RESEARCH ARTICLE

Genome packaging of reovirus is mediated by the scaffolding property of the microtubule network

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Summary
Reovirus replication occurs in the cytoplasm of the host cell, in virally induced mini-organelles called virus factories. On the basis of the serotype of the virus, the virus factories can manifest as filamentous (type 1 Lang strain) or globular structures (type 3 Dearing strain). The filamentous factories morphology is dependent on the microtubule cytoskeleton; however, the exact function of the microtubule network in virus replication remains unknown. Using a combination of fluorescent microscopy, electron microscopy, and tomography of high-pressure frozen and freeze-substituted cells, we determined the ultrastructural organisation of reovirus factories. Cells infected with the reovirus microtubule-dependent strain display paracrystalline arrays of progeny virions resulting from their tiered organisation around microtubule filaments. On the contrary, in cells infected with the microtubule-independent strain, progeny virions lacked organisation. Conversely to the microtubule-dependent strain, around half of the viral particles present in these viral factories did not contain genomes (genome-less particles). Complementarily, interference with the microtubule filaments in cells infected with the microtubule-dependent strain resulted in a significant increase of genome-less particle number. This decrease of genome packaging efficiency could be rescued by rerouting viral factories on the actin cytoskeleton. These findings demonstrate that the scaffolding properties of the microtubule, and not biochemical nature of tubulin, are critical determinants for reovirus efficient genome packaging. This work establishes, for the first time, a functional correlation between ultrastructural organisation of reovirus factories with genome packaging efficiency and provides novel information on how viruses coordinate assembly of progeny particles.

KEYWORDS
microtubules, Reovirus, virus assembly and packaging, Virus factory

1 INTRODUCTION

All positive-strand RNA viruses replicate in the host cytoplasm (Nagy, Strating, & van Kuppeveld, 2016). For these viruses, the cytoplasm represents a significant barrier as it induces the dilution of viral components limiting virus replication and assembly (Radkte, Dohner, & Sodeik, 2006). Therefore, RNA viruses have evolved to overcome this barrier by remodelling and repurposing host cellular elements to promote virus life cycle. The most commonly used cellular element is the endoplasmic reticulum (ER) (Romero-Brey & Bartenschlager, 2016). However, it is not the only source of membranes exploited by viruses. Lysosomes-, mitochondria-, and Golgi-derived membranes are also co-opted during RNA virus replication (Miller & Krijnse-Locker, 2008; Nagy et al., 2016). Collectively, these virus-derived compartments are known as replication organelles or virus factories (VFs).

The putative functions of VFs are (a) the protection of the viral genome from host innate immune recognition, (b) the concentration of viral proteins within discrete locations, (c) to coordinate the different steps of the viral replication cycle, and finally, (d) to provide a scaffold on which progeny virions can be assembled (reviewed in Harak & Lohmann, 2015; Miller & Krijnse-Locker, 2008; Nagy et al., 2016; Novoa et al., 2005). The best characterised examples are hepatitis C virus (HCV)
and dengue virus (Romero-Brey et al., 2012; Welsch et al., 2009), in which non-structural viral proteins participate in the remodelling of intracellular membranes. For instance, the HCV non-structural proteins remodel ER membranes, which results in the formation of a structure called the membranous web, which is composed of single and double membrane vesicles of varying sizes (Egger et al., 2002; Romero-Brey et al., 2012). Similar to HCV, Dengue viruses (DENVs) non-structural proteins also induce massive ultrastructural organisation of the host endomembrane system (Miller & Krijnse-Locker, 2008; Welsch et al., 2009). As such, virally modified cellular membranes constitute an essential building block necessary for the replication of virtually all positive-strand RNA viruses. In contrast, much less is known about negative-stranded and double-stranded RNA viruses also replicating in the cytoplasm.

Indeed, for many negative-stranded viruses such as Ebola virus (Baskerville, Fisher-Hoch, Neild, & Dowsett, 1985; Geisbert & Jahrling, 1995) and rabies virus (Lahaye et al., 2009), the VFs are composed primarily of viral proteins in which the involvement of cellular membrane appears less critical if not membrane independent. These VFs are formed in discrete locations within the infected cell. Similarly, viruses belonging to the family of Reoviridae such as reovirus (Broering, Parker, Joyce, Kim, & Nibert, 2002) and rotavirus (Silvestri, Taraporewala, & Patton, 2004) assemble VFs where the essential building block appears to be strictly made of viral proteins. How these membrane-independent VFs coordinate virus replication, particle assembly, genome packaging, and escape from the innate immune system is even less understood compared to the prototype membrane-based VFs.

Mammalian orthoreoviruses (MRV) are a group of non-enveloped, icosahedral viruses that belong to the family of Reoviridae. MRV are characterised by the presence of multi-segmented genome that is enclosed by concentric layers of protein shells (Metcalf, Cyrklaflf, & Adrian, 1991). The outermost coat proteins mediate the viral attachment to cellular receptors (Stettner, Dietrich, Reiss, Dermody, & Stehle, 2015) and are responsible for the penetration of the host membrane barrier (Chandran & Nibert, 2003; Danthi et al., 2010). The innermost coat proteins form the core of the virus particles. The reoviral core protects the viral genome from host degradation mechanisms. More importantly, once in the cytoplasm, the core initiates the virus replication cycle by de novo transcribing the daughter mRNA strands using the packaged double-stranded RNA genome segments as template (Shatkin & LaFlianda, 1972; Watanabe, Millward, & Graham, 1968).

The non-structural protein μNS is an 80 kDa protein translated from the M3 gene segment and is responsible for reovirus VF formation. Expression of μNS alone is sufficient to induce VF-like structures into cells (Broering et al., 2005). This function was mapped to the very C-terminal domain of μNS as expression of the last 250 amino acid stretch has been shown to be sufficient for VF formation (Broering et al., 2005). The N-terminal 221 amino acids of μNS were shown to be responsible for the formation of a complex viral protein network by interacting with all viral structural and non-structural proteins (Miller, Arnold, Broering, Hastings, & Nibert, 2010). As such, μNS constitutes the functional backbone of reovirus VFs. Most MRV VFs, including the prototype strain type 1 Lang (T1L; Ramos-Alvarez & Sabin, 1954), manifest as filamentous structures in infected cells (Broering et al., 2002; Parker, Broering, Kim, Higgins, & Nibert, 2002). However, VFs of the laboratory prototype strain type 3 Dearing (T3D) display globular VFs in infected cell's cytoplasm. These strain differences do not reside in μNS as expression of either T1L or T3D μNS alone results in the formation of globular VF-like structures. Instead, this morphology strain specificity was mapped into the minor structural protein μ2 (Broering et al., 2002; Parker et al., 2002). When μNS is co-expressed with the minor structural protein μ2 of T3D, the VF-like structures organise as globular VF-like structures. On the contrary, when expressed with T1L μ2, the VF-like structures assume a filamentous morphology (Broering et al., 2002; Parker et al., 2002). It was shown that these filamentous VF were colinear with microtubules and that μ2 of T1L acts as an “accessory” protein, anchoring the VF to the cytoskeleton by directly binding microtubules. The interaction of μNS with μ2 was mapped to the first N-terminal 41 amino acids of μNS (Miller et al., 2010). The strain difference in the ability of μ2 to bind microtubules is mapped to single point mutation at amino acid position 208. Indeed, substitution of the phenylalanine 208 to serine (F208S) in T1L strain's μ2 completely abrogates its association with microtubules (Parker et al., 2002). Despite the point mutation in T3D μ2, virus replication is unimpaired. However, given the observation that the great majority of reovirus strains exhibit microtubule-dependent VFs and that only a small subset including the laboratory prototype strain T3D reovirus grow independent of this association, we hypothesised that microtubule association may confer a replication advantage for reovirus.

Here, using a combination of fluorescent and electron microscopy, we investigated how reovirus VF association with microtubules participate and promote reovirus replication. We characterised its function in virus assembly and genome packaging. Additionally, by using gain-of-function experiments, we addressed whether the biochemical or the scaffolding nature of the microtubule network is important for reovirus replication.

## 2 RESULTS

### 2.1 Temporal characterisation of reovirus factory formation in BSC-1 cells

The functions of reovirus factory association to the microtubule cytoskeleton remain unclear. To investigate whether this association could confer an advantage to reovirus during infection, we compared two reovirus strains that are either associated (T1L) or not (T3D) with the microtubule cytoskeleton. We first characterised the dynamics of VF formation during the course of reovirus infection. BSC-1 cells were infected with T1L or T3D reovirus, and the kinetics of VF formation was addressed at different times postinfection using fluorescence confocal microscopy. VFs were detected by immunostaining against the non-structural proteins μNS. Reovirus T1L VFs manifested as small bright speckles starting at 5 hr postinfection (hpi). The speckles resolved into foci by 8 hpi and attained filamentous morphology by 12–18 hpi (Figure 1a, top panel and Figure 1b). For the reovirus T3D VFs, at 5 hpi, μNS had a diffuse distribution in the cytoplasm that was reminiscent of the ER. This diffuse staining resolved into speckles followed by foci and finally distinct larger globules by 18 hpi (Figure 1a bottom panel and Figure 1b). We quantified the appearance of the different morphologies of reovirus VFs as a function of time and observed that the switch to the final pattern was obtained around 12 hpi for both strains (Figure 1b). The observed filaments in reovirus
T1L-infected cells were reminiscent of the microtubule network (Figure 1c). Using antibodies directed against tubulin, a perfect colocalization between microtubules and the reovirus T1L filamentous VFs was not obviously observed. It was previously proposed that μNS coating the microtubules could result in the masking of the tubulin epitope (Parker et al., 2002). To test this hypothesis, we labelled the microtubules (MTs) by overexpressing tubulin fused to the green fluorescent protein (GFP; microtubule targeted GFP, MT-GFP). Using this approach, we could observe the colocalization between T1L μNS and microtubule targeted GFP, whereas immunostaining against tubulin failed to demonstrate the colocalization (Figure S1).

Taken together, these results show that reovirus VF formation initiates from discrete speckle-like structures that grow in size over time to form a final pattern. The reovirus T3D strain forms globular VFs that are not associated with microtubules, whereas the T1L strain assembles filamentous VFs in a microtubule-dependent manner.

2.2 Ultrasctructural organisation of microtubule-dependent and -independent reovirus factories

Next, we aimed at analysing the possible association of the reovirus VF with microtubules at the ultrastructural level by transmission electron microscopy.
microscopy (TEM). Analysis of multiple infected cells at times post-infection ranging from 12 to 24 hpi revealed that T1L reovirus factories present as contiguous stretches of electron dense inclusions with progeny virions arranged in paracrystalline arrays. These virus particles were organised in a parallel manner along densely coated microtubule filaments (Figure 2a, inset, red arrowhead). The densely coated microtubules appeared thicker (~50 nm) than normal. The reason for their markedly thicker diameter is thought to be the result of the accumulation of μ2 and μNS and other viral proteins around microtubule filaments (Broering et al., 2002; Dales, 1963; Miller et al., 2010; Parker et al., 2002; Fields et al, 1971, Mora et al, 1987, Spendlove et al, 1963). Indeed, in reovirus T3D-infected cells, where VFs are not associated

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**FIGURE 2** Ultrastructural characterisation of type 1 Lang (T1L) and type 3 Dearing (T3D) reovirus virus factories (VFs). (a–c) BSC-1 cells were infected with reovirus T1L or T3D. At 24 hpi, cells were fixed and observed using transmission electron microscopy (TEM). (a) Ultrathin section EM analysis of T1L and T3D reovirus-infected cells. Microtubule filaments (green arrow, inset), μ2-coated microtubule filaments (red arrow, inset), genome-containing particles (yellow arrow, inset), and genome-less particles (blue arrow, inset). Scale bar = 0.5 μm. (b) Three-dimensional ultrastructural characterisation of reovirus VFs using TEM tomography. Tilt-series and three-dimensional reconstruction were performed as described in Section 4. (Upper panel) One representative virtual slice of the VF three-dimensional reconstruction (Movies S1 and S2). Microtubule filaments (green arrow, inset), μ2-coated microtubule filaments (red arrow, inset), genome-containing particles (yellow arrow, inset), and genome-less particles (blue arrow, inset). Scale bar = 0.1 μm. (Lower panel) Three-dimensional reconstruction of T1L and T3D reovirus VFs. Microtubule filaments (green), μ2-coated microtubule filaments (red), genome-containing particles (yellow), genome-less particles (blue), and endoplasmic reticulum membrane (pink). Inset regions highlight the spiral organisation of reovirus T1L particles around μ2-coated microtubule filaments. (c) The percentage ratio of genome-containing to genome-less particles was quantified by ultrathin section TEM. Average and standard deviation are shown. Experiment was performed in triplicate. The number of genome-containing and genome-less particles in VFs was quantified from 10 sections each for T1L- and T3D-infected cells. Ten thousand nine hundred thirteen genome-containing (T1L = 8,666 and T3D = 2,247) and 1,472 (T1L = 139 and T3D = 1,333) genome-less particles were counted.
with microtubules, they display the expected size of ~20 nm (Figure 2a, inset green arrowhead). Moreover, in reovirus T3D-infected cells, progeny virions are randomly ordered within the matrix of the VF where numerous large cytosolic inclusions are observed (Figure 2a).

To better determine the ultrastructural organisation of the VFs and the three-dimensional arrangement of viral particles, we performed electron tomography of reovirus T1L- and T3D-infected BSC-1 cells. In reovirus T1L VFs, viral particles are arranged in a tiered manner organised spirally around microtubules (Figure 2b, inset and Movie S1). In contrast, in reovirus T3D-infected cells, progeny virions in VF had no obvious organisation. Interestingly, ER-derived membranes were often found (80%) in close apposition to VFs as well as to non-coated MT filaments (Figure 2b). The average distance between the ER and the VFs was 530 nm +/- 327 nm, thus confirming earlier findings on reovirus T3D VF morphology (Fernández de Castro et al., 2014). In both strains, two species of virus particles were observed, electron dense particles (Figure 2a,b, yellow arrow head) and electron lucent particles (Figure 2a,b, blue arrow head). These particles correspond to genome-containing particles and empty, genome-less particles, respectively (Dales, Gomatos, & Hsu, 1965; Ooms, Jerome, Dermody, & Chappell, 2012). The proportion of genome-containing and genome-less particles was quantified for both strains at 16 hpi using TEM. We observed that nearly 100% of the progeny virus particles produced during reovirus T1L infection corresponded to genome-containing virions. On the contrary, only 50% of the particles contained genome in the context of reovirus T3D infection (Figure 2c).

Taken together, our results highlight major differences between T1L and T3D reovirus in the ultrastructure of their VFs and in the ultrastructural organisation of the progeny virions within these VFs. More importantly, our observations suggest strain differences in the efficiency of reovirus genome packaging.

2.3 Correlation between linearity of the microtubule network and genome packaging efficiency

To investigate the origins of these strain differences, BSC-1 cells were infected with a multiplicity of infection of 1 of either T1L or T3D reovirus, and the efficiency of genome packaging was addressed during the course of reovirus infection using TEM and by direct purification of viral particles. TEM montages of reovirus-infected cells were collected to quantify the proportion of genome-containing and genome-less virus particles in the whole cell. In T1L reovirus-infected cells, 1 day postinfection (dpi), VFs display mostly genome-containing particles arranged in paracrystalline arrays (Figures 3a,b and S2). In reovirus T3D-infected cells, an equal proportion of genome-containing and genome-less particles were observed (Figure 3a). These observations were in full agreement with our thin section TEM analyses (Figure 2c). Unexpectedly, although the ratio of genome-containing and genome-less particles did not change at 2 dpi for T3D reovirus-infected cells, this ratio was significantly different in reovirus T1L-infected cells. We observed a significant increase in the proportion of genome-less particles from 1 to 2 dpi along with a reduction in genome-containing particles (Figure 3b). Quantification of the TEM montages revealed that VFs displaying paracrystalline arrays of genome-containing particles represented around 70% of the total area occupied by VFs in cells infected by T1L reovirus at 1 dpi (Figure S2a, left panel, green area and S2c). Interestingly, at 2 dpi, T1L-infected cells show a dramatic change of their VFs with only around 30% of the VFs having paracrystalline organisation of genome-containing particles (Figure S2a, right panel, green area and S2c). No difference was observed for T3D-infected cell in which all VFs show lack of organisation at both 1 and 2 dpi (Figure 3 and data not shown). To better address whether genome packaging efficiency decreases over the course of infection, we purified viral particles from cells infected with either T1L or T3D reovirus on a caesium chloride gradient. We quantified the relative proportion of viral particles containing genome versus genome-less particles at 1 and 2 dpi. When purified on a caesium chloride gradient, reovirus particles segregate into two distinct bands, top and bottom bands, that are well known to correspond to genome-less and genome-containing particles, respectively (Fernández de Castro et al., 2014). The results of the quantification demonstrate that the filamentous T1L strain produces more genome-containing virus than genome-less virus at 1 dpi. However, by 2 dpi, significantly more genome-less particles are isolated. Comparatively, the relative ratio of virus particles isolated from the top and bottom fractions of the globular T3D strain was unaffected from 1 to 2 dpi (Figure 4c).

Taken together, our results suggest that reovirus T1L strain is more efficient in genome packing within the first day of replication compared to T3D. However, at 2 dpi, genome packaging appears partially impaired, but the origin of this decreasing efficiency remains unclear. Interestingly, detailed analysis of our TEM montages revealed that, even at 1 dpi, genome-less particles were observed specifically in regions of the cell where the microtubules were not linear (Figure S2a,b). Similarly, in regions where no discernible microtubules were detected, the paracrystalline order of the particles was disrupted, and this was concomitant with the appearance of genome-less particles (Figure S2a,b). Complementarily at 2 dpi, although global quantification shows a great proportion of genome-less particles in T1L reovirus-infected cells (Figure 3b), local analysis by TEM revealed the presence of continuous stretches of paracrystalline genome-containing particles at proximity to linear microtubules (Figure S2a,b).

All together, these observations strongly suggest that genome packaging efficiency, for T1L reovirus, directly correlates with the presence of microtubules. The linearity of the microtubules appears to be critical to support efficient genome packaging.

2.4 Microtubules are key determinants for efficient genome packaging

To address the function of microtubules in promoting genome packaging during reovirus T1L infection, we disrupted the microtubule network using nocodazole. At the onset of reovirus VF formation at 6 hpi, (Figure 1a), cells were treated with nocodazole. At 10–16 hr post-treatment, cells were fixed and analysed by immunofluorescent staining and TEM. In nocodazole-treated cells, T1L reovirus VFs lose their filamentous nature and phenocopy the globular T3D reovirus VFs (Figure 5a). No significant modification of the globular organisation was observed for T3D reovirus-infected samples in the presence or absence of the drug (Figure 5a). Comparative TEM analysis of ultrathin
sections of cells infected in the presence or absence of nocodazole confirmed the impact of microtubule disruption on reovirus VFs (Figure 5b). In the presence of nocodazole, both the filamentous nature of reovirus T1L VF and the tiered paracrystalline organisation of the viral particles were lost. All T1L VFs, in nocodazole-treated cells, lose their typical paracrystalline ultrastructural organisation. Importantly, when microtubules were disrupted, a higher incidence of genome-less particles was observed in reovirus T1L-infected cells as compared to dimethylsulfoxide-treated cells (Figure 5b). No significant difference was observed for reovirus T3D-infected samples in the presence or absence of nocodazole (Figure 5b). To estimate the extent to which the microtubule disruption reduced the amount of genome-containing particles, we quantitated the ratio of genome-containing to genome-less virus particles. Unlike mock-treated reovirus T1L-infected cells, where nearly 100% of the viral particles contained genome, disrupting the microtubule network with nocodazole reduced the genome-
containing particles in half (Figure 5c). As expected, this ratio remained unchanged for reovirus T3D infected cells in the presence or absence of nocodazole (Figure 5c). Because disruption of the microtubule network was associated with significant accumulation of genome-less particles, we hypothesised that this increase was associated with a decrease in the number of infectious particles produced. To test this, BSC-1 cells infected with either TIL or T3D in the presence or absence of nocodazole were lysed, and the amount of infectious virus particles produced was titrated on naïve cells. We observed that disruption of the microtubule network induces a 50% reduction of the amount of infectious particles produced compared to mock-treated cells (Figure 5d). No significant differences were observed with T3D reovirus (Figure 5d).

Taken together, our results strongly suggest that the association of T1L reovirus with the microtubule network has an advantageous effect on T1L reovirus replication by promoting better genome packaging and as such producing more infectious viral particles.

2.5 Virus factories can be induced to grow on actin filaments

The observation that genome packaging efficiency decreases when microtubules lose their linearity (Figure S2a,b) suggests that the scaffolding properties provided by the microtubule network are key to promote genome packaging. To test this hypothesis, we decided to uncouple reovirus VFs from microtubules and reroute them onto a different cellular scaffolding network: the actin cytoskeleton. To this end, we fused the actin binding peptide Lifeact, a 17 amino acid peptide sequence derived from the actin binding protein Abp140 (Riedl et al., 2008), to μNS (μNS-Lifeact). Although expression of μNS alone resulted in the formation of globular VF-like structures, expression of μNS-Lifeact manifested with the formation of filamentous structures that were colinear with actin filaments and mainly localised to regions rich in cortical actin (Figure S3).

BSC-1 cells expressing either μNS-Lifeact or wild-type (wt) μNS were infected with T1L reovirus. In cells overexpressing μNS, VFs had the classical filamentous organisation and were colinear with microtubules (Figure 6). Of note, larger globular structures were observed compared to cells infected only with T1L reovirus. These globular structures are likely due to the overexpression of μNS favouring VFs to fuse together. Interestingly, in cells expressing μNS-Lifeact, we detected hybrid VFs that associated with the microtubules as well as with the actin cytoskeleton (Figure 6 DMSO panel and inset). Microtubule filaments in these factories were poorly detectable, very likely due to the occlusion of the tubulin epitope by the μNS protein (Figure S1). Therefore, to uncouple VFs from microtubule filaments
and force VFs to assemble on actin, we treated T1L reovirus-infected cells with nocodazole. As expected, disruption of microtubules resulted in the formation of globular VFs (Figure 6). Overexpression of μNS did not affect VF morphology (Figure 6). Conversely, when μNS-Lifeact was overexpressed, large actin bundles, positive for μNS, were observed in T1L-infected cells (Figure 6 nocodazole panel and inset). Such large actin bundles were not observed in cells expressing only μNS-Lifeact (Figure S3) suggesting that this bundling was induced by reovirus infection, likely due to the additional endogenous μNS proteins driving actin bundling.

To address the impact of actin-driven VF formation on virus assembly and genome packaging, we performed correlative light and electron microscopy to unambiguously identify and ultrastructurally characterise VFs that were assembled on actin filaments (Figure S4). Both T1L-infected cells expressing or not μNS-Lifeact were identified by fluorescence microscopy and further observed by TEM using correlative approaches. As expected, ultrastructural analyses revealed that unperturbed T1L-infected cells display VFs containing mostly genome-containing viral particles (Figure 7a,b). As reported above (Figure 5), disruption of the microtubule network severely impaired genome packaging efficiency (Figure 7a,b). Importantly, when T1L reovirus VF were assembled on the actin cytoskeleton scaffold, we found that around 90% of the virus particles present in the actin-based VFs contained genome (Figure 7a,b). Interestingly, although genome
packing was rescued when VFs were assembled on the actin scaffold, the paracrystalline arrangement of the daughter virions was not rescued. To fully demonstrate that the scaffolding properties of the cytoskeleton promote reovirus assembly and genome packaging, we generated a mutant of μNS-Lifeact that lost ability to bind actin filaments. We substituted both the leucine and the isoleucine at positions 13 and 14 with proline, which resulted in the loss of the critical alpha-helix responsible for actin binding (Figure S5). These substitutions abrogated the ability of the μNS-Lifeact to bind actin and resulted in the formation of globular VFs similar to wt-μNS as confirmed by immunofluorescence analysis (Figure S5). TEM was performed in cells expressing the μNS-Lifeact-null construct and infected with T1L reovirus. As expected, VFs were not found associated to actin filaments, and reovirus particles were observed in globular VFs with ~50% of the particles being genome-less (Figure 7a,b).

Taken together, these results demonstrate that efficient virus assembly and genome packaging can be rescued by rerouting VF on actin filaments.
DISCUSSION

The observation that some strains of reovirus assemble VFs in a microtubule-independent manner and the fact that mutation of the minor protein \( \mu_2 \) renders VF microtubule-independent without significantly affecting reovirus replication lead to the conclusion that \( \mu_\text{NS} \) association to microtubule has a moderate role in the reovirus life cycle. Here, using a combination of fluorescence and advanced electron microscopy approaches, we reveal that reovirus VFs hijack the microtubule cytoskeleton scaffold to coordinate and promote virus assembly and genome packaging events. We show that disruption of the microtubule network severely impacts genome packaging efficiency and that this could be rescued by rerouting VFs to the actin cytoskeleton. As such, we demonstrate that the scaffolding properties of the microtubule, and not the biochemical nature of tubulin, are critical determinants for reovirus assembly and genome packaging. This work provides, for the first time, functional evidence correlating the ultrastructural organisation of reovirus VFs with virus assembly and genome packaging efficiency.

All RNA viruses rely on the formation of VFs that act as replication compartments (Nagy & Pogany, 2012). Although it is fully accepted that VFs promote viral replication by spatially concentrating/sequestering viral proteins and viral genomes and by protecting viral replication intermediates against the cellular intrinsic innate immune system, the mechanisms leading to these specific functions remain mostly unknown. To identify and characterise these mechanisms, it is mandatory to manipulate the specific function of the viral protein responsible for VF formation but without affecting both the capacity of this protein to induce the formation of these compartments and without altering other fundamental functions. In the case of reovirus, prior studies have intended to shed light on the mechanisms by which VFs promote viral replication. It was shown that specific silencing of

**FIGURE 7** Rerouting reovirus virus factories to actin filaments rescues genome packaging efficiency. BSC-1 cells were transduced with BacMAM expressing \( \mu_\text{NS-Lifeact} \), \( \mu_\text{NS-Lifeact null} \) or left untransduced. Two days post-transduction cells were infected with type 1 Lang (T1L) in the presence or absence of nocodazole. At 16 hpi, cells were fixed and observed by transmission electron microscopy (TEM). (a) Representative TEM micrographs. Scale bars = 0.5 and 0.1 \( \mu \text{m} \) (inset). (b) Quantification of the genome-containing and genome-less particles. The percentage ratio of genome-containing to genome-less particles was quantified from 5, 12, 8, and 5 ultrathin section TEM micrographs of dimethylsulfoxide (DMSO)-treated, nocodazole-treated, \( \mu_\text{NS-Lifeact} \), and \( \mu_\text{NS-Lifeact-null} \) transduced cells, respectively [as shown in (a)]. Average and standard deviation are shown. Experiment was performed in triplicate. Fourteen thousand two hundred nineteen genome-containing (DMSO = 11,660, nocodazole = 1,175, nocodazole + \( \mu_\text{NS-Lifeact} \) = 876, and nocodazole + \( \mu_\text{NS-Lifeact-null} \) = 508) and 1,838 non-genome containing particles (DMSO = 101, nocodazole = 1,125, nocodazole + \( \mu_\text{NS-Lifeact} \) = 115, and nocodazole + \( \mu_\text{NS-Lifeact-null} \) = 497) were counted. Student’s t-test *** = \( p < .001 \), ** = \( p < .01 \); n.s. = not significant
the expression of μNS and μ2 proteins during reovirus infection inhibits viral replication (Carvalho, Arnold, & Nibert, 2007; Kobayashi, Ooms, Chappell, & Dermody, 2009). This approach, by inducing a loss of VF in the case μNS silencing, demonstrated that μNS and as such, VFs were critical for reovirus replication but could not conclude on the mechanisms by which μNS protein promotes de novo virus production (Carvalho et al., 2007). In the current work, we have successfully uncoupled the association of VF with microtubules without affecting the primary function of μNS to form these replication compartments. This approach has allowed us to unambiguously conclude on the function of VF microtubule association in promoting reovirus assembly and genome packaging.

Our ultrastructural analysis on T1L and T3D reovirus-infected BSC-1 cells strongly suggest that the microtubule-dependent T1L strain packages its genome more efficiently than the globular T3D strain (Figure 2). By disrupting the microtubule network using nocodazole, we could show that VF association with microtubules promotes viral assembly and genome packaging (Figure 5). Interestingly, in the T1L strain, progeny viruses are arranged in paracrystalline arrays around microtubule filaments. This tiered organisation is possibly due to the diameter (~50 nm) of the coated microtubule that allows for this perfect symmetrical arrangement of virions around the microtubule filaments (Figure 2). Assembly of virions in a paracrystalline manner seem to be an important factor for efficient genome packaging. Indeed, in regions of the cells where the microtubule linearity was disrupted, the paracrystalline organisation was lost, and more importantly, genome packaging was severely impaired (Figure S2). It is interesting to note that this phenotype was further exacerbated at later times post-infection as the area occupied by the VF increased and lesser microtubule involvement was observed (Figures 3 and S2). The reason for the accumulation of genome-less particles late in the infection cycle is unknown, but it is tempting to speculate that the increasing size of the factory coupled with the loss of microtubule involvement could reduce the accessibility to viral and cellular factors necessary for genome packaging.

An earlier study had proposed that association of VFs to the microtubule network would provide an evolutionary advantage to reovirus by reducing the volume of the VF thus facilitating access to small molecules required for assembly and packaging (Parker et al., 2002). To directly test this hypothesis, we rerouted VFs from the microtubule network to the actin cytoskeleton through the fusion of μNS to the actin binding protein peptide Lifeact (μNS-Lifeact) and subsequently infected them with T1L. Immunofluorescence analysis demonstrated that VF assembled on bundles of actin filaments. Because we failed to detect actin bundling when only μNS-Lifeact was expressed into cells, we believe that the actin bundling observed during viral infection is likely due to the increased expression of μNS and of other protein participating in virus replication (i.e., μ2). Interestingly, TEM analyses revealed that rerouting VFs to actin was associated with a strong rescue of genome packaging efficiency (Figure 7). Using correlative light and electron microscopy approaches, we could clearly show that VFs that are assembled on actin bundles show a high ratio of genome-containing to genome-less (around 90%). On the contrary, factories that were not assembled on actin show a defect in genome packaging efficiency (Figures 7 and S4). This observation strongly supports a model where the scaffolding nature of the cytoskeleton is more important to support virus assembly and genome packaging than the biochemical nature of the cytoskeleton (tubulin). The notion that cellular factors are important to form a physical scaffold to support VF formation and virus replication has been proposed for viruses that form membrane-based VF. Flock house virus VFs can be retracted from mitochondrial membranes to ER membranes by swapping the mitochondria-binding domain of protein A (the VF-inducing protein) by an N-terminal ER targeting sequences of host cytochrome as well as HCV NS5B polymerase (Miller, Schwartz, Dye, & Ahlquist, 2003). To our knowledge, this study constitutes the first report demonstrating the importance of a cellular scaffold in promoting virus replication for a non-membrane VF. Although rerouting VF to actin could rescue genome packaging, we observed that the paracrystalline order of the virus particles was not restored. The lack of a neat three-dimensional organisation might be the result of the following: (a) the actin bundles in the VF are heterogeneous in size (Figure 7) and do not allow for paracrystalline array formation like coated microtubules do (Figures 2 and 3 and Movie S1) or (b) actin-bound VFs are premade in cells prior viral infection, and it might be necessary to assemble de novo virus particles as the VF is formed and assembled around the cytoskeleton. This observation suggests that the paracrystalline organisation is a consequence of efficient assembly and not the cause. Most importantly, it further supports the notion that the scaffolding property of the cytoskeleton network is key for efficient viral assembly and genome packaging.

We propose that reovirus VF association to microtubule helps coordinating virus assembly and genome packaging. μ2 associates with microtubules, which in turn recruit μNS, forming the building block of reovirus VFs. The association of μ2 with microtubules might represent a critical component that helps coordinating virus assembly, likely by directing the inward/outward flow from the cytosol to the core of the VF of viral and molecular compounds necessary for viral replication. μNS, via its N-terminal domain, is responsible for the formation of a complex viral protein network by interacting with all viral structural and non-structural proteins (Miller et al., 2010) and has been well demonstrated to mediate the recruitment of multiple cellular factors (Ivanovic et al., 2011). As such, μNS would co-opt together with μ2 to coordinate virus assembly. The precise principles by which these two proteins coordinate de novo virus formation and genome packaging remain to be identified. However, our results suggest that active mechanisms are established to promote efficient genome packaging and these mechanisms seem to benefit from association with a cellular cytoskeleton scaffold. Indeed, such active mechanisms coordinating genome packaging was reported for blue tongue virus (Sung & Roy, 2014), another member of the Reoviridae family.

In addition to the involvement of the microtubule scaffolding property for reovirus genome packaging, our TEM analysis revealed the presence of ER-derived membranes at proximity of reovirus VFs (Figure 2b). Quantification over multiple infected cells showed that these membranes are located on average at 500 nm from the VFs. Presence of ER membranes in close proximity to the reovirus VFs was previously reported (Desmet, Anguish, & Parker, 2014; Fernández de Castro et al., 2014). Although these membranes do not form a complex membranous web surrounding the replication compartments, like observed for HCV or dengue virus (Romero-Brey et al., 2012; Welsch
et al., 2009), multiple cisternae contact sites between the ER and the VFs were observed and proposed to be a site for incorporation of new synthesised viral protein in VFs (de Castro, Volonté, & Risco, 2013). However, how, during infection, VFs and ER membranes become juxtaposed and how this juxtaposition helps in reovirus replication remain to be determined.

Our study has not been able to uncover assembly intermediates of reovirus, thus making it difficult to exactly explain how the microtubule scaffolds coordinates genome packaging. However, our current understanding of reovirus assembly and genome packaging could be further enhanced through the use of advances in single molecule fluorescence in situ hybridisation techniques to label specific genome segments to track genome packing events (Wichgers Schreur & Kortekaas, 2016). Recent developments in cryo-electron microscopy, namely, the introduction of direct electron detectors and aberration-corrected TEMs (Bai, Fernandez, McMullan, Scheres, & Kühlbrandt, 2013) and image reconstruction (Schur et al., 2016) further coupled to cryo-correlative work flow (Kukulski et al., 2012), could help us further refine our model and help to uncover replication intermediates within an infected cell.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Cells, viruses, and antibodies

BSC-1 cells were cultured and maintained in Dulbecco’s modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). MRV strains, T1L, and T3D originally derived from stocks obtained from B. N. Fields were purified from L-cell suspensions by ultrasonic disruption of cells and freon (1,1,2-trichloro-1,2,2-trifluoroethane; Sigma) extraction and stored in virus buffer (150-mM NaCl, 10-mM MgCl2, and 10-mM Tris-HCl, pH 7.5) (Furlong, Nibert, & Fields, 1988). Reovirus μNS was detected by using an antibody against T1L-μNS peptide sequence QPFPTPSVDMQSREC in rabbit and used at 1:300 dilution for indirect immunofluorescence assay (GenScript). Tubulin was detected with monoclonal alpha-tubulin Ab (Sigma Aldrich) and was used at 1:1000 dilution. Actin was labelled with Phalloidin (Molecular Probes) conjugated to Alexa Fluor 647 and used at 1:100 dilution. Secondary IgG antibodies raised against mouse and rabbit coupled to Alexa conjugated dyes were obtained from Molecular Probes and used at 1:1000 dilution for immunofluorescence assay.

### 4.2 | Generation of μNS-Lifeact constructs

The μNS-Lifeact plasmid was generated by swapping the GFP in pEGFP-C1 plasmid with Lifeact oligonucleotide between the Age1 and BspE1 restriction sites. This plasmid was designated pLifeact. The T1L M3 gene encoding the μNS protein was cloned into the multiple cloning site of the pLifeact plasmid between the BamH1 and Xho1 restriction sites. The Gateway cloning system (Invitrogen) was used to shuffle the μNS-Lifeact gene fragment into a gateway compatible baculovirus expression system according to manufacturer instructions (Invitrogen). To generate the μNS-Lifeact-null mutant, point mutations in the μNS-Lifeact entry plasmid were introduced using the QuikChange II site directed mutagenesis kit following the manufacturer’s protocol (Agilent Technologies). The wt-μNS, μNS-Lifeact, and μNS-Lifeact-null plasmids were delivered into cells using the Virapower BacMam expression system (Life Technologies). Bacmids encoding wt-μNS, μNS-Lifeact, and μNS-Lifeact-null sequences were engineered using the Gateway system and following the manufacturer’s instructions (Life Technologies). Baculoviruses, were obtained by transfecting SF9 cells with the bacmids and passing the virus three times in SF9 cells to sufficiently high titres. Baculoviruses obtained from the third passage were used to transduce BSC-1 cells for 48 hr before infection with reovirus.

### 4.3 | Indirect immunofluorescence assay

BSC-1 cells seeded on ultraviolet sterilised glass coverslips (Thermo Fisher) were fixed either using 2% paraformaldehyde in phosphate-buffered saline (PBS) solution for 10 mins at room temperature (RT) or with cold methanol for 10 mins at −20 °C. Cells were then rinsed three times with PBS and in the case of paraformaldehyde-fixed samples permeabilised with 0.05% Triton-X100 for 10 mins at RT. Post permeabilisation cells were blocked with 1% bovine serum albumin in PBS for 30 mins at RT. Primary antibodies diluted in PBS containing 1% bovine serum albumin were applied to the samples and incubated at RT for 1 hr. Coverslips were washed three times with PBS and incubated with the secondary antibody for 1 hr at RT. Coverslips were washed extensively in PBS, rinsed once with distilled H2O and mounted in ProLong Gold Antifade mounting medium supplemented with 4,6-diamidino-2-phenylindole (Molecular Probes).

### 4.4 | Spinning disc confocal microscopy and structured illumination microscopy

Images were acquired on a PerkinElmer spinning disc confocal microscope on a Nikon Eclipse Ti-E microscope using a Nikon PlanApo 100 × 1.4 objective onto a Hamamatsu EM-CCD camera (C9100-02), as described in Stanifer et al. (2016). For super-resolution microscopy, we used a structured illumination microscope (Nikon N-SIM) using a Nikon TIRF Apo Objective with NA 1.49 as described previously (Engel, 2014). Channels were captured sequentially with single bandpass emission filters: enhanced GFP was excited with 488 nm, detected with 520/45 nm, Alexa 568 was excited with 561 nm and detected at 610/60 nm, and Alexa 647 excited with 640 nm and detected at 700/700. Images were acquired and reconstructed in NIS-Elements 4.3. On the basis of multichannel image stacks recorded on TetraSpeck beads, templates for subpixel alignment were created in NIS-Elements. In xy, a three-point registration tool (Nis-Elements channel registration) was used. The axial shift of the far-red channel was corrected by shifting it 200 nm up (away from the objective) using subpixel alignment. After correction, precision of alignment was <100 nm in xy and z. Final adjustment of contrast (maximum and minimum values displayed) and conversion to 8-bit images was performed in FIJI (Schindelin, 2008).

### 4.5 | Transmission electron microscopy

Cells seeded on coverslips and infected with reovirus were fixed in 2.5% glutaraldehyde in sodium cacodylate (CaCO) buffer (50-mM
sodium CaCO3, 2% sucrose, 1-M KCl, 100-mM CaCl2, and 100-mM MgCl2 for 30 mins at RT. Cells were then rinsed with 0.1 M Na-CaCO3 and post-fixed with 1% OsO4 (pH 7.2) on ice for 40 mins. Samples were then rinsed three times with water followed by incubation with 0.5% aqueous uranyl acetate solution for 30 mins at RT, in the dark. The samples were then dehydrated through an ethanol gradient (40%, 60%, 80%, 90%, and 96%) for 2 mins each. Following two times rinsing in 100% ethanol, samples were embedded in Epoxy resin and polymerised at 65 °C overnight. Ultratrin sections with a nominal thickness of 60 nm were obtained from the Leica Ultracut UC7 (Leica, Germany) using a diamond knife (Diatome, Biel, Switzerland) and collected on 1% Formvar coated 100 mesh copper grids (Electron Microscopy Services). The sections were then contrasted with 4% aqueous uranyl acetate for 10 mins followed by further staining in 1% Reynolds citrate solution for 5 mins. Sections were observed on Zeiss EM 900 (Carl Zeiss AG) TEM operating at 80 kV. Montaging of cells was performed on a JEOL JEM1400 (Jeol Ltd., Japan) operated at 80 kV and equipped with a 2K × 2K TVIPS (TVIPS, Gauting, Germany) EM-CCD camera. Montages were stitched from 3 × 3 images at a nominal magnification of 4000× leading to a resolution of 2.8 nm/pixel on the detector. The montaged images were then analysed in FIJI (Schindelin, 2008) and combined in Adobe Photoshop.

4.6 | Negative staining of reovirus particles

A 3 μl of purified reovirus particles were adsorbed onto freshly glow-discharged 400 mesh carbon-coated grids (Electron Microscopy Services) and stained with 3% aqueous uranyl acetate solution for 1 min. Post staining, grids were washed three times in distilled water, air dried, and imaged using the EM 900 at an accelerating voltage of 80 kV.

4.7 | High-pressure freezing, freeze substitution, and tomography

BSC-1 cells were grown on glow-discharged, carbon-coated 50-μm thick sapphire discs (Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland) in 24 wells and infected with either T1L or T3D reovirus for 18 hr. Cells were subsequently fixed with 2% glutaraldehyde in CaCO3 buffer. The sapphire discs were clamped between two aluminium planchettes soaked with 1-hexadecane (Buser & Walther, 2008; Studer, Michel, & Müller, 1989) and high-pressure frozen (HPM 010, Bal-Tec). Samples were freeze substituted in a temperature-controlling device (AFS2, Leica, Vienna, Germany) with a substitution medium consisting of acetone supplemented with 0.1% uranyl acetate, 0.2% osmium tetroxide, and 5% water over 16 hr and further processed as described previously (Höhn et al., 2011). Sections of 240 nm were cut using the Leica UC7 microtome and collected on 1% Formvar coated grids (Electron Microscopy Services) coated with 1% Formvar. Protein A coupled to 15-nm gold was adsorbed on both sides of the sections as fiducials for reconstruction. Tilt series over a range of +50° to −50° were collected on a Tecnai F20 (FEI, Eindhoven, Netherlands) with field emission gun and operated at an accelerating voltage of 200 kV. Tomograms were calculated using IMOD (Kremer, Mastronarde, & McIntosh, 1996) using the weighted back-projection algorithm. Objects from the tomograms were segmented in Microscopy Image Browser (Belevich, Joensuu, Kumar, Vihinen, & Jokitalo, 2016), and the final visualisation was performed in Amira (FEI, Eindhoven).

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SUPPORTING INFORMATION
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