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Abstract (max 250)

Influenza A virus (IAV) is an enveloped virus of the *Orthomyxoviridae* with a negative-sense single-stranded RNA genome. During virus cell entry, viral and cellular cues are delivered in a stepwise manner within two distinct cellular compartments – the endosomes and the cytosol. Endosome maturation primes the viral core for uncoating by cytosolic host proteins and host-mediated virus disaggregation is essential for genome import and replication in the nucleus. Recent evidence shows that two well-known cellular proteins - histone deacetylase 6 (HDAC6) and karyopherin-β~~β~~2 (k~~K~~apβ~~β~~2) - uncoat influenza virus. HDAC6 is one of eleven HDACs and an X-linked, cytosolic lysine deacetylase. Under normal cellular conditions HDAC6 is the tubulin deacetylase. Under proteasomal stress HDAC6 binds unanchored ubiquitin, dynein and myosin II

to sequester misfolded protein aggregates for autophagy. Kapβ~~β~~2 is a member of the importin β ~~β~~ family that transports RNA-binding proteins into the nucleus by binding to disordered nuclear localization signals (NLSs) known as PY-NLS. Kapβ~~β~~2 is emerging as a universal uncoating factor for IAV and human immunodeficiency virus type 1 (HIV-1). Kap β 2 can also reverse liquid-liquid phase separation (LLPS) of RNA-binding proteins by promoting their disaggregation. Thus, it is becoming evident that key players in the management of cellular condensates and membraneless organelles are potent virus uncoating factors. This emerging concept reveals implications in viral pathogenesis, as well as, the promise for cell-targeted therapeutic strategies to block universal virus uncoating pathways hijacked by enveloped RNA viruses.

Fundamentals of IAV entry

Influenza virus is an enveloped virus with a negative-sense single-stranded RNA genome. As shown in Figure 1, two viral glycoproteins decorate the viral membrane surface; the fusion protein and receptor-binding hemagglutinin (HA) and neuraminidase (NA). Inside the viral membrane is the viral capsid made of matrix protein (M1), inside which the eight segments of viral ribonucleoproteins (vRNPs) are encapsidated. Each RNP contains the RNA genome, and the viral polymerase. IAV undergoes a series of stepwise uncoating events during cell entry as the virus particle is assisted by facilitators and a complex set of temporally and spatially regulated cellular cues (Yamauchi and Greber, 2016). Entry begins with multivalent attachment of the viral HA to sialic acid containing cell surface receptors (Skehel and Wiley, 2000). The half-life of HA-sialic acid binding is 0.8–5.5 sec (Sieben et al., 2012), whereas that of IAV internalisation is 10–15 min (Matlin et al., 1981). Thus, multivalent binding of HA to sialic acid-containing molecules

such as epidermal growth factor receptor (EGFR) is necessary to trigger signalling and uptake of the viral particle into early endosomes via receptor-mediated endocytosis (Eierhoff et al., 2010). More recently it was shown that IAV infection activates G coupled protein receptor kinase 2 (GRK2) which promotes downstream viral uncoating (Yanguez et al., 2018).

Single-molecule experiments have shown that the cellular plasma membrane is partitioned into 50-300 nm wide domains by the combined action of actin-based membrane cytoskeleton "fences" and anchored-transmembrane protein "pickets" (Kusumi and Sako, 1996). On the cell surface, organization and interaction of proteins and lipids have been proposed to occur on different time and length scales, from direct molecular interactions to transient association within nanoscopic domains. Lipid raft microdomains are critical for entry and budding of enveloped viruses and assumed to function as selective concentration devices for viruses and proteins and to serve as platforms for signal transduction for endocytosis (K. Simons, D. Toomre, 2000). Blocking one endocytic pathway is typically insufficient to reduce IAV infection, and the virus is capable of eliciting two or more endocytic pathways. The endocytic pathway may depend on the cell membrane context such as abundance of receptor tyrosine kinases (RTKs) and facileness to induce lipid raft clustering upon multivalent HA binding to receptor(s) (Grecco et al., 2011, Eierhoff et al., 2010). Clathrin-coated pits are 120-150 nm in diameter in human epithelial cells (Bretscher et al., 1980). Most IAV entry experiments have been conducted using lab adapted, pleomorphic, spherical virions with a diameter ranging from about 80-120 nm (Noda, 2011). Filamentous influenza virions, which are 100 nm x 20 μm in size, are thought to predominantly trigger macropinocytic uptake in cell culture (Rossman and Lamb, 2011).

Following binding, a signalling circuit involving Rho kinase and phosphatidylinositol 4-phosphate 5-kinase (PIP5K)-phopholipase C (PLC) which is regulated by intracellular Ca^{2+} regulates the clathrin-dependent and -independent uptake of IAV (Fujioka et al., 2013). A sialylated voltage-dependent Ca^{2+} channel (Cav1.2) acts as a functional receptor by binding to IAV PR8 HA (Fujioka et al., 2018). In Lec2 Chinese hamster ovary (CHO) cells that are deficient for sialic acids, infection is impaired but attachment and entry recovered when Ca^{2+}-dependent (C-type) lectins that bind carbohydrate structures on viral glycoproteins [i.e. dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN)] were expressed (Londrigan et al., 2011). IAV does not efficiently infect Lec1 CHO cells deficient for N-acetylglucosaminyl transferase 1 (GnT1) indicating that the virus requires N-linked glycoprotein for entry (Chu and Whittaker, 2004). Using African green monkey kidney BSC-1 cells it was shown that 65% of influenza virus uptake is clathrin-dependent (Rust et al., 2004), and that the non-clathrin pathway is macropinocytosis-like and serum-inducible (de Vries et al., 2011). Epsin 1 is a cargo-specific adaptor for IAV, and a clathrin-, ubiquitin-, and phospholipid-interacting protein. Its depletion specifically blocks clathrin-mediated IAV uptake, however, the resulting infectivity remains the same because IAV particles can divert to a non-clathrin pathway (Chen and Zhuang, 2008). Thus, it is clear that IAV can alternate between clathrin and non-clathrin pathways.[MCK1][YY2]

In GnT1-deficient CHO cells, virus macropinocytic uptake was blocked whereas dynamin-dependent uptake was not (de Vries et al., 2012). Serum can induce dynamin-independent micropinocytosis-like uptake (de Vries et al., 2011). At the same time,— serum contains high levels of sialic acids and can act as decoy receptors that compete with virus binding to the cell surface.[MCK3] Such competitive binding can be neutralised by NA activity - a mechanism thought

to be relevant for *in vivo* infections along the mucus-rich airway epithelium (Matrosovich et al., 2004). ~~Using African green monkey kidney BSC-1 cells it was shown that 65% of influenza virus uptake is clathrin-dependent (Rust et al., 2004), and that the non-clathrin pathway is macropinocytosis-like and serum-inducible (de Vries et al., 2011). Epsin 1 is a cargo-specific adaptor for IAV, and a clathrin-, ubiquitin-, and phospholipid-interacting protein. Its depletion specifically blocks clathrin-mediated IAV uptake, however, the resulting infectivity remains the same because IAV particles can divert to a non-clathrin pathway (Chen and Zhuang, 2008). Thus, it is clear that IAV can alternate between clathrin and non-clathrin pathways.~~[MGK4]

Following endocytosis, IAV resides in a vesicle that undergoes three stages of motility accompanied by a maturation process that leads to late endosome (LE) and multivesicular body formation (Huotari and Helenius, 2011). Stage I is an actin-dependent movement in the cell periphery; stage II is a rapid directed motion towards the nucleus; stage III is a bi-directional, microtubule-dependent type of movement (Lakadamyali et al., 2003). Microtubules are important for influenza entry and infection is halved upon their depolymerisation by nocodazole (Yamauchi et al., 2011, Lozach et al., 2010).

[insert Figure 1 here, in color]

Multistep IAV uncoating in endosomes

Viral uncoating is promoted by cellular cues, facilitators, and built-in mechanisms for uncoating (Yamauchi and Greber, 2016). Cellular cues come in many shapes and forms; from cell surface receptors to enzymes, scaffolds and chemicals that exist extracellularly or in endocytic vesicles, or in the cytosol. ~~Enveloped~~ Many enveloped and non-enveloped viruses including influenza, Ebola, Lassa, Rhino and Adeno link their uncoating program to the endocytic machinery (Staring et al., 2018, Yamauchi and Helenius, 2013, Yamauchi and Greber, 2016). Furthermore, the endocytosis machinery provides an environment for IAV that allows separation of uncoating and assembly. The inner shell of the IAV virion is represented by a membrane-associated scaffold of matrix proteins, M1, that makes contacts with both vRNPs and ~~the lipid envelope with~~ the cytoplasmic tails of HA and NA in the lipid envelope (Lamb and Choppin, 1983). In endosomes, IAV takes advantage of cues such as cathepsin cleavage, low pH and high K^+ to promote viral fusion and uncoating (Stauffer et al., 2014, Edinger et al., 2015, Martin and Helenius, 1991). The NA of IAV H5N1 was shown to affect LAMP2 glycosylation at low pH, thereby potentially destabilizing the lysosomal glycocalyx and promoting endosomal escape (Ju et al., 2015).

Uncoating can be split into two steps: priming within the endosomal compartment, then uncoating by host factors on the cytosolic surface or in thw close vicinity of LEs. During endocytic transit of incoming IAV particles, the M2 ion channel opens and the influx of protons and K^+ ions affects M1 conformation, which subsequently dissociates interactions between HA C-terminus ~~and~~ M1, M1-M1 and M1-vRNPs, resulting in reduced viral particle rigidity (Stauffer et al., 2014, Li et al., 2014, Martin and Helenius, 1991). Once the endosomes reach an acidic environment of pH<6.0, M1 oligomers dissociate irreversibly into dimers resulting in a softening of the viral particle (Li et al., 2014, Zhang et al., 2012).This *priming* step is dependent on ~~the~~ a functional M2 channel and is completely stalled by the M2 blocker amantadine. Thus, priming in

endosomes is an essential step in the IAV life cycle that instigates the full infectious capacity of the IAV particle **(Fig. 1)**.

Many enveloped viruses use an acid-activated fusion mechanism to create a fusion pore at the endosomal membrane and gain cytosolic access. Fusion is mediated by specialised transmembrane proteins on the viral envelope (Harrison, 2008). At a low pH (≅5.0-5.5) IAV HA undergoes conformational changes that brings viral and endosomal membranes in extreme proximity, triggering membrane fusion and generating ~~micropits that transform into~~ narrow connections that eventually form a fusion pore (Kanaseki et al., 1997, Lee, 2010, Chlanda et al., 2016). However, IAV fusion is not sufficient for M1 uncoating and for vRNPs to enter the nucleus (Martin and Helenius, 1991). Viral fusion can be effectively performed at the plasma membrane if the low pH trigger is supplied to virus bound on the cell surface (Stauffer et al., 2014, Helenius et al., 1980, White et al., 1981, Kielian, 2014). Experiments have shown that in the case of IAV, acid-triggered fusion alone is very inefficient in releasing the genome and that stepwise uncoating signals must be provided to make the viral core (M1 and vRNPs) uncoating-competent. Infectivity can be restored, by mimicking endocytic transit by pre-acidification of IAV particles in vitro (Stauffer et al., 2014). Furthermore, the high K^+ concentration (≅120 mM) in LEs increases solubility of the vRNP bundle (Stauffer et al., 2014). The tetraspanin CD81, a top hit from multiple siRNA screens, was shown to be required for IAV fusion and endosomal escape (He et al., 2013). Here, incoming IAV particles fused in CD81-positive endosomes, and CD81 depletion blocked viral entry (He et al., 2013).[MCK5][YY6] CD81 associates with tetraspanins and other tetraspanin-interacting proteins to form tetraspanin-enriched microdomains on cellular membranes (Hemler, 2005), and is also incorporated into budding virions (He et al., 2013). Such

micodomains may contribute to viral fusion at LEs, however, the precise mechanism of how the presence of CD81 promotes viral fusion is unclear.

IAV fusion and cytosolic uncoating

RNAi and proteomic screening approaches have identified cellular proteins required for IAV fusion and uncoating (Yamauchi et al., 2011, Huotari et al., 2012, Gschweitl et al., 2016, Konig et al., 2010, Edinger et al., 2015, Pohl et al., 2014, Konig and Stertz, 2015, Edinger et al., 2014, Yanguez et al., 2018, Su et al., 2013, Banerjee et al., 2013, Miyake et al., 2019a, He et al., 2013). These are histone deacetylase 6 (HDAC6) (Banerjee et al., 2014), the nuclear import factor karyopherin-β2 (kKapβ2) (Miyake et al., 2019a), SPOPL/Cullin 3 (Gschweitl et al., 2016), ITCH (Su et al., 2013), EGFR substrate protein 8 (EPS8) (Larson et al., 2019), GRK2 (Yanguez et al., 2018) and LY6E (Mar et al., 2018). The E3 ubiquitin ligase ITCH localises to LEs and ubiquitinates viral M1 to promote uncoating (Su et al., 2013). LY6E, a member of the LY6/uPAR family, is an interferon-stimulated gene (ISG) that enhances viral entry at the uncoating step (Mar et al., 2018). GRK2 is activated by IAV and was identified from phosphoproteomic-based kinase profiling of IAV infected cells. GRK2 is required for efficient IAV uncoating but its cellular target is unclear (Yanguez et al., 2018). EPS8 physically associated with incoming IAV viral cores and its depletion reduced uncoating (Larson et al., 2019). Eps15 is a ubiquitination target of $CRL3^{SPOPL}$ E3 ubiquitin ligase complex required for IAV uncoating and regulates intraluminal vesicle formation during endosome maturation (Gschweitl et al., 2016). Likewise cullin 3 depletion interferes with endosome maturation and as a consequence IAV uncoating is blocked (Huotari and Helenius, 2011). Cytosolic IAV uncoating uses the aggresome processing pathway and HDAC6 for M1 shell breakage **(Fig. 2)**, and kKapβ2 for removal of residual M1 from the surface of vRNPs, which leads

to vRNP debundling and nuclear import (Banerjee et al., 2014, Kawaguchi et al., 2003, Hao et al., 2013, Miyake et al., 2019a).

After replication in the nucleus, progeny vRNPs are exported to the cytoplasm. This requires the assembly of a nuclear export complex consisting of the vRNP, M1 protein, and the viral nuclear export protein (NEP),[MCK7] which contains two nuclear export signals (NESs), in mediating the association of exportin1/XPO1/CRM1 with vRNPs in a so-called daisy chain arrangement (Akarsu et al., 2003, Huang et al., 2013, Neumann et al., 2000, O'Neill et al., 1998, Paterson and Fodor, 2012).

Could it be that incoming IAV cores retain the characteristics of membraneless liquid organelles?[MCK8][YY9] Recently, it was shown that during IAV replication viral inclusions display characteristics of liquid organelles **(Fig. 1)** (Alenquer et al., 2019). Using Fluorescence Recovery After Photobleaching (FRAP), viral inclusions formed by either PA-GFP or GFP-NP dynamically exchanged material with the exterior in a manner consistent with nucleoli and stress granules (SGs) [MCK10][YY11](Nikolic et al., 2016). These inclusions develop close to endoplasmic reticulum exit sites, segregate vRNPs from the cytosol and are thought to promote RNA-RNA interactions. Rab11, a marker for recycling endosomes, also plays a critical role in vRNP egress towards the plasma membrane (de Castro Martin et al., 2017, Eisfeld et al., 2011, Amorim et al., 2011). Biogenesis of IAV liquid inclusions enriched in vRNPs and Rab11 is dependent on continuous cycles of material between the ER and the Golgi, indicating that their distribution is spatially regulated (Alenquer et al., 2019). ~~Could it be that incoming IAV cores retain the characteristics of membraneless liquid organelles?~~[MCK12]

[insert Figure 2 here]

Histone deacetylases (HDACs) in IAV entry: what sets HDAC6 apart from other HDACs

Acetylation on lysine residues of histone and other proteins has been recognised as a major post-translational modification that affects multiple aspects of protein function. Protein acetylation is regulated by the balance of enzymes with opposing activities: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Yang and Seto, 2008). HDACs are enzymes that catalyze the removal of acetyl groups from lysine residues located on histone and non-histone proteins. As shown in Figure 3, HDACs have 18 isofo[MCK13][YY14]rms that use either Zn^{2+}- or nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent mechanisms to deacetylate lysine substrates; the former 11 enzymes are of the histone deacetylase family, the latter 7 enzymes are of the silent information regulator 2 (Sir2) family (Seto and Yoshida, 2014). ~~and~~ HDACs can be categorised into four classes: class I HDACs are 400-500 amino acids long, and include HDAC1, HDAC2, HDAC3 and HDAC8. Class II HDACs are ~1000 amino acids long; class IIa comprises HDAC4, HDAC5, HDAC7 and HDAC9, and class IIb comprises HDAC6 and HDAC10 (Valenzuela-Fernandez et al., 2008). Class III comprises the Sir2-like deacetylases Silent Information Regulator (SIRT1)-SIRT7, ranging in size from 310-757 amino acids (Marks et al., 2003), while Class IV (HDAC11) HDAC~~s are~~is a ~~Zn^{2+} dependent~~ metalloproteases (Gregoretti et al., 2004, Haigis and Guarente, 2006) (**Fig. 3**[MCK15][YY16]).

[insert Figure 3 here]

Class I HDACs influence IAV infection in complex ways: a pan class I HDAC inhibitor increases IAV infectivity; HDAC1 and 2 are antiviral whereas HDAC3 and 8 are pro-viral (Yamauchi et al., 2011). HDAC8 depletion induced centrosome splitting, an aberrant microtubule network and scattered LEs, impacting IAV entry and infection (Yamauchi et al., 2011). HDAC6, ~~an~~ a critical IAV uncoating factor, is mostly cytoplasmic with unique characteristics that distinguish it from the other HDACs: tandem catalytic domains with tubulin deacetylation activity and the presence of a zinc finger domain ~~with~~ that has homology to ubiquitin-specific proteases (ZnF-UBP)~~, which~~ and binds to unanchored ubiquitin **(Fig. 4)** (Zhang et al., 2006, Grozinger et al., 1999, Verdel et al., 2000, Miyake et al., 2016). HDAC6 ZnF-UBP binds to ubiquitin at its C-terminal Gly-Gly residues unlike most other ubiquitin binding domains, which interact with the hydrophobic core of ubiquitin (Ouyang et al., 2012, Pai et al., 2007).

[insert Figure 4 here]

HDAC6 regulates cellular stress granules, aggresomes and phase separation

HDAC6 is a versatile cytosolic deacetylase and some of its non-histone substrates are α-tubulin ~~cortactin~~, Hsp90, cortactin, HIV-1 Tat ~~and~~, DEAD box RNA helicase 3, and X-linked (DDX3X) [MCK17][YY18](Saito et al., 2019, Zhang et al., 2016, Huo et al., 2011, Hubbert et al., 2002, Kovacs et al., 2005, Zhang et al., 2007)(Reviewed in (Moreno-Gonzalo et al., 2018)). Most importantly, HDAC6 functions in the management of the cellular stress response and the management of misfolded proteins, phase separation, autophagy, pathogen sensing and neurodegeneration (Matthias et al., 2008, Miyake et al., 2016, Saito et al., 2019, Zhang et al., 2008, Boyault et al., 2007,

Kawaguchi et al., 2003, Iwata et al., 2005, Hubbert et al., 2002, Kwon et al., 2007, Lee et al., 2010a, Lee et al., 2010b, Moreno-Gonzalo et al., 2018).

HDAC6 participates in the formation of SGs and aggresomes (Kawaguchi et al., 2003, Kwon et al., 2007). Misfolded proteins that result from genetic mutations, defective protein maturation, or environmental stress are polyubiquitinated and degraded by the proteasome system. Protein aggregates form when the proteasome is inadequate, and are harmful to cell survival and must be eliminated (Tran and Miller, 1999). In the cell, misfolded protein aggregates are transported and removed from the cytoplasm by dynein motors via microtubules to a membraneless organelle called the aggresome **(Fig. 5)**(Kawaguchi et al., 2003). Aggresomes were first identified in the characterisation of a mutant form of the aggregation-prone cystic fibrosis transmembrane conducting regulator CFTR-ΔF508 (Johnston et al., 1998). CFTR-ΔF508 is normally polyubiquitinated and degraded by the proteasome (Jensen et al., 1995, Ward et al., 1995). However, the proteasome cannot degrade aggregates but stimulates autophagy-dependent aggregate clearance by generating unanchored K63-linked ubiquitin chains via Poh1, a proteasomal deubiquitinase (Nanduri et al., 2015, Hao et al., 2013). Inhibition of Hsp90 also suppresses K63-linked ubiquitin chain formation and aggresome clearance (Nanduri et al., 2015). Here, HDAC6 plays a central role by regulating both the concentration and autophagic clearance of protein aggregates or aggresomes (Lee et al., 2010a, Iwata et al., 2005, Kawaguchi et al., 2003).

Unanchored ubiquitin is a hallmark of misfolded proteins in the cell and is typically generated together with cell stress-induced misfolded proteins that are polyubiquitinated by the cellular ubiquitination machinery. These misfolded proteins are normally linked with ubiquitin K-48 (see details below) and degraded by the proteasome (Kawaguchi et al., 2003, Hao et al., 2013, Ouyang

et al., 2012). When this fails due to negative conditions of the cellular environment, the protein waste complexes undergo further rounds of polyubiquitination and deubiquitination. This leads to generation of polyubiquitin chains that are 'unanchored' and not conjugated to any substrate protein. Poh1 – a deubiquitinase from JAMM/MPN+ family – is associated with the 26S proteasome (Yao and Cohen, 2002), and plays a role in the recycling of ubiquitin. During aggresome processing, unanchored ubiquitin that regulates HDAC6 function is generated by deubiquitinases such as Ataxin-3 (Ouyang et al., 2012), which associate with protein aggregates and the proteasome-residing Poh1 (Hao et al., 2013).

The unanchored ubiquitin chains are recognised by the ZnF-UBP of HDAC6 (Ouyang et al., 2012). This binding activates recruitment of the molecular motor dynein to the dynein-binding region of HDAC6 in between the two catalytic domains, and myosin II. Hydrophobic protein-protein interactions retain the unanchored ubiquitin chains associated with the misfolded protein. Thus, the action of dynein motors transports the misfolded protein aggregate towards the microtubule-organising centre (MTOC), leading to aggresome formation. The action of myosin II and Poh1 promotes the disassembly of the aggresome. The catalytic activity of HDAC6 modulates aggresome clearance (Hao et al., 2013) and eventually the autophagic machinery or UPS degrades the misfolded protein (Moreno-Gonzalo et al., 2018).

[Insert Figure 5 here, in color]

HDAC6 uncoats IAV via ubiquitin chains and the aggresome pathway

The aggresome pathway and IAV uncoating share many similarities. Following viral fusion, HDAC6 is recruited to the fusion site on LEs via viral unanchored ubiquitin, colocalising with M1-positive vesicles (Banerjee et al., 2014). M1 binds to the HDAC6 N-terminal domain to form an M1-HDAC6-polyubiquitin chain complex **(Fig. 5)**. HDAC6 activation and triggering of the aggresome pathway in turn connects the M1-HDAC6-ubiquitin chain complex to molecular motors dynein (via the HDAC6 dynein-binding region) and myosin II **(Fig. 4, 5)**. The mechanism of myosin II recruitment remains to be solved. When both microtubules and actin are depolymerised, IAV uncoating is completely inhibited (Banerjee et al., 2014).

The co-existence of deacetylase-dependent and -independent functions of HDAC6 adds layers of complexity to the IAV infection phenotype observed upon full knockout of HDAC6. During viral entry the ZnF is important for uncoating, however, after vRNP import the deacetylase's antiviral functions come into play. For example, HDAC6 restricts IAV replication by deacetylating the viral RNA polymerase PA subunit (Chen et al., 2019), and deacetylation of Lys909 of retinoic-acid inducible gene I (RIG-I) by HDAC6 promotes RIG-I oligomerisation and viral RNA sensing, activating mitochondrial antiviral signalling protein (MAVS)-IRF3-NF-kB and IFN-β (Choi et al., 2016). Overexpression of HDAC6 leads to diminished viral budding due to induction of hypo-acetylated tubulin (Husain and Cheung, 2014, Husain and Harrod, 2011). Therefore, the complex involvement of HDAC6 in the IAV life cycle must be interpreted with care (Zheng et al., 2017).

How are ubiquitin chains made and packed into virions?

Ubiquitination is a prevalent post-translational ~~modification~~ addition of ubiquitin (a small 8.5 kDa protein composed of 76 amino acids) to other proteins, ~~that~~ thus ~~alters~~ altering ~~the~~ protein

function, localisation, trafficking and ~~its~~ degradation by the proteasome. Ubiquitin itself can be ubiquitinated on 8 different sites, namely seven lysines and the N-terminal methionine. The most prevalent types of ubiquitin linkages are K-48 and K-63 linkages. The former is known to lead to proteasomal degradation of the substrate protein, and the latter is involved in endocytosis, trafficking and enzyme activity (Komander and Rape, 2012, Kulathu and Komander, 2012).

Mechanistically, the process of protein ubiquitination involves a three-step enzymatic cascade, which starts with the ubiquitin-activating enzyme E1, followed by the ubiquitin-conjugating enzyme E2, and the ubiquitin ligase E3. Ubiquitin is activated in an ATP-dependent manner, when a high-energy thioester bond is formed between the C-terminus of ubiquitin and an internal cysteine residue of the ubiquitin-activating enzyme E1. Activated ubiquitin is then transferred onto the active site cysteine of one of the E2-conjugating enzymes. Finally, the formation of an isopeptide bond is catalyzed by E3 ubiquitin ligases, which link ubiquitin moieties to target proteins or elongate a polyubiquitin chain (Ciechanover, 2015, Rudnicka and Yamauchi, 2016). Unanchored ubiquitin chains[MCK19][YY20] ranging from mono- to hepta-ubiquitin are packaged into IAV virions in producer cells and can be detected in roughly 60 % of individual particles by super-resolution fluorescence microscopy **(Fig. 6)** (Banerjee et al., 2014). It is remarkable that IAV virions package such unanchored ubiquitin chains, and they are mostly packed between the viral envelope and M1 layer to ensure cytosolic exposure following viral fusion at LEs **(Fig. 5)** (Banerjee et al., 2014). Open questions remain: Which ubiquitin modifying enzymes generate the ubiquitin chains? How are the chains packaged into the virion? Is packaging passive or active? What about other enveloped viruses?[MCK21]

[insert Figure 6 here]

Genome-wide siRNA screening, OMICs and other approaches have identified E3 ubiquitin ligases that are important for the replication cycle of IAV. These are Cullin 3, ITCH, TRIM 25, NEDD4, and UBR4 (Huotari et al., 2012, Gschweitl et al., 2016, Su et al., 2013, Tripathi et al., 2015, Meyerson et al., 2017, Chesarino et al., 2015). Cullin 3, a member of the RING ubiquitin protein ligase family, is critical for proper functionality of the endosome maturation program and thus its effect on IAV uncoating is indirect. ITCH, a HECT-type family of E3 ubiquitin ligases, is important for IAV uncoating and was shown to ubiquitinate M1 in *in vitro* ubiquitination assays (Su et al., 2013). Its localisation to LEs suggests that ITCH can be present or recruited to viral sites of fusion on the LE surface in order to ubiquitinate M1. An influenza OMICs study of the hit genes from eight independent RNAi screen datasets identified UBR4, an UBR-box containing N-recognin family of E3 ubiquitin ligases required for efficient autophagy and membrane morphogenesis (Tasaki et al., 2013, Parsons et al., 2015). UBR4 interacts with viral M2 and promotes its translocation to the plasma membrane during late stages of viral replication (Tripathi et al., 2015). Interestingly, in UBR4 depleted cells, M2 is unable to reach the plasma membrane and is degraded by autophagy, ~~the consequences being~~ thus resulting in ~~inhibited~~ inhibition of viral budding from the plasma membrane (Tripathi et al., 2015).

Whether packaging of ubiquitin chains is promoted by any of these identified E3 ubiquitin ligases, or any of the viral proteins, is unknown. However, it is likely that ubiquitin modifiers involved in aggresome processing are important. Initial exposure of ubiquitin chains during early viral infection recruits HDAC6 via the ZnF-UBP to the surface of LEs. A single amino acid substitution (W1182A in human HDAC6, W1116A in mouse) blocks unanchored ubiquitin binding to the ZnF and inhibits aggresomes and viral uncoating (Hao et al., 2013, Banerjee et al., 2014). That the

ubiquitin binding capacity of HDAC6 is necessary and sufficient to promote IAV uncoating indicates the importance of the packaged ubiquitin chains. The linkages and various heterotypes ~~heterotypicity~~ [MCK22][YY23]of the ubiquitin chains await elucidation. It is also clear that a fraction of IAV uncoating occurs without the aid of HDAC6 (Banerjee et al., 2014).

Karyopherin-β 2 (transportin-1/TNPO1): a cellular condensate surveillance factor

M1 uncoating and vRNP debundling ~~is~~ are promoted by ~~karyopherin-β 2 (k~~Kap β 2~~)~~ - also called transportin-1 (TNPO1) - an import factor of RNA-binding proteins such as heterogenous nuclear ribonucleoprotein A1 (hnRNP A1), FUS and HuR (Twyffels et al., 2014). Kap β 2 was identified as the import factor for hnRNP A1 in mammalian cells (Pollard et al., 1996, Fridell et al., 1997, Nakielny et al., 1996). A very similar protein named karyopherin-β 2B or transportin-2 was discovered (Siomi et al., 1997, Shamsher et al., 2002) and later shown to have two isoforms A and B that share 84% and 92% sequence similarity with karyopherin-β 2 (Rebane et al., 2004). Structural analysis of complexes between k~~K~~ap β 2 and its nuclear localisation signals (NLSs) showed common patterns among various k~~K~~ap β 2-dependent NLSs and ~~was~~ these were collectively termed PY-NLS **(Fig. 7, 8)**. Canonical PY-NLSs respond to physical rules: they should be included in a basic and structurally disordered region. Cargoes that bear the PY-NLS are mostly RNA-binding proteins with many of them having RNA processing or transcription activity (Lee et al., 2006). The PY-NLS of hnRNP A1 is also known as the "M9" sequence.

[insert Figure 7 here]

The PY-NLS is defined by loose sequence motifs (N-terminal hydrophobic or basic motifs and a C-terminal (R/K/H)X2–5PY motif), structural disorder, and an overall basic charge (Lee et al., 2006). Recently it was shown that kKapβ2 binds with high affinity to the N-terminal tail of histone H3 even though it lacks a recognisable PY-NLS. The N-terminal tail of H3 contacts many of the same kKapβ2 residues as that of a typical PY-NLS (Soniat and Chook, 2016). Combinatorial mixing of energetically weak and strong motifs, PY-NLS Epitopes 1, 2, and 3, results in a range of kKapβ2 affinities suitable for nuclear import and generates large sequence diversity of PY-NLSs **(Fig. 8)** (Suel et al., 2008). For example, H3 uses a very strong EEpitope 1[MCK24][YY25] (^{12}GGKAPRK^{18}) to compensate for the lack of the often-conserved PY epitope (Soniat and Chook, 2016). There are many other cargos without a PY sequence ~~NLS~~ that binds kKapβ2 such as N-terminal tail of histone H4 and ribosomal protein rpL23A , the RNA-editing enzyme ADAR1 (Barraud et al., 2014), IAV M1 (Miyake et al., 2019a), ~~, viral proteins IAV M1 (Miyake et al., 2019a),~~ HIV-1 REV ~~**(Fig. 8)**~~ (Arnold et al., 2006), HIV-1 CA **(Fig. 8)** (Fernandez et al., 2019), and HPV E6 (Le Roux and Moroianu, 2003). The IAV M1 PY-NLS contains Epitopes 1 (^{18}GPL^{20}) and 2 [MCK26][YY27](^{24}IAQR^{27}), of which Epitope 1 overlaps with the binding motif of HB-64, an M1 monoclonal antibody that detects M1 uncoating **(Fig. 8)** (Miyake et al., 2019a). The HIV-1 CA PY-NLS Epitope 1 (^{88}AGPI^{91}) overlaps with the unstructured non-canonical cyclophilin A (CypA) [MCK28]binding site (^{88}AGP^{90}) **(Fig. 8)** (Liu et al., 2016).

[insert Figure 8 here (fit to half of page-width)]

The link between IAV uncoating and management of cellular condensates

Viruses hijack ubiquitous processes to execute the viral uncoating program. Protein disaggregation is central to the maintenance of a healthy state inside the cell and to ensure longterm cell survival. To understand viral uncoating it is critical to dissect the cell biological mechanisms of protein disaggregation and degradation (Yamauchi and Greber, 2016). To uncoat, IAV uses aggresome processing (Banerjee et al., 2014), vaccinia virus uses the ubiquitin proteasome system (UPS) (Schmidt et al., 2013b, Schmidt et al., 2013a), polyomaviruses use endoplasmic reticulum-associated degradation (ERAD) (Geiger et al., 2011, Inoue and Tsai, 2011, Schelhaas et al., 2007), and IAV and HIV-1 use nuclear import receptor (NIR)-mediated disaggregation (Guo et al., 2018, Yoshizawa et al., 2018, Miyake et al., 2019a, Fernandez et al., 2019).

Liquid-liquid phase separation (LLPS) is a common mechanism contributing to formation of membrane-less organelles (Banani et al., 2017, Hyman et al., 2014). SG[MCK29]s and aggresomes are membrane-less organelles that form when cells are under specific environmental stresses. SGs represent a pool of mRNAs stalled in translation and are composed of ribonucleoproteins (RNPs) (Protter and Parker, 2016). HDAC6 colocalises with the SG-marker Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP-1) under various stress conditions (Tourriere et al., 2003). It was recently found that HDAC6 preferentially targets acetylated lysines in intrinsically disordered regions (IDRs) of the SG component DDX3X (Saito et al., 2019). Stresses such as oxidative and osmotic stress~~, and~~ or inhibition of translation induced the acetylation of lysines in IDRs by the acetyltransferase CBP. Acetylation of DDX3X-IDR1 inhibited LLPS, and its deacetylation by HDAC6 was necessary for the assembly of large, mature SGs (Saito et al., 2019).

Aggresomes are misfolded protein aggregates that are enriched at microtubule organising centres by microtubule-dependent motors and subsequently processed by autophagy (Tyedmers et al., 2010). Recent studies have shown that LLPS driven by multivalent macromolecular interaction is an important organising principle for biomolecular condensates. Classic organelles, like the endoplasmic reticulum or Golgi apparatus are compartments defined by surrounding lipid bilayer membranes. Many cellular compartments such as Cajal bodies and PML bodies, SGs and aggresomes are not separated by membranes (Banani et al., 2017). The physical processes that promote the formation of membraneless compartments such as P granules (perinuclear membraneless compartments composed of proteins and RNAs found in germ cells of *Caenorhabditis elegans*) are liquid-like. As such, P granules fuse with one another and relax back into a spherical shape (Brangwynne et al., 2009). RNA granules are condensates that require dynamic regulation by chaperones, ATP-dependent disaggregates and molecular motors (Kroschwald et al., 2015, Jain et al., 2016).

What is most striking about the cellular function of k~~K~~ap β 2 is its significant role in the regulation of LLPS that is also connected to HDAC6 deacetylase activity and maturation of SGs **(Fig. 9)**. ~~Kap β 2 suppresses phase separation and SG association of FUS [MCK30] by chaperoning it and promoting solubility – a function that is absent in the neurodegenerative disease amyotrophic lateral sclerosis (ALS) in which disease-lined mutations in the FUS NLS impair Kap β 2 binding (Hofweber et al., 2018).~~ It is thought that NLSs constitute disaggregation signals in the cytoplasm and that nuclear import receptors such as k~~K~~ap β 2 disaggregate NLS-bearing cargo and can reverse phase separation of RNA-binding proteins (Guo et al., 2018, Yoshizawa et al., 2018). Kap β 2 promotes RNA-binding protein solubility and suppresses their phase separation and SG

association. An example is the fused in sarcoma (FUS) gene which is a component of the hnRNP complex that contains a PY-NLS recognised by k~~K~~ap β 2 (Hofweber et al., 2018) **(Fig. 8)**.[YY31]

[Insert Figure 9 here, in color]

How does k~~K~~ap β 2 uncoat incoming influenza viruses? As an importin, k~~K~~ap β 2 recognises a PY-NLS on its cargo. The IAV M1 ~~N-terminus contains~~ epitopes 1 and 2 [MCK32]~~of a PY-NLS of which Gly18 is critical for recognition by Kap β 2 **(Fig. 8)** (Miyake et al., 2019a).~~ Ser17 and Pro19 of the PY-NLS are buried interface residues of the N domains of the neutral pH M1 dimer (Harris et al., 2001). The M1 N-terminal residues 18-GPL-20 are also important for recognition by HB-64, a monoclonal anti-M1 antibody that reacts preferentially to uncoated M1 during virus entry (Miyake et al., 2019a, Banerjee et al., 2013). Priming in LEs (pH<6.0) induces conformational changes within the viral core resulting in the exposure of M1 G18, facilitating k~~K~~ap β 2 binding and promoting disaggregation of M1-M1 and vRNP-M1 complexes, leading to vRNP debundling (Miyake et al., 2019a). Overexpression of M9-NLS (from hnRNP A1) competed with M1 uncoating (Miyake et al., 2019a). In the absence of endosomal priming, the incoming vRNPs-M1 do not dissociate and vRNPs fail to enter the nucleus (Martin and Helenius, 1991, Stauffer et al., 2014). This indicates that the acidification cue during priming is critical for M1 PY-NLS exposure and k~~K~~ap β 2-mediated uncoating. Newly synthesised M1 monomers, however, ~~did~~ do not interact with k~~K~~ap β 2, reflecting that k~~K~~ap β 2-M1 interaction is dependent on acidification of high-order M1 oligomers that ~~is~~ are present in virions (Miyake et al., 2019a). This mechanism prevents vRNP debundling or M1 uncoating by k~~K~~ap β 2 from happening during assembly.

492

493 *Challenges in live imaging IAV endosomal escape (Box)*

494

495 During virus entry conformational changes within the influenza viral capsid or HA are induced
496 by cellular cues (reviewed in (Yamauchi and Greber, 2016)). Conformational changes can be
497 detected by specific monoclonal antibodies (such as one recognising the HA acid-form) used for
498 immunofluorescence quantification by automated microscopy and fluorescence activated cell
499 sorting (FACS) (Banerjee et al., 2013, Yanguez et al., 2018, Miyake et al., 2019a, Larson et al., 2019,
500 Gschweitl et al., 2016, Mar et al., 2018). HA acidification triggers lipid mixing between
501 membranes of the virus and LEs, and formation of a fusion pore (White et al., 1981, Helenius,
502 2013, Harrison, 2008). The hemifusion/fusion of a viral envelope with the endosomal membrane
503 can be detected by viral labelling with lipophilic dyes administered at a self-quenching
504 concentration. The dye dequenches upon low pH-induced lipid mixing, resulting in increased
505 fluorescence that can be readily detected by fluorescence microscopy or fluorescence activated
506 cell sorting (FACS). This method has been used successfully for a variety of virus entry studies
507 (Banerjee et al., 2013, Sakai et al., 2006, Krzyzaniak et al., 2013, Pohl et al., 2014, Rowse et al.,
508 2015, Zaitseva et al., 2010, Lakadamyali et al., 2003).

509

510 Virus fusion assays using liposomes, lipid droplets, or lipid bilayers have been combined with
511 spectrofluorimetry or total internal reflection fluorescence (TIRF) microscopy equipped with a
512 microfluidic device. These methods allow measurement of fusion kinetics and viral content
513 release (Floyd et al., 2008, Wessels et al., 2007). In addition, cryo electron tomography can be
514 used to quantify sequential membrane remodelling during influenza virus-liposome fusion (Gui et
515 al., 2016, Lee, 2010, Chlanda et al., 2016). Specifically, these steps include HA mediated dimpling

of the liposomal membrane, pinching, formation of a tightly docked interface of viral and liposomal membranes followed by M1 dissociation, hemifusion and full fusion (Gui et al., 2016). It is thought that small pores on the viral/endosomal membranes may initially form to proceed into a pore large enough for viral penetration (Zimmerberg et al., 1994). It is also thought that receptor-bound HAs are not able to carry out fusion (Dobay et al., 2011). Increased lability of the particle by endosomal priming could increase the number of non-receptor-bound HA trimers that come into proximity of the limiting membrane of the LE.

Discrimination between hemifusion/fusion in cells requires a virus content release assay in which a dye or fluorophore within the particle is released into the cytosol. An example is HIV-1 grown in ~~cyclophilin A (~~CypA~~)~~-DsRed expressing cells that incorporate CypA into the viral capsid, thus allowing the progeny particles to be used for virus content release imaging studies (Francis et al., 2016). GFP-Vpr has also been used to detect content release during HIV-1 entry into host cells (Desai et al., 2014). For IAV there are no robust content release assays compatible with live cell imaging, apart from a recently reported quantum dot (QD) labelling approach of the viral RNA polymerase subunit (Qin et al., 2019). To circumvent this influenza virus-like particles (VLPs) with a β lactamase fused to the N-terminus of M1 (BlaM1) have been used to detect full fusion and pore formation in cells (Tscherne et al., 2010). In this approach, CCF2/4 (a green dye) is cleaved by β lactamase released into the cytosol which alters the dye to a blue fluorescence. These VLPs, however, are not suited to study post-fusion uncoating and nuclear import events.

The interferon induced transmembrane protein 3 (IFITM3) inhibits the cytosolic entry of many enveloped and some non-enveloped viruses (Smith et al., 2014). The antiviral properties of IFITM1, 2 and 3 were only discovered in 2009 in an RNAi screen for regulators of influenza

infection (Brass et al., 2009). IFITM3-mediated inhibition of IAV entry and replication is of particular importance. Two single nucleotide polymorphism (SNPs); one in the coding region (rs12252-C) and another in the promoter region (rs34481144) was shown to regulate severity of influenza in human patients (Allen et al., 2017, Everitt et al., 2012). The rs34481144 allele regulates IFITM3 expression by determining methylation-dependent binding of the CTCF transcriptional repressor (Allen et al., 2017). The mechanism by which IFITM3 restricts virus entry remains controversial. Li and colleagues showed that IFITM3 blocks viral hemifusion by increasing membrane rigidity in IFITM3-overexpressing cells (Li et al., 2013). Desai and colleagues showed using single virus particle analysis of Alexa Fluor 488- and DiD-labeled IAV and influenza pseudovirus that IFITM3 inhibited fusion pore formation at late endosomes but lipid mixing happened normally (Desai et al., 2014).

(end of Box)

vRNP nuclear import

The end-point of influenza virus entry is the nuclear import of the uncoated vRNPs, which is an importin α/β-dependent process regulated by ~~a~~ classical NLSs (Boulo et al., 2007). Therefore, IAV hijacks two distinct nuclear import machineries during viral entry. The viral RNP is made of oligomeric NP, the RNA genome and viral polymerase (Moeller et al., 2012). One NP molecule binds 24-27 nucleotides *in vivo* (Area et al., 2004, Martin-Benito et al., 2001) and also has two sets of NLS, ensuring robust nuclear import of uncoated vRNPs (Eisfeld et al., 2015, Wu et al., 2007). When influenza vRNPs free of M1 were microinjected into cells, their nuclear accumulation was observed after 1 h of injection. Nuclear uptake occurred whether the vRNPs were prepared at neutral pH or extracted from viruses at acidic pH (pH 5.5) (Kemler et al., 1994). Cores that

consisted of vRNPs and M1 could not be ~~uptaken~~ imported into the nucleus. Thus the removal of M1 by k~~K~~ap β 2 causes the dissociation of vRNPs from each other, ensuring that they are small enough to pass through the nuclear pore (Bui et al., 1996, Kemler et al., 1994, Miyake et al., 2019a). During cell entry the accumulation of vRNPs in the nucleus can be detected by immunofluorescence or fluorescence in situ hybridisation (FISH) (Chou et al., 2013, Banerjee et al., 2013, Pohl et al., 2014) or by microinjection of purified vRNPs (Kemler et al., 1994, Babcock et al., 2004). Using single-particle tracking of microinjected, fluorescently labelled vRNPs, it was shown that vRNPs undergo multiple rounds of binding and release before finally being translocated through the NPC (Babcock et al., 2004). A recent study used quantum dot (QD)-bound IAV polymerase subunit PA to image incoming IAV PR8 strain vRNPs. In MDCK cells, incoming vRNPs exited from Rab7-positive LEs 30 to 90 minutes after infection (Qin et al., 2019). Here, separation of different vRNP segments were observed simultaneously or soon after the viral particles separated from Rab7-positive LEs, suggesting that M1 uncoating and vRNP debundling are events that take place rapidly at the LE surface. Following LE exit the QD-vRNPs translocated into the nucleus in a three-stage movement and exhibited two types of diffusion patterns in the nucleus (Qin et al., 2019).

How IAV prevents uncoating during assembly

In a series of elegant experiments Bui et al. showed that recombinant M1 expressed via a Semliki Forest Virus vector in cultured cells associates with vRNPs and inhibits their nuclear import. This interfering activity was eliminated after transient acidification of the cytosol to pH values approaching that of the late endosome (pH 5.0 to 5.5) using a NH_4Cl-prepulse protocol, after which the incoming vRNPs were able to enter the nucleus (Bui et al., 1996).

After replication in the nucleus, progeny vRNPs are exported to the cytoplasm via M1 and NEP. Here, the exported vRNPs are prevented from re-import into the nucleus due to binding to M1 protein (Whittaker et al., 1996, Babcock et al., 2004).

How can a virus be assembled in an infected cell and disassemble during entry into an uninfected cell? The first possibility is that the virus capsid is assembled as a stable structure in an infected cell and rendered metastable, for example by limited proteolysis, such that it can receive cues from the host (Greber et al., 1994). The second possibility is that the virus particle remains the same during assembly and egress but the infected and uninfected cells are different i.e. an uncoating factor may be activated during viral entry but rendered inactive during viral assembly. A third possibility is that the virus is unchanged but assembly and uncoating are spatially separated - viruses that undergo endocytosis use this strategy among others. [MCK33]

Low pH in LEs can prime influenza virus cores for disassembly by specifically causing M1 dissociation and allowing the vRNPs to enter the nucleus (Martin and Helenius, 1991). Remarkably, newly assembled progeny M1-vRNP complexes in the cytosol of infected cells are also dissociated by acidification. Using a heterokaryon system, Bui et al. showed that the removal of M1 from progeny vRNPs (that have exited the nucleus of an infected cell) by brief acidification allows vRNPs to re-enter the nucleus of a non-infected cells (Bui et al., 1996)[MCK34][YY35]. Thus, acidic pH inside endosomes serves as a critical switch that allows M1 to carry out its multiple functions in the uncoating, nuclear transport, and assembly of vRNPs. Finally, Figure 10 summarises the stepwise entry of IAV into host cells from attachment, uptake (clathrin-mediated endocytosis or macropoinocytosis), priming in endosomes by endosome maturation, viral fusion

with LEs and fusion pore formation, M1 shell uncoating by HDAC6, vRNP debundling by kKapβ ~~β~~2, and vRNP import into the nucleus **(Fig. 10)**.

[insert Figure 10 here, in color, rotate 90 degrees counter clock-wise to fill one page]

Kapβ2 is a ~~The emerging concept of uu~~niversal uncoating factor~~s~~ [MCK36]

As shown in Figure 11, ~~Karyopherin-~~ ~~β~~2 ~~is emerging as a universal uncoating factor for enveloped RNA viruses~~It ~~is logical that multiple viruses hijack Kap~~ ~~β~~2, ~~a key player in the regulation of LLPS, for uncoating by containing a PY-NLS in their capsid protein.~~ ~~and has been shown to use a similar mechanism to uncoat IAV and HIV-1 **(Fig. 11)** (Miyake et al., 2019a, Fernandez et al., 2019).~~ there is a remarkable similarity between the uncoating mechanisms of IAV and HIV-1 ~~uncoating suggests that usage of PY-NLS is a common RNA virus uncoating strategy~~. ~~Similar~~ Potential PY-NLS ssequences with a loose consensus of -ϕ-G/A/S-ϕ-ϕ (where ϕ is a hydrophobic side chain) (Lee et al., 2006) are found in the coat proteins of flaviviruses and filoviruses.~~, however,~~Whether ~~if these~~such sequences contribute to viral uncoating remains to be seen. Kapβ2 binds unstructured NLSs (Lee et al., 2006). For ~~Both viruses possess a PY-NLS in their shell protein **(Fig. 8)** which is recognised by Kap~~ ~~β~~2 ~~in the cytosol.~~ IAV M1, Gly18 and its adjacent hydrophobic residues ~~is~~ are important for ~~is critical for viral infectivity, and primed G18A mutant IAV virions failed to interact with~~ kKap β 2 interaction. G18A mutant virions exhibit a dual negative effect on viral assembly and uncoating, which greatly compromises infectivity (Miyake et al., 2019a). Interestingly, X-ray crystallography and structural analysis of M1 G18A showed ~~the~~ disappearance of a cavity present in the WT M1 (L1/L3 loop region).

~~indicating that~~ ~~T~~this structural alteration likely impacts M1 binding to k~~K~~ap β 2 ~~, as well as, HB-64 monoclonal antibody M1 binding~~ (Miyake et al., 2019a). ~~Kapβ 2 is known to bind unstructured NLSs (Lee et al., 2006) . It is unclear which part of M1 dimer becomes disordered for optimal Kapβ 2 binding~~T~~. ~~For example, t~~he disordered linker region between the N- and C-terminal domains of M1 ~~is a candidate, due to its~~becomes ~~increasing~~ susceptible to limited protoelysis ~~disordered nature and~~ after low pH/high K^+ treatment ~~priming induced exposure~~ (Stauffer et al., 2014). Though the linker is not part of the canonical PY-NLS **(Fig. 8)** it may contribute to kap β 2 binding due to its ~~physical~~ proximity to ~~G18~~the PY-NLS in the three-dimensional M1 structure. ~~Thus, the PY-NLS of M1 is most likely to be unconventional and may recruit kapβ 2 only under a specific structural context that is active during viral entry.~~ That n~~N~~ewly synthesised M1 fails~~ed~~ to interact with kap β 2 suggests that the PY-NLS of M1 recruits kapβ2 only under a structural context that is present during viral entry (Miyake et al., 2019a). ~~This would separate viral uncoating and assembly and be beneficial for IAV.~~

For HIV-1, G~~lycine~~ 89 of ~~HIV-1~~ CA overlaps with the unstructured non-canonical c~~C~~ypA [MCK37]binding site (^{88}A~~88~~ G~~89~~ P^{90}) **(Fig. 8)** (Liu et al., 2016). Since a single c~~C~~ypA bridges two CA molecules from adjacent hexamers, a sub- stoichiometric level of c~~C~~ypA stabilizes the capsid and protects premature uncoating, thus promoting reverse transcription in all cell types (Sayah and Luban, 2004). Glycine 89 is also crucial for k~~K~~ap β 2 binding to the CA lattice, and for uncoating and subsequent nuclear import of the HIV-1 genome. The G89V mutant HIV-1 disposed of the k~~K~~ap β 2-dependent phenotype for cell entry albeit with deleterious effects on viral infectivity. Recombinant k~~K~~ap β 2 protein ~~induced structural damage to~~disassembles purified wt HIV-1

capsid/nucleocapsids ~~(CANC[MCK38])~~ whereas ~~-~~G89V mutant [YY39]capsid/nucleocapsids ~~CANC~~ ~~was~~were unaffected. Molecular docking simulations suggested that k~~K~~ap β 2 may insert itself between capsid hexamers, which could induce strong steric hindrance and uncoating (Fernandez et al., 2019). ~~To summarise, kapβ2 is emerging as a universal viral uncoating factor.~~ Thus,~~It is logical that, for efficient viral uncoating and disaggregation of viral capsids,~~ RNA viruses have evolved to hijack kap β 2, a key player in the regulation of LLPS, for efficient viral uncoating and disaggregation of incoming capsids. ~~It is logical that multiple viruses hijack Kapβ2, a key player in the regulation of LLPS, for uncoating by containing a PY-NLS in their capsid protein.~~

[insert Figure 11 here]

Concluding remarks

Influenza viruses are pathogens of enormous medical and economic impact. According to the World Health Organization (WHO), annual influenza epidemics result in an estimated 3–5 million cases of severe illness and 290,000 to 650,000 deaths worldwide each year. The virus' zoonotic nature, its segmented genome, and the error-prone viral RNA polymerase means that IAV can undergo antigenic alterations and genetic reassortment that can lead to an unexpected pandemic. Current seasonal influenza virus vaccines do not provide sufficient protection to alleviate the annual impact of influenza and cannot confer protection against potentially pandemic influenza. The lack of protection is due to rapid changes of the viral epitopes targeted by the vaccine and the often suboptimal immunogenicity of current immunization strategies (Nachbagauer and Palese, 2019). Current and past IAV antivirals target the viral proteins NA

(oseltamivir, Tamiflu®), ~~the~~ RNA polymerase (baloxavir marboxil, Xofluza) or M2 channel (amantadine). That amantadine was historically effective (until viral resistance spread) indicates the importance of priming and uncoating in a physiological context and for IAV pathogenicity. During the last decade, genome-wide screens using siRNA, haploid cells, and CRISPR/Cas identified a multitude of host cell factors that promote viral entry and infection. However, not much is known about the precise mechanism of action. For such findings to develop into viable cell-targeted antiviral therapies, they require mechanistic dissection of the interplay that occurs at the interface of virus and host. In the future, we may be able to develop antiviral strategies that complement influenza vaccines, and also identify universal uncoating pathways used by enveloped RNA viruses. The 2020s hold~~s~~ promise in the conceptual advancement of broadband antiviral therapeutics that target cellular machineries essential for the entry and uncoating of enveloped RNA viruses.

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Figure legends

Figure 1

The influenza A virus life cycle. After virion binding to cell surface sialic-acid containing receptors the particle is endocytosed into vesicles. As the endosome matures, virions undergo priming by acidic pH followed by viral fusion with the late endosomal membrane, M1 uncoating, and vRNP debundling. The vRNPs are nuclear imported by importin α/β after which viral replication takes place inside the nucleus. After replication, progeny vRNPs are trafficked to the plasma membrane in a Rab11- and endoplasmic reticulum-dependent manner as the vRNPs separate into membraneless organelles and eight vRNPs are bundled. Budding takes place from lipid rafts into the extracellular space. Note that M1 and vRNP uncoating can take place on the cytosolic surface of LEs or in their vicinity. (Goodsell et al., 2019). Three-dimensional structure data: M1 (PDB ID: 1EA3); HA (PDB ID: 2IBX); NA (PDB ID: 6CRD); M2 (PDB ID: 3BKD); Ubiquitin (PDB ID: 1UBQ).

Figure 2

Mechanistic model of HDAC6-mediated IAV M1 uncoating. After endocytosis, the IAV particle is trafficked towards the nucleus. As endosomes mature, their internal pH ~~reduces~~ becomes acidic, ~~and~~ trigger~~s~~ing HA-mediated viral fusion with the limiting membrane of the late endosome. This leads to fusion pore formation, and the viral core consisting of M1 and vRNPs gains access to the cytoplasm. The virally packaged unanchored ubiquitin is exposed to the cytosolic surface of the endosomes, acting as bait to recruit the HDAC6 protein by its ZnF-UBP which activates

aggresome processing, and the dynein and myosin II binding capacity of HDAC6. The IAV M1 also binds to the N-terminus of HDAC6, resulting in the linking of M1 to microtubule motors and the actomyosin network. The shearing force generated by the molecular motors eventually leads to the dismantling of the viral core and release/exposure of the vRNPs to the cytoplasm after which they are further debundled by Karyopherin-β 2 (not shown) (Banerjee et al., 2014, Miyake et al., 2019a). Debundling is then followed by nuclear import of the vRNPs into the nucleus. MTOC, microtubule organising centre. The figure was adopted from (Banerjee et al., 2014)[MCK40].

Figure 3

Different classes of histone deacetylases (HDACs) and their domain composition. HDACs family contains 18 isoforms, which can be categorized into four classes: class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9 and 10) and class IV (HDAC11) HDACs are Zn^{2+} dependent metalloproteases, and are mechanistically distinct from the NAD^{+} dependent class III HDACs called SIRT ((Sirtuins) 1–7)(not shown). MEF, myocyte-specific enhancer factor 2A; SE14, Ser-Glu-containing tetradecapeptide repeats. Modified from (Ellmeier and Seiser, 2018).

Figure 4

Domains of HDAC6 and their major functions. HDAC6 has tandem catalytic domains (CD1, CD2) with the capacity to deacetylate tubulin and the presence of a zinc finger domain with that has homology to ubiquitin-specific proteases (ZnF-UBP), which and binds unanchored ubiquitin. Binding to ubiquitin occurs by recognition of the C-terminal diglycine motif in unattached chains or monoubiquitin. DDX3X, DEAD box RNA helicase 3, X-linked. Three-dimensional structure data: HDAC6 (PDB ID: 5G0J).

Figure 5

How HDAC6 reacts to cellular stress and virus entry. (Top panel) Cellular stress: misfolded protein aggregates are poly-ubiquitinated, after which they are deubiquitinated by DUBs (not shown), generating unanchored ubiquitin chains. The C-terminal-free ubiquitin is recognised by HDAC6 ZnF-UBP and activates HDAC6 binding to dynein. The protein aggregate undergoes retrograde transport on microtubules towards the MTOC, generating an aggresome. (Bottom panel) Virus entry: IAV packages unanchored ubiquitin chains that are exposed to the cytosol after low pH-mediated viral fusion at LEs. HDAC6 binds both to the unanchored chains and to M1, forming an M1-HDAC6-polyubiquitin chain complex. HDAC6 activation and binding to dynein and myosin II generates the shearing force needed to uncoat the M1 shell.

Figure 6

A rendered structured illumination microscopy (SIM) [MCK41]image of individual IAV X31 particles stained ~~against~~ for HA (red) and unanchored ubiquitin (green). Scale bar; 100 nm.

Figure 7

Domain composition of k~~K~~ap β 2/TNPO1. Kap β 2 is a superhelical stacking of 20 HEAT repeats (H1-H20). The RanGTP-binding domain locates at the N-terminal HEAT repeats 1-7. The H8 loop (residues 312-374) is crucial for substrate dissociation in the presence of Ran. HEAT repeats 9-20 comprise the substrate binding sites (adapted from (Lee et al., 2006, Twyffels et al., 2014))(Yoshizawa et al., 2018, Miyake et al., 2019a, Fernandez et al., 2019, Saito et al., 2019, Hofweber et al., 2018). Three-dimensional structure data: k~~K~~ap β 2 (PDB ID: 5YVG).

Figure 8

Sequence alignment of known PY-NLSs with the conserved IAV M1 N-terminal sequence and the HIV-1 CA. Sequences for epitopes 1 (a hydrophobic patch, containing glycine (highlighted in light pink), 2 (a basic patch) and 3 (PY) were adapted from (Lee et al., 2006, Soniat and Chook, 2016). FUS, fused in sarcoma. Adapted from (Fernandez et al., 2019, Miyake et al., 2019b)

Figure 9

HDAC6 and kKap β 2 are at the interface of cellular condensates and involved in regulation of stress granules, protein aggregates and liquid-liquid phase separation (LLPS). Cellular condensates: intrinsically disordered regions (IDRs) are acetylated upon cellular stress, and acetylated lysines are subsequently deacetylated by HDAC6. Positively charged lysine residues in the IDR promotes LLPS and allows other IDR-containing proteins as interaction partners of IDR to engage in the formation of large mature SGs (Saito et al., 2019). Kap β 2 can bind to PY-NLSs in disordered regions of condensates and chaperone their disaggregation (Yoshizawa et al., 2018, Hofweber et al., 2018). Aggresomes: HDAC6 recognizes unanchored ubiquitin chains generated from deubiquitination, such as by ataxin-3, of polyubiquitinated misfolded proteins, which binds to HDAC6 and activates its interaction with the dynein motor. The misfolded proteins undergo retrograde traffic on microtubules to form aggresomes.

Figure 10[MCK42]

Model of stepwise IAV uncoating by HDAC6 and kKap β 2. (1)- Attachment to sialic acids on receptor proteins on the cell surface triggers (2) virus uptake by clathrin-mediated endocytosis (top) or macropinocytosis (bottom). (3) Endosome maturation, acidification and ~~viral core~~ priming in endosomes via M2 induces conformational changes in the viral core. (4) Viral fusion at low pH

exposes ubiquitin chains to the cytosol recruiting HDAC6. (5) HDAC6 binding to ubiquitin chains ~~HDAC6 is recruited to the fusion pore, binds the ubiquitin chain thereby activating~~activate dynein and myosin II interaction with HDAC6 (red arrows)~~s~~, . ~~This~~ generat~~ing~~es a shearing force that ~~breaks apart the M1 shell~~uncoats the M1 shell ~~and vRNP bundle release~~. Note that M1 uncoating and vRNP bundle release/debundling can take place on the LE cytosolic surface or in the cytoplasm. In this scheme, vRNP release and debundling is shown in the cytoplasm for clarity of representation. (6) Kap β 2 binds to PY-NLS on M1 (colored in red) and disaggregates the vRNP-M1 bundle, leading to ~~the debundling of~~ vRNP debundling~~s~~ into individual segments. (7) vRNPs are nuclear imported by importin□□□□□after which viral replication takes place.

Figure 11

PY-NLS is a viral cue that triggers IAV and HIV-1 uncoating. M1 dimers and CA hexamers are the smallest unit of the viral shell. Recognition by Kap β 2 ~~binds PY-NLSs via~~is dependent on the hydrophobic side chains of substrate residues adjacent of G18 (IAV M1) or G89 (HIV-1 CA)[MCK43] ~~on the capsid surface and promotes their disaggregation~~. The cue for ~~Low pH is the cue for~~ PY-NLS exposure of IAV M1 is low pH exposure in endosomes~~, whereas the cue for HIV-1 is unclear~~ (Rose et al., 2018, Fernandez et al., 2019, Miyake et al., 2019b). Three-dimensional structure data: M1 (PDB ID: 1EA3), CA (3J3Q), CA (3GV2), kap β 2 (5YVG).