



## Review

## Revisiting influenza A virus life cycle from a perspective of genome balance

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## ABSTRACT

Influenza A virus (IAV) genome comprises eight negative-sense RNA segments, of which the replication is well orchestrated and the delicate balance of multiple segments are dynamically regulated throughout IAV life cycle. However, previous studies seldom discuss these balances except for functional hemagglutinin-neuraminidase balance that is pivotal for both virus entry and release. Therefore, we attempt to revisit IAV life cycle by highlighting the critical role of “genome balance”. Moreover, we raise a “balance regression” model of IAV evolution that the virus evolves to rebalance its genome after reassortment or interspecies transmission, and direct a “balance compensation” strategy to rectify the “genome imbalance” as a result of artificial modifications during creation of recombinant IAVs. This review not only improves our understanding of IAV life cycle, but also facilitates both basic and applied research of IAV in future.

## 1. Introduction

Influenza A virus (IAV) is a negative RNA virus with eight genome segments. Each viral RNA (vRNA) segment harbors one or two open reading frames (ORFs), and the segments were simply named after the major protein they encode, including polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M) and non-structural proteins (NS). The segmented genome allows reassortment occur frequently (Li et al., 2010; Sun et al., 2011; Zhang et al., 2013; Shao et al., 2017), which in combination with the inherent high mutation rate of RNA viruses contributes to the rapid evolution of IAV (Shao et al., 2017). Moreover, there are 18 HA (H1–H18) and 11 NA (N1–N11) subtypes, making the reassortment process more sophisticated (Tong et al., 2013; Shao et al., 2017). Although human influenza viruses have only limited subtypes of HA and NA, including H1, H2, H3 and N1, N2, other HA/NA combinations might transmit across species barrier causing sporadic human infections that are of high mortality, e.g., the avian influenza viruses H5N1, H7N3, H7N9, H5N6, H9N2 and H10N8 (Gambotto et al., 2008; Gao et al., 2013; Yiu Lai et al., 2013; Chen et al., 2014; Sun and Liu, 2015; Beerens et al., 2020; Gu et al., 2022).

Vaccines and antiviral drugs are two major countermeasures against IAV infection. However, as seasonal influenza vaccines only comprise

circulating IAV H1N1 and H3N2 strains in addition to one or two influenza B virus strains, it is necessary to develop novel universal influenza vaccines that provide broader and lasting protection against pan-subtype IAVs (Du et al., 2021). On the other hand, increasing emergence of drug resistance emphasizes the urgent need to develop novel antivirals (Lackenby et al., 2018; Takashita, 2021). Expanding our understanding of IAV infection would greatly promote drug development and improve vaccine production. In this review, we focus on current advances in the delicate and dynamic regulation of IAV life cycle.

## 2. Overview of IAV life cycle

The IAV virions are enveloped with a lipid bilayer that contains three viral transmembrane proteins, including HA, NA and M2. HA is the most abundant surface protein that comprises two subunits, HA1 and HA2 (Wilson et al., 1981; Chen et al., 2021). HA1 mediates receptor binding to sialic acids (SA) residues at the surface of host cells, followed by virus uptake through endocytosis. The low pH condition of the maturing endosomes then induces a series of conformational changes of HA2, including exposure of the hydrophobic fusion peptide and “loop-to-helix” transition, which is responsible for fusion of viral and host endosomal membranes, allowing release of internal genetic contents into the cytoplasm (Luo, 2012; Chen et al., 2021).

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The genome of IAV exists in the form of viral ribonucleoprotein (vRNP) complexes, which comprises the eight vRNA segments, each binding to multiple copies of viral NP and a trimeric viral RNA dependent RNA polymerase (RdRp) complex (PA, PB1 and PB2). After release into the cytoplasm, the vRNPs traffic into the nucleus by using the classical importin  $\alpha/\beta$ 1 (IMP $\alpha/\beta$ 1)-dependent nuclear import pathway, while various other host factors are also involved (Eisfeld et al., 2015; Zhang et al., 2016; Luo et al., 2018).

Within the nucleus, the transcription of vRNAs into messenger RNAs (mRNAs) occurs by hijacking the host's transcription machinery using a mechanism termed "cap snatching" for its own benefits, followed by transport of viral mRNAs into cytoplasm and translation into varied viral proteins (Plotch et al., 1981; Reich et al., 2014). The newly synthesized NP and RdRp constituents were then imported to the nucleus to initiate vRNA replication through a complementary RNA (cRNA) intermediate (Deng et al., 2006). These new vRNA copies can either be used as templates for transcription and replication, or be exported from the nucleus via the chromosome region maintenance 1 (CRM1)-dependent pathway through the nuclear pores, where viral M1 and nuclear export protein (NEP) are involved (Martin and Helenius, 1991; Shimizu et al., 2011; Huang et al., 2013).

Subsequently, the progeny virions assemble in the cytoplasm and bud at the host cell's plasma membrane (Nayak et al., 2009). At last, the viral NA at the plasma membrane would cleave the SA residue from glycoproteins and glycolipids, facilitating release of mature virions from host cells (Palese et al., 1974).

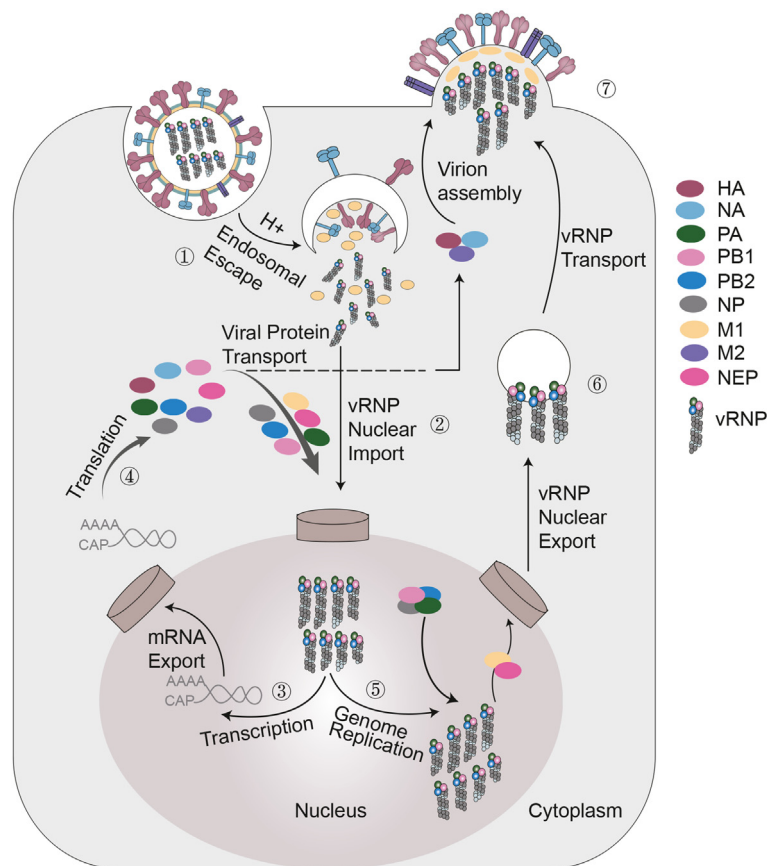
The model of IAV life cycle is summarized in Fig. 1.

### 3. Functional balance between HA and NA

The functional balance between HA and NA in influenza viruses has been illustrated extensively (Gaymard et al., 2016; Du et al., 2019). In brief, as the IAV virions attach to cell surface via binding of HA to the SA receptors, while NA cleaves the SA residues from glycoproteins and glycolipids enabling virus detachment from host cells, a balance between the antagonistic activities of HA and NA on SA binding is pivotal for both virus entry into and virus release from host cells (Du et al., 2019; Reiter-Scherer et al., 2019). Otherwise, in the case that HA binding affinity is high while NA cleavage is less efficient, the virus might get trapped by decoy receptors, blocking virus movement at cell surface and entry. Oppositely, if receptor binding activity of HA is overwhelmed by the receptor destroying activity of NA, every binding attempt of IAV via HA to host cells will be disrupted by NA cleavage, resulting in failed binding (Du et al., 2019). Moreover, the HA-NA balance is closely related to the interspecies transmission, host adaptation, and pathogenicity (Matrosovich et al., 2000; Chutinimitkul et al., 2010; Zhu et al., 2013; Byrd-Leotis et al., 2017).

### 4. Equilibrium of viral polymerase-vRNA interplay during transcription and replication

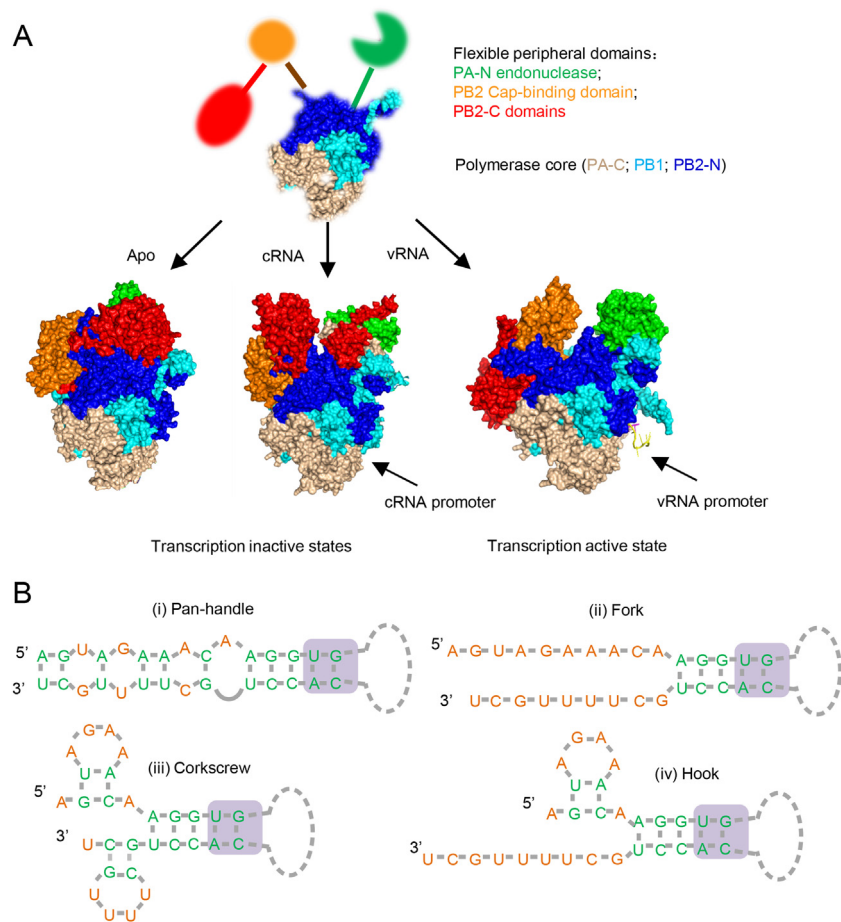
After entry into host cells, the vRNAs are used as template to transcribe into mRNAs as well as replicate themselves. Moreover, the viral RdRp complexes are responsible for both transcription and the two-step replication. It's therefore essential to illustrate how IAV regulates the initiation of transcription versus replication.



**Fig. 1.** Influenza A virus (IAV) life cycle. The virus entry into host cell is initiated by hemagglutinin (HA) mediated receptor binding followed by endocytosis and membrane fusion, releasing internal genetic contents into the cytoplasm (1). The incoming viral RNAs (vRNPs) are then transported into nucleus (2), where genome transcription occurs (3). After export of viral mRNAs from nucleus to cytoplasm, viral proteins are expressed (4) and the newly synthesized RdRp constituents and NPs are imported into nucleus, facilitating vRNA replication and vRNP formation (5). Progeny vRNPs are then transported into cytoplasm, assemble (6) and bud at plasma membrane (7). Eventually, progeny virions are released from host cell surface via viral neuraminidase (NA) mediated receptor destroying.

So far, it has been well acknowledged that the RdRp complexes that are bound to incoming vRNAs in the context of vRNPs could catalyze transcription only, while the vRNAs would not start to replicate themselves until functional RdRp complexes are available in trans (Te Velthuis and Fodor, 2016; Fay et al., 2020). Although the underlying mechanism remains elusive, the inherent flexibility of influenza polymerase is one of the most critical factors (Fig. 2A). Multiple conformations of the polymerase have been captured by X-ray crystallography and most likely represent two different functional states. First, the RNA-free (apo form) and cRNA-bound polymerases show similar conformation, in which PB2 cap-binding and PA endonuclease domains are oriented in a way that is incompatible with cap-snatching, corresponding to a transcriptionally inactive state (Hengrung et al., 2015; Thierry et al., 2016; Fan et al., 2019). Second, in the presence of vRNA, the polymerase would undergo substantial rearrangements to form a transcription pre-initiation state, in which the cap-binding domain makes little contact with the other domains and can rotate freely *in situ*, while the endonuclease is stabilized by the PB1 C-terminus and PB2 N-terminus interaction motif; the rearrangement allows IAV polymerase ready for cap-snatching, indicating a transcription active state (Cianci et al., 1995; Fan et al., 2019). Interestingly, some IAV-derived regulatory small vRNAs may also be involved in viral switch from transcription to replication through interactions with the viral polymerase machinery (Perez et al., 2010; Resa-Infante et al., 2011).

In addition to the flexible conformation of virus polymerase, the diverse secondary structures of IAV vRNAs also contribute a lot to the regulation of replication versus transcription. Generally, the genome vRNAs in mature virions and IAV infected cells are held in a circular conformation, while the secondary structure formed from the 3' and 5'-terminal acts as the promoter for viral RdRp complexes (Hsu et al., 1987). Interestingly, the promoter structures have been a controversial topic in the literature, and as many as four models have been proposed, including “pan-handle”, “fork”, “corkscrew” and “hook”-like structures (Fig. 2B) (Fodor et al., 1995; Flick et al., 1996; Bae et al., 2001). All the four models share a duplex formed by 10–12 bases at the 3' end and 11–13 bases at the 5' end, which can be further extended by 1–4 base-pairs (bp) in a segment-specific manner (Hsu et al., 1987; Shin et al., 2021). Although it remains a question regarding the precise promoter structures that are relevant for transcription or replication, the promoter structure may change upon binding to RdRp complexes and interactively influence the arrangement of viral polymerase. For instance, the promoter may fold as a panhandle initially in the absence of the polymerase (Noble et al., 2011; Liu et al., 2015), and its binding to the apo form of polymerase allows initiation of replication; however, this replication-capable state should exist in transient and soon after the binding, the polymerase rearranges to the transcription-active form while the panhandle



**Fig. 2.** Alternative configurations of influenza polymerase and viral RNA (vRNA) promoters. **A** The peripheral domains (PA-N endonuclease, PB2 cap-binding domain and PB2-C domains) of influenza polymerase complex are flexible and rearrange upon binding to template vRNA and cRNA. The apo form (PDB ID: 5d98) and cRNA binding form (PDB ID: 5epi) of influenza polymerase represent transcription inactive states, while the vRNA binding form (PDB ID: 4wsa) could initiate “cap-snatching” which is critical for transcription. **B** Different vRNA promoter structure models proposed over time. Unpaired nucleotides are shown in brown, while paired nucleotides are shown in green. The segment specific duplex extensions are highlighted in grey.

promoter converts to a corkscrew or hook conformation accordingly (Flick et al., 1996; Pflug et al., 2014).

The interplay between the flexible polymerase conformations and promoter structures cooperatively regulates vRNA transcription and replication in order to produce desired amounts of mRNAs as well as cRNA/vRNAs. In addition, it is worth noting that the compatibility between each of the influenza RdRp constituents is also critical during IAV replication (Taubenberger and Kash, 2010; Mänz et al., 2013).

## 5. Balance of multiple segments during IAV infection

Since the IAV genome consists of eight segments, how the virus balances the replication/transcription of these multiple segments is another important issue. It is not surprising that the multiple segments compete with each other to recognize RdRp complexes (Widjaja et al., 2012). As a consequence, at early stages of infection when RdRp complexes are of limiting amounts, the replication of one segment should be negatively regulated by the presence of other seven counterparts; while as RdRp complexes accumulate according to the course of infection, the competition could be gradually relieved (Widjaja et al., 2012). This competition confers delicate and dynamic balance of multiple segments during IAV infection, and each segment follows individual dynamics in the levels of vRNA, cRNA and mRNA (Phan et al., 2021).

Several factors are involved in the competition, including the aforementioned promoter structures, the length of coding regions, as well as the inherent activity and template preference of IAV polymerase (Widjaja et al., 2012). As the length of each segment is relative fixed, the segment-specific non-coding regions (NCRs) are evolved to accommodate the varied length and desired transcription/replication efficacy. For instance, the segment-specific extended duplex region of promoter plays critical role to ensure optimal replication/transcription of individual segment in the multi-segment environment (Ma et al., 2013; Zhao et al., 2014; Wang et al., 2017; Xiao et al., 2021). In addition, a U/C polymorphism at position 4 within the highly conserved region of 3'-NCR also contributes a lot to fine tune the balance of multiple segments (Sun et al., 2014).

## 6. Differentiated regulation of viral proteins expression

Different viral proteins function at different stages during virus life cycle, and many viruses have developed diverse strategies to regulate the time-phase and amount of each viral protein expression.

During IAV infection, as NP and RdRp constituents are required immediate early for vRNA transcription and replication and throughout the whole life cycle, NA and HA are involved at the late budding stage. Moreover, as each vRNP constitutes multiple copies of NP compared to one copy of RdRp constituent each, the virus needs to express much more NP compared to PA, PB1 and PB2. To this end, the virus controls the delicate balance of multiple segments during infection using a mechanism of dynamic competition as described above, and as a consequence, different groups of viral genes express following varied kinetics (Phan et al., 2021).

Besides, all the viral segments except for HA and NA encode for more than one protein (Chauhan and Gordon, 2022). For example, segments M and NS encode for two proteins each through mRNA splicing. Segment M encodes for M1 and M2, whereas segment NS encodes for NS1 and NEP. As spliced products, M2 and NEP are generally much less abundant than NS1 and M1 due to the inefficient splicing (Samji, 2009; Mor et al., 2016). Notably, it has been reported that the NS transcript maintains a similar ratio of non-spliced and spliced transcripts throughout infection and the slow accumulation of NEP is critical to coordinate the timing of IAV infection (Inglis and Brown, 1984; Chua et al., 2013), whereas the ratio of the spliced M transcripts (encoding M2) has been shown to increase during infection, ensuring the biased M2 expression toward the later stages of infection (Valcárcel et al., 1991).

## 7. Selective packaging of multiple segments into virions: stubborn or compromised?

To guarantee productive infection, an infectious IAV particle should consist of at least one copy of each segment, which makes the packaging process sophisticated. In order to explain the packaging of multiple segments into virions, two models have been hypothesized (Fig. 3A): a random packaging model assumes that the various viral RNA segments are arbitrarily incorporated into virions (Enami et al., 1991; Bancroft and Parslow, 2002); whereas a selective packaging model predicts that each segment has a distinct packaging signal differentiating it from the others, ensuring the integration of eight unique vRNP segments into each virus particle (Fujii et al., 2003).

So far, growing evidence from reverse genetics studies, EM observations and other advanced macromolecular interaction analysis has favored the selective packaging model. For instance, the packaging signals of individual segments have been identified to play essential role for their efficient incorporation into progeny virus particles (Hutchinson et al., 2010; Li et al., 2021). Besides, it was characterized that a single copy of each of the eight different vRNP segments is bundled following a “1 + 7” configuration within progeny virions (Noda et al., 2006, 2018; Chou et al., 2012; Haralampiev et al., 2020). Moreover, the inter-vRNP interactions and pathways that are decisive for vRNP bundling have been widely illustrated (Fournier et al., 2012; Gavazzi et al., 2013; Bolte et al., 2019; Haralampiev et al., 2020; Miyamoto and Noda, 2020).

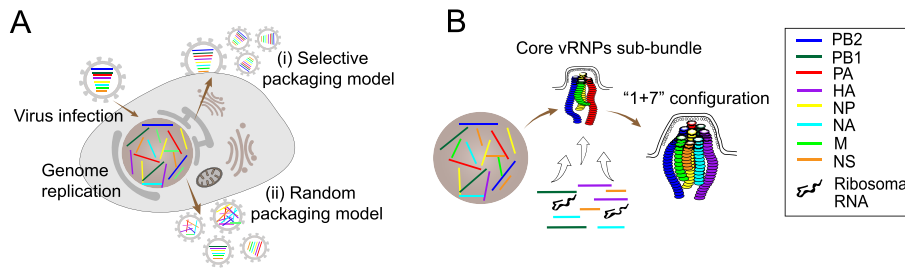
However, increasing controversies that challenge the selective packaging model have been reported. First, several recombinant IAVs harboring nine instead of eight unique segments have been successfully constructed by reverse genetics (Enami et al., 1991; Gao et al., 2010; Wu et al., 2012). Second, the eight segments are not equally important in their role for vRNP bundling, and it is likely that PB2, PA, NP and M play a more crucial role than other segments in the packaging procedure (Muramoto et al., 2006; Gao et al., 2012). In addition, Noda et al. reported that most budding progeny virions incorporated eight RNPs arranged in a “1 + 7” pattern even in the presence of only seven distinct vRNAs lacking HA (Noda et al., 2018). Intriguingly, the progeny virions incorporate 18S or 28S ribosomal RNAs (rRNAs) but not other available segments to occupy the place of HA segment (Noda et al., 2018). All these together impel the highly selective packaging model to be modified.

It is reasonable that the highly “selective” model is fine-tuned to the most at the expense of viral genome to code the desired information, while “random” model sacrifices packaging efficacy to save the coding capacity. We herein propose a compromised packaging model that prefers half selective and half random, which balances the cost of coding capacity and packaging efficacy. After exporting from nucleus, a highly selective core sub-bundle containing at least PB2, PA, NP and M is formed initially, which is sufficient for success budding. Nevertheless, the core sub-bundle is prone to form a more stabilized “1 + 7” configuration by recruiting more vRNPs. This process occurs at random and is less selective, allowing incorporation of the additional ninth segment or rRNAs by chance (Fig. 3B). Of note, self-repulsion at this random packaging stage is probably involved to avoid iterate integration of identical segments into the same progeny virions (Venev and Zeldovich, 2013).

## 8. Conclusions and perspectives

### 8.1. Balance regression during virus evolution

The delicate balance described above might get disturbed during IAV evolution, e.g., when newly coupled HA-NA subtypes emerge, after interspecies transmission, or in presence of antiviral drugs. Once the imbalance threatened the viral viability, selection of mutations that help to rectify the imbalance would continuously occur to achieve balance regression. This balance regression phenomenon has been well documented (Glaser et al., 2005; Hossain et al., 2008; Sorrell et al., 2010; Hoffmann et al., 2012; Park et al., 2017; Arai et al., 2020).



**Fig. 3.** Diagrams of the proposed models of influenza A virus (IAV) genome packaging. **A** Conflicting hypothesis of the genome packaging model. The viral genome replication takes place in the nucleus. The viral RNA (vRNA) segments, in the form of RNPs, are transported to the plasma membrane, followed by packaging and budding. In the selective packaging model (i), eight unique vRNA segments are consistently packaged into every progeny virus particle. The random packaging model (ii) proposes that vRNA segments are arbitrarily packaged into virus particles, and the progeny virus particles are infectious only when at least one copy of each segment is incorporated. **B** A compromised model that accommodates the “selective” and “random” packaging models. After transport from nucleus, a highly selective sub-bundle of vRNPs comprising of at least PB2, PA, NP and M segments is formed initially. Although the core sub-bundle is sufficient for virus budding, a more stabilized “1 + 7” configuration is prone to form by recruiting more vRNPs. This process occurs at random and contaminants like ribosomal RNAs might be incorporated by chance.

For example, as HA-NA mismatch is a common consequence of IAV interspecies transmission, balance regression of HA-NA is usually required for host-adaption (Fig. 4). The dichotomy in receptor specificity is a general paradigm in IAV biology, that avian IAVs bind to SAs linked by an  $\alpha$ 2,3-linkage to the penultimate galactose and human IAVs bind to  $\alpha$ 2,6-linked SAs (Rogers and Paulson, 1983; Matrosovich et al., 2000). The binding specificity is in accordance with the dominant SA-linkage type at the preferred infection sites, being the enteric tract of avian hosts and upper respiratory tract of humans (van Riel et al., 2007; França et al., 2013; Walther et al., 2013). Therefore, occasional transmission of avian IAVs to humans is usually unlivable unless HA mutates to enhance its binding affinity for human type SA receptor (Matrosovich et al., 2000; Glaser et al., 2005; Teng et al., 2016). Alternatively, the HA-NA mismatch can also be amended by adjusting the SA-destroying activity of NA, for instance, by NA-stalk truncations that interfere with NA availability to HA-SA bonds (Castrucci and Kawaoka, 1993; Hossain et al., 2008; Sorrell

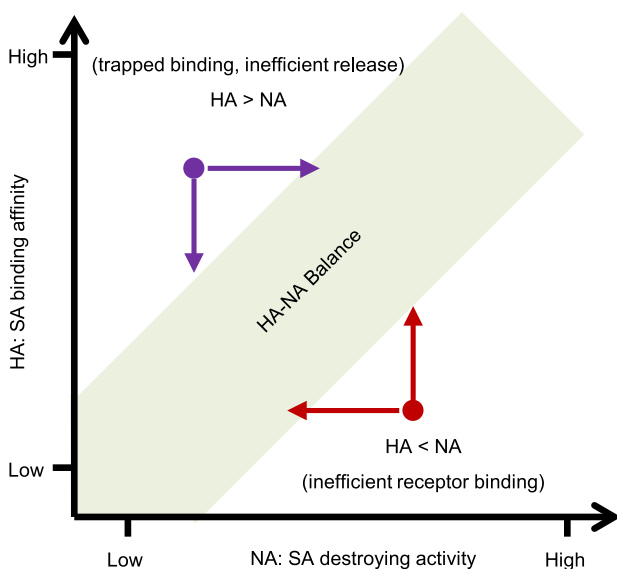
et al., 2010; Hoffmann et al., 2012; Park et al., 2017; Arai et al., 2020). Most strikingly, Ferraris et al. have isolated two IAV strains that are defective in NA to escape from NA inhibitors. Meanwhile, the HAs were evolved to harbor very low affinity for SA receptors, in order to maintain the HA-NA balance (Ferraris et al., 2006; Richard et al., 2012).

Another representative example of balance regression occurs to the heterotrimeric viral polymerase components of avian influenza virus during adaptation in mammals. Generally, avian IAV polymerases are usually suboptimal in mammalian cells, due to the species-specific difference in host protein ANP32A which is dispensable for the activity of IAV polymerase (Long et al., 2016; Zhang et al., 2019). The low polymerase activity attributed to avian PA in mammalian cells would drive rapid acquisition of mutations in polymerase components, such as K356R in PA itself or E627K in PB2, to re-establish a balanced state of viral polymerase complex (Xu et al., 2016; Liang et al., 2019).

## 8.2. Balance compensation—a strategy for artificial manipulation of IAV genome

Besides natural factors that drive virus evolution, the balance of segmented IAV genome is also vulnerable to artificial modifications. With the rapid development of reverse genetics, various recombinant IAVs expressing foreign genes have been engineered as tools, including diverse reporter IAVs (Manicassamy et al., 2010; Heaton et al., 2013; Pan et al., 2018; Nogales et al., 2019; Wang et al., 2019), recombinant influenza vaccines (Li et al., 2014; Nogales et al., 2016; Zeng et al., 2018; Vandoorn et al., 2022), live-attenuated influenza virus-vectored vaccines (Kotomina et al., 2019; Matyushenko et al., 2020), as well as therapeutic transgenic vectors (Hamilton et al., 2018). However, since foreign insertions into an individual segment inevitably elongated the length of its coding region, the replication capacity of modified segment reduced accordingly in competition with the other seven counterparts (Zhao et al., 2018). Subsequently, the delicate balance of segmented genome during virus infection was impaired (Zhao et al., 2018). As a consequence, the recombinant viruses usually replicate less efficiently than the parental strains *in vitro* and are attenuated *in vivo*. Moreover, the foreign insertions are usually unstable and might loss after several rounds of replication (Manicassamy et al., 2010; Heaton et al., 2013; Pan et al., 2018; Nogales et al., 2019; Wang et al., 2019).

Although it could be anticipated that directed evolution of attenuated recombinant virus to a wildtype-like fitness could be achieved by serially passing in cell culture or animals, following the balance-regression mechanism of natural evolution (Cai et al., 2018; Furusawa et al., 2019), the procedure is time-consuming and the anticipated outcome is not always guaranteed. Recently, we have developed a “balance compensation” strategy for generation of recombinant IAVs as desired



**Fig. 4.** Schematic representation of hemagglutinin (HA)-neuraminidase (NA) balance regression during influenza A virus (IAV) evolution. HA-NA mismatch usually occurs when new coupled HA-NA subtypes emerge via reassortment or after interspecies transmission, resulting in either overwhelmed receptor binding affinity of HA by receptor destroying activity of NA, or vice versa. Both kinds of HA-NA mismatch would threaten the viral viability by interfering with virus entry into and release from host cells, compelling directed virus evolution to achieve HA-NA rebalance. SA, sialic acid.

(Zhao et al., 2022). Briefly, the genome imbalance of recombinant IAVs can be successfully rectified by introducing appropriate compensatory enhancement at initial construction. Using this strategy, we successfully generated genomic stable recombinant IAVs carrying foreign genes with a range from 500 to 1600 bp in length. Our strategy substantially expands the tolerance of the segmented IAV genome, and will greatly facilitate creation and exploitation of more valuable recombinant IAVs as tools.

### Conflict of interest

The authors declare that they have no conflict of interest.

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