Filamentous Influenza Viruses

Bernadeta Dadonaite[1], Swetha Vijayakrishnan[2], Ervin Fodor[1], David Bhella[2] and Edward C Hutchinson[1,2*].

[1]Sir William Dunn School of Pathology, University of Oxford
[2]MRC-University of Glasgow Centre for Virus Research, University of Glasgow
*edward.hutchinson@glasgow.ac.uk

Abstract

Clinical isolates of influenza virus produce pleomorphic virus particles, including extremely long filamentous virions. In contrast, strains of influenza that have adapted to laboratory growth typically produce only spherical virions. As a result, the filamentous phenotype has been overlooked in most influenza virus research. Recent advances in imaging and improved animal models have highlighted the distinct structure and functional relevance of filamentous virions. In this review we summarise what is currently known about these strikingly elongated virus particles and discuss their possible roles in clinical infections.

Introduction

Influenza viruses are serious human and animal pathogens which have been intensively studied for decades. Despite such close attention, one of the most striking features of influenza infections is typically overlooked. Although influenza viruses are often described as producing spherical virions, natural infections are characterised by the additional presence of filaments: extremely elongated virions which can reach microns in length.

This oversight can be explained by the ease with which influenza viruses adapt to laboratory culture, a trait which has allowed so many other advances in their study. Passage in embryonated chicken eggs, which has been used to produce many commonly studied strains of influenza virus, rapidly selects against the production of filaments (Fig 1) (Choppin, 1963; Hayase *et al.*, 1995; Seladi-Schulman *et al.*, 2013). Filaments are also less physically robust during laboratory purification methods than spherical virions, further complicating their

characterisation (Ada & Perry, 1958; Burnet & Lind, 1957; Roberts *et al.*, 1998; Valentine & Isaacs, 1957; Vijayakrishnan *et al.*, 2013). As a result, although influenza filaments have been recognised since 1946 (Mosley & Wyckoff, 1946), their study has until recently been sporadic.

It is now clear that mixtures of spherical and filamentous virions can be produced by influenza A, B and C viruses (Chu *et al.*, 1949; Mosley & Wyckoff, 1946; Nishimura *et al.*, 1990). Filament production has been repeatedly observed with low-passage clinical and veterinary influenza A virus isolates (Basu *et al.*, 2012; Choppin *et al.*, 1960; Chu *et al.*, 1949; Elton *et al.*, 2013; Hayase *et al.*, 1995; Itoh *et al.*, 2009; Kilbourne & Murphy, 1960; Lang *et al.*, 1968; Seladi-Schulman *et al.*, 2013; Shortridge *et al.*, 1998) as well as in lung sections from a fatal human case (Nakajima *et al.*, 2010). A similar mixture of filaments and spherical virions has been observed for other orthomyxoviruses: in a low-passage isolate from a fatal human thogotovirus infection (Kosoy *et al.*, 2015) and for infectious salmon anaemia viruses in tissue cultures and the tissues of infected fish (Crane & Hyatt, 2011; Kibenge *et al.*, 2001; Koren & Nylund, 1997). This suggests that the ability to produce filaments may be a general feature of the orthomyxovirus family.

Many observations of filament structure have been limited by the need to use electron microscopy (EM) methods such as negative staining, metal shadowing and ultrathin sectioning of resin embedded material. Although informative these depend on heavy metal contrasting agents, and often chemical fixation, and are therefore prone to artefacts including sample deformation and shrinkage. Following the development of cryo EM, it has been possible to determine the structure of filaments to higher resolution in a close-to-native environment without sample preparation artefacts (Calder *et al.*, 2010; Vijayakrishnan *et al.*, 2013; Wasilewski *et al.*, 2012). This has shown that filaments have a distinctive and highly ordered ultrastructure.

The importance of filaments in natural infections is highlighted by recent experimental studies with influenza A viruses in animal models. These showed that despite being selected against in egg passage, filament production is selected for during serial intranasal passage of a highly laboratory-adapted spherical strain in guinea pigs (Seladi-Schulman *et al.*, 2013). Furthermore, filament formation correlates with transmissibility between co-housed guinea pigs and by respiratory droplets in ferrets (Campbell *et al.*, 2014a; Lakdawala *et al.*, 2011).

Taken together with the many observations of recently-isolated strains producing filaments, these data indicate that the filamentous phenotype is an important but neglected feature of natural influenza infections.

In this review we summarise seven decades of work on influenza virus filaments, with a particular emphasis on recent structural and molecular biology studies. We also discuss possible functions of this often neglected trait in the virus lifecycle.

Filament Structure

Influenza infections do not produce virions of a single, well-defined size. However, the virions produced by laboratory-adapted 'spherical' influenza viruses have broadly consistent dimensions. The majority (typically 65-75%) are spherical (axial ratio < 1.2), with a mean outer diameter of 120 nm (Harris *et al.*, 2006; Yamaguchi *et al.*, 2008). Irregularly-shaped virions are often observed (Almeida & Waterson, 1967a; b; Harris *et al.*, 2006; Ruigrok *et al.*, 1986; Stevenson & Biddle, 1966; Wrigley, 1979), but it appears that many of these result from damage during ultracentrifugation, storage and sample preparation for electron microscopy (Noda, 2011; Sugita *et al.*, 2011). 'Spherical' strains also produce a minority of well-preserved but elongated virions, which for the most part are still less than 250 nm in length – too short to be described as filaments (Calder *et al.*, 2010; Harris *et al.*, 2006; Wasilewski *et al.*, 2012; Yamaguchi *et al.*, 2008). These intermediate-length virions, which often appear to be ellipsoidal, capsular or kidney-bean shaped, have been described as bacilliform (Vijayakrishnan *et al.*, 2013).

Influenza viruses that retain their natural morphology produce not only spherical and bacilliform virions, but also a class of highly elongated virions, or filaments (Figs 1, 2) (Ada *et al.*, 1958; Calder *et al.*, 2010; Roberts *et al.*, 1998; Vijayakrishnan *et al.*, 2013). These striking structures are typically more than 250 nm in length and can reach many microns (Fig 3). Filaments reaching or exceeding 30 μm in length have been reported (Cox *et al.*, 1980; Roberts *et al.*, 1998), though their exact range of size is hard to determine as they are fragile (Burnet & Lind, 1957; Valentine & Isaacs, 1957), comparatively hard to purify (Ada *et al.*, 1958; Sugita *et al.*, 2011), prone to aggregation (Cox *et al.*, 1980) and are hard to capture complete when cutting thin sections for transmission EM. The

proportion of filaments in a given sample varies widely and depends on the virus strain used, tissues infected and the handling of virions (Bourmakina & Garcia-Sastre, 2003; Rossman *et al.*, 2012; Seladi-Schulman *et al.*, 2013; Vijayakrishnan *et al.*, 2013).

During the budding of filamentous influenza A and C viruses cord-like associations of multiple filaments have often been observed (Beale *et al.*, 2014; Bialas *et al.*, 2014; Bruce *et al.*, 2010; Elton *et al.*, 2013; Morgan *et al.*, 1956; Muraki *et al.*, 2007; Muraki *et al.*, 2004; Nishimura *et al.*, 1990; Simpson-Holley *et al.*, 2002). End-to-end association of filaments has also been reported (Calder *et al.*, 2010; Vijayakrishnan *et al.*, 2013), though it is unclear if this is due to separate filaments associating, concatemers of filaments arising from incomplete budding, or a single filament fragmenting.

Filaments can be distinguished from spherical virions not just by their great length, but because their width, around 80 nm, is less than that of spherical virions (Fig 3a; Morgan *et al.*, 1956; Vijayakrishnan *et al.*, 2013). Infectious salmon anaemia virus, another orthomyxovirus, also produces narrower filamentous and wider spherical virions (Koren & Nylund, 1997). Bacilliform virions have an intermediate width of around 95 nm (Calder *et al.*, 2010; Harris *et al.*, 2006; Vijayakrishnan *et al.*, 2013; Wasilewski *et al.*, 2012; Yamaguchi *et al.*, 2008), and show an inverse correlation between length and diameter (Fig 3b) (Vijayakrishnan *et al.*, 2013). Particles can therefore be categorised based on their axial ratio (< 1.2 for spherical virions, > 1.2 for bacilliform virions and filaments) and length (> 250 nm for filaments; Fig 3). Particle dimensions do not provide sharp distinctions between these categories but they are useful, as closer examination shows that each category of virion has a characteristic composition and structure.

Filament Composition

Viral Components

Haemagglutinin (HA) and neuraminidase (NA) are the two major viral glycoproteins present in the envelope of influenza virus. HA binds to sialic acid, the viral receptor, and is required for entry of the virus into the host cell. NA mediates the release of viral progeny from the cell by cleaving sialic acid from cell surface proteins. The glycoproteins have a characteristic fringe-like appearance in electron micrographs of virions (Fig 2) and tomography shows that both spherical virions and filaments incorporate an abundance of HA along with smaller

quantities of NA (Calder *et al.*, 2010; Harris *et al.*, 2006). NA forms clusters, which in bacilliform and filamentous virions tend to be at the pole proximal to the budding site on the host cell surface, at the opposite end of the virion to the viral genome (Fig 3a; Calder *et al.*, 2010; Chlanda *et al.*, 2015; Harris *et al.*, 2006; Murti & Webster, 1986; Wasilewski *et al.*, 2012). It is possible that this clustering of NA may play a role in the formation of cord-like bundles of budding filaments – with NA sequestered at the poles, sufficient sialic acid may remain attached to surface proteins along the length of the filaments to allow HA on adjacent filaments to bind. The viral glycoproteins appear to be more regularly distributed on filaments than on spherical virions, suggesting interactions with a more ordered matrix layer beneath (Wasilewski *et al.*, 2012).

The matrix layer, which is bound to the internal surface of the viral membrane, is made of the M1 protein. M1 multimerises to form a helical matrix, the organisation of which appears to influence virion morphology (Calder *et al.*, 2010). Multimerised M1 can form lattices with a range of curvatures: along the length of filaments it forms a rigid cylindrical helix, whereas in spherical particles and at the poles of filaments it appears to form a less-ordered spherical spiral (Calder *et al.*, 2010). The poles of filaments sometimes form enlarged oval structures. Where these enlarged structures have a diameter greater than 200 nm they are termed Archetti bodies (Fig 2c) (Archetti, 1955; Vijayakrishnan *et al.*, 2013). Archetti bodies retain a contiguous matrix layer and can sometimes contain coils of M1-like material that are not membrane-associated (Vijayakrishnan *et al.*, 2013). Similar coils are observed within filaments as their structure transforms and becomes disorganised at low pH (Calder *et al.*, 2010), a change which mimics the fragmentation of filaments in acidifying endosomes during viral entry (Rossman *et al.*, 2012), suggesting that Archetti bodies may arise from a partial breakdown of filament structure.

The genome of influenza viruses consists of segments of viral RNA bound to the viral polymerase proteins (PB2, PB1 and PA/P3) and nucleoprotein (NP). It can be clearly visualised in spherical virions as a complex of rod-shaped segments, the longest spanning the internal diameter of the virion (Fig 3a; Calder *et al.*, 2010; Noda *et al.*, 2006). Studies of mutant viruses suggest that genome packaging is not strictly necessary for virion assembly, although it can make assembly more efficient (Gavazzi *et al.*, 2013; Hutchinson *et al.*, 2008), and in practice most virions do not have a full complement of functionally active genome segments (Brooke *et al.*, 2014; Brooke *et al.*, 2013; Heldt *et al.*, 2015). Images of the genome

have been obtained in bacilliform virions (Fig 2a) and occasionally in filaments, particularly shorter ones (Fig 2b) (Calder *et al.*, 2010; Noda *et al.*, 2006; Vijayakrishnan *et al.*, 2013; Wasilewski *et al.*, 2012). In such cases, the viral genome appears to remain associated with one pole of the virion (Calder *et al.*, 2010; Vijayakrishnan *et al.*, 2013; Wasilewski *et al.*, 2012) – the distal tip when virions bud from the cell membrane (Fig 3a; Noda *et al.*, 2006). The rod-shaped genome segments associate with the virion pole through their tips, and in filaments they appear to remain closely associated in a parallel array (Calder *et al.*, 2010; Vijayakrishnan *et al.*, 2013). In spherical virions this ordered clustering of genome segments can also be observed, though disordered arrangements of the genome appear to be more common (Harris *et al.*, 2006).

At present the efficiency with which filaments package the viral genome is unclear. Early observations suggested that filaments might incorporate more viral genome, and be more infectious, than spherical virions (Ada & Perry, 1958; Ada *et al.*, 1958; Ada *et al.*, 1957; Burleigh *et al.*, 2005; Roberts *et al.*, 1998), a hypothesis consistent with the greater resistance of filament-containing stocks to ultraviolet inactivation (Smirnov *et al.*, 1991), and recalling the polyploid filamentous virions of Ebola virus (Beniac *et al.*, 2012). However, these observations could also be explained by the tendency of multiple filaments to form cord-like bundles. Other negative data do not support the hypothesis that filaments can package multiple copies of the genome: clear images of the genome have only been obtained in a minority of longer filaments, multiple copies of the genome have not been clearly visualised within a single virion (Morgan *et al.*, 1956; Vijayakrishnan *et al.*, 2013), and fragmentation of filaments using a number of methods does not increase the infectious titre (Ada & Perry, 1958; Burnet & Lind, 1957; Donald & Isaacs, 1954; Valentine & Isaacs, 1957).

The ion channel M2 has not been detected by immunofluorescence in filaments, suggesting that it is not an abundant component (Rossman *et al.*, 2010a). However, its presence can be inferred as an M2-binding antibody causes filaments to fragment, while an M2 inhibitor allows filaments to resist fragmentation at low pH (Rossman *et al.*, 2010a; Rossman *et al.*, 2012). NS1 and NEP are known to be present at low levels in spherical virions (Hutchinson *et al.*, 2014), but their presence in filaments has not been assessed.

Host Components

All influenza virions incorporate membrane from the host cell. As with spherical virions, the envelopes of filaments are resistant to low-temperature nonionic detergent extraction and contain material with a low buoyant density, implying the incorporation of lipid rafts (Simpson-Holley *et al.*, 2002). Cholesterol also appears to be important for filament stability (Rossman *et al.*, 2010a). Spherical virions incorporate a substantial quantity of host-encoded proteins, resembling those incorporated into exosomes (Hutchinson *et al.*, 2014; Shaw *et al.*, 2008). It is reasonable to assume that filaments also incorporate such host proteins – quite possibly more than spherical virions, due to their larger membrane area and internal volume – but this has not been assessed in detail. Fibrillar material, which does not have the appearance of the viral genome, has been observed inside filamentous particles but its identity is still unclear (Vijayakrishnan *et al.*, 2013).

Filament Formation

General Requirements for Virion Formation

All influenza virions are formed at the cell surface in a concerted process which requires both viral and host components (Fig 3a; Hutchinson & Fodor, 2013; Noda *et al.*, 2006; Rossman & Lamb, 2011). Viral glycoproteins accumulate at the apical plasma membrane and are individually sufficient to cause budding when over-expressed (Chen *et al.*, 2007; Chlanda *et al.*, 2015). M1 is neither necessary nor sufficient for the production of virus-like particles, but it does appear to be required for the production of infectious virions (Chen *et al.*, 2007). This may be due to its interactions with genome segments: genome packaging increases the efficiency of budding, though the importance of this appears to depend on cell type (Hutchinson *et al.*, 2008). Finally, abscission of the budded virion is mediated by M2 in an ESCRT-independent process (Rossman *et al.*, 2010b). While defects in normal virion assembly can produce irregular virions which superficially resemble filaments – for example the elongated and distended virions resulting from mutations in the cytoplasmic tails of NA or HA (Jin *et al.*, 1997; Mitnaul *et al.*, 1996) or the beads-on-string structures due to M2 scission mutants (Rossman *et al.*, 2010b) – ‘well-formed’ filaments appear to result from a process which involves a number of host and viral determinants.

Host Determinants of Filament Formation

Virions require host processes to assemble and interfering with these processes can impair filament formation. Drugs targeting the actin cytoskeleton, depletion of Rab11-family interacting protein 3 (FIP3, which regulates actin dynamics and membrane trafficking) and cholesterol depletion have all been shown to specifically reduce filament formation, while depletion of or mutation of Rab11 (a GTPase involved in endocytic recycling) affects both spherical and filamentous particle production (Bruce et al., 2010; Roberts et al., 1998; Rossman et al., 2010a; Simpson-Holley et al., 2002). It has been posited that all of these processes affect the supply of lipid-raft enriched membrane required to form the extensive surfaces of filaments. In addition, mutation of an LC3-interacting region (LIR) in the viral M2 protein, a motif which allows interaction with autophagosomal membranes via LC3, or depletion of ATG16L1, which is required for LC3 activation, reduces filament formation and decreases the stability of filamentous virions. This suggests that autophagosomal membranes are recruited to support filament formation (Beale et al., 2014).

Even without active intervention, some cell lines are less permissive to filament formation than others. The same strain of virus can have different morphologies in different cell lines (Al-Mubarak *et al.*, 2015; Bialas *et al.*, 2012; Itoh *et al.*, 2009; Lakdawala *et al.*, 2011), though whether a particular cell line is permissive for filaments can vary between different strains of the virus (Al-Mubarak *et al.*, 2015). Generally, polarised cell types are more permissive to filament formation compared to nonpolarised cells, consistent with a role for the cytoskeleton in determining viral morphology (Roberts *et al.*, 1998).

Viral determinants of filament formation

Filament formation is a heritable trait. Spherical virions purified from filament-forming stocks can form filamentous progeny (Chu *et al.*, 1949) and the selection against filament formation during passage in embryonated chicken eggs can be slowed by passaging only the minimal amount of virus necessary for an infection, thereby excluding low-frequency mutants from the stock (Burnet & Lind, 1957). Although viral genes clearly contribute to filament formation the trait is complex. There appear to be multiple pathways to filament formation, with some loci relevant only in particular genetic backgrounds while others suppress filament formation in genotypes which would normally support it. A summary of loci in influenza A virus genes known to affect filament formation is given in Table 1.

Unsurprisingly, considering its role in maintaining virion structure (Calder *et al.*, 2010), the majority of mutations affecting filament formation have been mapped to M1. Reverse-genetic studies using reassortant viruses showed that the M1 gene of influenza A/Udorn/301/72 virus (Udorn), one of the few strains to retain filament-forming ability after laboratory passage (Roberts *et al.*, 1998), can confer filament-forming ability on spherical strains such as the influenza A/WSN/33 (WSN) or A/Puerto Rico/8/1934 (PR8) viruses (Bourmakina & Garcia-Sastre, 2003; Noton *et al.*, 2007). Reciprocally, the M1 gene from the spherical WSN strain abrogated filament production by the normally filament-forming influenza A/Victoria/3/75 virus (Elleman & Barclay, 2004). While most studies of filament formation have considered influenza A viruses, a key role for M1 has also been identified in an influenza C virus (Muraki *et al.*, 2007).

To date, mapping filament determinants in M1 has not produced a clear mechanistic model of filament formation. Difficulties in interpretation arise from the multiple structural roles of M1, which include membrane binding, homo-oligomerisation and interactions with other viral proteins (Burleigh *et al.*, 2005), as well as from the overlap of the M1 gene with other viral genes, notably for the ion channel M2. For example M1 residue 41 was one of the first residues to be experimentally associated with filament formation (Campbell *et al.*, 2014b; Roberts *et al.*, 1998; Zebedee & Lamb, 1989); it is also known to have mutated during adaptation of the WSN strain, which is now spherical, to mouse brain passage (Ward, 1995). However, mutations affecting this position have also been shown to create a splice-variant form of the M2 protein, whose role in morphology remains to be determined (Wise *et al.*, 2012).

One mechanism underlying filament formation can be inferred from studies of helix six, a basic alpha helix in M1. Scanning alanine mutagenesis shows that a number of residues in this region are required for the production of regularly-shaped virions and one mutation, M1 K102A, confers filament-forming abilities on spherical viruses (Burleigh *et al.*, 2005). This mutation, which is adjacent to a proposed M1-M1 interaction site (Harris *et al.*, 2001), causes M1 in virions to form a helix with similar symmetry to that observed in the filamentous Udorn strain, emphasising the importance of an ordered M1 helix in maintaining filament structure (Calder *et al.*, 2010).

Other loci influencing filament formation have been mapped to the HA, NP, NA and M2 proteins (Table 1). Most strikingly, an appropriate NA can enhance filament formation in a laboratory-adapted virus and even induce limited filament formation when overexpressed alone (Campbell *et al.*, 2014a; Chlanda *et al.*, 2015). The influence of these proteins on filament formation has generally been attributed to altered interactions with M1 (Bialas *et al.*, 2014; Chen *et al.*, 2008; Liu *et al.*, 2002) or by their influence on processes upstream of virion assembly. For example, it has been suggested that mutations in M2 and NA influence filament production by altering the recruitment of lipid rafts required for the virion membrane (Enami & Enami, 1996; Jin *et al.*, 1997; Mitnaul *et al.*, 1996; Rossman *et al.*, 2010a; Zhang *et al.*, 2000).

Filament Function

Decades of observational work, and more recent experimental studies in animal transmission models (Campbell *et al.*, 2014a; Lakdawala *et al.*, 2011; Seladi-Schulman *et al.*, 2013), clearly show that filament-forming viruses have a selective advantage in natural influenza infections. While it is possible that filament production itself is a 'spandrel' – a conspicuous by-product of some underlying trait (Gould & Lewontin, 1979) – the more obvious explanation is that filaments act directly to increase viral fitness in their natural hosts. However, the particular functions of filaments have been difficult to determine due to the difficulty in completely separating spherical and bacilliform virions from filaments during analysis, and as filaments do not typically provide an advantage in embryonated eggs or in the tissue culture systems most suited to detailed functional studies. Despite these difficulties, a number of properties of filaments have been identified which suggest functional roles.

The Costs of Making Filaments

Filamentous strains have a clear selective disadvantage in embryonated chicken eggs (Seladi-Schulman *et al.*, 2013). This may be due to the specific constraints of egg passage. For example, virions grown in eggs incorporate a different profile of host proteins to those grown in mammalian cells, notably by utilising different members of the tetraspanin family of membrane proteins (Hutchinson *et al.*, 2014). It is possible that these egg-specific proteins, which are relatively abundant in virions, may increase the costs of filament production.

Alternatively, it may be that filaments have intrinsic costs which are not compensated for during laboratory passage. This also applies to passage in tissue cultures, in which the rate at which filamentous strains replicate varies, but does not typically exceed that of spherical strains. Filaments require a greater amount of membrane and viral proteins to form each virion and to the route of filament entry is different to that of spherical virions. Although filaments have receptor binding activity (Ada *et al.*, 1958; Burnet & Lind, 1957; Chu *et al.*, 1949; Donald & Isaacs, 1954; Seladi-Schulman *et al.*, 2014), they are too large to enter cells through the canonical clathrin-mediated endocytic pathway that can take up spherical and bacilliform virions. Instead, filaments undergo delayed uptake through macropinocytosis, and break apart into smaller fragments as endosomal acidification triggers conformational changes in the virion (Rossman *et al.*, 2012; Sieczkarski & Whittaker, 2005). The comparative efficiency of this process is unclear. While these issues may account for some of the fitness cost of filaments in laboratory culture, the structure of filaments suggests two advantages which may overcome these costs during a natural infection.

Filaments May Be More Robustly Infectious

Firstly, some studies suggest that filaments may have higher specific infectivities than spherical virions (Ada & Perry, 1958; Ada *et al.*, 1958; Ada *et al.*, 1957; Burleigh *et al.*, 2005; Roberts *et al.*, 1998). As discussed above, the implication that this is due to packaging multiple genomes is contentious, but the same effect could also be achieved by the frequently-observed association of individual filaments into higher-order cord-like structures (Beale *et al.*, 2014; Bialas *et al.*, 2014; Bruce *et al.*, 2010; Elton *et al.*, 2013; Morgan *et al.*, 1956; Muraki *et al.*, 2007; Muraki *et al.*, 2004; Nishimura *et al.*, 1990; Simpson-Holley *et al.*, 2002). Physically associating multiple genomes, in a single particle or a cluster of particles, is unlikely to be advantageous in the high-multiplicity infections that characterise laboratory growth and (presumably) foci of infection within the host. However, it would be expected to increase the efficiency of low-multiplicity infections during the spread of viruses within and between hosts, given that virions typically lack at least one functional gene segment (Brooke *et al.*, 2013; Heldt *et al.*, 2015). Redundant copies of the genome may also provide some resistance to ultraviolet inactivation during between-host passage (Smirnov *et al.*, 1991). Packaging additional genomes which lack a full complement of functional segments even raises the intriguing possibility of variable gene dosage for each segment within the context of a high-multiplicity infection (Brooke *et al.*, 2014).

Secondly, it has recently been noted that influenza genomes can pass directly between cells though an actin-dependent, NA-independent process without packaging into virions (Mori *et al.*, 2011; Roberts *et al.*, 2015). Although this has not been demonstrated for filamentous influenza viruses, it is plausible that filaments could enhance cell-associated spread and provide an advantage in natural infections, for example by evading mucociliary clearance. However, direct transmission of a filamentous influenza virus has not been demonstrated.

Filaments May be an Adaptation to Spread Through Mucus

Influenza virions bind to sialic acid, which is cleaved by the viral NA. This neuraminidase activity is required during the initiation and within-host spread of an infection, to prevent virions being sequestered by sialic acid moieties on the mucins in respiratory mucus, as well as on the surfaces of infected cells when new virions are produced (Chlanda *et al.*, 2015; Cohen *et al.*, 2013; Matrosovich *et al.*, 2004; Yang *et al.*, 2014). Filaments appear to have an elevated NA activity, which would potentially enhance infectivity in natural hosts (Campbell *et al.*, 2014a; Campbell *et al.*, 2014b; Seladi-Schulman *et al.*, 2014). Surprisingly, this activity is increased by mutations in M1 that allow filament formation, even when NA itself is unaltered (Campbell *et al.*, 2014a; Campbell *et al.*, 2014b; Seladi-Schulman *et al.*, 2014). It is unclear whether this is due to the clustering of NA at the tips of filaments (Calder *et al.*, 2010; Chlanda *et al.*, 2015) or to the abundance of NA in filaments, which has not been determined.

There are only limited data available on the relative stability of filaments and spherical virions within mucus. Filaments do appear to be fragile during laboratory manipulations, but despite this they appear to be no more susceptible to heat inactivation than spherical strains (Beale *et al.*, 2014; Seladi-Schulman *et al.*, 2014). They can be bound by antibodies (Chu *et al.*, 1949), but they appear to be no more susceptible to neutralisation than spherical virions in *in vitro* assays (Seladi-Schulman *et al.*, 2014). Conversely, it has been suggested that non-infectious filaments may serve as an immune decoy by sequestering IgA antibodies away from smaller particles during infection (Vijayakrishnan *et al.*, 2013).

Finally, as well as being a barrier to infection and within-host spread, mucus is ultimately the vehicle for between-host spread. Longer filaments would be able to extend through the low-viscosity periciliary layer, which coats the airway epithelium to a depth of around 7 μm (Button *et al.*, 2012; Fahy & Dickey, 2010), potentially providing more efficient access to the

gel-like airway mucus beyond and increasing the likelihood of their respiratory transmission. Respiratory transmission of influenza genomes in ferrets occurs most efficiently in large (> 4 μm) droplets of mucus. While the same distribution between droplet sizes is observed for both filamentous and spherical strains, it is notable that these droplets are large enough to contain intact filaments (Lakdawala *et al.*, 2011).

Conclusion

Influenza viruses naturally exhibit a range of morphologies from small spherical particles to extremely long filamentous structures. It appears that filament production may be a common feature of orthomyxoviruses as it has been observed in influenza A, B and C viruses as well as in thogotovirus and infectious salmon anaemia virus. It is also notable that a number of unrelated respiratory viruses can form filamentous virions, including respiratory syncytial virus and certain paramyxoviruses (Compans *et al.*, 1966; Liljeroos *et al.*, 2013; Shaikh *et al.*, 2012; Yao & Compans, 2000), although other respiratory viruses form exclusively spherical virions.

For influenza viruses filament formation is a heritable trait which is selected for in natural transmission. Despite this, long filaments have often been neglected in laboratory studies, and the reason for their production remains uncertain. Recent technical advances have allowed the structure of filaments to be analysed in detail and the conditions which select for them to be studied in a laboratory setting. This has strengthened the case for filaments as a class of well-formed viral structures which provide functional benefits during natural influenza infections. What these benefits are though remains uncertain, and in clarifying them we will need to fundamentally revise our models of how influenza virus genomes are transmitted outside of a laboratory setting.

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442 **Tables**

443

444 Table 1: Influenza A virus mutations known to influence filament production.

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Segment (gene)	Residues	Phenotype	Context	Reference
7 (M1)	R95K E204D	Reduces filament formation	WSN with Ud M1 gene; residues changed to match WSN.	(Bourmakina & Garcia-Sastre, 2003)
	A41V	Reduces filament formation	Ud, selected for resistance to an anti-M2 antibody.	(Roberts *et al.*, 1998)
	P41A	Reduces filament length	SNP04 and PR8:SPN04 reassortants	(Campbell *et al.*, 2014b)
	V41A+K95R+T218A	Confers filamentous morphology	Vic with WSN M1 gene; residues changed to match Ud.	(Elleman & Barclay, 2004)
	S85N N231D	Confers filamentous morphology	PR8 with Miami M1 gene; residues changed to match Nkt.	(Elton *et al.*, 2013)
	K102A	Confers filamentous morphology	WSN	(Burleigh *et al.*, 2005)
7 (M2)	S71A+M72A+R73A	Reduces filament formation	Ud	(Rossman *et al.*, 2012)
5 (NP)	R214K and I217S F253I	Reduces filament formation	WSN with Aichi M1 gene; NP residues changed to match Aichi.	(Bialas *et al.*, 2014)

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447 Morphology (filamentous or spherical) is as originally defined in the cited studies.
448 Abbreviations: Aichi: A/Aichi/2/68; Miami: A/equine/Miami/63; Nkt:

449 A/equine/Newmarket/11/03; PR8: A/PR8/34; SPN04: A/swine/Spain/53207/2004; Udorn:
450 A/Udorn/72; Vic: A/Victoria/3/75; WSN: A/WSN/33.
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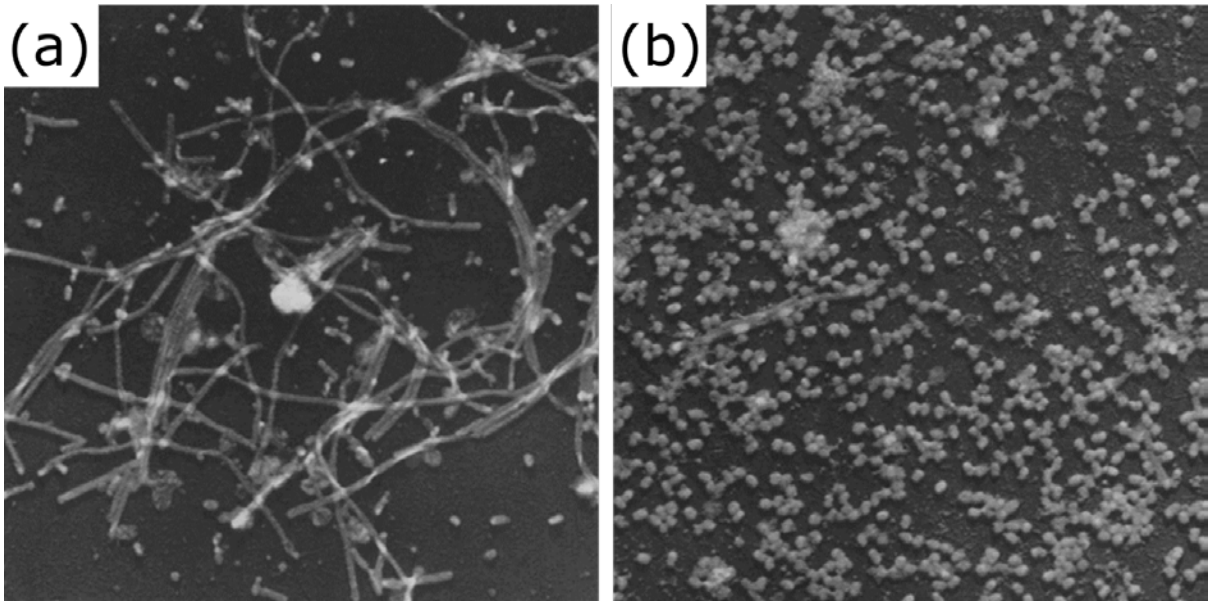
452

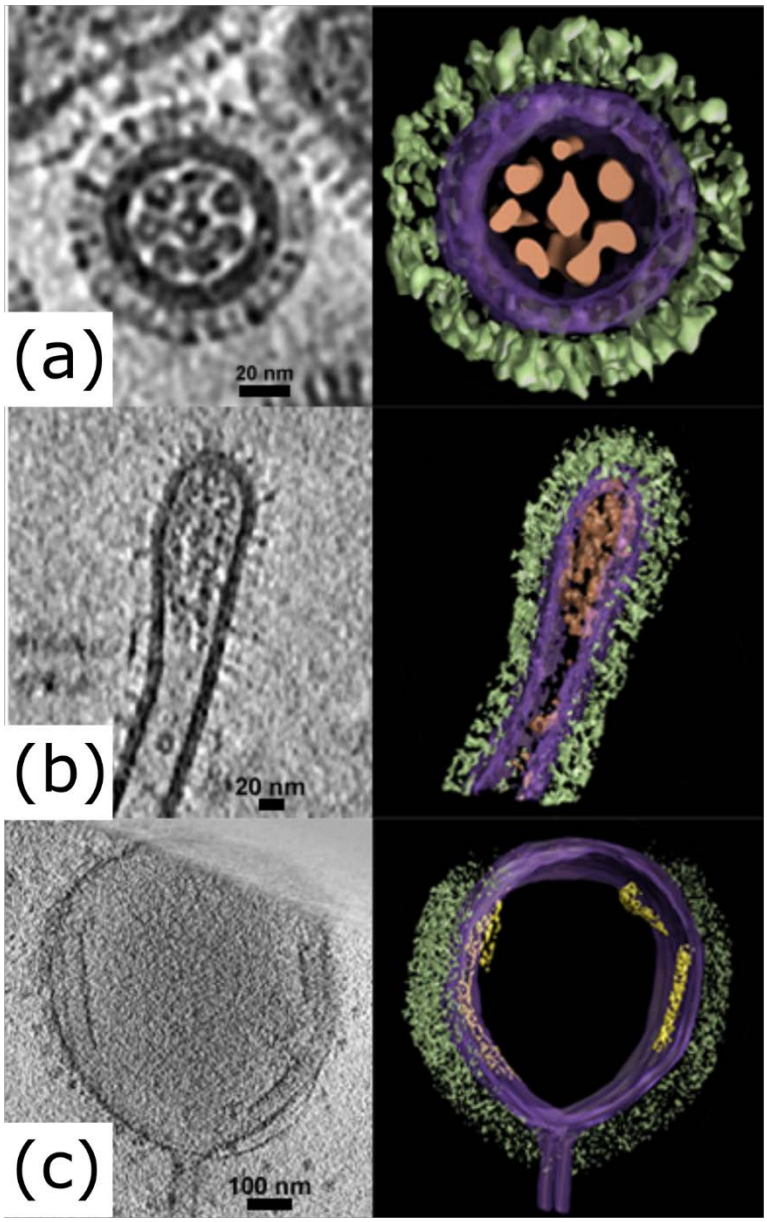
Figures

Figure 1: Filamentous influenza virions are lost on laboratory passage.



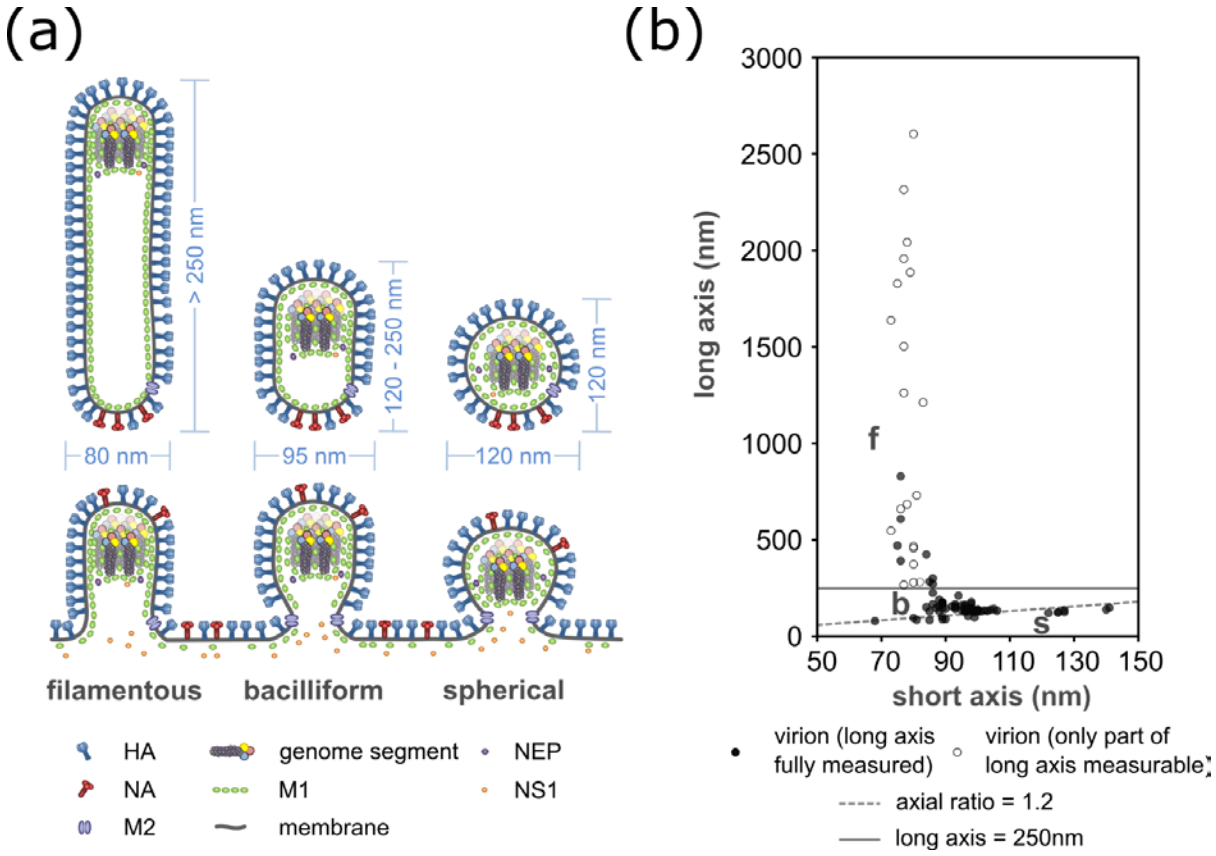
Filamentous influenza virions are clearly visible after two passages of the clinical isolate influenza A/Rockefeller Institute/1/1957 (H2N2) virus in embryonated chicken eggs (a) but are lost following twelve passages (b). Electron micrographs © Choppin *et al.*, 1960. Originally published in *THE JOURNAL OF EXPERIMENTAL MEDICINE.* 112:945-52.

Figure 2: Bacilliform and filamentous influenza virions at high resolution.



Electron tomograms of influenza virions, showing slices (left panels) and segmented images (right panels) of (a) a transverse section of a bacilliform virion, (b) a longitudinal section of the tip of a filamentous virion and (c) a longitudinal section of an Archetti body at the end of a filamentous virion. Images were manually segmented and coloured to show viral glycoproteins (green), membrane and associated matrix (purple), genome (brown) and putative free M1 sheets (yellow). Tomograms were obtained as part of a previous study (Vijayakrishnan *et al.*, 2013) and manually segmented using Amira (TGS).

Figure 3: Dimensions of influenza virions.



The dimensions of influenza virions, shown (a) as a schematic of budding and released virions, with typical sizes indicated, and (b) as measurements of purified influenza A/Udorn/72 virions. For (a) it should be noted that the incorporation of NS1 and NEP has so far only been examined in spherical virions, and their general incorporation is inferred from this. For (b) measurements of 96 virions were taken by cryoelectron microscopy (data replotted from Vijayakrishnan *et al.*, 2013). Open circles indicate filaments which extended beyond the field of view and so are longer than measured. Spherical virions (s) are distinguished from bacilliform virions (b) by having an axial ratio less than 1.2 (dashed line); filaments (f) are distinguished from bacilliform virions by having a length greater than 250 nm (solid line).

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