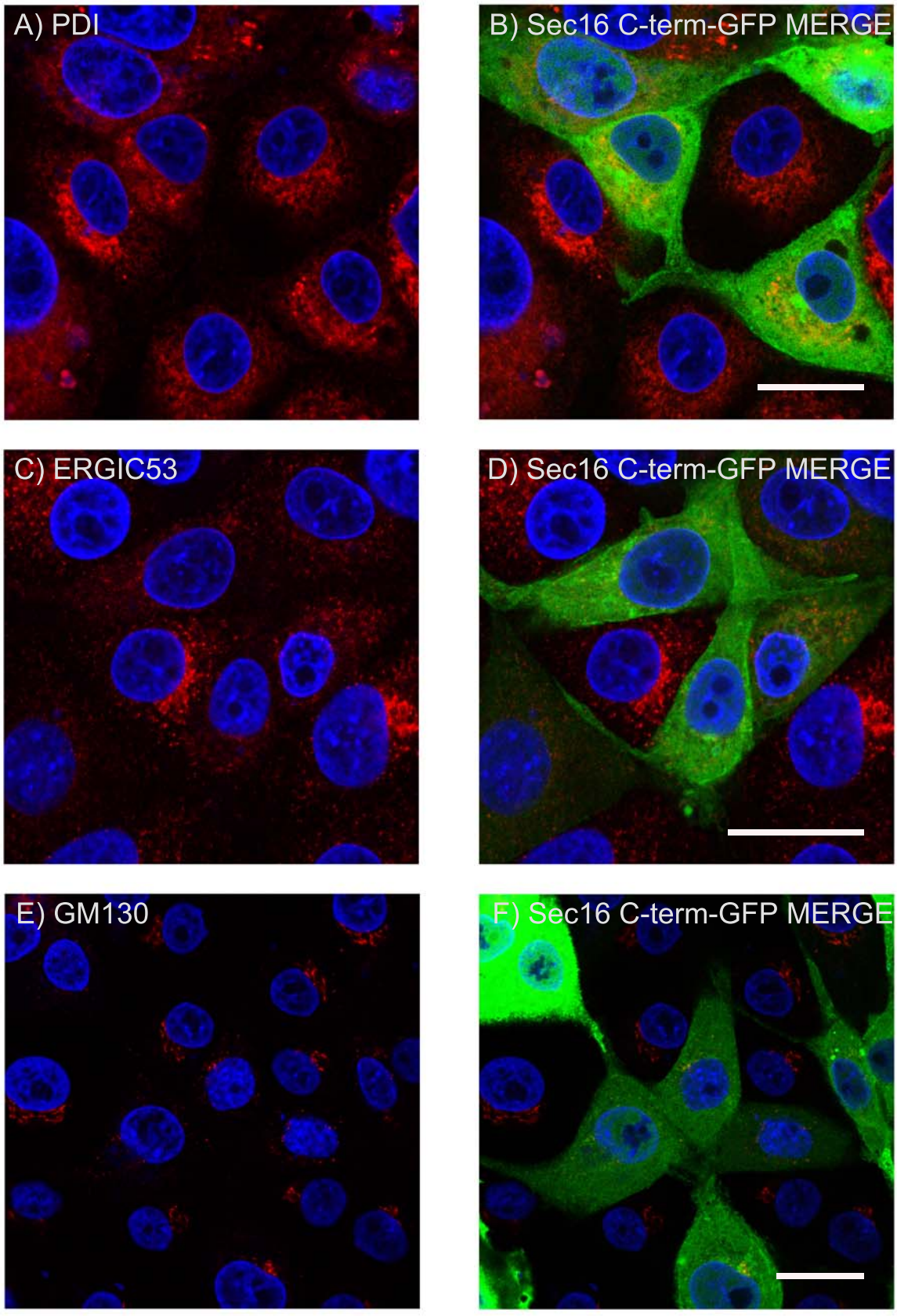


Figure 4.21 The effects of dn Sec16 C-term expression on the ER, ERGIC and Golgi
IBRS-2 cells were transfected to express dn Sec16 C-term-GFP for 14 hours and labelled for the ER (PDI; panels A & B), the ERGIC (ERGIC53; panels C & D) or the Golgi (GM130; panels E & F). Cells expressing Sec16 C-term are shown as green; the ER, ERGIC and Golgi are shown in red. Panels B, D and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

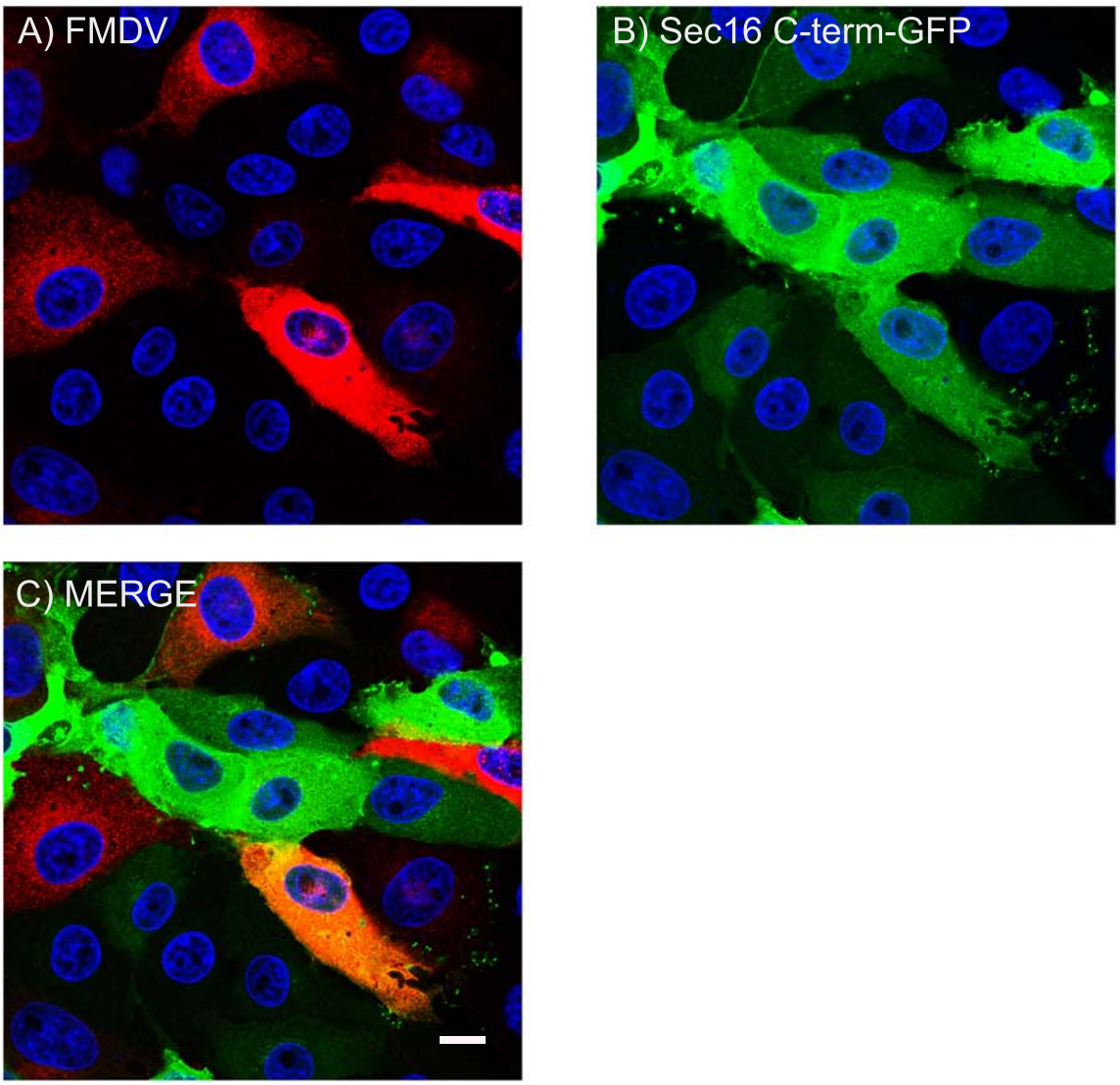


Figure 4.22 FMDV Infection of cells expressing dn Sec16 C-term
IBRS-2 cells were transfected to express dn Sec16 C-term-GFP for 14 hours and then infected with FMDV. Cells expressing Sec16 are shown as green. Infected cells are shown as red. Panel A shows labelling for FMDV. Panel B shows the signal for Sec16-GFP. Panel C shows merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**36**
Sec16L GFP	**Expressing**	36	30
	Non-expressing	64	42
Sec16 C-terminus GFP	**Expressing**	40	29
	Non-expressing	60	43

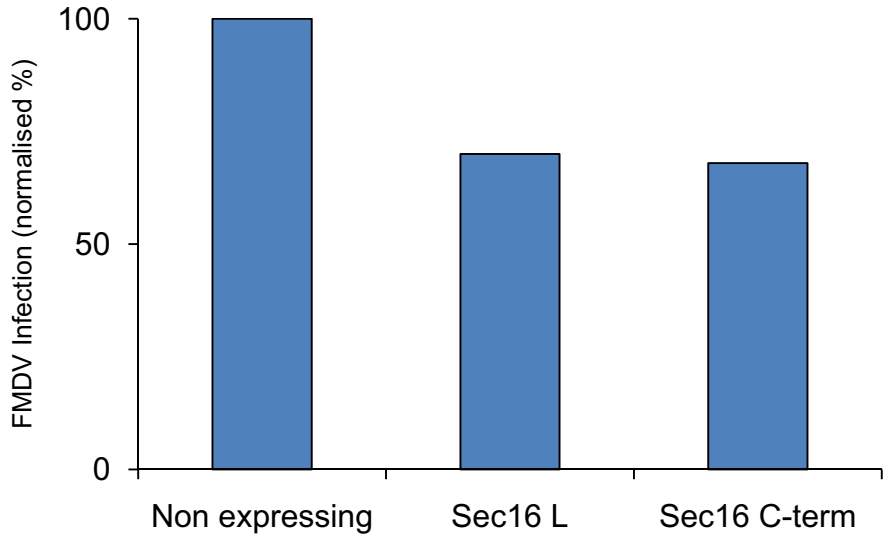


Figure 4.23(A): Experiment 1. The effects of dominant-negative Sec16 on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express Sec16L-GFP or dn Sec16 C-term-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sec16 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sec16 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**34**
Sec16L GFP	**Expressing**	41	26
	Non-expressing	59	26
Sec16 C-terminus GFP	**Expressing**	44	16
	Non-expressing	56	19

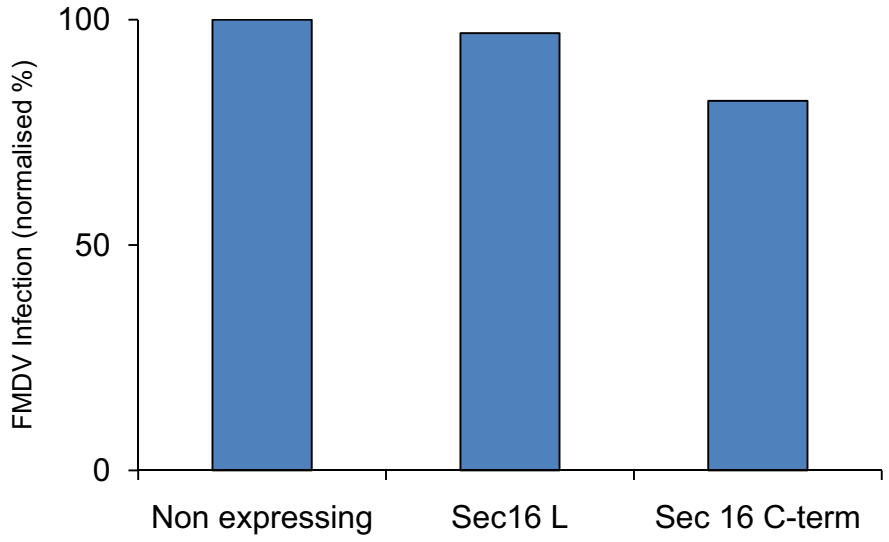


Figure 4.23(B): Experiment 2. The effects of dominant-negative Sec16 on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express Sec16L-GFP or dn Sec16 C-term-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sec16 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sec16 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**32**
Sec16L GFP	**Expressing**	40	44
	Non-expressing	60	34
Sec16 C-terminus GFP	**Expressing**	62	32
	Non-expressing	38	29

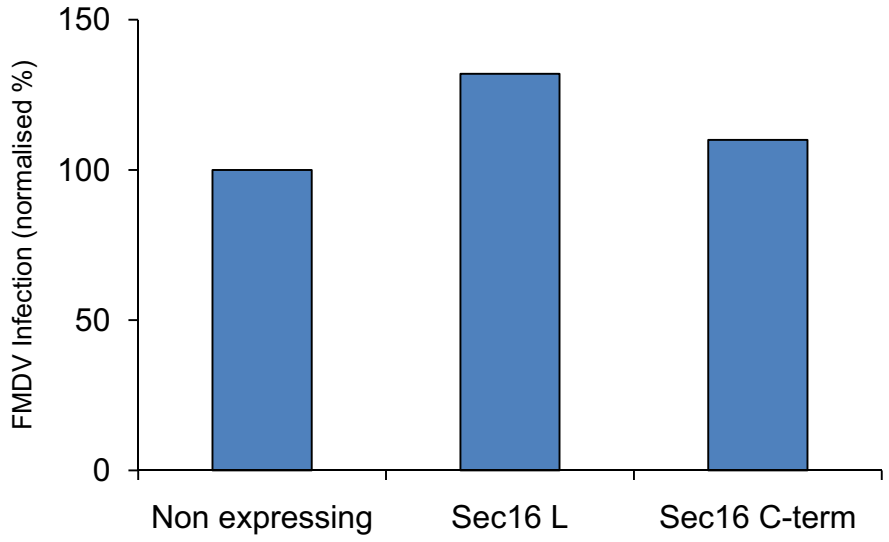


Figure 4.23(C): Experiment 3. The effects of dominant-negative Sec16 on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express Sec16L-GFP or dn Sec16 C-term-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sec16 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower figure*: For each transfection, the frequency of infection of cells expressing Sec16 was normalised to the results for the non-expressing cell population.

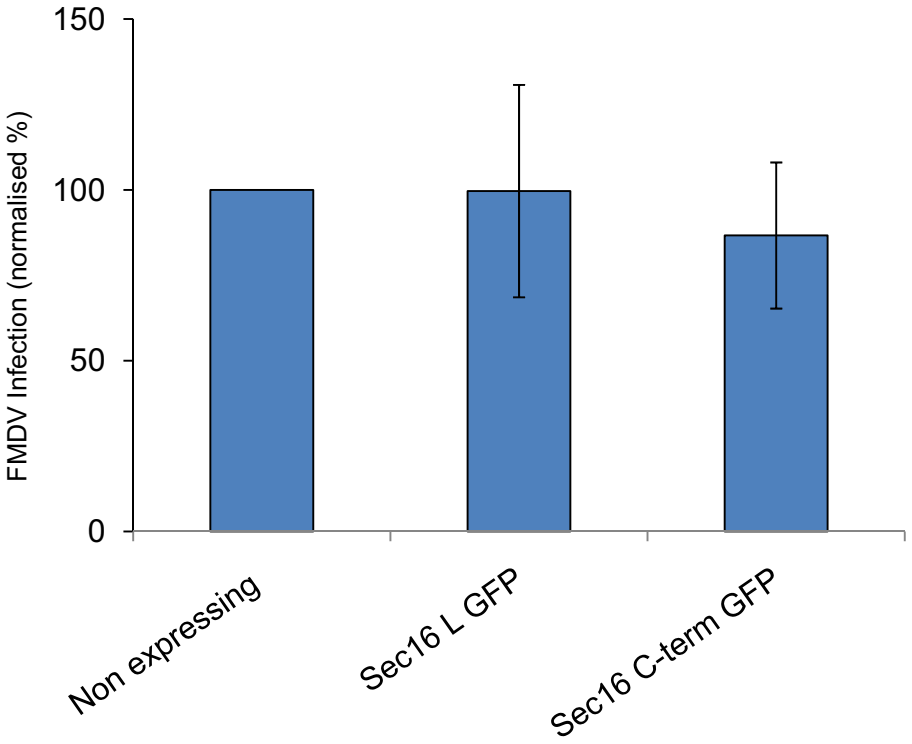


Figure 4.23(D): The effects of dominant-negative Sec16 on FMDV infection of IBRS-2 cells
Shown is the mean ± SD for the experiments shown in figure 4.23 (A-C). For each transgene (Sec16 L or C-term) the frequency of infection of cells expressing Sec16 was normalised to the results for the non-expressing cell population.

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The mean level of infection for the three experiments is shown in Figure 4.23 (D). Overall, it appears Sec16L-GFP did not have any substantial effect on FMDV infection, as the frequency of infection remains equal to that of infection seen in cells of the non-expressing population. In cells expressing dn Sec16 C-term-GFP, ~20% reduction was observed. Although, Sec16 is thought to be involved in COPII vesicle formation at the ERES, it has not been shown to be essential for this process. This could explain why FMDV infection is largely unaffected by the expression of the dn Sec16 mutant.

4.4 Investigating the role of Sar1 in FMDV infection using RNA interference

4.4.1 shRNA

The above experiments suggest a role for Sar1 in FMDV infection. Sar1 exists as two isoforms a and b, and both are involved in COPII vesicle formation. Sar1a and Sar1b differ in sequence by approximately 20 amino acids, but separate functions have not been assigned to the different isoforms (Charcosset et al., 2008). RNA interference was targeted to both a and b isoforms. These experiments should confirm our findings from the dominant-negative experiments.

As multiple published sequences for porcine Sar1 were found on the NCBI database, cDNA for both a and b isoforms was cloned from IBRS-2 cells and then sequenced. These sequences were required to gain an accurate match for the design of siRNA. Total RNA was extracted from IBRS-2 cells using a mRNA direct kit (Dynal) and reverse transcribed into single-stranded cDNA using an oligo dT primer (see methods 2.6.3 - 2.6.4). Three PCR primers were designed, based on sequence homology with Sar1 from other mammalian species; a single forward primer was used for amplification of both the a and b

isoforms, whereas two different reverse primers were used for the different isoforms (see methods 2.3.3.3). The PCR products were ligated into a p-GEM-T vector (Promega) and transformed into JM109 cells. Ampillicin resistant cultures were grown overnight at 37^{o}C and DNA extracted using a mini-prep kit (Qiagen). EcoR1 digests of mini-prep DNA were run on a 1.2% agarose gel to confirm the correct plasmid and insert size (700bp) for each sample. Plasmid DNA containing correct sized inserts were sequenced using p-GEM-T vector primers as per manufacturer's instructions (see methods 2.5.9). Sequence analysis and BLAST searches indicated a 99% -100% match for both of the Sar1 isoforms to one of the published pig genomes (accession number NM_001031786).

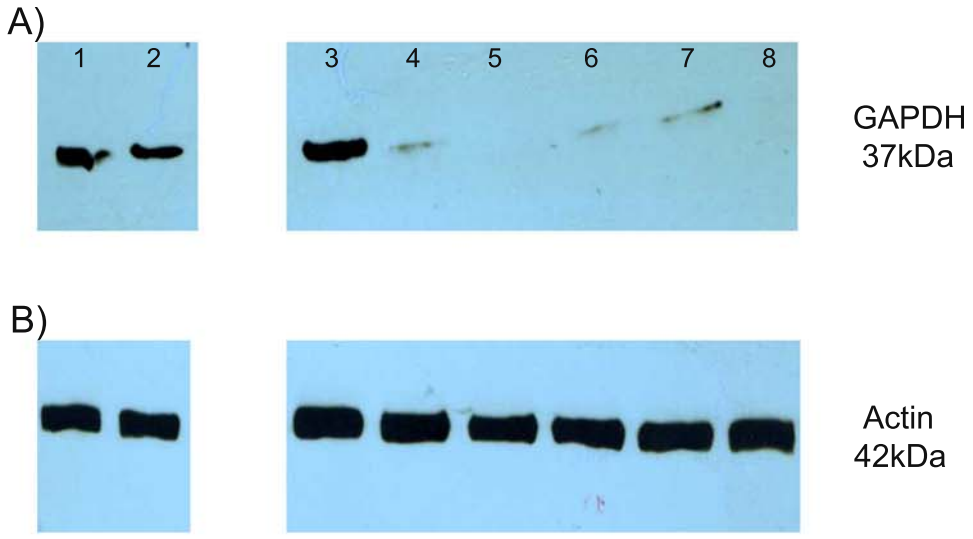
RNA interference (RNAi) is widely used to analyse the function of mammalian genes. RNAi can take the form of small dsRNA RNAs (siRNA) which are 21-23 base pairs in length, or a plasmid or viral vector system that expresses short hairpin RNAs (shRNA) within the cell, which are subsequently processed to form siRNA molecules. Initially, Sar1a and Sar1b shRNA lentiviral vectors (pLKO1) were obtained from Open Biosystems (see appendix III). The advantage of using shRNA as opposed to siRNA is that it is believed to more accurate than siRNA, as shRNAs enter the RNAi pathway at an earlier stage than siRNA molecules, and therefore are less prone to off target effects. Also, inclusion of a selectable marker on the plasmid (such as puromycin resistance) makes the process more versatile, allowing for both transient transfection and the potential to create a stable cell line through marker selection.

Three shRNA constructs for Sar1a (Plasmids D5, D7 and E9) and two constructs for Sar1b (plasmids C3 and C4) were selected to increase the potential for knock down of the protein. shRNA constructs were also selected

for GAPDH (plasmids GC3 and GC4) to serve as a control. Although these shRNA were originally designed for mouse or human Sar1 they show a high degree of complement to the porcine sequences.

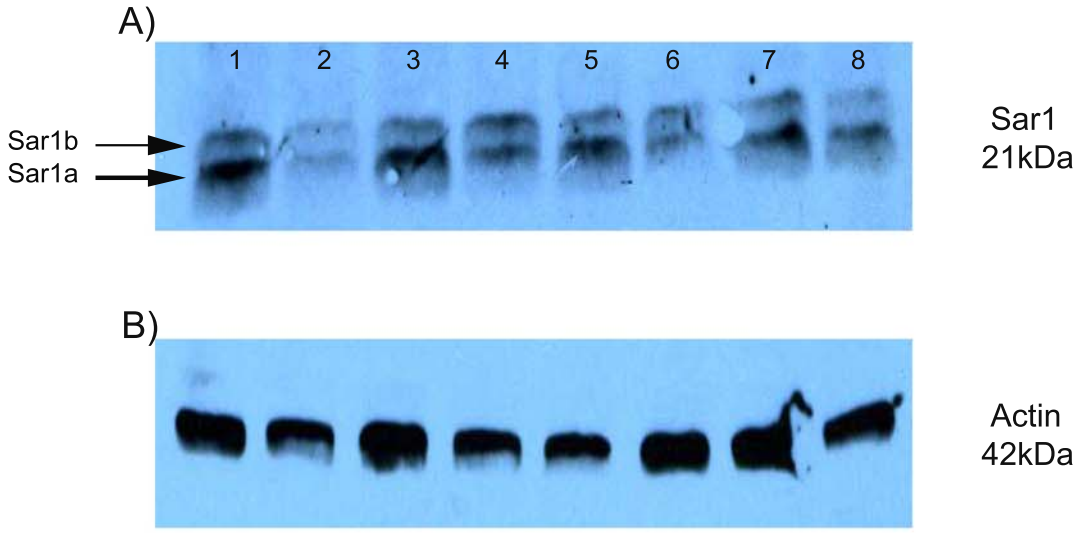
In the first experiment, the methods were optimised for IBRS-2 cells and GAPDH. Cells were seeded in 24 well plates, grown until 50% confluent and then transfected with1μl Lipofectamine and 0.5μg-1μg of GAPDH plasmid GC4. The transfected cells were incubated for 48 hours prior to the addition of puromycin (0.5, 1.0 or 2.0 μg/ml), then left for three days to select for resistant cells (i.e. cells expressing shRNA). Cells were then collected by trypsinisation and re-plated into a fresh 24 well plates and returned to 37^{o}C for 12-16 hours in the absence of puromycin. The cells were lysed and processed for Western blotting using an anti-GAPDH primary antibody and the appropriate HRP secondary antibodies (see methods section 2.11). Figure 4.24, panel A shows the knockdown of GAPDH in IBRS-2 cells. Lane 1 shows untreated cells and lane 2 mock transfected cells. Both lane 1 and lane 2 contain a positive band of 37kDa which is the expected size for GAPDH. Lanes 3-8 show cells transfected with GAPDH shRNA (GC4) using different amounts of plasmid DNA and different puromycin concentrations. Expression of GAPDH was efficiently reduced under all conditions except for lane 3 which used the lowest amount of DNA and puromycin concentration. The samples were also probed with an anti-actin antibody to ensure even loading of protein across the gel (Figure 4.24, panel B).

Figure 4.25, panel A shows a similar experiment using shRNA to Sar1a and b either as individual plasmids or in defined combinations. Lane 1 shows untreated cells and positive bands (~20-22kDa) for Sar1. The upper band is Sar1b and the lower band is Sar1a. Lane 2 shows cells transfected with all



1) Cell Lysate (-Puromycin)
2) Mock transfected lysate (-Puromycin)
3) 0.5μg DNA + 0.5μg Puromycin
4) 0.5μg DNA + 1μg Puromycin
5) 0.5μg DNA + 2μg Puromycin
6) 1μg DNA + 0.5μg Puromycin
7) 1μg DNA + 1μg Puromycin
8) 1μg DNA + 2μg Puromycin

Figure 4.24 Knockdown of GAPDH in IBRS-2 cells by shRNA
IBRS-2 cells were transfected with GAPDH targeted shRNA (plasmid GC4). At 48h after transfection the cells were incubated with puromycin at the indicated concentration (0.5 or 1.0μg/ml) for 3 days. Panel A shows a Western blot for GAPDH. Lane 1 shows untreated cells and lane 2 mock-transfected cells without puromycin treatment and a positive band for GAPDH. Lanes 3-8 show cells transfected with different amounts of plasmid DNA and treated with different puromycin concentrations (see lane order). Panel B shows the same samples probed for Actin.



1) Cell lysate
2) Sar1a shRNA (all constructs)
3) Sar1b shRNA (all constructs)
4) Sar1a shRNA D5
5) Sar1a shRNA D7
6) Sar1a shRNA E9
7) Sar1b shRNA C3
8) Sar1b shRNA C4

Figure 4.25 Knockdown of Sar1 in IBRS-2 cells by shRNA
IBRS-2 cells were transfected with Sar1a (D5, D7 or E9) or Sar1b (C3 and C4) targeted shRNA either individually (1μg of plasmid DNA) or in combinations (i.e. all 3 Sar1a plasmids or both Sar1b plasmids). At 48h after transfection the cells were incubated with puromycin (1.0μg/ml) for 3 days. Panel A shows a Western blot for Sar1. Lane 1 shows untreated cells and positive bands for Sar1. The upper band is Sar1b and the lower band Sar1a. Lane 2 show cells co-transfected with all 3 Sar1a plasmids. Lane 3 show cells co-transfected with 2 Sar1b plasmids. Lanes 4-8 show cells transfected with individual plasmids (see lane order). Panel B shows the same samples probed for Actin.

three Sar1a plasmids (D5, D9 and E9) and that the level of both Sar1a and Sar1b are effectively reduced. Lane 3 shows cells transfected with the two plasmids for Sar1b (C3 and C4) and an effective reduction of Sar1b only (the upper band). Lanes 4-8 show each shRNA plasmid transfected individually and variable reductions in the levels of Sar1. From these experiments it was concluded that the most effective reduction in Sar1 would be achieved using all five shRNA plasmids in combination.

However, subsequent attempts to reproduce the Sar1 knock down shown in figure 4.25 were unsuccessful. To overcome these difficulties a number of alternative approaches were tried. Initially, a time course was carried out to determine at what point after transfection Sar1 knock down was optimal. Cells transfected with shRNA plasmids were incubated with puromycin for 24h, 48h and 72h, however this did not appear to make a substantial improvement to the level of Sar1 knockdown. Subsequently, two rounds of transfection were tried; the plasmids were transfected into cells for a second time 24 hours after the initial transfection, however this did not appear to produce an improvement in Sar1 knockdown. Next, the influence of transfection efficiency and puromycin selection were investigated. To determine transfection efficiencies, a GFP plasmid was co-transfected into IBRS-2 along with the shRNA constructs, and the GFP signal viewed by fluorescent microscopy at 24h post transfection. This analysis showed that ~30% of the cells were expressing detectable GFP. Cells were also scored for GFP expression after puromycin treatment. This showed that only 20% of the cells expressed GFP. These findings suggest that both transfection efficiency and puromycin selection have a major influence on the extent of Sar1 knockdown and could explain the failure to reproducibly see an effective reduction in Sar1 protein. In a final attempt to optimise Sar1

knockdown the Sar1 shRNA plasmids were transfected into the human HeLa cell line, as the shRNAs were originally designed for human Sar1. However no knockdown of Sar1 was observed. It was concluded that transfection with shRNA plasmids was not able to give a reproducible knockdown of Sar1 and alternative RNAi methods were investigated (see below).

4.4.2 Sar1 siRNA

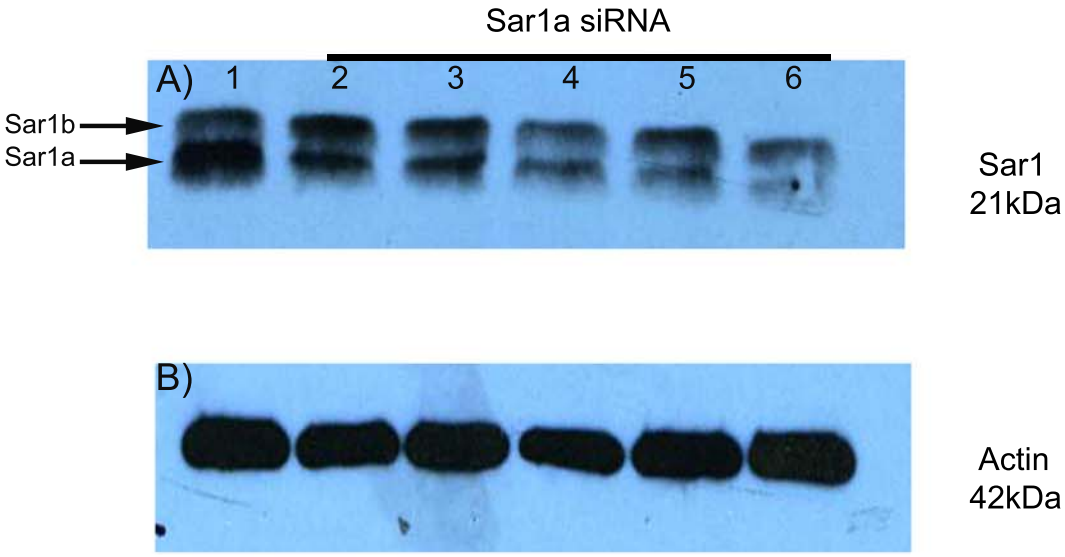
Due to the inconsistent knockdown of Sar1 using shRNA, I tried siRNA as an alternative. Four Sar1a (Duplex A, B, C & D) and three Sar1b (Duplex E, F & G) siRNA duplexes were custom made (Thermo scientific; Dharmacon). Transfections were optimised in IBRS-2 cells using the siGLO transfection indicator (Dharmacon), which are fluorescent oligonucleotides that localise to the nucleus. This showed that a high transfection efficiency (>70%) could be readily obtained. Next, cells in a 24 well plate were transfected with siRNA targeting either Sar1a individually or in combination, or with non-target negative-control siRNA duplexes (Qiagen). After six hours incubation at 37^{o}C the transfection media was replaced with normal cell culture media (without antibiotics). Cells were incubated for 48 hours before lysis for western blot analysis to determine the level of Sar1a knockdown. The amount of protein in each sample was quantified using a BCA assay (see methods 2.11) to ensure that equal amounts of protein were loaded onto the gel. In addition, a duplicate western blot was performed for each experiment and labelled for actin, to further ensure the equal loading of protein across the gel.

Figure 4.26 shows a western blot of Sar1a in cells transfected with 5pmol of each siRNA duplex (Duplex A, B, C and D) individually, and in cells co-transfected with all four siRNAs. Lane 1 shows untreated cells and bands representing Sar1a and Sar1b. Lanes 2-5 show that when used individually

only a limited reduction to Sar1a was seen. However, when used in combination (lane 6) the band for Sar1a (the lower band) was greatly reduced indicating specific targeting of Sar1a. Increasing the amount of Sar1a siRNA to 10pmol did not appear to make a significant improvement to Sar1 knockdown (data not shown). These results indicate that transfection with a combination of all four siRNAs (5-10pmol) was the most effective at knocking down Sar1a.

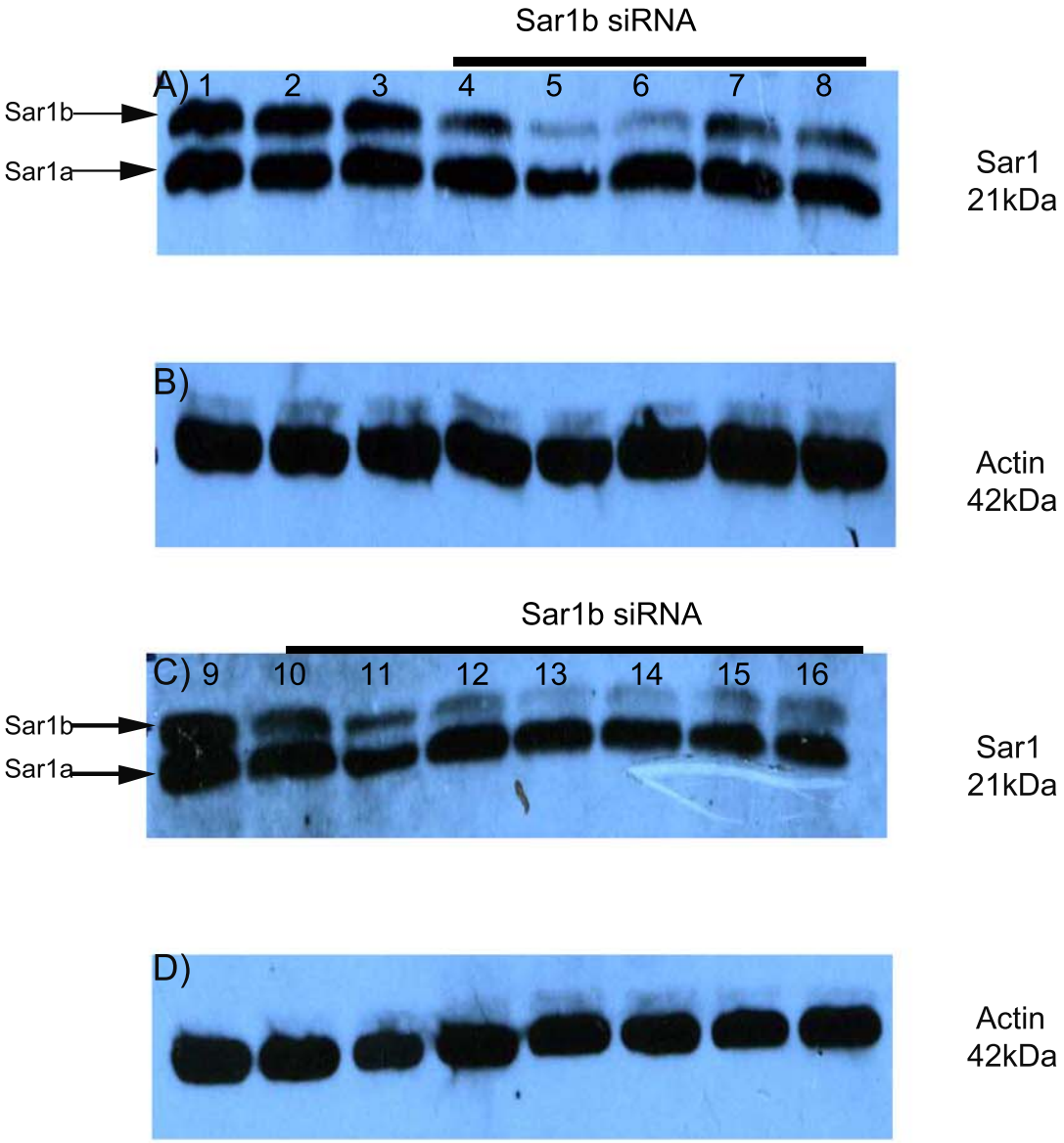
The above experiment was repeated using Sar1b siRNAs. For this experiment three different siRNA amounts were used (5, 10 or 25 pmol). Figure 4.27 shows a western blot for Sar1 with Sar1b siRNA transfected cells. Lane 1 (panel A) and lane 9 (panel C) show control cells and the expected bands for Sar1a and Sar1b. Lanes 2-3, panel A show cells transfected with non-target siRNA and a band for Sar1. Lanes 4-6 (panel A) show cells transfected with duplex E (5, 10 or 25 pmol) and that with higher amounts of siRNA, a good knock down of Sar1b (the upper band) is achieved. Lanes 7, 8 (panel A) and 10 (panel C) show that a poor knockdown of Sar1b is achieved with duplex F; Lanes 11, 12 and 13 (panel C) show that a good knockdown of Sar1b is achieved when duplex G is used at 25pmol. Finally, lanes 14-16 (panel C) show that a good knockdown of Sar1b is achieved when all three siRNA duplexes are used in combination. Panels B and D show duplicate samples probed for actin.

Next cells were transfected with the best combinations of Sar1a (duplex C and D) or Sar1b (Duplex E and G) siRNAs identified above, plus combinations of Sar1a with Sar1b siRNAs. Figure 4.28 shows a western blot of IBRS-2 cells transfected with different combinations of siRNAs. Lanes 1 and 2 (panel A) show untreated and mock transfected control cells (i.e. non-targeted siRNA). Lane 3 shows cells transfected with two Sar1a siRNAs (duplex C and D) and lane 4 with two siRNAs to Sar1b (duplex E and G). Lanes 6-9 show the results



1-2) IBRS-2 cell lysate
3) Sar1a siRNA duplex A
4) Sar1a siRNA duplex B
5) Sar1a siRNA duplex C
6) Sar1a siRNA duplex ALL

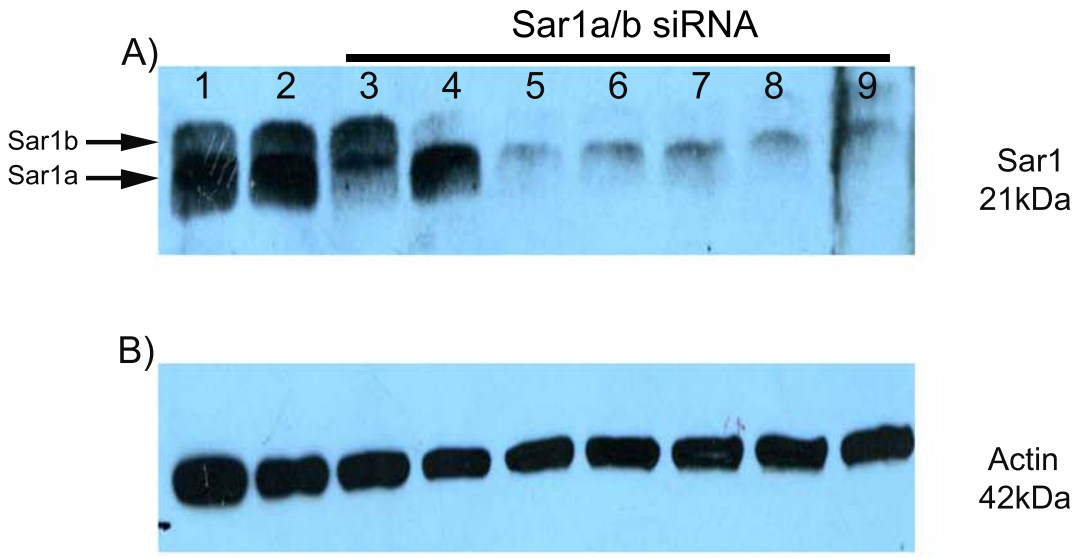
Figure 4.26 Knockdown of Sar1a in IBRS-2 cells by siRNA
IBRS-2 cells were transfected with Sar1a (Duplex A, B, C and D) targeted siRNA either individually (5pmol) or in combinations (i.e. all 4 Sar1a duplexes). At 48h after transfection the cell lysates were prepared. Panel A shows a Western blot for Sar1. Lanes 1-2 show untreated cells and positive bands for Sar1. The upper band is Sar1b and the lower band Sar1a. Lanes 3-5 show cells transfected with individual duplexes (see lane order). Lane 6 shows cells co-transfected with all 3 Sar1a siRNAs. Panel B shows the same samples probed for Actin.



Blot (A-B)
1) Cell lysate
2) Non-target siRNA (5pmol)
3) Non-target siRNA (10pmol)
4) Sar1b (E) siRNA (5pmol)
5) Sar1b (E) siRNA (10pmol)
6) Sar1b (E) siRNA (25pmol)
7) Sar1b (F) siRNA (5pmol)
8) Sar1b (F) siRNA (10pmol)

Blot (C-D)
9) Cell lysate
10) Sar1b (F) siRNA (25pmol)
11) Sar1b (G) siRNA (5pmol)
12) Sar1b (G) siRNA (10pmol)
13) Sar1b (G) siRNA (25pmol)
14) Sar1b (ALL) siRNA (5pmol)
15) Sar1b (ALL) siRNA (10pmol)
16) Sar1b (ALL) siRNA (25pmol)

Figure 4.27 Knockdown of Sar1b in IBRS-2 cells by siRNA
IBRS-2 cells were transfected with Sar1b (Duplex E, F and G) targeted siRNA either individually or in combinations (i.e. all 3 Sar1b duplexes). A range of concentrations were used for each duplex (5, 10 and 25pmol). At 48h after transfection the cell lysates were prepared. Panel A and C shows Western blots for Sar1. Lane 1 and 9 show untreated cells. Lanes 2-3 show cells transfected with the control non-target siRNA. Positive bands for Sar1 are seen in all control lanes. The upper band is Sar1b and the lower band Sar1a. Lanes 4-8 and 10-13 show cells transfected with individual duplexes at various concentrations (see lane order). Lanes 14-16 show cells co-transfected with all 3 Sar1b siRNAs (see lane order). Panels B and D shows the same samples probed for Actin.



1) IBRS-2 cell lysate
2) Non-Target siRNA cells
3) Sar1a siRNA (C + D)
4) Sar1b siRNA (E + G)
5) Sar1a/b siRNA (ALL)
6) Sar1a/b siRNA 1a (C) + 1b (E)
7) Sar1a/b siRNA 1a (C) + 1b (G)
8) Sar1a/b siRNA 1a (D) + 1b (E)
9) Sar1a/b siRNA 1a (D) + 1b (G)

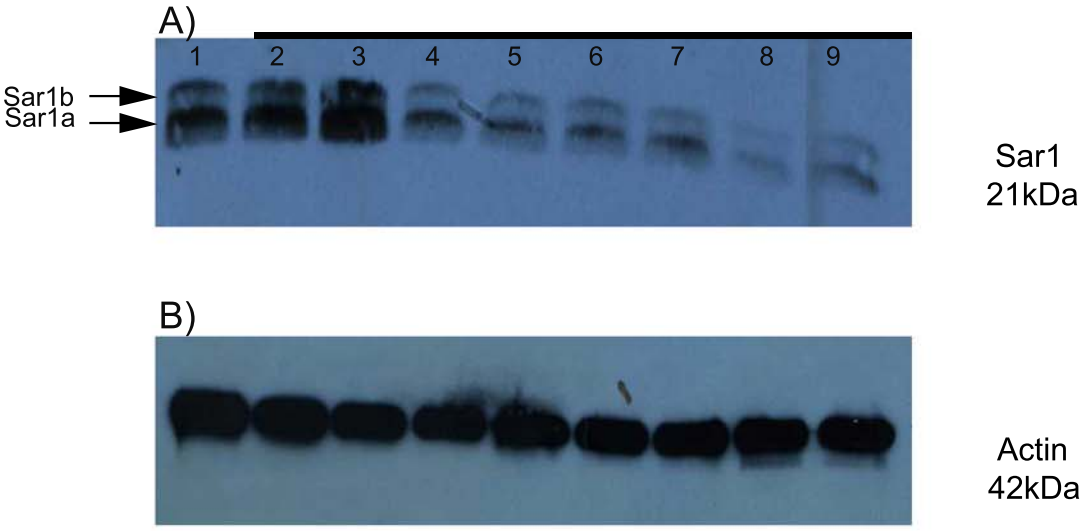
Figure 4.28 Knockdown of Sar1a and Sar1b in IBRS-2 cells by siRNA
IBRS-2 cells were transfected with various combinations of Sar1a (Duplex C and D) and Sar1b (Duplex E and G) targeted siRNA (5pmol). At 48h after transfection the cell lysates were prepared. Panel A shows a Western blot for Sar1. Lane 1 shows untreated cells and lane 2 shows cells transfected with non-target siRNA, both lanes show positive bands for Sar1. Lane 3 shows cells transfected with both Sar1a duplexes (C and D) while lane 4 shows cells transfected with Sar1b duplexes (E and G). In lane 5 are cells transfected with all four duplexes (C, D, E and G). Lanes 6-9 show cells transfected with various combinations of the duplexes (see lane order). Panel B shows the same samples probed for Actin.

for transfections with mixed siRNAs for either Sar1a or Sar1b. Lane 5 shows results for transfection with all 4 siRNAs (i.e. 2 for Sar1a and 2 for Sar1b) and that this combination gave an effective knockdown of both Sar1 isoforms. Figure 4.29 shows a further experiment where cells were transfected with all 4 siRNAs (i.e. 2 for Sar1a and 2 for Sar1b) but using different amounts of siRNA (2.5, 5.0 or 10 pmol of each duplex). This showed that the most effective knockdown was achieved using 10pmol amounts of each duplex. These results confirmed the previous findings shown in figure 4.28. These conditions (i.e. all 4 siRNA duplexes at 10pmol of each duplex) were used in all subsequent experiments.

4.4.2.1 The effect of Sar1 knockdown on the early secretory pathway

4.4.2.2 The effect of Sar1 siRNA on the Golgi

The above results show that an efficient knockdown of Sar1 could be achieved using Sar1a and Sar1b targeted siRNA. Next, the effect of Sar1 knockdown on the Golgi was investigated to indicate the effect of Sar1 knockdown on the early secretory pathway. Depletion of Sar1 from cells would be expected to inhibit COPII formation and result in a block in membrane and protein traffic through the early secretory pathway, and hence Golgi disruption (see section 1.8.1). Figure 4.30 shows IBRS-2 cells transfected with Sar1 siRNA (Duplex C, D, E and G) for 48 hours before being processed for Golgi labelling (with GM130) as described above. Panel A shows that cells transfected with the non-target siRNA have normal peri-nuclear Golgi labelling. The majority of cells transfected with Sar1 targeted siRNA showed varying degrees of Golgi fragmentation (B-D). Around 70% of the cells showed Golgi fragmentation which is consistent with the transfection efficiency determined



1) IBRS-2 cell lysate
2) Non-target siRNA
3) Non-target siRNA
4) 2.5pmol Sar1(C, D, E and G)
5) 2.5pmol Sar1(C, D, E and G)
6) 5pmol Sar1 (C, D, E and G)
7) 5pmol Sar1 (C, D, E and G)
8) 10pmol Sar1 (C, D, E and G)
9) 10pmol Sar1 (C, D, E and G)

Figure 4.29 Optimisation of the knockdown of Sar1 in IBRS-2 cells by siRNA
IBRS-2 cells were co-transfected with Sar1a/b (Duplex C, D, E and F) targeted siRNA at various concentartion (2.5, 5 or 10pmol) using 1μl Lipofectamine 2000. At 48h after transfection the cell lysates were prepared. Panel A shows a Western blot for Sar1. Lane 1 shows untreated cells and lanes 2-3 show cells transfected with control non-target siRNA, all show positive bands for Sar1. Lanes 4-9 show cells co-transfected with Sar1a/b duplexes (C, D, E and F) at various concentrations (see lane order). Panel B shows the same samples probed for Actin.

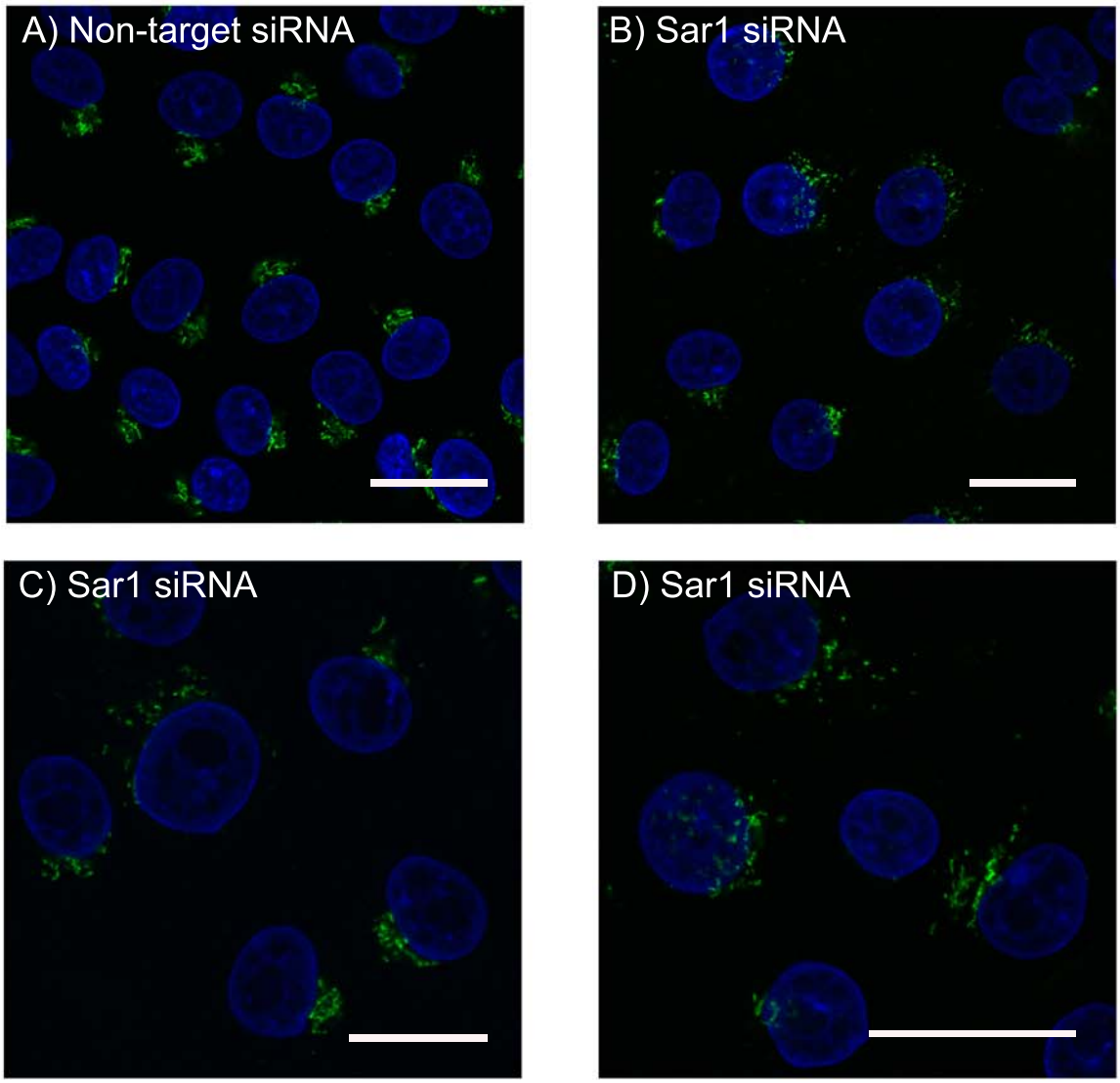


Figure 4.30 The effects of Sar1 knockdown on the Golgi
IBRS-2 cells were co-transfected with Sar1 siRNA (Duplexes C, D, E and F) for 48 hours and labelled for the Golgi (GM130 panels A-D). The Golgi is shown as green. Panel A shows cells transfected with non-target siRNA. Panels B-D show cells transfected with Sar1 siRNA. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

from siGLO transfections performed in parellel. These observations show that depletion of Sar1a and Sar1b by siRNA leads to fragmentation of the Golgi and therefore it can be assumed that Sar1 siRNA is preventing both membrane and protein traffic through the early secretory pathway. However, complete loss of Golgi labelling was rarely observed, this indicated there was a severe disruption, but not a complete loss of Golgi membranes.

4.4.2.2.1 The effect of Sar1 siRNA on FMDV induced cell membrane rearrangements

Next, the effect of Sar1 knockdown on FMDV induced membrane rearrangements was investigated. IBRS-2 cells were transfected with Sar1 siRNA (duplexes C, D, E and G) and then infected with FMDV O1Kcad$_2$ (MOI 0.8). Uninfected cells and siRNA transfected cells (without infection) were included for comparison. At 3.5h post-infection the cells were processed for conventional TEM (see methods 2.10). Non-transfected, uninfected cells (i.e. normal cells) showed a normal complement of organelles in the cytosol whereas non-transfected infected cells showed typical membrane rearrangements (see Fig 3.1 A and B for examples). Figure 4.31 (A-B) shows the effect of Sar1 knockdown on the ER, which appears swollen (arrowed on the figure) suggesting export of proteins from the ER was inhibited. Cells treated with Sar1 siRNA were infected with FMDV. One hundred cells were observed for membrane rearrangements. Interestingly, these cells showed membrane rearrangements that were indistinguishable from control infected cells (Figure 4.32). The percentage of cells observed with membrane rearrangements by TEM was compared to the percentage of FMDV infected cells (depleted of Sar1) determined by IF, this was found to be equal (~60%) for

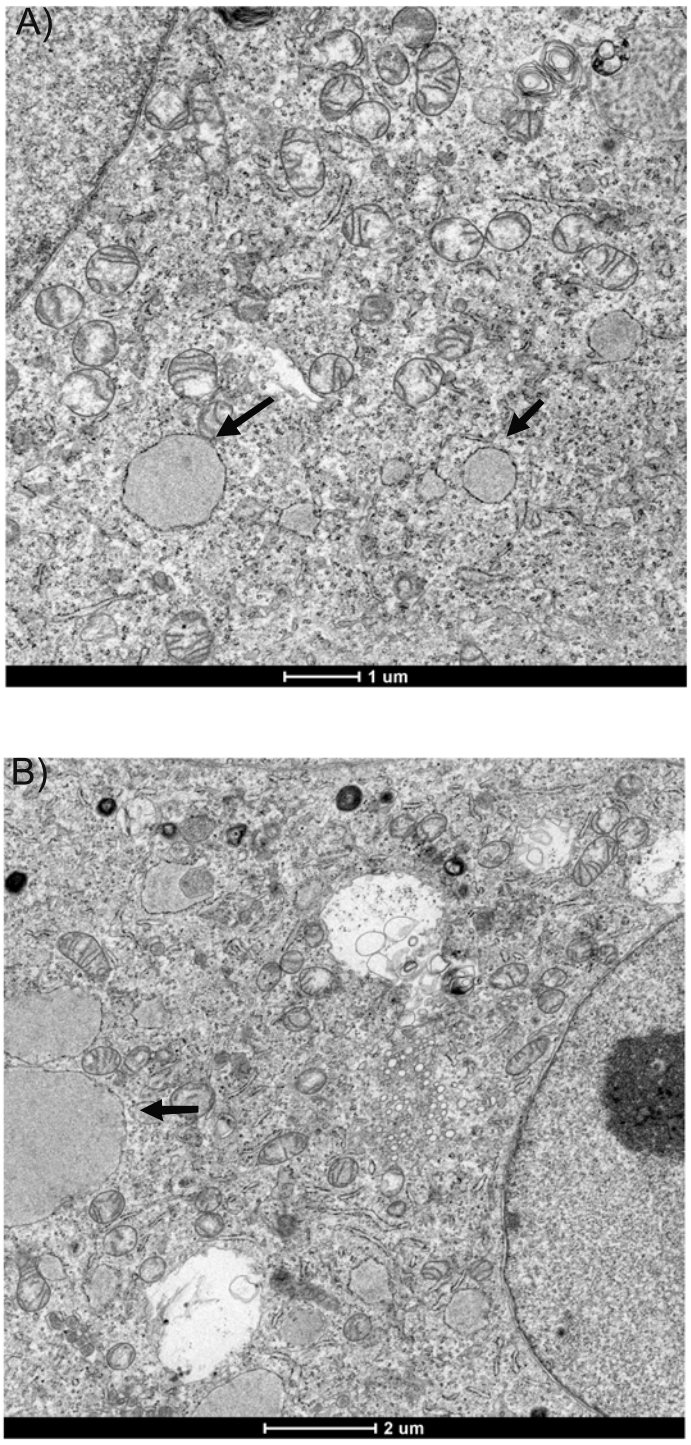


Figure 4.31 Knockdown of Sar1 by siRNA induces ER swelling
IBRS-2 cells were co-transfected with Sar1 siRNA (duplexes C, D, E and F) for 48 hours. Cells were fixed and processed for TEM. Panels (A-B) show ER swelling (black arrows). Scale bars: (A) 1μm and (B) 2μm.

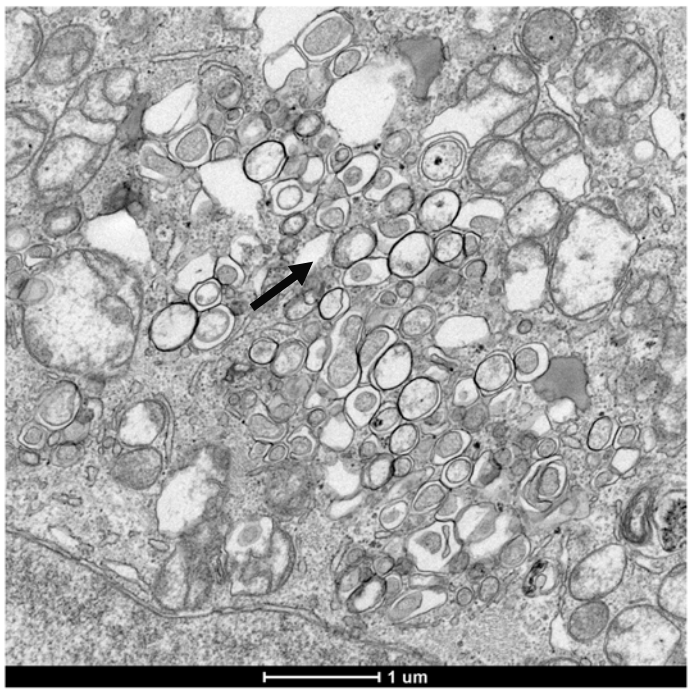


Figure 4.32 The knockdown of Sar1 by siRNA does not inhibit the formation of viral replication complexes
IBRS-2 cells were co-transfected with Sar1 siRNA (duplexes C, D, E and F) for 48 hours. Cells were infected with FMDV (MOI 0.8) for a total of 4 hours. Cells were fixed and processed for TEM study. n=100 cells observed for membrane rearrangements. Scale bar = 1μm.

both samples, therefore indicating membrane rearrangements were being observed in Sar1 depleted cells. This observation is discussed in section 4.5.

4.4.2.3 The effects of Sar1 knockdown of FMDV infection

Next, the effect of Sar1 knockdown on FMDV infection was investigated. IBRS-2 cells in 24 well plates were transfected with Sar1a and Sar1b siRNA (Duplex C, D, E, G) for 48 hours. The cells were then infected with FMDV O1Kcad$_2$ (MOI 0.5) for 4 hours. Cells were fixed in 4% pfm and processed for immunofluorescence confocal microscopy. Infected cells were detected using a rabbit anti-FMDV antibody followed by an anti-Rabbit Alexa 568 secondary. Similarly, transfected cells were infected with BEV and processed for immunofluorescence confocal microscopy using an anti-BEV antibody.

Figure 4.33 shows cells transfected with either the control non-target siRNA (panels A and C) or Sar1 targeted siRNA (duplexes C, D, E and G) (panels B and D) and infected with FMDV (shown as red, panels A-B) or BEV-1 (shown as red, panels C-D). The proportion of cells infected after Sar1 siRNA transfection appeared similar to the cells transfected with the control non-target siRNA, suggesting that Sar1 knockdown had no affect on the number of infected cells for either virus. A western blot of Sar1 was run in parallel to the above experiment which confirmed a knockdown of Sar1, similar to that shown in figures 4.28–4.29.

The experiment described above was repeated three times using FMDV or BEV and Figures 4.34 (FMDV) and 4.35 (BEV) show the mean levels of infection for the three experiments, normalised to the control cells (non-targeted siRNA). These results showed that for both FMDV and BEV there was no

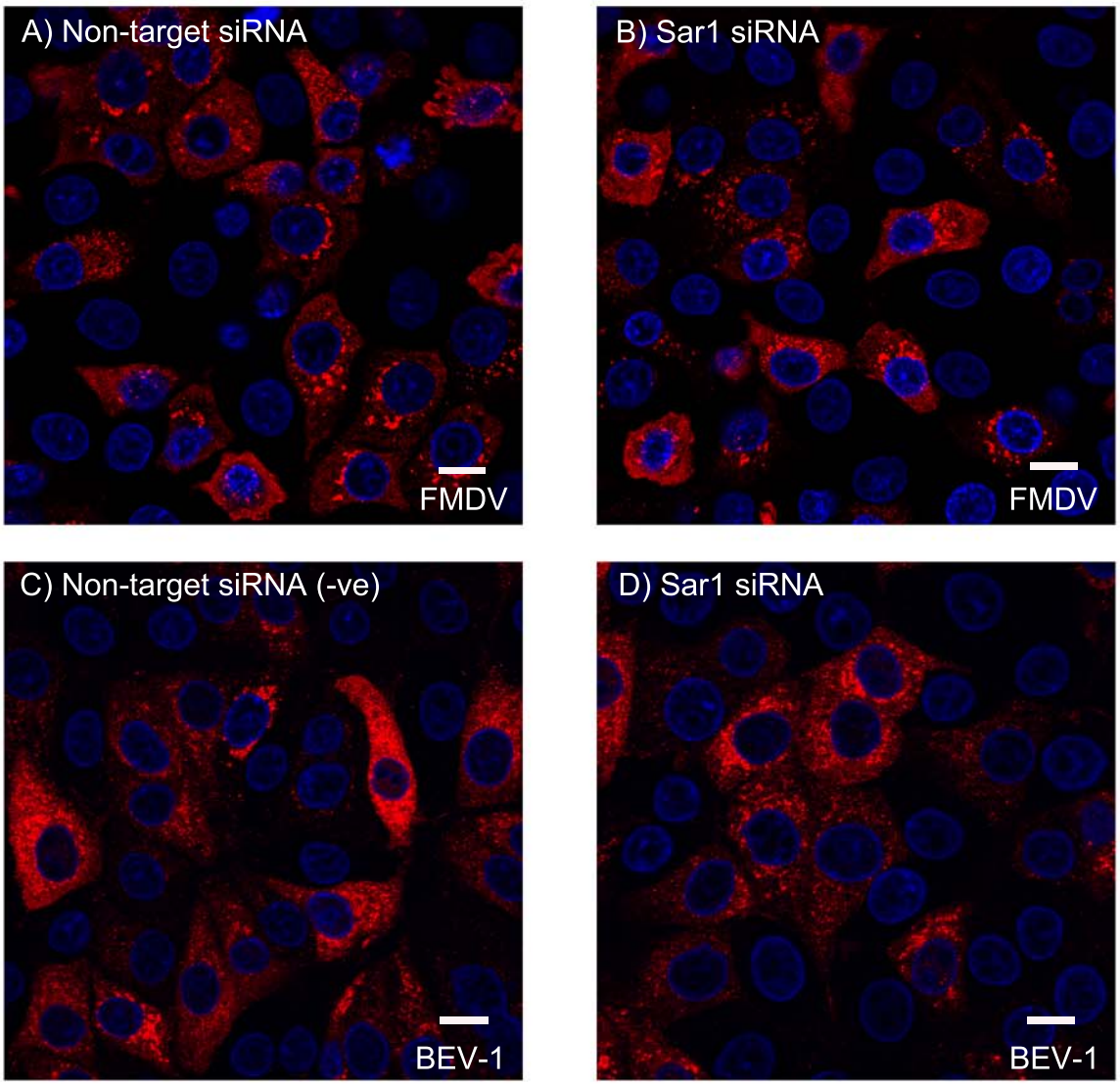


Figure 4.33 The effect of Sar1 knockdown by siRNA on FMDV and BEV-1 Infection
IBRS-2 cells were transfected with non-target siRNA (panels A & C) or co-transfected with Sar1 siRNA (duplexes C, D, E) (panels B & D) for 48 hours and then infected with FMDV or BEV-1. Infected cells are labelled in red. Panels A-B show labelling for FMDV. Panels C-D show labelling for BEV-1. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

	Infectedcells (%)	
Experiment	**Non-target**	**Sar1 siRNA**
1	55	48
2	24	29
3	54	48

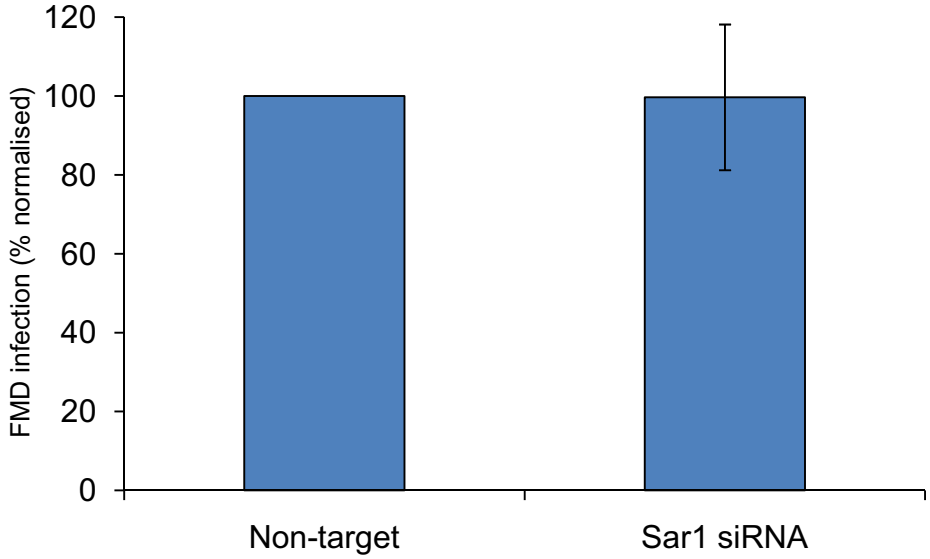


Figure 4.34: The effects of Sar1 knockdown on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected with non-target siRNA or Sar1 siRNA (Duplexes C, D, E and G) for 48 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined. *Lower Figure*: The proportion of infected cells transfected with Sar1 siRNA was normalised to the results for cells transfected with the non-target siRNA. The proportion of infected cells is shown as the mean +/- SD of triplicate samples.

	Infected cells (%)	
Experiment	**Non-target**	**Sar1 siRNA**
1	38	40
2	38	35
3	40	44

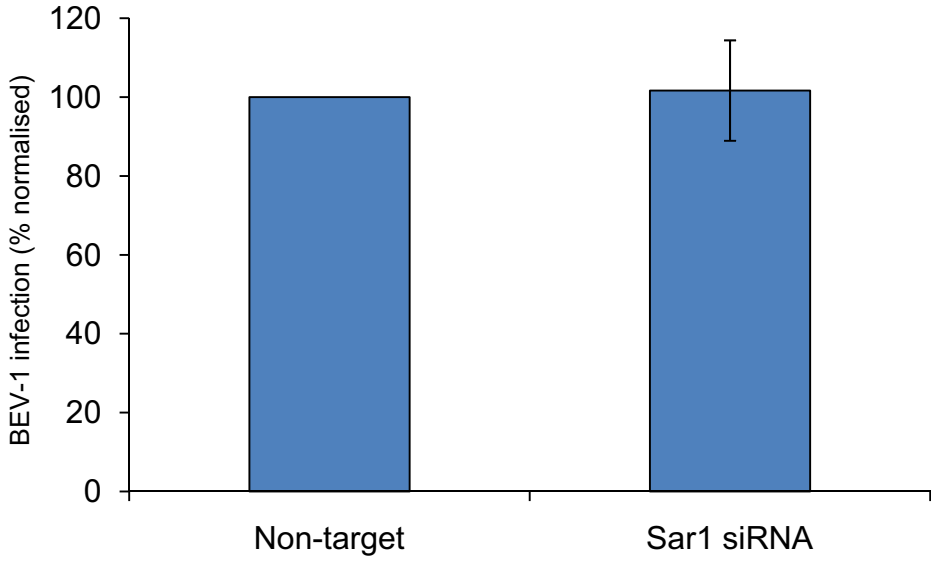


Figure 4.35: The effects of Sar1 knockdown on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected with non-target siRNA or Sar1 siRNA (Duplexes C, D, E and G) for 48 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined. *Lower Figure*: The proportion of infected cells transfected with Sar1 siRNA was normalised to the results for cells transfected with the non-target siRNA. The proportion of infected cells is shown as the mean +/- SD of triplicate samples

	pfu/ml		
Experiment	**Non-target**	**Sar1 siRNA**	**Normalised data (%)**
1	4.8. x 10^4	3.5.x10^3	7%
2	8.3.x 10^3	2.0.x 10^3	25%
3	1.3. x 10^3	4.6.x 10^2	44%

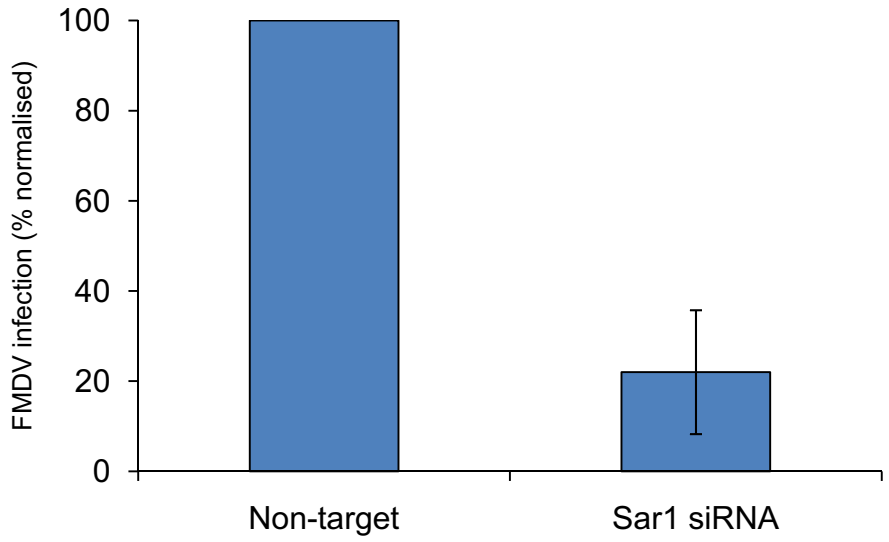


Figure 4.36 The effect of Sar1 knockdown on FMDV virus yield
IBRS-2 cells were transfected with non-target siRNA or Sar1 siRNA (Duplexes C, D E and F) for 48 hours and then infected with FMDV. *Upper Table*: Shows the average pfu/ml over three independent experiments and the normalised data. *Lower Figure*: The viral yield of cells transfected with Sar1 siRNA was normalised to the yield for cells transfected with non-target siRNA. The viral yield is shown as the mean +/- SD of triplicate samples.

	pfu/ml		
Experiment	**Non-target**	**Sar1 siRNA**	**Normalised data**
1	9.4.x 10^4	7.4.x 10^4	79%
2	1.8.x 10^4	1.4.x 10^4	80%
3	1.7.x 10^4	1.2.x 10^4	71%

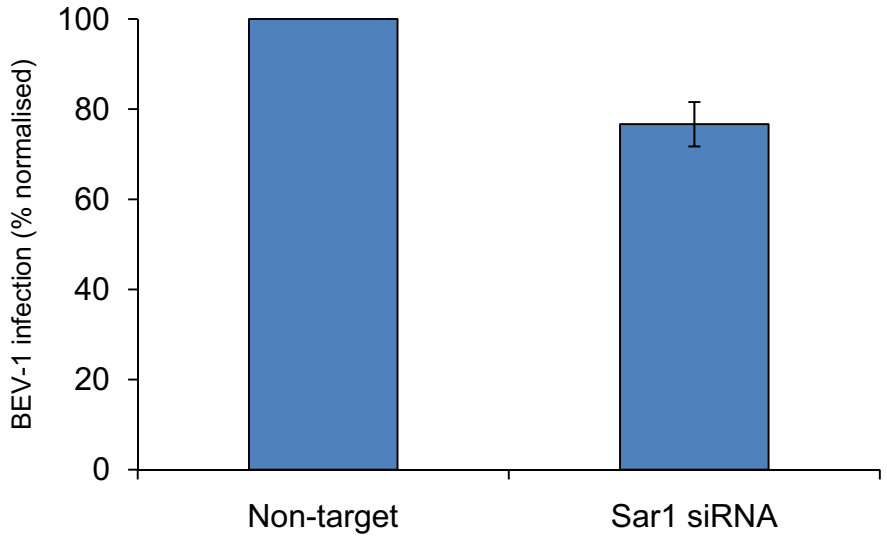


Figure 4.37 The effect of Sar1 knockdown on BEV virus yield
IBRS-2 cells were transfected with non-target siRNA or Sar1 siRNA (Duplexes C, D E and F) for 48 hours and then infected with BEV. *Upper Table*: Shows the average pfu/ml over three independent experiments and the normalised data .*Lower Figure*: The viral yield of cells transfected with Sar1 siRNA was normalised to the yield for cells transfected with non-target siRNA. . The viral yield is shown as the mean +/- SD of triplicate samples.

significant difference between the proportions of infected cells in Sar1 depleted and control cells.

To determine if Sar1 knockdown reduced virus yield, samples of cell supernatants from the above experiments were collected at 0 hours and 4 hours post-infection. A plaque assay was performed using serial dilutions of the supernatants on BHK21 (see methods 2.2.3), and the results normalised to the yield for cells transfected with the non-target siRNA. At 0 hours, FMDV virus was present in the supernatant at >10 pfu/ml. The results show that at 4h post-infection, Sar1 knockdown reduced FMDV yield by ~80% (Figure 4.36). Figure 4.37 shows the results of the experiment with BEV. At 0 hours BEV virus was present in the supernatant at >200 pfu/ml and that Sar1 knockdown resulted in a 25% reduction in viral yield in comparison to non-target transfected cells. These results support the conclusion that Sar1 is important for FMDV infection, but appear to be less important for BEV.

4.5 Discussion

This chapter aimed to determine the effects of dominant negative Sar1 on secretory membranes and FMDV infection. Expression of wt Sar1-CFP did not appear to disrupt the ERGIC or Golgi extensively but did cause a slight loss to ER labelling. The observed loss of ER labelling may be due to over-expression of the protein, which may then exert a dn effect on ER exit. It was also observed that dn Sar1 T39N-CFP did not disrupt membranes efficiently when expressed below a certain threshold, however when over-expressed, a loss to the ER and dispersal of the ERGIC and Golgi membranes was observed, which may indicate a successful block in protein transport at ERES. Expression of dn Sar1a H79G-CFP (the constitutively active version of Sar1) appeared to create a strong block in transport. The loss of GM130 and 23C

labelling in cells expressing this dn protein indicates disruption of the Golgi, similar to that observed with BfA treatment (see Fig 3.4).

Expression of wt Sar1-CFP reduced FMDV infection by ~50%. This result may be explained by the observation that over-expression of the protein may have a dominant-negative effect. The dn Sar1a T39N-CFP also reduced FMDV infection suggesting that COPII vesicle formation or Sar1 activity is necessary for virus replication. In contrast dn Sar1a H79G-CFP, did not appear to inhibit FMDV infection. This mutant of Sar1 acts as a dn by stabilising COPII coats after transport vesicle formation at ERES. This suggests that FMDV may target COPII vesicles for replication, but not their subsequent maturation. However, when the experiments were repeated in MAX cells, dn Sar1a H79G-CFP did appear to have a inhibitory effect on infection (down by 25 %). In general it was observed that expression levels of the Sar1 protein were higher in MAX cells, which suggests a greater inhibition to Sar1 activity. Expression of dn Sar1 H79G-CFP is thought to hold the COPII coat on vesicles and Sar1 is not released back into the cytosol to allow transport to continue. It is possible that FMDV infection is more efficient when COPII is released, resulting in free membranes at ERES.

For BEV, expression of the wt and dn Sar1 proteins produced a 25% reduction in infection. This suggests a potential involvement of Sar1/COPII vesicles in BEV-1 infection, however the inhibition is not as great as that observed in FMDV infected cells. BEV-1 is sensitive to BfA (Monaghan et al., 2004) and therefore likely to require Arf1 for infection. There are no reports published indicating BEV-1 infection requires Sar1 function or COPII vesicle formation. However, this possibility cannot be ruled out as early reports on PV suggest a role for the COPII complex in replication complex formation, although

it is not considered essential (see section 1.10). Alternatively, dn Sar1 may prevent the flow of membranes through the early secretory pathway and this could reduce infection by limiting the available membranes for Arf1 dependant replication.

Sec16 has been reported to interact with components of the COPII complex and aid in the formation of transport vesicles by providing a platform for COPII assembly (Iinuma et al., 2007). The dn Sec16 used in the above experiments (Sec16 C-term-GFP) is thought to prevent normal ERES organisation (Bhattacharyya and Glick, 2007) and thereby reduce protein transport by inhibiting formation of secretory vesicles. The dispersal of the peri-nuclear region of the ERGIC and fragmentation of the Golgi in cells expressing dn Sec16 C-term-GFP, shown in figure 4.21, supports the observation that membrane flow between the ER and ERGIC is inhibited. However, expression of Sec16 C-term-GFP only appeared to have a mild inhibitory effect on FMDV infection (~20% reduction). Some studies investigating Sec16 function have reported that Sec16 is not essential for vesicle formation at ERES, as depletion of the protein only resulted in a mild disorganisation of ERES which delayed, but did not block, COPII formation and the transport of proteins out of the ER (Iinuma et al., 2007). The failure of Sec16 to completely inhibit COPII vesicle formation could explain the low inhibition to FMDV infection observed in these experiments.

In order to confirm the findings of the dominant negative data, the effect of siRNA targeting Sar1 was investigated. Initially, the effect of Sar1 siRNA on membranes of the early secretory pathway was determined. The Golgi appeared to become fragmented in transfected cells, however this was not complete which suggests the block to transport is incomplete. Cells treated

with Sar1 siRNA and infected with FMDV were also observed using TEM, interestingly this showed membrane rearrangements that were indistinguishable from control infected cells (Figure 4.30). Although membrane rearrangements are evident in Sar1 depleted cells it cannot be assumed that replication is continuing unaffected as it is thought that the actual properties of the RC's allow viral replication to proceed rather than the presence of the vesicles themselves (Miller and Krijnse-Locker, 2008) .

Further investigation of Sar1 using siRNA showed that depletion of Sar1 had no significant difference on the proportion of FMDV or BEV infected cells when compared to control cells, however when virus infected supernatants were titrated on BHK21 cells, up to an 80% decrease in FMDV yield was observed suggesting that Sar1 is involved FMDV replication. The proportion of FMDV infected cells was not affected by Sar1 depletion, this suggests that successful entry and translation of viral proteins is occurring. In contrast, the reduction to viral yield indicates the amount of progeny virus is affected, which suggests an inhibition of replication. In BEV infected supernatants around a 25% reduction was seen, this figure seemed to agree with the dn data.

Taking into account both dn and siRNA data, it appears Sar1 or Sar1 activity is required for efficient FMDV replication in IBRS-2 cells. It is possible the Sar1 GTPase is recruited onto membranes to help form vesicles, as is seen with PV and Arf1, however whether this is a direct or indirect interaction remains to be determined. Another possibility is that the COPII vesicles themselves may be involved as membrane donors for formation of the viral RC.

Chapter Five: Investigating the Role of Arf1 in FMDV Infection

5.1 Introduction

Arf1 co-localises to the Golgi complex (Stearns et al., 1990) and is responsible for the recruitment of COPI coats to Golgi membranes and formation of COPI transport vesicles (see section 1.8.2). Arf1 is converted to its GTP-bound form by interactions with multiple GEFs including GBF1, BIG1 and BIG2. Arf1 GEFs are known to be sensitive to BfA which results in the inhibition if Arf1 activity and therefore COPI formation (see section 1.6.4.1). As previously discussed in section 1.6.4.1, several picornaviruses are sensitive to BfA treatment which suggests they require an Arf1-GTP for successful replication. Due to FMDV's insensitivity to BfA it seems unlikely that Arf1-GTP is necessary for its replication.

To determine if Arf1 has any influence on FMDV infection the role of Arf1 was investigated through the use of dn proteins, Arf1 T31N and Arf1 Q71L. Arf1 T31N has a preferential affinity for GDP and therefore remains in an inactive state. Expression of Arf1 T31N results in a block to protein secretion, the release of β–COP into the cytoplasm and the subsequent disruption of the Golgi (Dascher and Balch, 1994). However, ER exit sites are present and COPII vesicles are formed (Puri and Linstedt, 2003; Scales et al., 1997; Ward et al., 2001). Expression of dn Arf1-T31N has similar effects on membranes of the secretory pathway as BfA and prevents maturation of the ERGIC (Altan-Bonnet et al., 2004).

In contrast, Arf1 Q71L is locked in its GTP-bound form and is therefore constituently active. Expression of Arf1 Q71L actively recruits β-COP to both the

ERGIC and Golgi membranes but blocks COPI coat disassembly, and hence protein transport, at the ERGIC and the Golgi stacks (Dascher and Balch, 1994).

5.2 Investigating the role of Arf1 on FMDV infection using Dominant-negative proteins

5.2.1 The effects of dominant-negative Arf1 on membranes in the early secretory pathway

The effect of dn Arf1 on membranes of the early secretory pathway was investigated by immunofluorescence confocal microscopy (see methods, section 2.7) using IBRS-2 cells. IBRS-2 cells were transfected to express either wt Arf1, dn Arf1 Q71L as fusions with GFP or dn Arf1 T31N fused to a HA tag for 14 hours (GFP and HA allow for detection of transfected cells). The cells were fixed with PFM and permeabilised with 0.1% Triton-X, then blocked in goat serum prior to labelling with antibodies to the ER (PDI), the ERGIC (ERGIC-53) or the Golgi (GM130) and the appropriate Alexa-Fluor labelled secondary antibody. Cells expressing a transgene (i.e. Arf1 protein) are shown as green on the figures.

Figure 5.1 shows cells expressing wt Arf1-GFP (shown as green) and labelled for the ER (shown as red on panels A-B), the ERGIC (shown as red on panels C-D) and the Golgi (shown as red in panels E-F). No effect was observed on ER, ERGIC or Golgi labelling in cells expressing the wt Arf1-GFP, which appeared to localise to the same peri-nuclear region as the Golgi labelling (Fig 5.1 E-F).

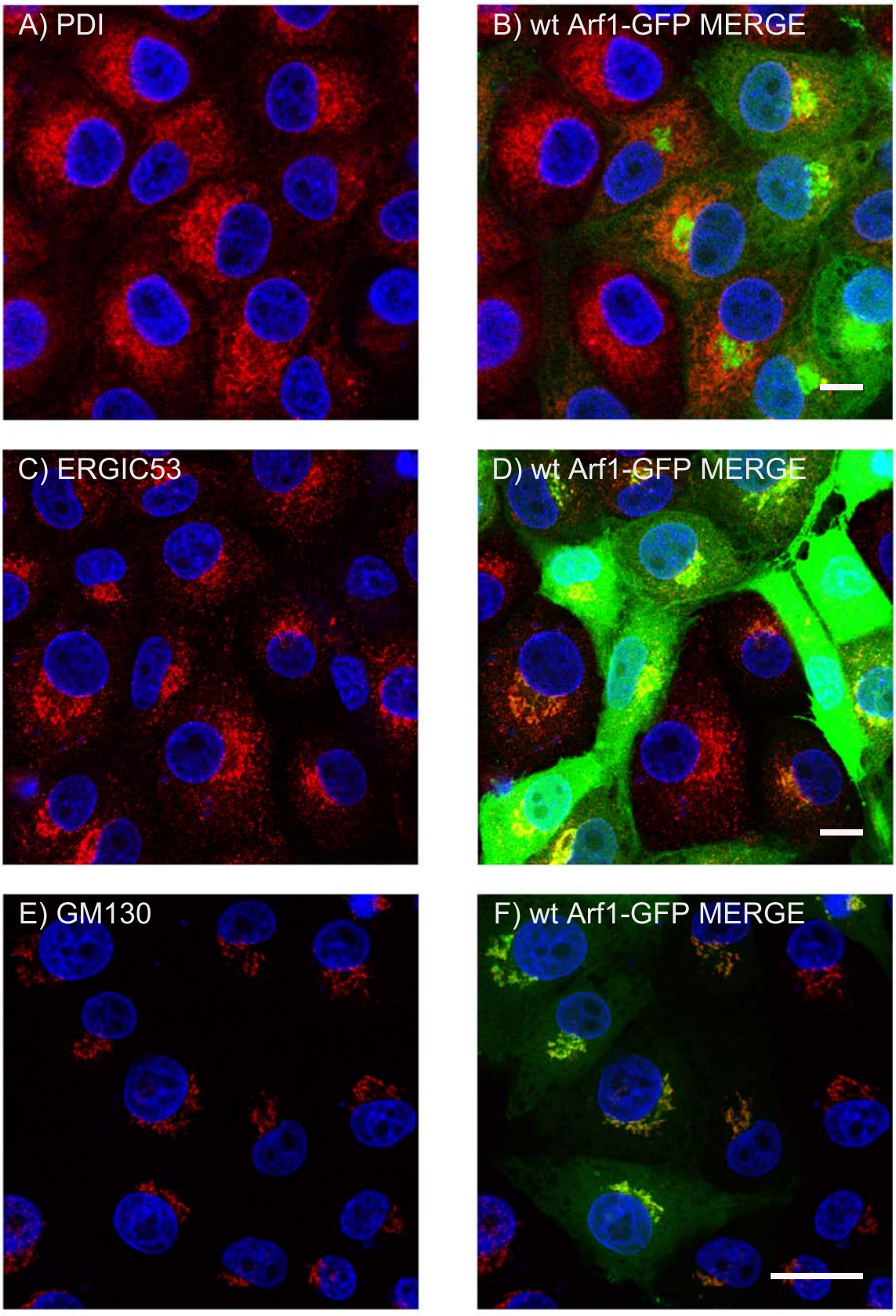


Figure 5.1 The effects of wt Arf1 expression on the ER, ERGIC and Golgi
IBRS-2 cells were transfected to express wt Arf1-GFP for 14 hours and labelled for the ER (PDI; panels A-B), the ERGIC (ERGIC53; panels C-D) or the Golgi (GM130; panels E-F). Cells expressing Arf1 are shown as green; the ER, ERGIC and Golgi are shown in red. Panels B,D and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

Figure 5.2 shows cells expressing dn Arf1 T31N-HA (shown as green) and labelled for the ER (shown as red in panels A-B), the ERGIC (shown as red in panels C-D) and the Golgi (shown as red in panels E-F). A reduction of ER labelling is observed in cells expressing dn Arf1 T31N-HA. The ERGIC labelling appears to be dispersed and more diffuse than in non-expressing cells (Fig 5.2 panels C-D). Similarly, Golgi labelling was greatly reduced and disrupted in cells expressing the dn protein (Fig 5.2 panels E-F). The disruption of membranes in the early secretory pathway in cells expressing dn Arf1 T31N-HA suggests this dn protein is a potent inhibitor of transport in the early secretory pathway.

Figure 5.3 shows cells expressing the dn Arf1 Q71L-GFP (shown in green) and labelled for the ER (shown in red panels A-B), the ERGIC (shown in red panels C-D) and the Golgi (shown in red panels E-F). Expression of dn Arf1 Q71L-GFP appears to reduce ER labelling (Fig 5.3 panels A-B) and in most cells dispersed the ERGIC from the concentrated peri-nuclear region (Fig 5.3 panels C-D). However the Golgi labelling was largely unaffected and only absent in cells over-expressing the dn protein (Fig 5.3 panels E-F). Overall, expression of dn Arf1 Q71L-GFP does not appear to have a major effect on the Golgi as it largely remains intact, however expression of this dn may affect retrograde transport from the Golgi back to the ER and ERGIC, resulting the observed alteration to ER and ERGIC membranes.

5.2.2 The effect of dominant-negative Arf1 on FMDV infection

In the next set of experiments the effects of dn Arf1 on FMDV infection were investigated. IBRS-2 cells were transfected with either wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP for 14 hours. The transfected cells were then infected with FMDV O1Kcad_2 at a low MOI (MOI 0.5). Infection was

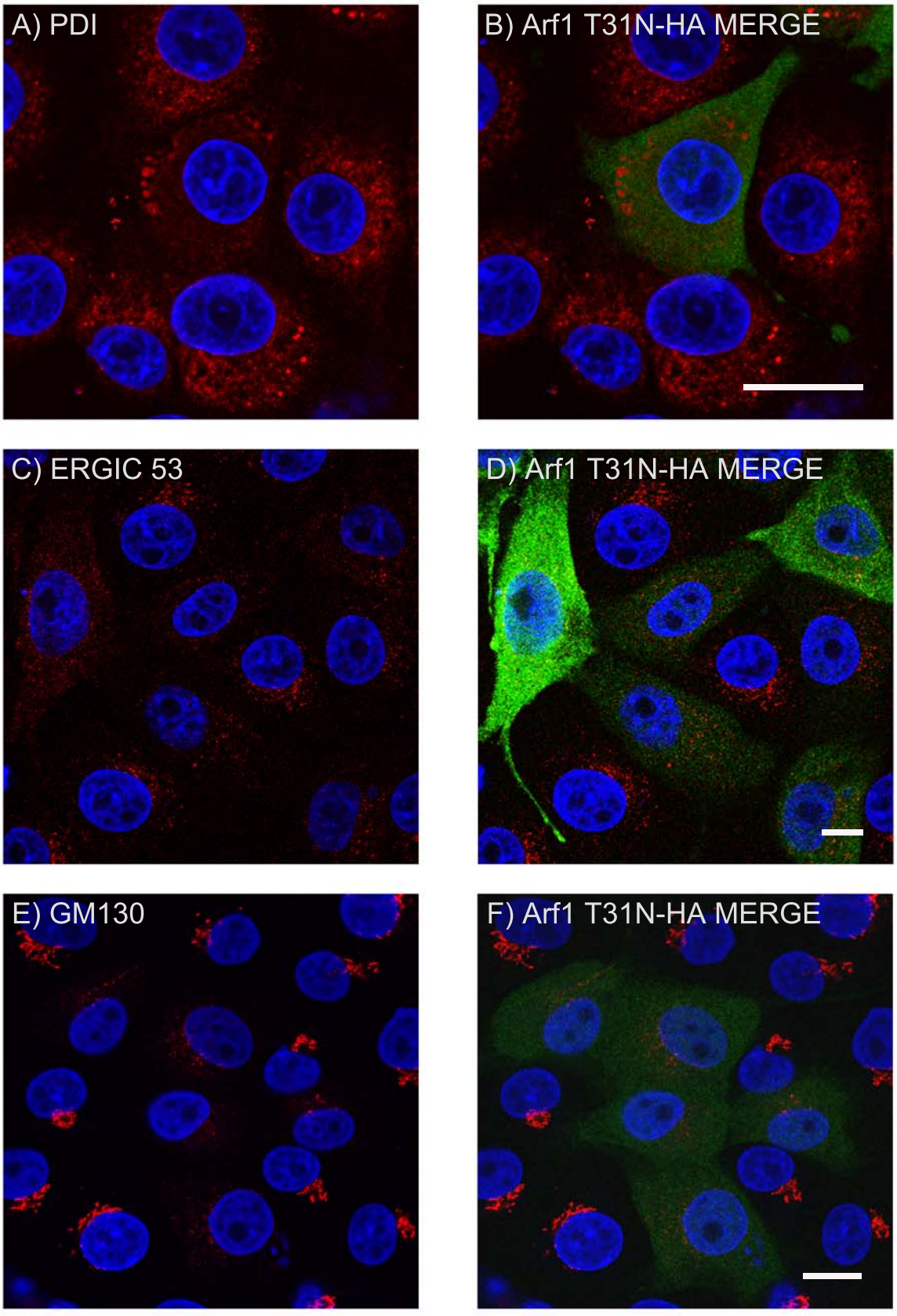


Figure 5.2 The effects of dn Arf1 (T31N) expression on the ER, ERGIC and Golgi
IBRS-2 cells were transfected to express dn Arf1 T31N-HA for 14 hours and labelled for the ER (PDI; panels A-B), the ERGIC (ERGIC53; panels C-D) or the Golgi (GM130; panels E-F). Cells expressing Arf1 are shown as green; the ER, ERGIC and Golgi are shown in red. Panels B, D and F show merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

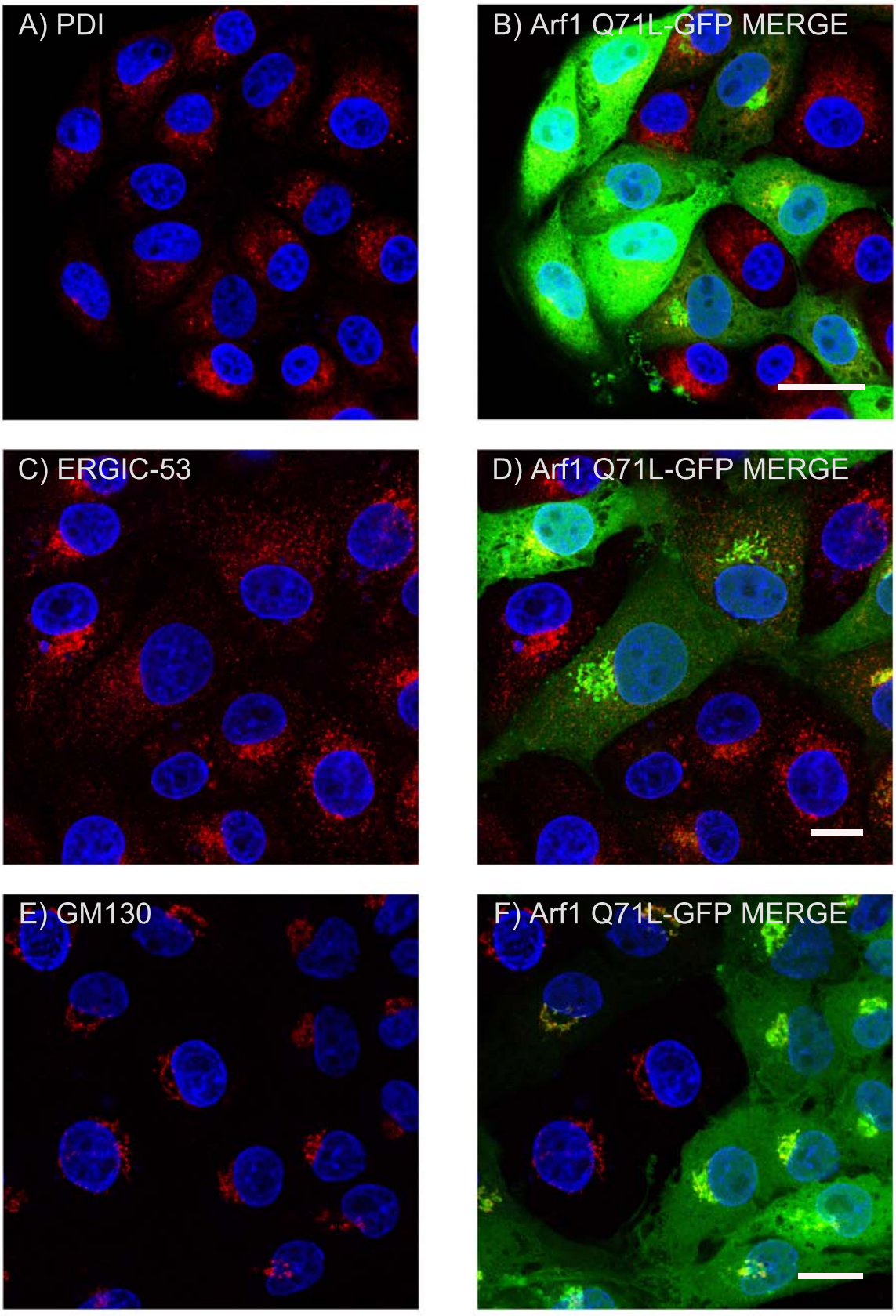


Figure 5.3 The effects of dn Arf1 (Q71L) expression on the ER and ERGIC
IBRS-2 cells were transfected to express dn Arf1 Q71L-GFP for 14 hours and labelled for the ER (PDI; panels A-B), the ERGIC (ERGIC53; panels C-D) or the Golgi (GM130; panels E-F). Cells expressing Arf1 are shown as green; the ER and ERGIC are shown in red. Panels B, D and F show merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

allowed to proceed for 4 hours before the cells were fixed with 4% PFM, permeabilised and labelled for virus using a rabbit anti-FMDV antibody and a goat anti-rabbit Alexa-fluor 568 secondary antibody. Infected cells are shown as red on the figures.

Figure 5.4 shows cells expressing dn Arf1 Q71L-GFP (shown in green panels B and E) and infected with FMDV (shown in red panels A and D). The frequency of infection appeared similar for cells expressing dn Q71L in comparison to cells of the non-expressing population. Figure 5.5 shows cells expressing dn Arf1 T31N-HA (shown in green panels B and E) and infected with FMDV (shown in red panels A and D). In contrast to the results above (Fig 5.4), the frequency of infection in cells expressing dn Arf1 T31N-HA appeared enhanced when compared to cells of the non-expressing populations.

The above experiments were repeated three times and the effect of the dn Arf1 proteins on infection quantified. The level of infection of cells expressing an Arf1 transgene (wt Arf1, dn Arf1 T31N or dn Arf1 Q71L) was normalised to the frequency of infection of cells of the non-expressing population (as described in section 4.2.2). This analysis would reveal if over-expression of the wt or dn proteins results in an inhibitory effect on infection.

Figure 5.6 (panels A-C) shows data for three independent experiments and the mean data (Fig 5.6 [D]) for cells expressing dn Arf1 T31N-HA. The first experiment (Figure 5.6A) shows ~25% reduction in the frequency of infection for cells expressing wt Arf1-GFP. In contrast, an increase of ~100% was observed for cells expressing the dn Arf1 T31N-HA. The second experiment (Figure 5.6 [B]) shows that expression of the wt Arf1-GFP had no effect on infection, whereas expression of dn Arf1 T31N-HA resulted in a ~150% increase to

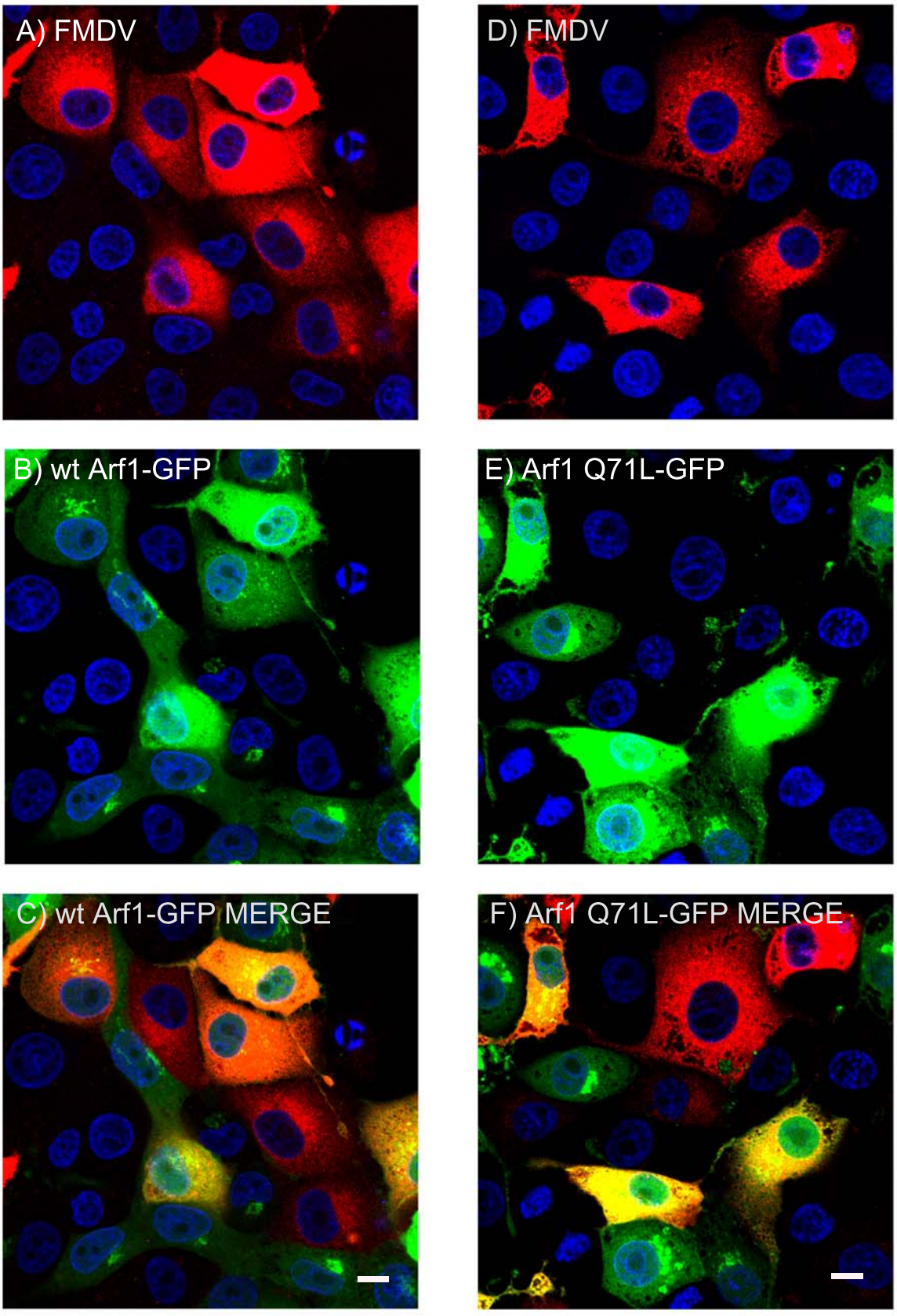


Figure 5.4 FMDV Infection of cells expressing wt or dn Arf1 (Q71L)
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. Cells expressing Arf1 are shown as green. Infected cells are shown as red. Panels A and D show labelling for FMDV. Panels B and E show the signal for Arf1-GFP. Panels C and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

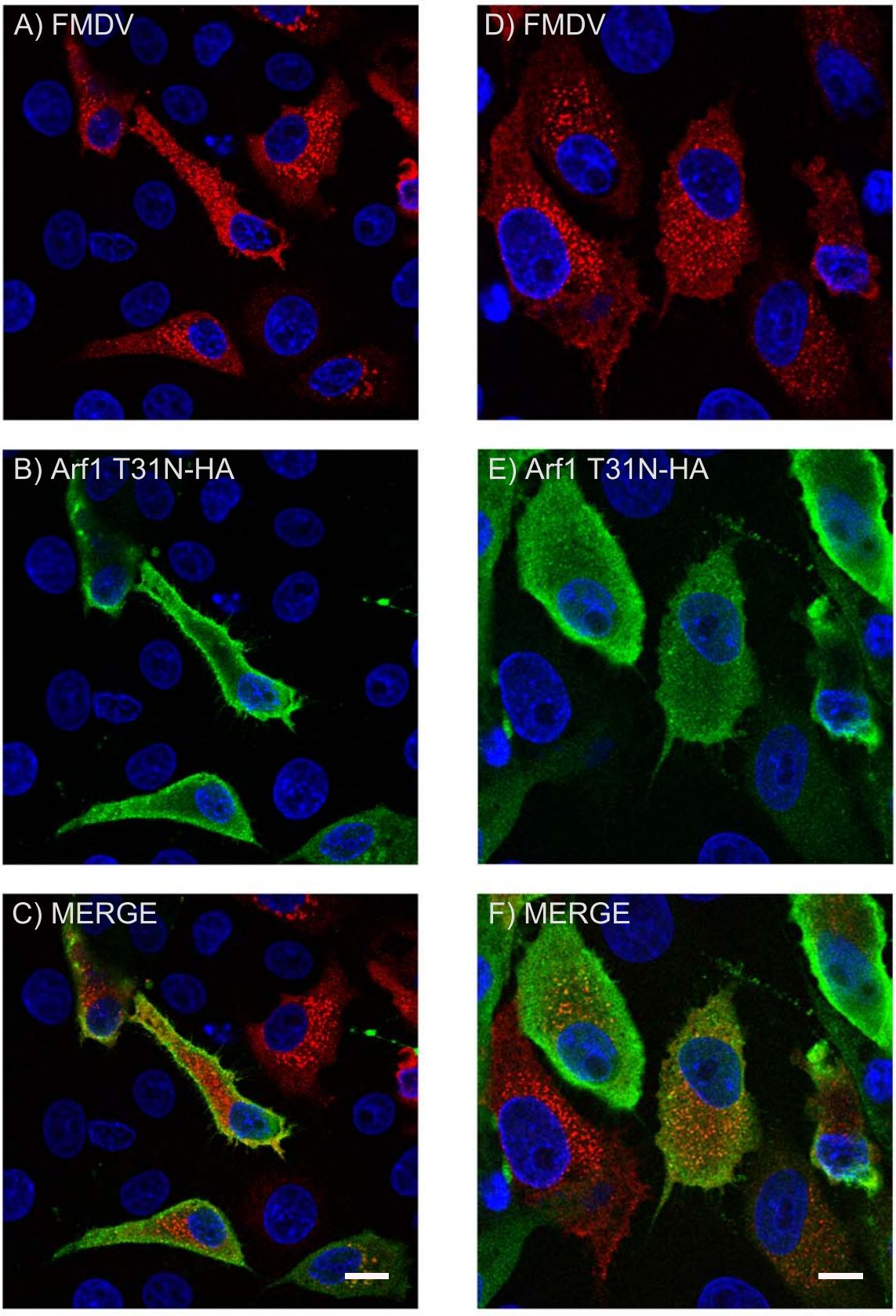


Figure 5.5 FMDV Infection of cells expressing dn Arf1 (T31N)
IBRS-2 cells were transfected to express dn Arf1 T31N-HA for 14 hours and then infected with FMDV. Cells expressing Arf1 are shown as green. Infected cells are shown as red. Panels A and D show labelling for FMDV. Panels B and E show the signal for Arf1-HA. Panels C and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**32**
ARF1 wildtype GFP	**Expressing**	64	22
	Non-expressing	36	28
ARF1 T31N GFP	**Expressing**	46	40
	Non-expressing	54	20

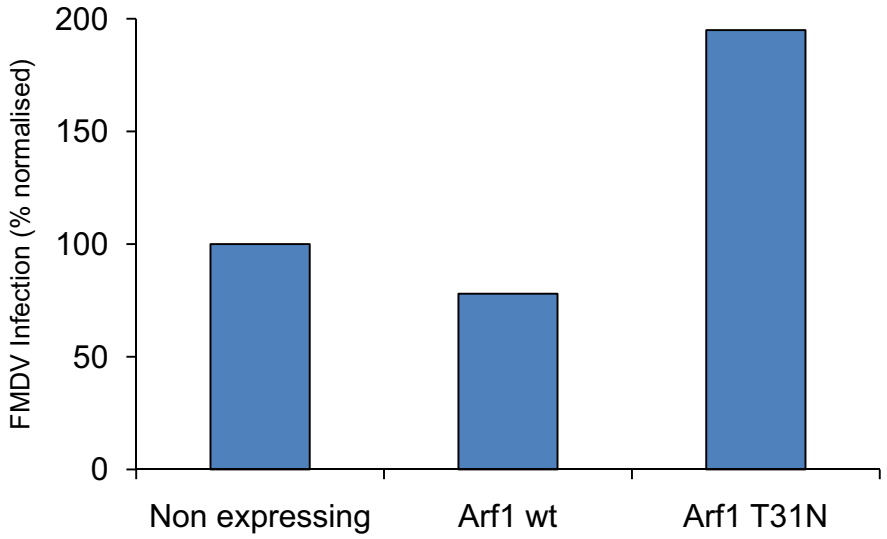


Figure 5.6(A): Experiment 1. The effects of dominant-negative Arf1 (T31N) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Sar1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**25**
ARF1 wildtype GFP	**Expressing**	55	19
	Non-expressing	45	19
ARF1 T31N GFP	**Expressing**	44	49
	Non-expressing	56	19

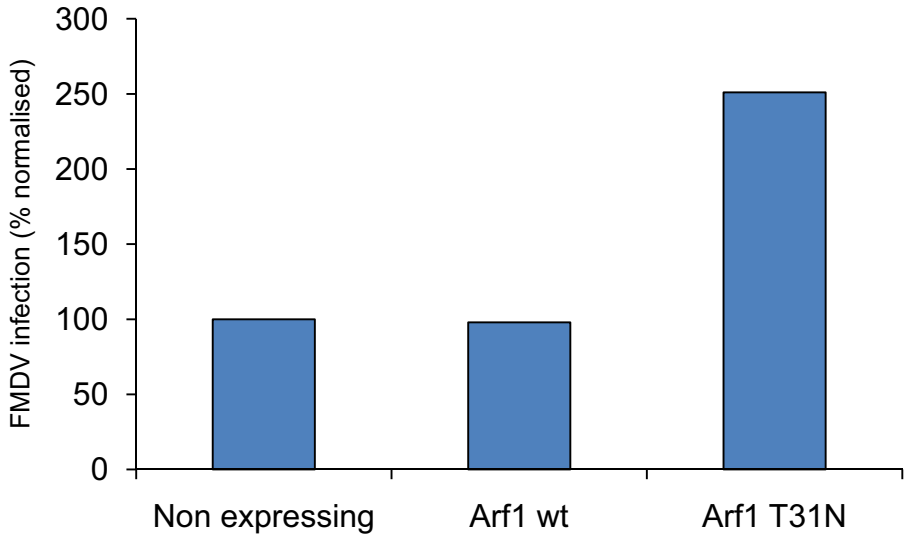


Figure 5.6(B): Experiment 2. The effects of dominant-negative Arf1 (T31N) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**31**
ARF1 wildtype GFP	**Expressing**	54	18
	Non-expressing	46	19
ARF1 T31N GFP	**Expressing**	41	43
	Non-expressing	59	16

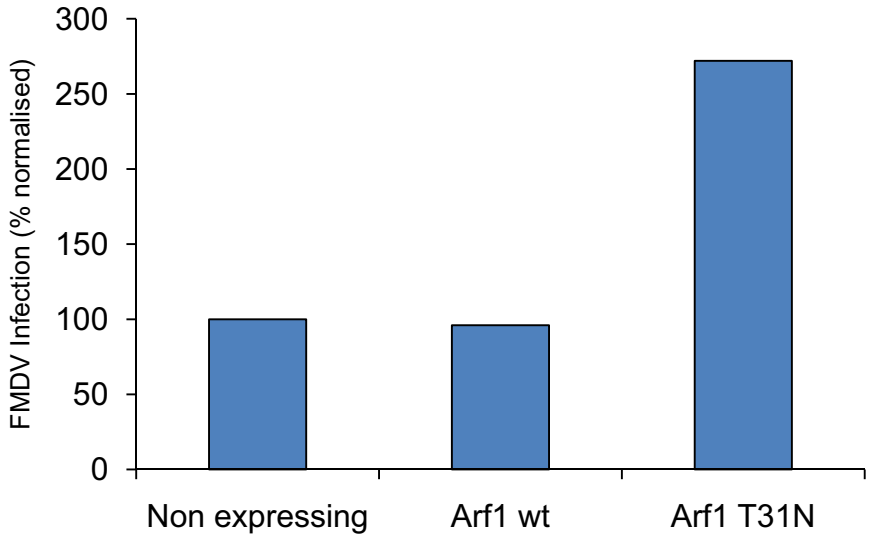


Figure 5.6(C): Experiment 3. The effects of dominant-negative Arf1 (T31N) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

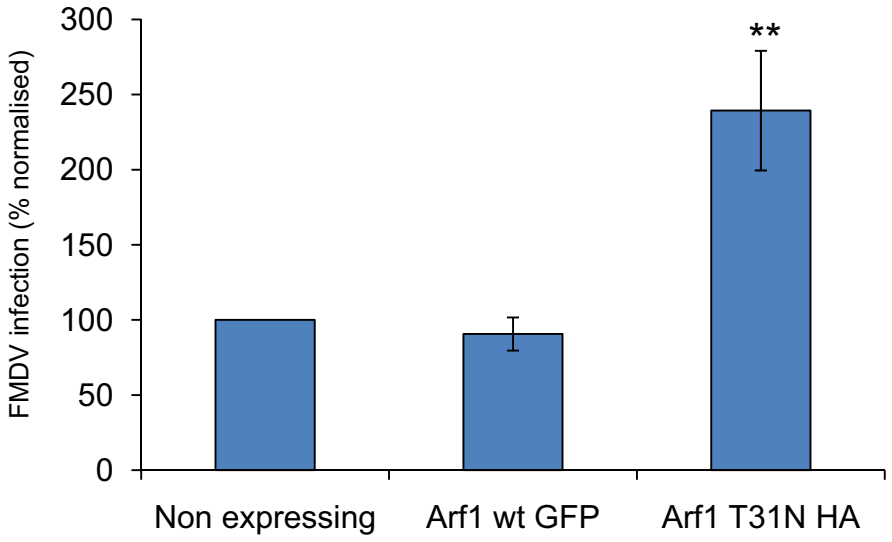


Figure 5.6(D): The effects of dominant-negative Arf1 (T31N) on FMDV infection of IBRS-2 cells

Shown is the mean ± SD for the experiments shown in Figure 5.6 (A-C). For each transgene (wt or dn T31N) the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population (P value: ** <0.01).

infection. Figure 5.6 (C) also shows that infection is not inhibited by wt Arf1-GFP, whereas expression of dn Arf1 T31N-HA results in a ~175% increase in infection.

The mean level of infection for the three experiments is shown in Figure 5.6(D). Expression of dn Arf1 T31N-HA enhanced the frequency of FMDV infection by nearly 2.5 fold, whereas expression of wt Arf1-GFP had no effect on infection. The dn Arf1 T31N-HA is the GDP-bound form of the Arf1 protein which is thought to block transport by preventing the recruitment of the COPI complex to Golgi/ERGIC membranes. Expression of this version of Arf1 has resulted in an increase to FMDV infection. This data not only suggests that FMDV does not require active Arf1 for infection, but that the loss of Arf1 activity is somehow advantageous to FMDV replication.

Figure 5.7 (panels A-C) shows data for three independent experiments and the mean data of the three experiments (Fig 5.7 panel D). The first experiment (Fig 5.7 [A]) shows that the frequency of infection in cells expressing dn Arf1 Q71L-GFP was not affected when compared to cells in the non-expressing population. Similarly, the second experiment shows (Figure 5.7 [B]) no effect in cells expressing dn Arf1 Q71L-GFP. The third experiment (Figure 5.7 [C]) shows expression of dn Arf1 (Q71L) decreases infection by ~30%.

The mean level of infection for the three experiments is shown in Figure 5.7 (D). Overall, it appears dn Arf1 Q71L-GFP only reduces the frequency of FMDV infection by ~15% having no substantial effect on FMDV infection levels.

		Transfection efficency (%)	% Infected cells
Mock			**27**
ARF1 Q71L GFP	**Expressing**	49	34
	Non-expressing	51	32

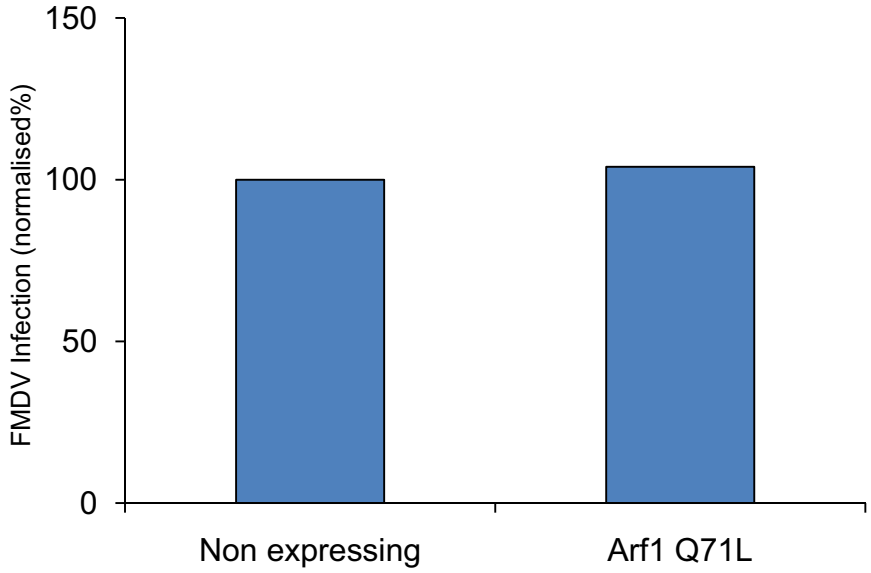


Figure 5.7(A): Experiment 1. The effects of dominant-negative Arf1 (Q71L) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**36**
ARF1 Q71L GFP	**Expressing**	56	26
	Non-expressing	44	26

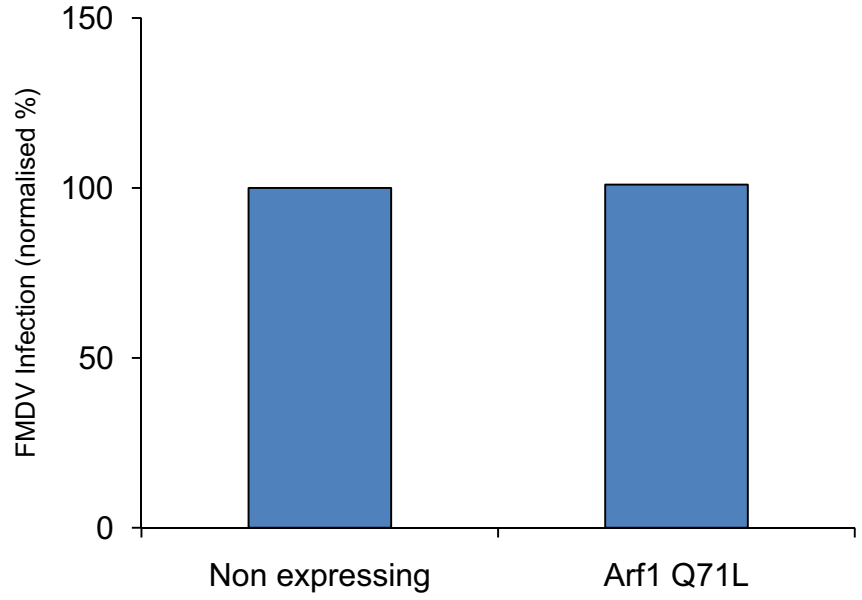


Figure 5.7(B): Experiment 2. The effects of dominant-negative Arf1 (Q71L) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**54**
ARF1 Q71L GFP	**Expressing**	55	36
	Non-expressing	45	57

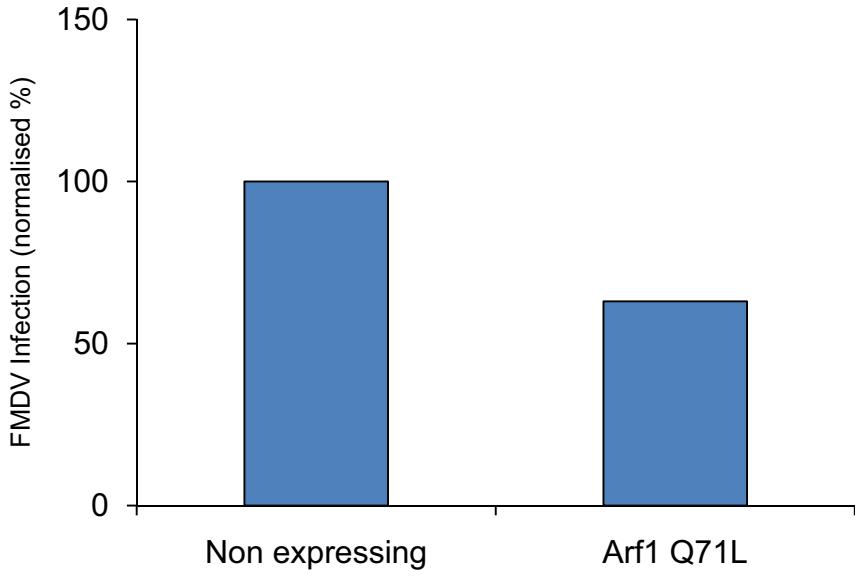


Figure 5.7(C): Experiment 3. The effects of dominant-negative Arf1 (Q71L) on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

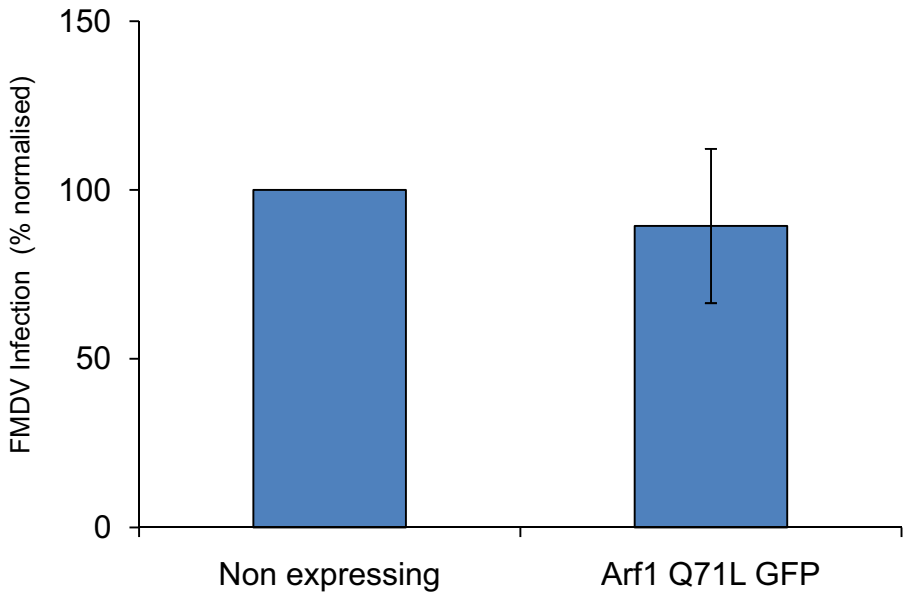


Figure 5.7(D): The effects of dominant-negative Arf1 (Q71L) on FMDV infection of IBRS-2 cells
Shown is the mean ± SD for the experiments shown in figure 5.7 (A-C). For cells expressing the transgene (dn Q71L). The frequency of infection of cells expressing dn Arf1 Q71L-GFP was normalised to the results for the non-expressing cell population.

5.3 The effect of dominant-negative Arf1 on BEV-1 infection

The above experiments were repeated using BEV-1 in place of FMDV. Due to BEV-1's sensitivity to BfA it can be assumed BEV relies on Arf1 and its GEF's for infection. Therefore, expression of the dn Arf1 T31N-HA, the GDP-bound version of protein is predicted to block BEV-1 infection.

Figure 5.8 shows cells expressing wt Arf1-GFP (shown in green panel B-C) or dn Arf1 T31N-HA (shown in green panel E-F) and infected with BEV (shown in red panels A, C, D, and F). A block to BEV-1 infection was observed in cells expressing dn Arf1 T31N-HA (Fig 5.8 panels D-F). A reduction in BEV infection was also observed in cells expressing wt Arf1-GFP, although this was not as strong as the block observed in cells expressing the dn Arf1 T31N-HA protein (Fig 5.8 panels A-C). Figure 5.9 shows cells expressing dn Arf1 Q71L-GFP (shown in green panels B-C) and infected with BEV (shown in red panels A and C). A reduction to the frequency of BEV infected cells was observed in cells expressing the dn Arf1 Q71L-GFP when compared to cells of the non-expressing population.

The experiments were repeated three times and the effect of the dn Arf1 proteins on BEV-1 infection quantified (see section 5.2.2). Figure 5.10 (A-C) shows three independent experiments and the mean values of the three experiments (Fig 5.10 [D]) in cells expressing dn Arf1 T31N-HA. The first experiment (Figure 5.10 [A]) shows that expression of wt Arf1-GFP resulted in ~45% reduction in the frequency of BEV infection in comparison to cells of the non-expressing population, whereas expression of dn Arf1 T31N-HA decreased the frequency of infection by ~80%. The second experiment (Figure 5.10 [B]), shows that expression of wt Arf1-GFP reduced the frequency of infection by ~50% and dn Arf1 T31N-HA reduced infection by ~90%. Similarly, the third

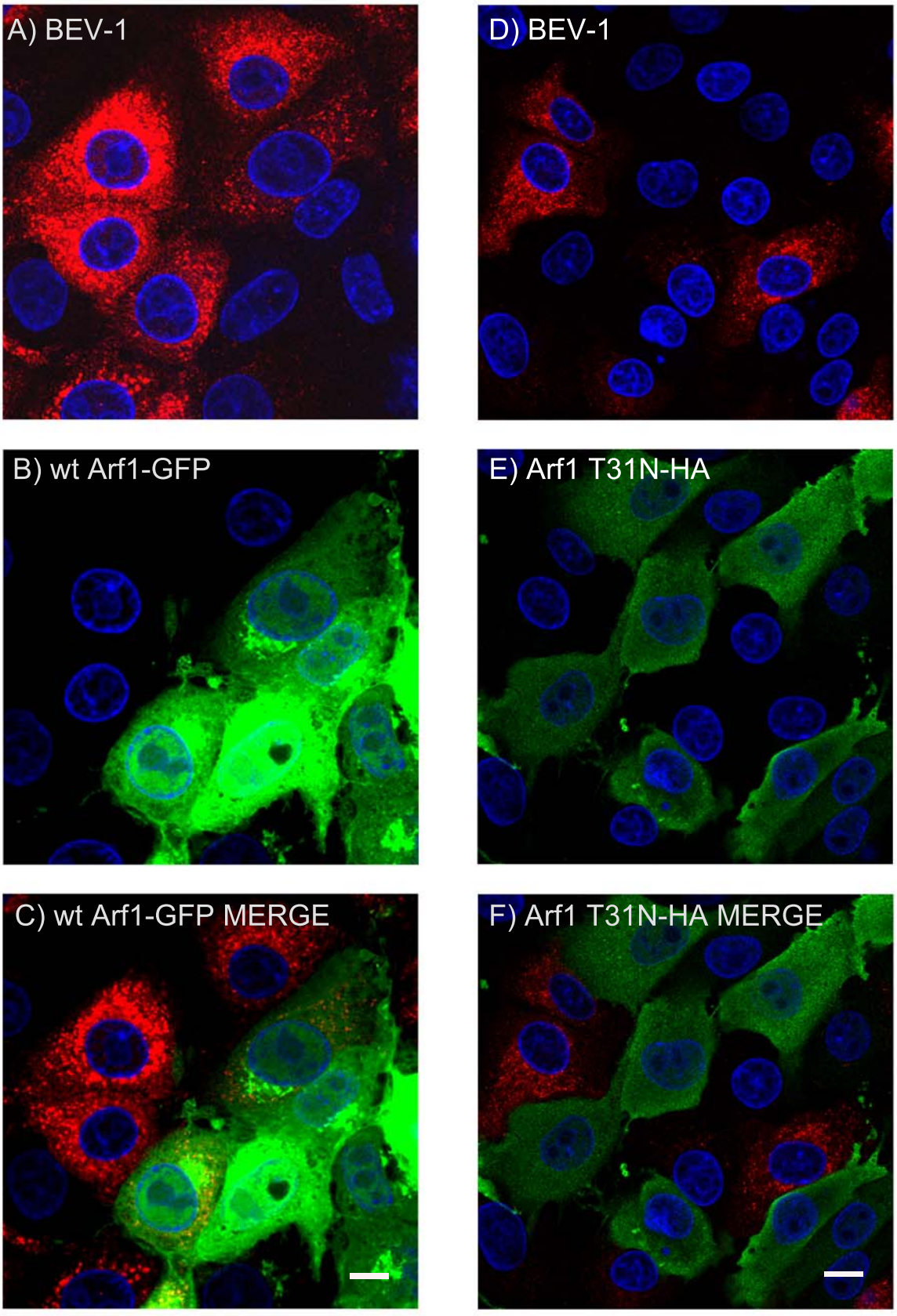


Figure 5.8 BEV Infection of cells expressing wt or dn Arf1 (T31N)
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with BEV. Cells expressing Arf1 are shown as green. Infected cells are shown as red. Panels A and D show labelling for BEV. Panels B and E show the signal for Arf1. Panels C and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

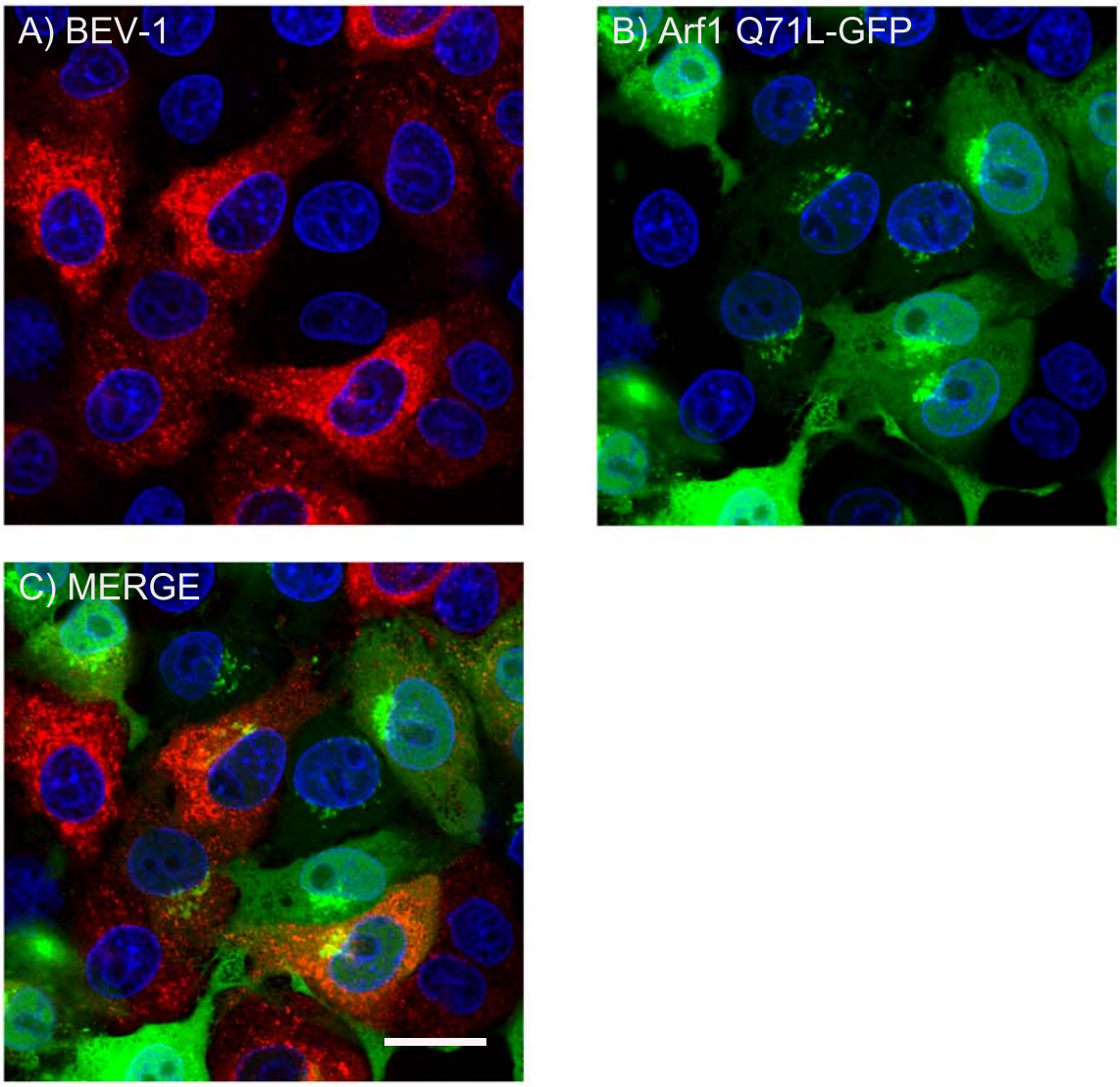


Figure 5.9 BEV-1 Infection of cells expressing dn Arf1 (Q71L)
IBRS-2 cells were transfected to express dn Arf1 Q71L-GFP for 14 hours and then infected with BEV. Cells expressing Arf1 are shown as green. Infected cells are shown as red. Panel A shows labelling for BEV. Panel B shows the signal for Arf1-GFP. Panels C and F show merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**60**
ARF1 wildtype GFP	**Expressing**	45	30
	Non-expressing	55	53
ARF1 T31N HA	**Expressing**	42	6
	Non-expressing	58	48

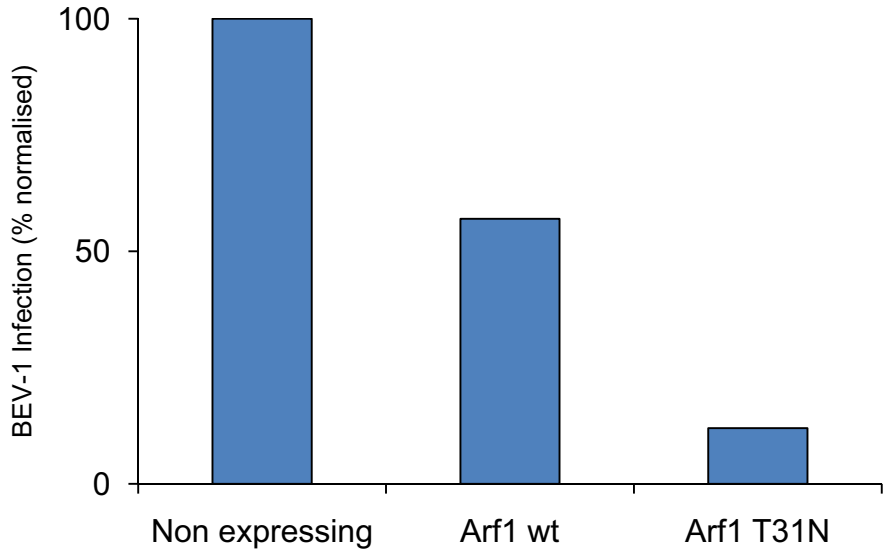


Figure 5.10(A): Experiment 1. The effects of dominant-negative Arf1 (T31N) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**69**
ARF1 wildtype GFP	**Expressing**	46	37
	Non-expressing	54	71
ARF1 T31N HA	**Expressing**	39	5
	Non-expressing	61	56

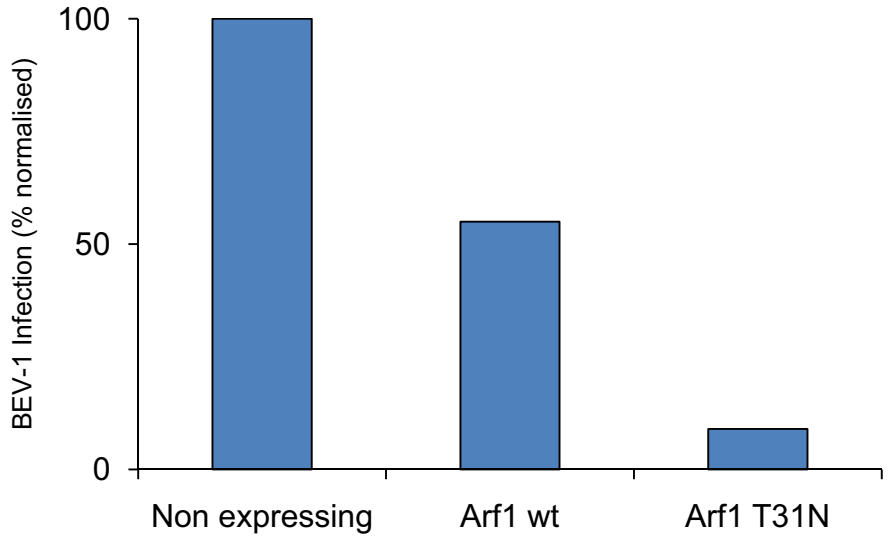


Figure 5.10(B): Experiment 2. The effects of dominant-negative Arf1 (T31N) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**60**
ARF1 wildtype GFP	**Expressing**	50	27
	Non-expressing	50	58
ARF1 T31N HA	**Expressing**	39	2
	Non-expressing	61	41

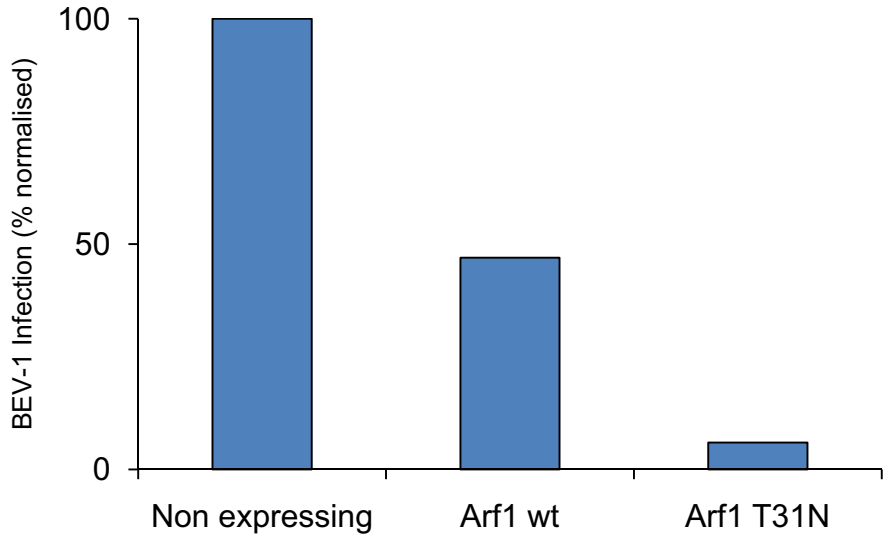


Figure 5.10(C): Experiment 3. The effects of dominant-negative Arf1 (T31N) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 T31N-HA for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

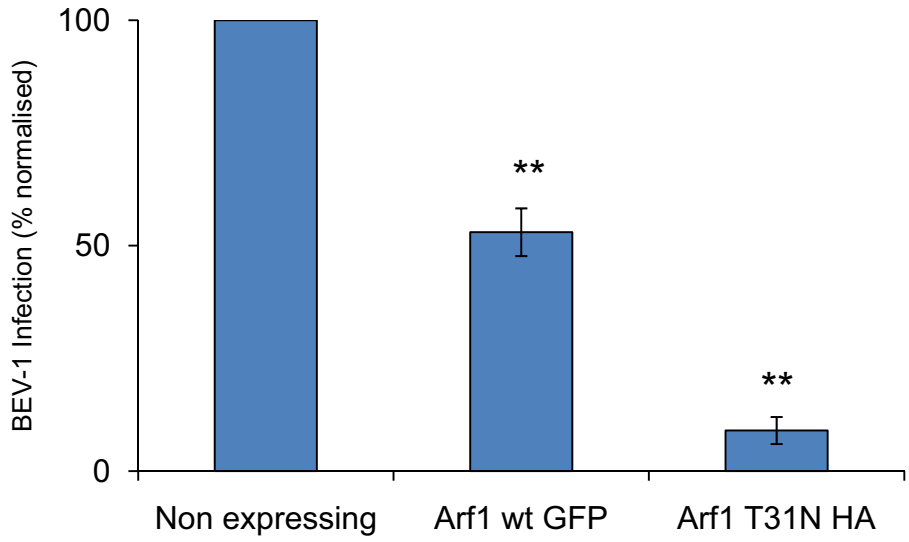


Figure 5.10(D): The effects of dominant-negative Arf1 (T31N) on BEV infection of IBRS-2 cells
Shown is the mean ± SD for the experiments shown in figure 5.10 (A-C). For each transgene (wt or dn T31N) the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population (P values: ** < 0.01).

experiment (Figure 5.10 [C]) shows a ~55% reduction to infection for cells expressing wt Arf1-GFP and a ~95% reduction to infection for cells expressing the dn Arf1 T31N-HA.

Figure 5.10 (D) shows the mean level of infection for the three experiments described above. The frequency of BEV infection was reduced by ~50% in cells expressing the wt Arf1-GFP and by ~90% in cells expressing dn Arf1 T31N-HA. The reduction of infection observed for cells expressing wt Arf1-GFP may be due to a potential dominant negative effect of the wt protein. The block to BEV infection observed for cells expressing dn Arf1 T31N-HA confirms that BEV relies on active Arf1 for replication. These observations also help validate the results seen in FMDV infected cells.

Figure 5.11 (panels A-D) shows three independent experiments for cells expressing dn Arf1 Q71L-GFP. The first experiment (Fig 5.11 [A]) shows a reduction in the frequency of infection in cells expressing wt Arf1-GFP by ~30% and dn Arf1 Q71L-GFP by ~25%. The second experiment (Fig 5.11 [B]) shows ~75% reduction in cells expressing wt Arf1-GFP and ~40% reduction in cells expressing the dn Arf1 Q71L-GFP. The final experiment (Fig 5.11 [C]) also shows ~60% reduction to infection in cells expressing wt Arf1-GFP and ~50% reduction in cells expressing the dn Arf1 Q71L-GFP.

Figure 5.11 (D) shows the mean level of infection for the three experiments described above. On average, the frequency of BEV infection was reduced by ~50% for cells expressing wt Arf1-GFP and by ~30% for cells expressing dn Arf1 Q71L-GFP. The over-expression of both wt Arf1-GFP and the constituently active version of Arf (dn Arf1 Q71L-GFP) appear to have an inhibitory effect on BEV infection.

		Transfection efficency (%)	% Infected cells
Mock			**48**
ARF1 wildtype GFP	**Expressing**	21	25
	Non-expressing	79	36
ARF1 Q71L GFP	**Expressing**	48	29
	Non-expressing	52	38

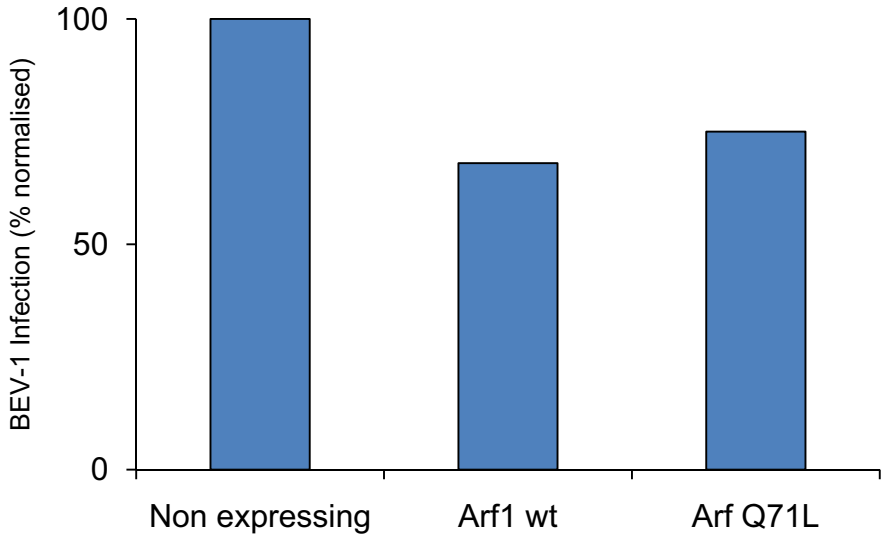


Figure 5.11(A): Experiment 1. The effects of dominant-negative Arf1 (Q71L) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 Q71L-GFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**52**
ARF1 wildtype GFP	**Expressing**	41	24
	Non-expressing	59	73
ARF1 Q71L GFP	**Expressing**	49	30
	Non-expressing	51	43

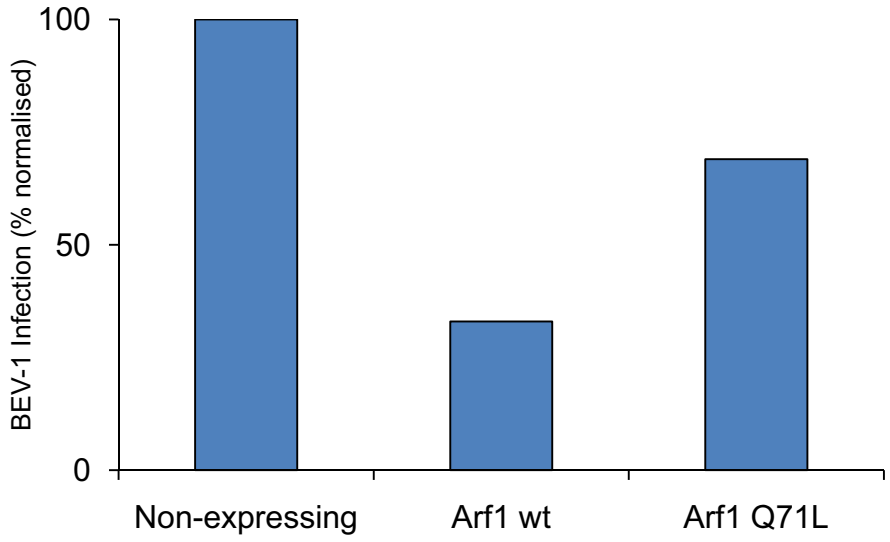


Figure 5.11(B): Experiment 2. The effects of dominant-negative Arf1 (Q71L) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 Q71L-GFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**38**
ARF1 wildtype GFP	**Expressing**	47	21
	Non-expressing	53	55
ARF1 Q71L GFP	**Expressing**	41	23
	Non-expressing	59	47

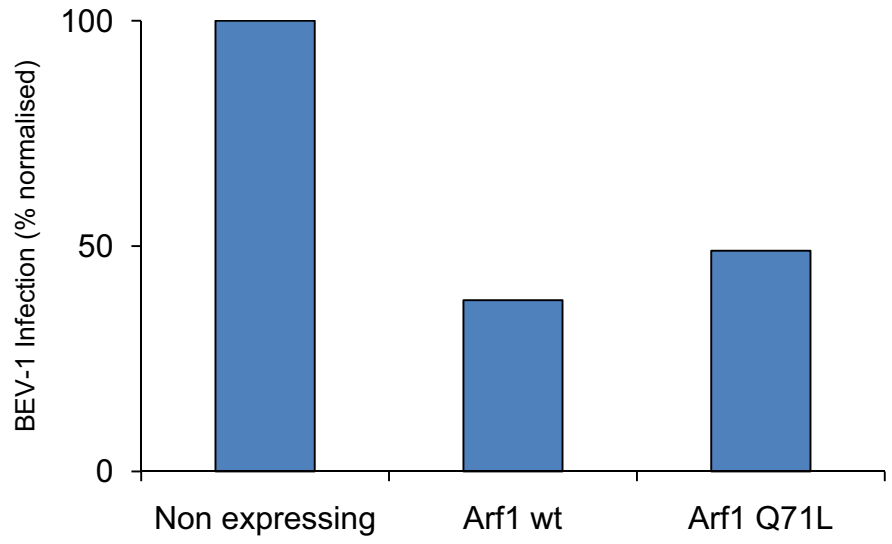


Figure 5.11(C): Experiment 3. The effects of dominant-negative Arf1 (Q71L) on BEV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Arf1-GFP or dn Arf1 Q71L-GFP for 14 hours and then infected with BEV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

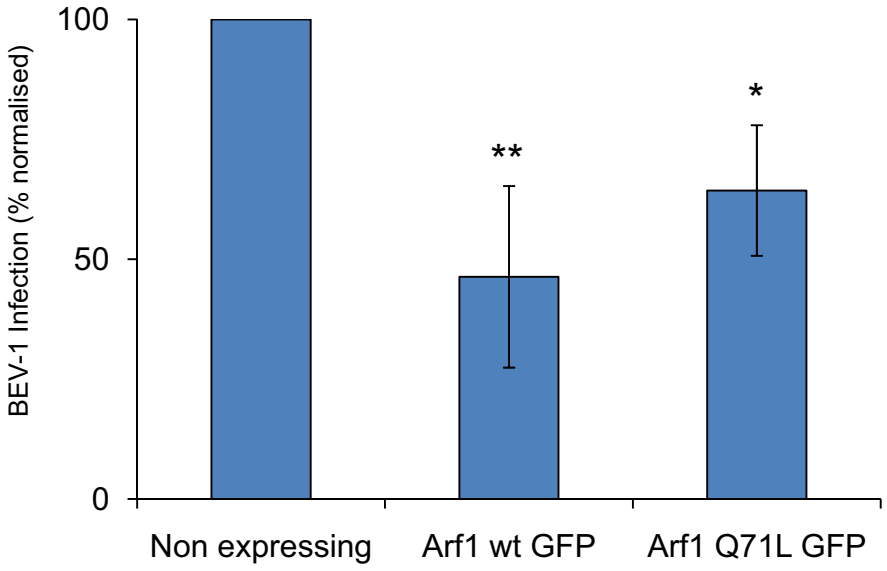


Figure 5.11(D): The effects of dominant-negative Arf1 (Q71L) on BEV infection of IBRS-2 cells

Shown is the mean ± SD for the experiments shown in figure 5.11 (A-C). For each transgene (wt or dn Q71L) the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population (P value: $*$ < 0.05, $**$ < 0.01).

5.4 The effect of dominant-negative Arf1 on FMDV infection in MAX cells

Next the effects of dn Arf1 on FMDV infection were investigated using MAX cells (see section 4.2.4). MAX cells transfected to express wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP were either (i) fixed using 4% PFM and labelled for the Golgi (for GM130) or (ii) infected with FMDV at an MOI of 0.5 . At 4 hours post-infection the cells were fixed in 4% PFM and labelled for FMDV with a rabbit anti-FMDV type O antibody as described above. Infected cells are shown as red on the figures.

MAX cells expressing the Arf1 proteins were labelled for the Golgi to confirm if expression of the dn proteins disrupted the Golgi in a similar manner to that observed in lBRS-2 cells. Figure 5.12 shows cells expressing wt Arf1-GFP (shown in green panel B), dn Arf1 T31N-HA (shown in green panel D) and dn Arf1 Q71L-GFP (shown in green panels F) labelled for the Golgi with GM130 (shown in red [A-F]). Expression of wt Arf1-GFP appears to result in the loss of the Golgi labelling only in cells over-expressing the protein. Cells expressing dn Arf1 T31N-HA showed a near complete loss of Golgi labelling, whereas expression of dn Arf1 Q71L-GFP did not cause a major disruption in Golgi labelling, however the cisternae appear less defined than in non-expressing cells. Disruption of the Golgi suggests that membrane flow through the secretory pathway and protein transport are blocked.

Figure 5.13 shows MAX cells expressing wt Arf1-GFP (shown as green, panels B-C) or dn Arf1 Q71L-GFP (shown as green, panels E-F) and infected with FMDV (shown as red, panels A, C, D and F). The frequency of infection of cells expressing wt Arf1-GFP or dn Arf1 Q71L-GFP was slightly reduced in comparison to cells in the non-expressing population (Fig 5.13, A-F). Figure 5.14 shows MAX cells expressing dn Arf1 T31N-HA (shown in green, panels B-

C) and infected with FMDV (A and C). There appears to be an increase in FMDV infection in cells expressing the dn protein, as seen in IBRS-2 cells (see section 5.2.2).

As described previously, the data for each transfection was normalised to the frequency of infection seen in the non-expressing cell population. Figure 5.15 (graphs A-C) shows three independent experiments and the mean effect of the Arf1 proteins on infection is shown in figure 5.15 (D). The results of the first experiment is shown in Figure 5.15 (A); a reduction in FMDV infection was observed in cells expressing wt Arf1-GFP (~50%) or dn Arf1 Q71L-GFP (~40%), while dn Arf1 T31N-HA expression increased FMDV infection by ~30%. The second experiment is shown in Figure 5.15 (B); similar results were seen with wt Arf1-GFP and dn Arf1 Q71L-GFP as infection was reduced by ~50%. For dn Arf1 T31N-HA, infection was enhanced by ~30%. The third experiment is shown in Figure 5.15 (C); expression of wt Arf1-GFP or dn Arf1 Q71L-GFP reduced infection by 50% and 40%, respectively, whereas for dn Arf1 T31N-HA, infection increased by ~75%.

Figure 5.15 (D) shows the mean for the three experiments. Both wt Arf1-GFP and dn Arf1 Q71L-GFP reduced FMDV infection by ~50%, while expression of dn Arf1 T31N-HA enhanced infection by ~50%. The increase to FMDV infection observed in dn Arf1 T31N-HA expressing cells agrees with the IBRS-2 cells data (see section 5.2.2), suggesting that a loss of Arf1 activity is advantageous to FMDV infection. However, the inhibitory effect of wt Arf1-GFP and dn Arf1 Q71L-GFP on infection in MAX cells appeared to be greater than that observed in IBRS-2 cells (see Discussion 5.5).

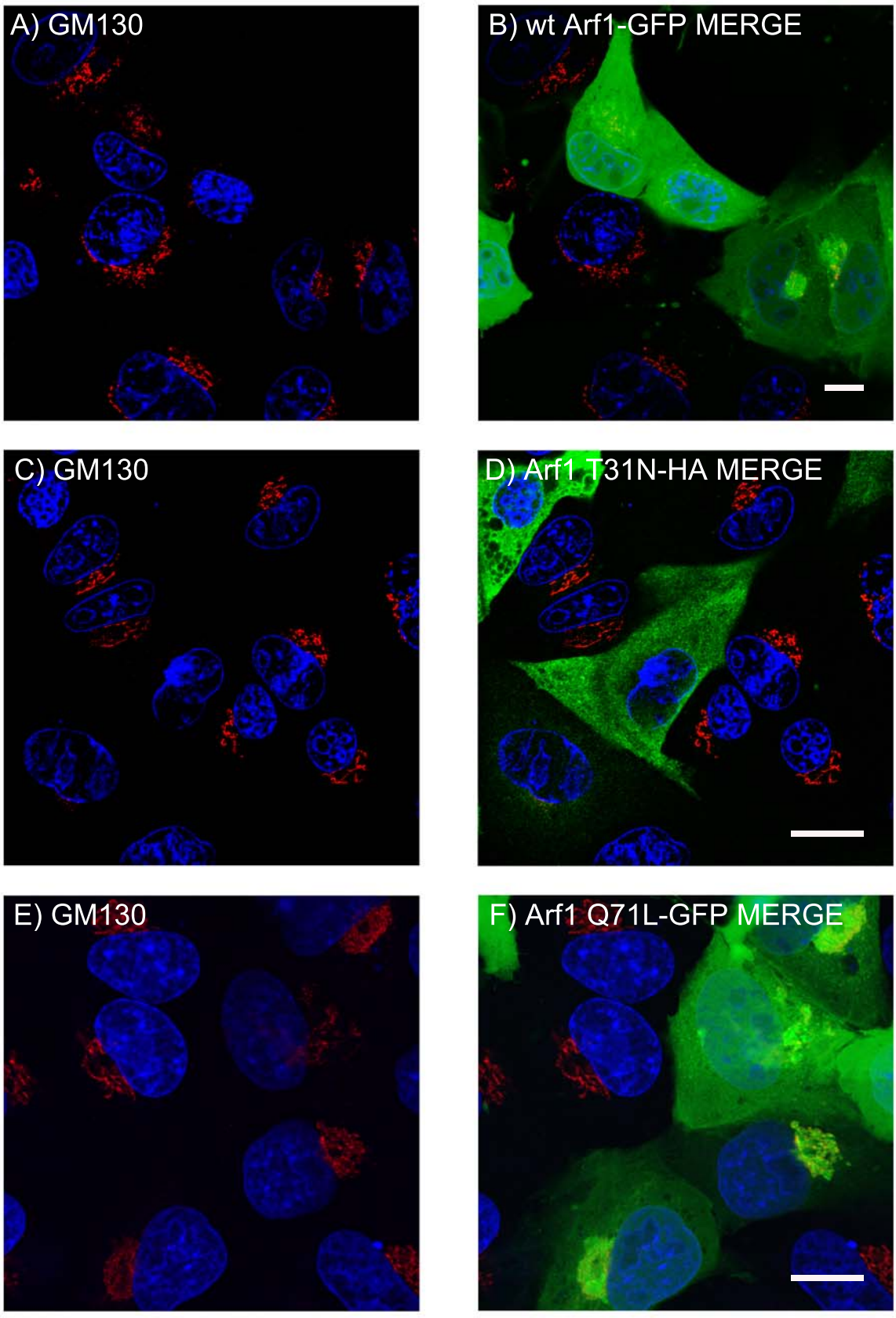


Figure 5.12 The effects of Arf1 expression on the Golgi in MAX cells
MAX cells were transfected to express wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP for 14 hours and labelled for the Golgi (GM130). Cells expressing Arf1 are shown as green; the Golgi is shown in red. Panels B, D and F shows merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

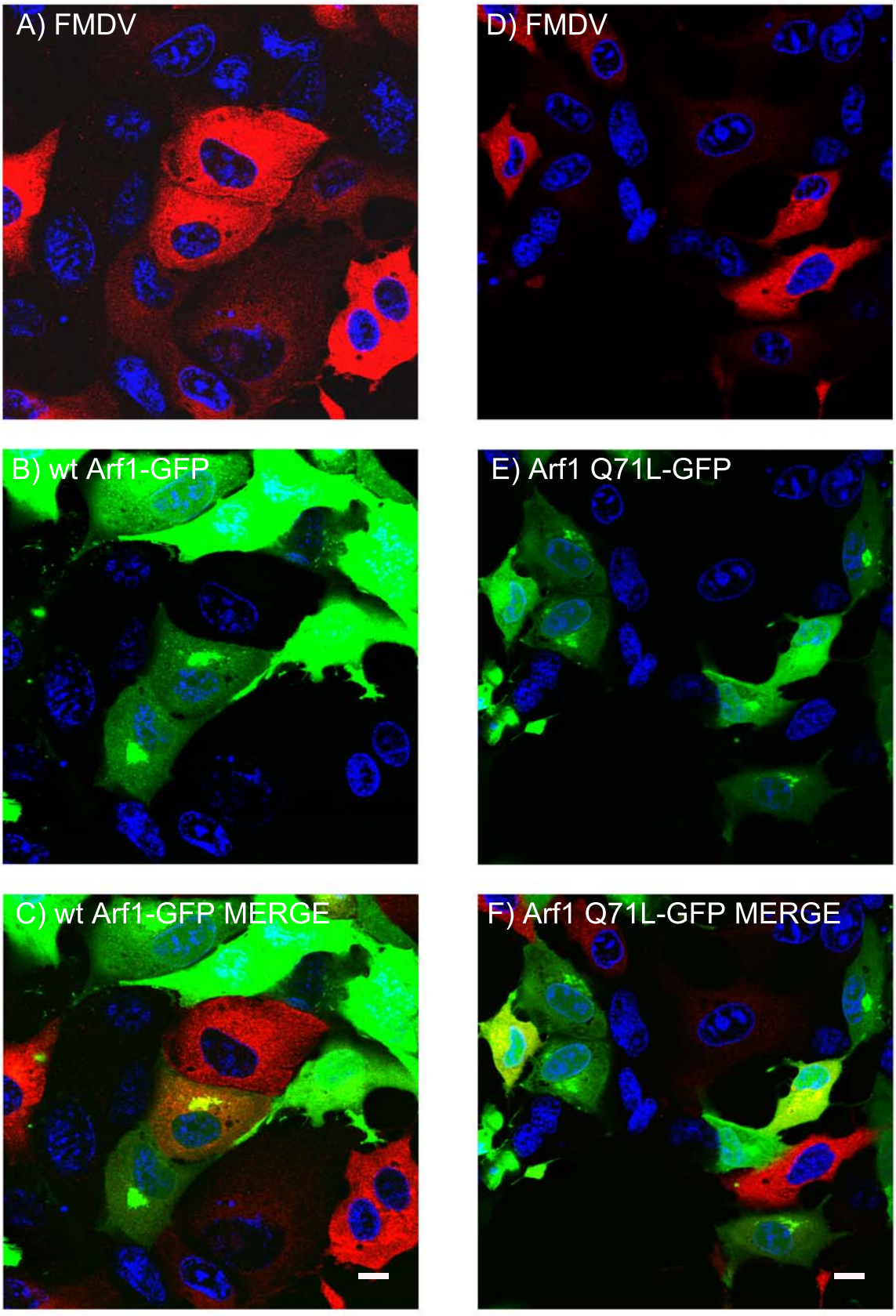


Figure 5.13 FMDV Infection of MAX cells expressing wt or dn Arf1 (Q71L)
MAX cells were transfected to express wt Arf1-GFP or dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. Cells expressing Arf1 are shown as green. Infected cells are shown as red Panels A and D show labelling for FMDV. Panels B and E show the signal for Arf1-GFP. Panels C and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

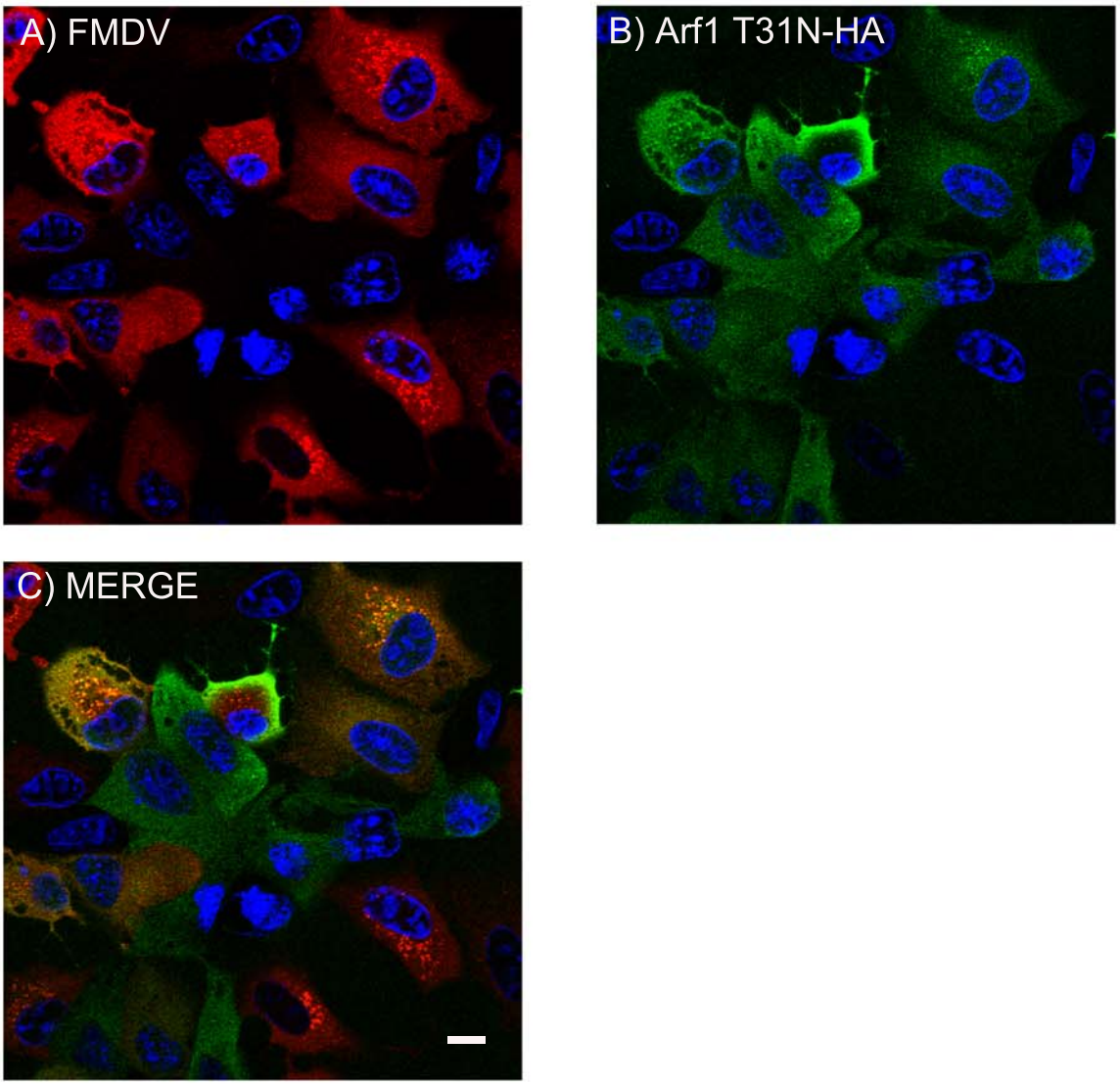


Figure 5.14 FMDV Infection of MAX cells expressing dn Arf1 (T31N)
MAX cells were transfected to express dn Arf1 T31N-HA for 14 hours and then infected with FMDV. Cells expressing Arf1 are shown as green. Infected cells are shown as red. Panel A shows labelling for FMDV. Panel B shows the signal for Arf1-HA. Panel C shows merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**41**
Arf1 wildtype GFP	**Expressing**	48	23
	Non-expressing	52	44
Arf1 Q71L GFP	**Expressing**	33	25
	Non-expressing	67	38
Arf1 T31N HA	**Expressing**	38	45
	Non-expressing	62	34

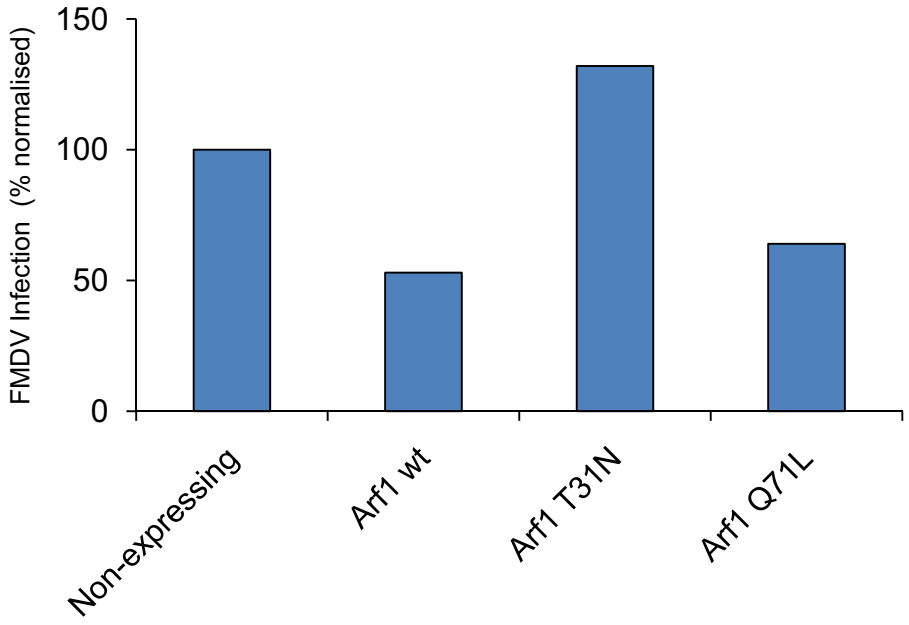


Figure 5.15(A): Experiment 1. The effects of dominant-negative Arf1 on FMDV infection of MAX cells
MAX cells were transfected to express wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**46**
Arf1 wildtype GFP	**Expressing**	43	24
	Non-expressing	57	43
Arf1 Q71L GFP	**Expressing**	37	23
	Non-expressing	63	43
Arf1 T31N HA	**Expressing**	34	50
	Non-expressing	66	37

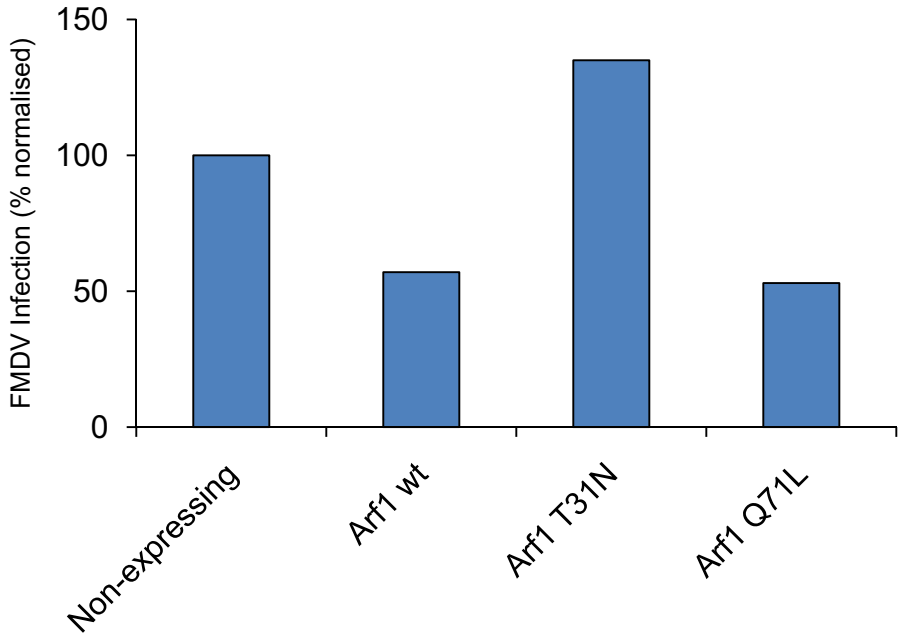


Figure 5.15(B): Experiment 2. The effects of dominant-negative Arf1 on FMDV infection of MAX cells
MAX cells were transfected to express wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

		Transfection efficency (%)	% Infected cells
Mock			**38**
Arf1 wildtype GFP	**Expressing**	46	25
	Non-expressing	54	46
Arf1 Q71L GFP	**Expressing**	37	29
	Non-expressing	63	46
Arf1 T31N HA	**Expressing**	32	44
	Non-expressing	68	25

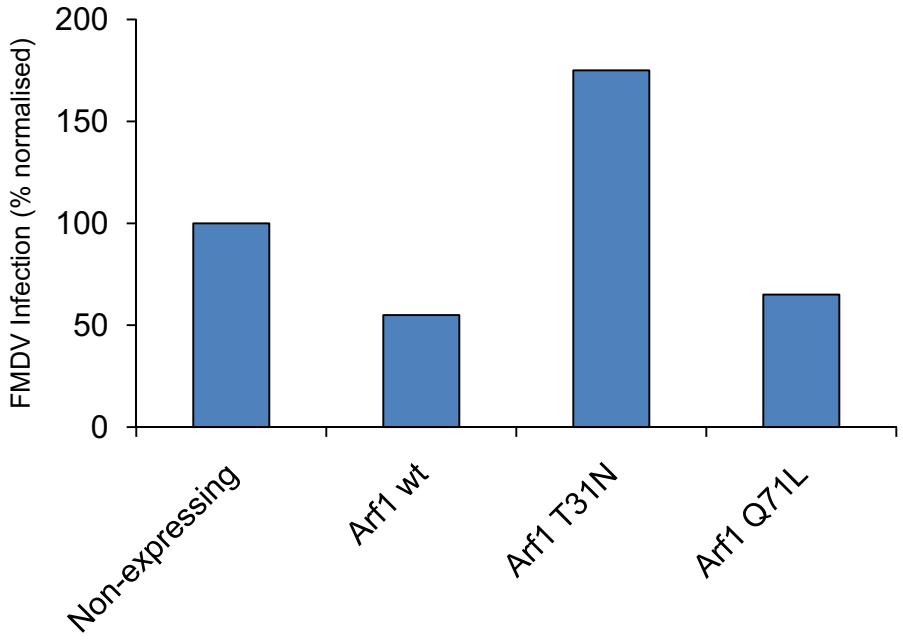


Figure 5.15(C): Experiment 3. The effects of dominant-negative Arf1 on FMDV infection of MAX cells
MAX cells were transfected to express wt Arf1-GFP, dn Arf1 T31N-HA or dn Arf1 Q71L-GFP for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Arf1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population.

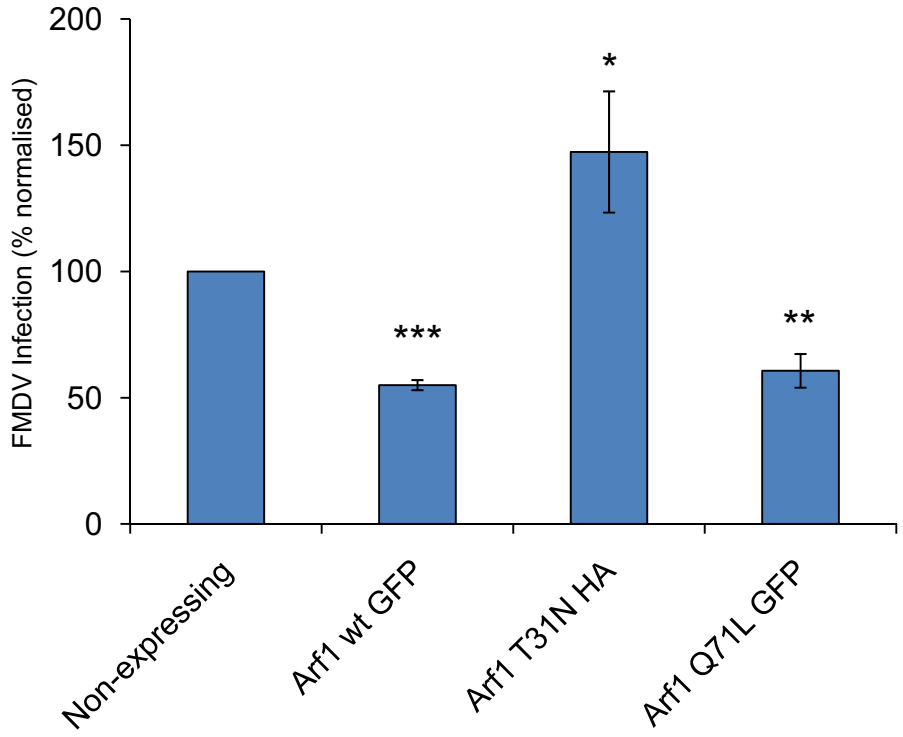


Figure 5.15(D): The effects of dominant-negative Arf1 on FMDV infection of MAX cells
Shown is the mean ± SD for the experiments shown in figure 5.15 (A-C). For each transgene (wt, dn T31N or dn Q71L) the frequency of infection of cells expressing Arf1 was normalised to the results for the non-expressing cell population (P values: * <0.05, ** <0.01, *** <0.001).

5.5 Discussion

The experiments in this chapter investigated the role of Arf1 in FMDV infection. Figure 5.1 shows that expression of the wt Arf1-GFP did not appear to cause major alterations to membranes in the early secretory pathway. In contrast, expression of dominant negative Arf1 T31N-HA resulted in a reduction of ER labelling, and dispersal of the peri-nuclear region of the ERGIC and Golgi labelling, which suggests this dn protein is a potent inhibitor of the early secretory pathway (Fig 5.2). Expression of the constituently active Arf1, dn Arf1 Q71L-GFP did not appear to alter the Golgi membranes but did reduce ER labelling and disperse the peri-nuclear region of the ERGIC (Fig 5.3). This particular dn is thought to block retrograde transport at the ERGIC and Golgi stack (Dascher and Balch, 1994), which may account for the observed alteration to ERGIC labelling, while the Golgi remained intact. A loss of ER labelling in cells expressing both dn proteins suggests the recycling of proteins back to the ER from the Golgi and ERGIC is inhibited.

In experiments investigating the effect of dn Arf1 on FMDV infection it appeared that expression of dn Arf1 T31N-HA enhanced the frequency of FMDV infection by over two fold, whereas expression of wt Arf1-GFP or dn Arf1 Q71L-GFP did not have any significant effect on infection (Fig 5.6 [D]). The dn Arf1 T31N-HA has an affinity for GDP, so remains inactive. This results in a block to protein transport as Arf1 cannot be recruited to Golgi/ERGIC membranes, and formation of COPI vesicles is inhibited. An increase in FMDV infection suggests that the activity of Arf1 is not required for infection, and this inactivity and/or block in transport may be advantageous for virus replication. Expression of this particular dn protein would prevent COPI formation however COPII transport is not thought to be affected. This could suggest that

prevention of COPI formation may be advantageous to infection. Expression of dn Arf1 Q71L did not appear to affect FMDV infection to any great extent, which may suggest that FMDV infection is still able to proceed regardless of a block in transport between the ERGIC and Golgi. These findings also strengthen the argument that recruitment of the COPI complex to membranes is not required for FMDV replication.

In contrast to FMDV, a dramatic reduction of BEV infection was observed in cells expressing dn Arf1 T31N-HA. BEV is sensitive to BfA treatment (Chapter 3, Fig 3.9) and therefore, like PV, may rely on the recruitment of Arf1 to membranes to form replication complexes. The results presented in this chapter would support this conclusion. A reduction to BEV infection was also observed in cells expressing the wt and dn Arf1 Q71L (Fig 5.3) versions of the protein. It is possible that over-expression of wt Arf1 may be producing a dominant negative effect on the endogenous Arf1; as the inhibitory effects of the wt and dn Arf1 Q71L on BEV were similar. The dn Arf1 Q71L remains GTP-bound and constituently active on Golgi and ERGIC membranes, this would result in a depletion of unbound Arf1 in the cytoplasm which could explain the inhibition of BEV infection.

In agreement with the results for expression of dn Arf1 T31N-HA in IBRS-2 cells, an increase in FMDV infection was also observed in MAX cells, supporting the finding that a loss of Arf1 activity is advantageous to FMDV infection. However the observed increase was not as dramatic as that observed in IBRS-2 cells. This may be due to different levels of expression between the cell lines. There appeared to be a stronger expression in IBRS-2 cells which was characterised by localisation of the dn to the plasma membrane (Fig 5.5 [A-C]). Whereas in MAX cells, the expression pattern appeared diffuse

throughout the cell and localisation of the dn protein to the plasma membrane was rarely evident (Fig 5.15 [A-C]). In contrast to IBRS-2 cells, an inhibition to FMDV infection was observed in cells expressing wt Arf1-GFP and dn Arf1 Q71L-GFP. This may be due to stronger expression levels of these proteins in MAX cells. It cannot be ruled out that the continual recruitment of the COPI complex to membranes may be resulting in a loss of free membranes which FMDV may rely on for replication, rather than a direct interaction with Arf-GTP itself. In addition it should also be taken into account that the dn Arf1 T31N possesses a very small HA tag (9 amino acids) whereas the wt Arf1 and the dn Arf1 Q71L are both fused to the much larger GFP protein (238 amino acids). The larger GFP reporter could potentially interfere with the function of the dn protein, which may explain some of the differences observed between the two dn proteins.

In conclusion these results suggest that inactivation of Arf1 enhances FMDV infection, whereas BEV infection is inhibited, highlighting the difference between BfA sensitive and insensitive viruses. This observation suggests that BEV may recruit Arf1 directly onto membranes to aid replication; however confirmation of this would require further investigation. In contrast it appears that the activity of Arf1 is not required for FMDV replication, and furthermore, inhibition of Arf1 could possibly be advantageous to (FMDV) replication.

Chapter 6: Investigating the effect of dominant-negative Rab proteins in FMDV infection

6.1 Introduction

Rab proteins are key regulatory proteins of the secretory pathway (Barr, 2009). They act as molecular switches that are converted from inactive GDP-bound to active GTP-bound states by specific GEF's (see section 1.9). Once activated, Rab proteins bind membranes and interact with specific effector molecules that carry out down-stream functions. Many Rabs are involved in the secretory pathway and regulate discrete transport steps along the pathway, for example Rab1, 2 and 6 act at the ER and Golgi and are involved in the secretory transport.

6.2 Rab1

Rab1 exists in two isoforms Rab1a and Rab1b and both are required for successful ER to Golgi transport. Evidence suggest the function of the two isoforms is interchangeable as both Rab1a and Rab1b wildtype proteins are able to antagonise the inhibitory effects of dn Rab1a and dn Rab1b on protein transport (Nuoffer et al., 1994). Rab1 and its effector molecule p115 are thought to be involved in tethering COPII vesicles to target membranes and help maintain the ERGIC (Allan et al., 2000; Alvarez et al., 1999; Bannykh et al., 1996). In accordance with this, Rab1 is seen to co-localise at the ERGIC (Plutner et al., 1991; Saraste et al., 1995). Rab 1 is also located on Golgi membranes and is thought to help recruit the COPI coat to membranes (Alvarez et al., 2003). Expression of dominant-negative versions of either isoform resulted in the fragmentation of the Golgi (Nuoffer et al., 1994; Plutner et al., 1991; Tisdale et al., 1992) or a block to protein secretion (Nuoffer et al., 1994; Wilson et al., 1994).

To investigate the role of Rab1 in FMDV infection, the dominant-negative Rab1a S25N myc was used to disrupt protein transport within the early secretory pathway. Rab1a S25N myc is constitutively bound to GDP and therefore inactive (Nuoffer et al., 1994; Wilson et al., 1994). Expression of dominant-negative Rab1a would be expected to inhibit protein transport by preventing COPI formation down-stream of the ERGIC; however COPII function is believed to remain unaltered (Alvarez et al., 2003; Tisdale et al., 1992).

6.2.1 The effects of dominant-negative Rab1 on membranes in the early secretory pathway

The effect of dn Rab1 on membranes of the early secretory pathway was investigated by immunofluorescence confocal microscopy (see methods, section 2.7). IBRS-2 cells were transfected to express either wt Rab1a or dn Rab1a S25N fused with myc tag (which allows for detection of transfected cells) for 14 hours. The cells were fixed with PFM and permeabilised with 0.1% Triton-X and blocked in goat serum prior to labelling with antibodies to the ER (PDI), the ERGIC (ERGIC-53) or the Golgi (Giantin/GM130) and the appropriate Alexa-Fluor labelled secondary antibody. Cells expressing a transgene (i.e. Rab1 protein) are shown as green on the figures.

The anti-myc and anti-ERGIC antibodies used in these studies are both mouse and could not be used to investigate the effect of dn Rab1 on ERGIC integrity. Figure 6.1 shows cells expressing wt Rab1a-myc or dn Rab1a S25N-myc (shown as green in panels B & D) and labelled for the Golgi (Giantin, shown in red). Wildtype Rab1a-myc appears to co-localise with the Golgi which remains intact in cells expressing the transgene (Fig 6.1 A-B). Expression of dn Rab1a S20N-myc appears to disperse Golgi labelling from its peri-nuclear location (Fig 6.1 C-D). The disruption of Golgi membranes in cells expressing

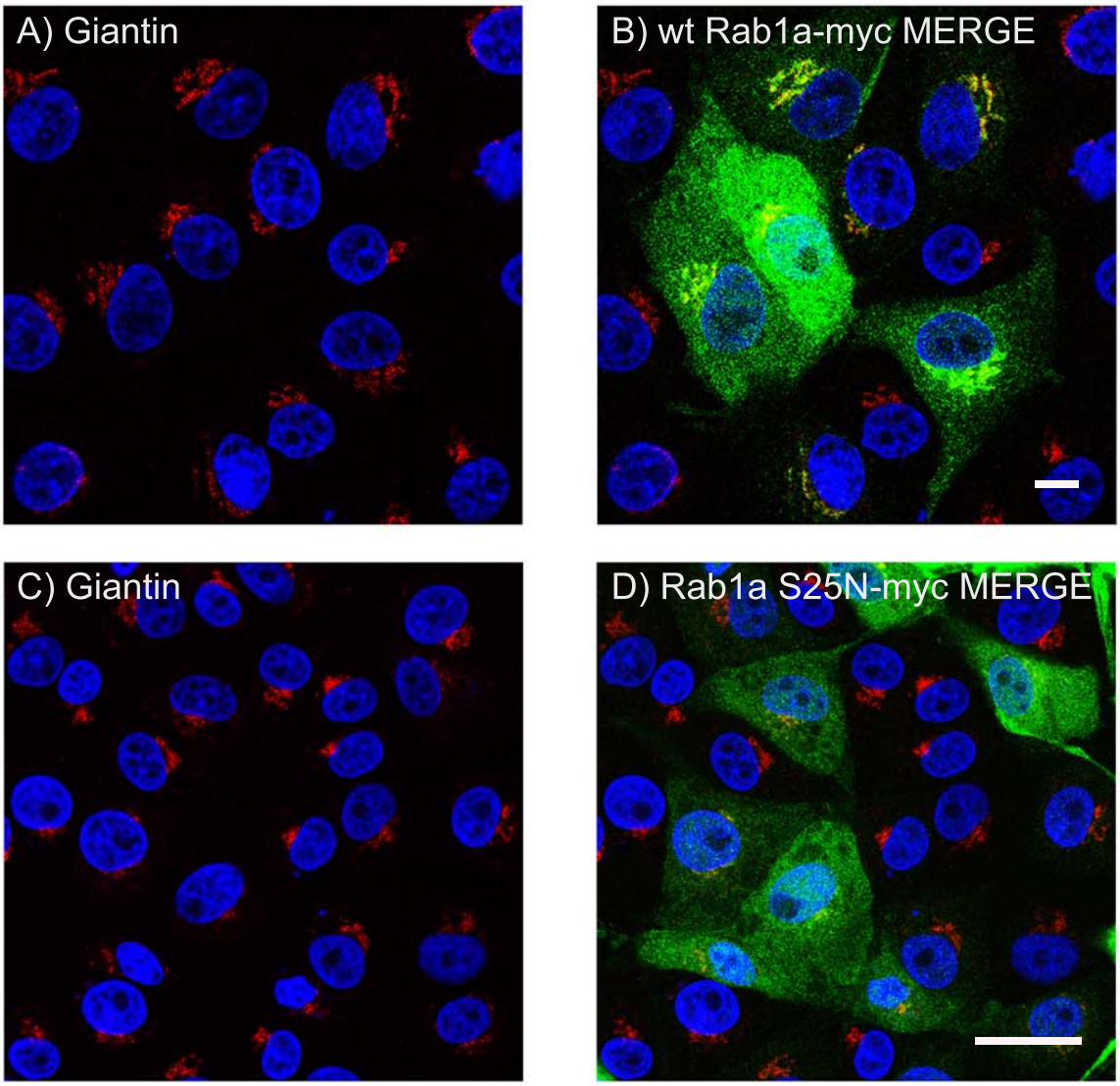


Figure 6.1 The effects of Rab1 expression on the Golgi
IBRS-2 cells were transfected to express wt Rab1a-myc (panels A-B) or dn Rab1a S25N-myc (panels C-D) for 14 hours and labelled for the Golgi (Giantin, panels B & D). Cells expressing Rab1 are shown as green; the Golgi is shown in red. Panels B and D showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

the dn Rab1a S25N-myc suggests this protein is successfully blocking transport within the early secretory pathway.

6.2.2 The effect of dominant-negative Rab1 on FMDV infection

In the next experiment the effect of dn Rab1 on FMDV infection was investigated. IBRS-2 cells were transfected with either wt Rab1a-myc or dn Rab1a S25N-myc for 14 hours. The transfected cells were then infected with FMDV O1Kcad$_2$ at a low MOI (MOI. of 0.5). Infection was allowed to proceed for 4 hours before the cells were fixed and processed for IFCM as previously described (see section 4.2.2). Infected cells are shown as red on the figures.

Figure 6.2 shows cells expressing wt Rab1a-myc or dn Rab1a S25N-myc (shown in green, panels B and E) and infected with FMDV (shown in red, panels A and D). There was no obvious change to the frequency of infection in cells expressing wt Rab1a-myc (Fig 6.2, merge, panel C) when compared to the non-expressing population, however an increase in infection levels was observed in cells expressing the dn Rab1 protein (Fig 6.2 merge, panel F). This suggested that expression of dn Rab1a S25N myc enhanced FMDV infection.

The above experiments were repeated three times and the effect of the dn Rab1a proteins on infection quantified. The frequency of infection was calculated for the cells expressing a Rab1 transgene (wt Rab1a, dn Rab1a S25N) and the data normalised to the results for the non-expressing cell population of the same cover-slip. Figure 6.3(A-C) shows the results for three independent experiments and the mean of the three experiments is shown in figure 6.3(D). Figure 6.3(A) shows ~ 20% reduction in the frequency of infection in cells expressing wt Rab1a-myc, whereas ~25% increase in infection is seen in cells expressing dn Rab1a S25N-myc. The second experiment (Figure 6.3 B)

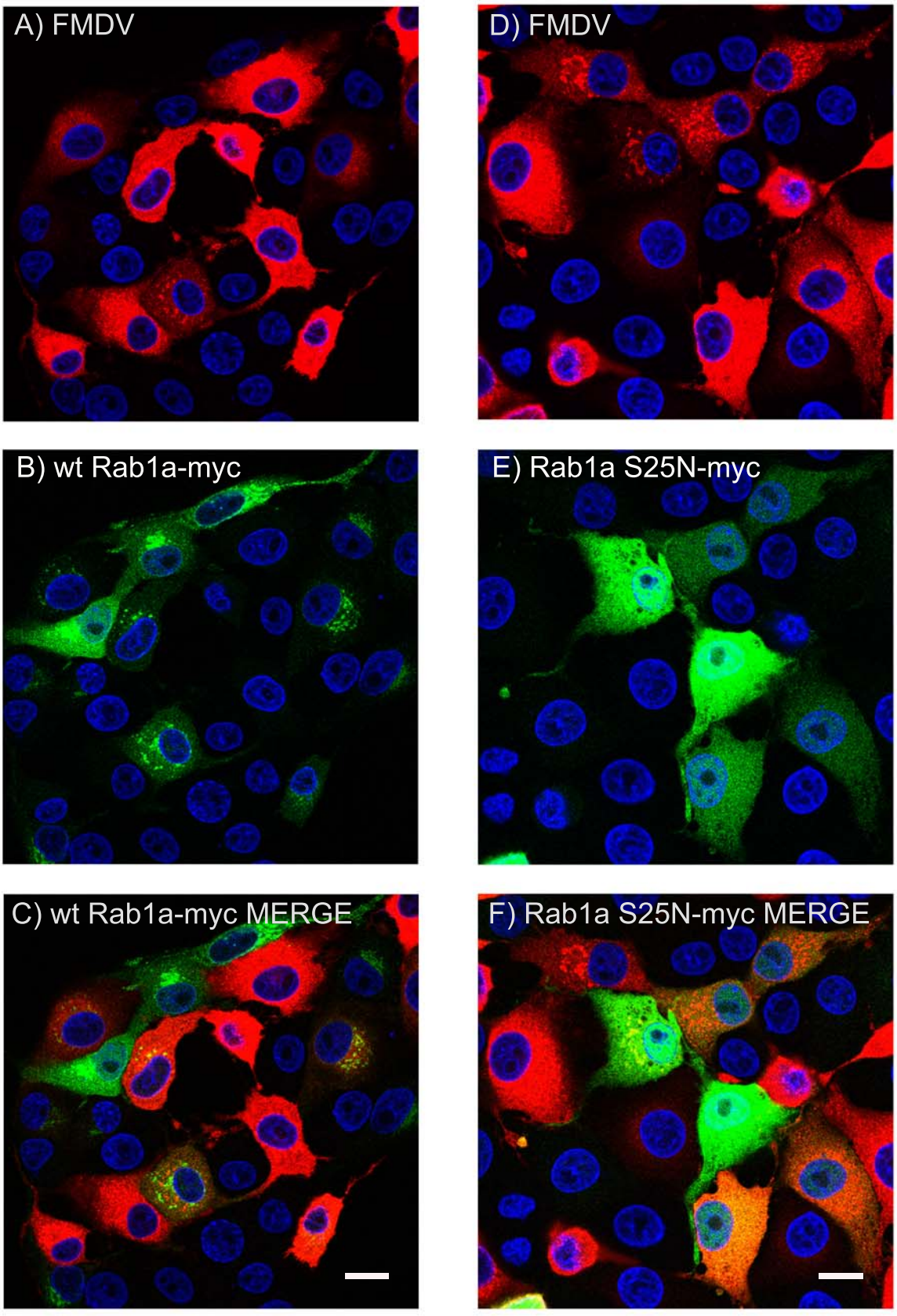


Figure 6.2 FMDV Infection of cells expressing wt or dn Rab1a (S25N)
IBRS-2 cells were transfected to express wt Rab1a-myc (A-C) or dn Rab1a S25N-myc (D-F) for 14 hour**s** and then infected with FMDV. Cells expressing Rab1a are shown as green. Infected cells are shown as red. Panels A and D show labelling for FMDV. Panels B and E show the signal for Rab1a-myc. Panels C and F showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.

		Transfection efficency (%)	% Infected cells
Mock			**27**
Rab1a wildtype myc	**Expressing**	37	26
	Non-expressing	63	30
Rab1a S25N myc	**Expressing**	30	36
	Non-expressing	70	29

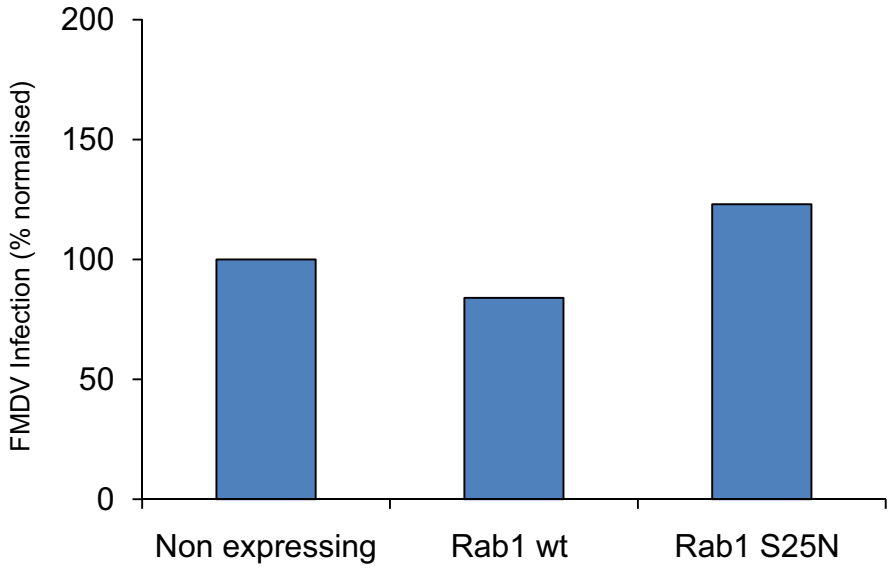


Figure 6.3(A): Experiment 1. The effects of dominant-negative Rab1 on FMDV infection of IBRS-2 cells
IBRS-2 cells were transfected to express wt Rab1a-myc or dn Rab1a S25N-myc for 14 hours and then infected with FMDV. *Upper Table*: For each transfection, the frequency of infection (% infected cells) was determined for the cells expressing the Rab1 transgene (i.e. green expressing cells) and for the non-expressing cell population (Non-expressing cells). The percentage of cells expressing a transgene is also shown (Transfection efficiency). *Lower Figure*: For each transfection, the frequency of infection of cells expressing Rab1 was normalised to the results for the non-expressing cell population.