

# Molecular Determinants of Pathogenicity in the Polymerase Complex

Gülsah Gabriel and Ervin Fodor

**Abstract** Viral pathogenesis involves numerous interactions between viral and cellular factors. In recent years, the influenza virus polymerase complex has emerged as a major determinant of interspecies transmission and pathogenicity. The viral RNA-dependent RNA polymerase, in concert with the nucleoprotein, mediates transcription and replication of the viral RNA genome in the nucleus of the infected cell. The activity by which the viral polymerase complex performs these processes in mammalian cells is considered to be a major contributor to viral pathogenicity in mammals. In this chapter, we summarise our current understanding on the pathogenicity determinants in the viral polymerase complex and highlight some of its cellular interaction partners. We particularly discuss the role of importin- $\alpha$  isoforms in host adaptation and pathogenesis as well as the role of the viral polymerase in regulating cellular responses to viral infection.

## Abbreviations

PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PA	Polymerase acidic protein
NP	Nucleoprotein
NEP	Nuclear export protein
NS1	Non-structural protein 1
NS2	Non-structural protein 2
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
cRNA	Complementary RNA
CRM1	Chromosome region maintenance 1
NLS	Nuclear localisation signal

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G. Gabriel (✉)

Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany  
e-mail: guelsah.gabriel@hpi.uni-hamburg.de

E. Fodor

Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

Hsp90	Heat shock protein 90
IFN	Interferon
RIG-I	Retinoic acid-inducible gene 1
MAVS	Mitochondrial antiviral signalling protein
CPSF30	Cleavage and polyadenylation specificity factor 30
Pol II	RNA polymerase II

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## 1 Introduction

Influenza viruses encode their own RNA-dependent RNA polymerase which consists of three subunits, PB1, PB2 and PA. The viral polymerase complex together with NP mediates transcription and replication of the viral genome in the nucleus of the infected host cell. The lack of proofreading activity results in high mutation rates during replication that facilitate viral adaptation to new host environments. Upon interspecies transmission, adaptive mutations in the viral polymerase complex and NP result in increased polymerase activity which may further facilitate enhanced pathogenesis and transmission in the mammalian host.

## 2 Transcription and Replication by the Viral Polymerase

### 2.1 Molecular Mechanisms of Transcription and Replication

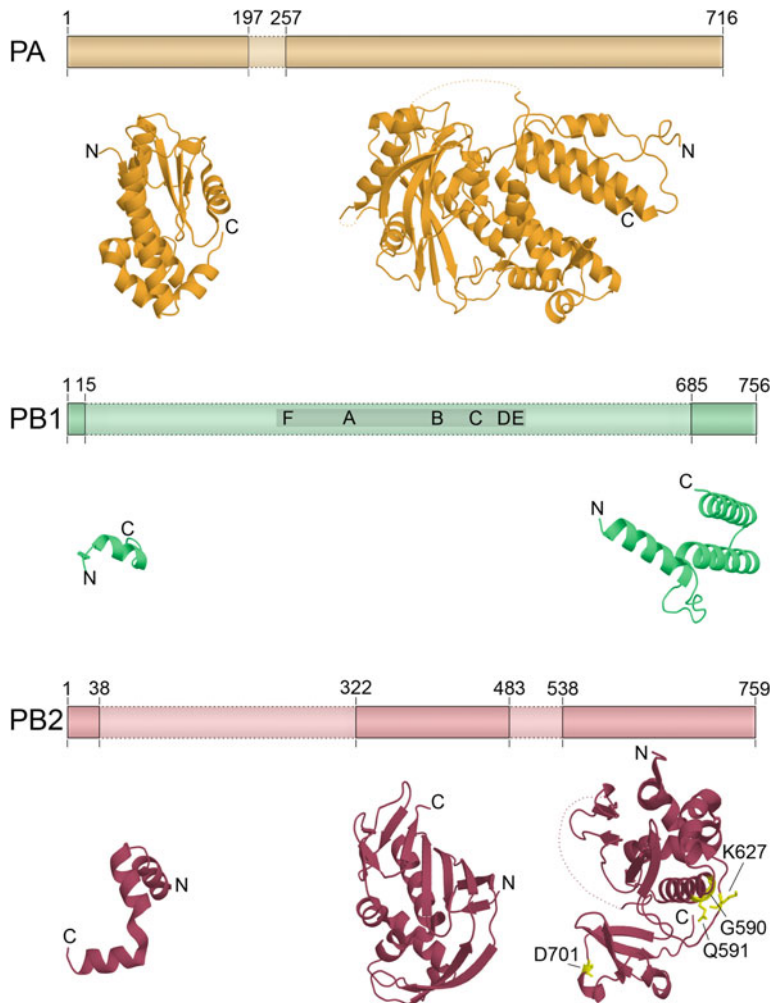
The influenza A virus genome consists of eight single stranded RNA segments of negative polarity (reviewed in (Krug and Fodor 2013; Shaw and Palese 2013)). These RNA segments associate with the trimeric RNA-dependent RNA

polymerase and oligomeric NP to form viral ribonucleoprotein (vRNP) complexes, which represent the minimal replication machinery of influenza virus (reviewed in (Fodor 2013; Resa-Infante et al. 2011)).

After virus internalisation by endocytosis and fusion of viral and endosomal membranes the vRNPs are released into the cytoplasm and are transported into the nucleus via the classical importin- $\alpha/\beta_1$  pathway (O'Neill et al. 1995; O'Neill and Palese 1995). Initially, vRNPs act as transcription complexes producing viral mRNAs. Transcription is a primer-dependent event and it involves the use of 5' capped RNA primers derived from host pre-mRNAs. These RNA primers are generated by the viral RNA polymerase. Association of the viral RNA polymerase with the cellular RNA polymerase II that synthesises capped pre-mRNAs is believed to facilitate the access of the viral RNA polymerase to cellular capped pre-mRNA (Engelhardt et al. 2005). Subsequently, the capped RNA primer is elongated by the viral polymerase using the vRNA as template. Transcription terminates by the addition of a poly(A) tail. Thus, viral transcription results in viral mRNAs that are structurally similar to cellular mRNAs containing a 5' cap structure and a poly(A) tail at the 3' end. This structural similarity enables viral mRNAs to be processed by cellular mRNA processing pathways and assembled into translation-competent mRNPs (reviewed in (York and Fodor 2013)). Translation of viral proteins is required for the onset of viral replication. vRNA is copied into a complementary RNA (cRNA) replication intermediate which then acts as a template for the synthesis of vRNA. Both these processes are primer-independent events resulting in RNAs containing 5' triphosphates. Both cRNA and vRNA exist as ribonucleoprotein complexes in which the RNA is associated with RNA polymerase and oligomeric NP (York et al. 2013). The viral nuclear export protein (NEP), formerly known as non-structural protein 2 (NS2) is implicated in the regulation of viral RNA genome replication (reviewed in (Paterson and Fodor 2012)). The resulting vRNPs can act as templates for both transcription and replication or, after nuclear export and transport across the cytoplasm, they can be assembled into progeny virions. NEP acts as an export factor for progeny vRNPs mediating their interaction with the cellular CRM1-dependent nuclear export pathway (reviewed in (Paterson and Fodor 2012)).

## 2.2 Structure and Function of the RNA Polymerase Complex

PB1 is the catalytic subunit of the RNA polymerase directly involved in RNA synthesis (reviewed in (Mehle and McCullers 2013; Ruigrok et al. 2010)). Apart from short N-terminal and C-terminal fragments, which have been co-crystallised with a C-terminal fragment of PA and an N-terminal fragment of PB2, respectively, structural information is not available for PB1 (He et al. 2008; Obayashi et al. 2008; Sugiyama et al. 2009). Sequence alignments show that the central region of PB1 contains the classical polymerase motifs (Fig. 1). A nuclear

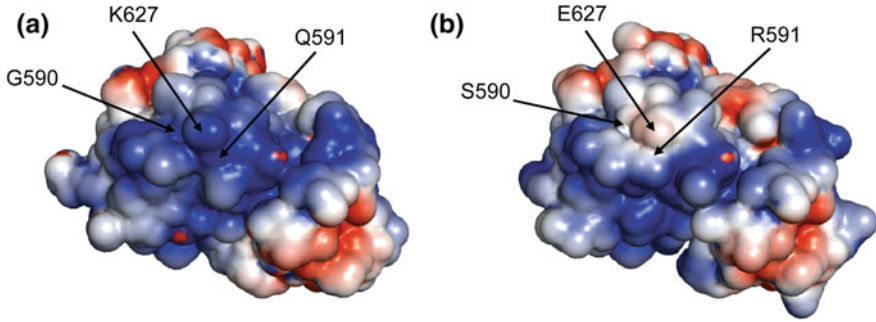


**Fig. 1** Structure and functional domains of the influenza A virus polymerase. A diagram of the three subunits and domains with known three-dimensional structure is shown. These include the N-terminal endonuclease and C terminal domains of PA (Dias et al. 2009; He et al. 2008; Obayashi et al. 2008; Yuan et al. 2009) and the central cap-binding and C-terminal 627-NLS domain of PB2 (Guilligay et al. 2008; Kuzuhara et al. 2009a, b; Tarendeau et al. 2008; Yamada et al. 2010). Key adaptive positions are indicated in the 627-NLS domain of PB2. Structures of the N-terminal and C-terminal regions of PB1, as well as of the N-terminal region of PB2, are also shown (He et al. 2008; Obayashi et al. 2008; Sugiyama et al. 2009). The polymerase signature domains (A–F) are indicated in the central region of PB1 (Biswas and Nayak 1994; Poch et al. 1989). Structures were generated with PyMOL using the following PDB accession numbers: PA endonuclease domain, 3EBJ; PA C-terminal domain and PB1 N-terminus, 2ZNL; PB1 C-terminus and PB2 N-terminus, 2ZTT; PB2 cap-binding domain, 4CB4 and PB2 627-NLS domain, 2VY6

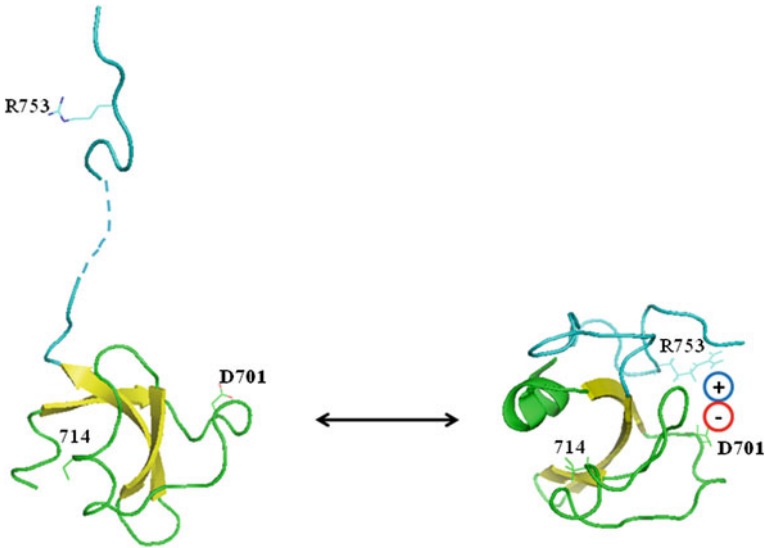
localisation signal (NLS) has been identified in the N-terminal third of PB1, although when PB1 is expressed in the absence of PB2 and PA, it predominantly accumulates in the cytoplasm (Fodor and Smith 2004). However, in the presence of co-expressed PA, it accumulates efficiently in the nucleus and RanBP5 and Hsp90 have been implicated in the nuclear import of a PB1-PA dimer (Deng et al. 2006; Naito et al. 2007). Hsp90 has also been implicated in the nuclear import of a PB1-PB2 dimer (Naito et al. 2007).

PB2 is a cap binding protein with the cap-binding domain located in the central region of the polypeptide as shown by structural analysis (Guilligay et al. 2008). Structural information is also available for the N-terminal fragment that interacts with PB1, the so called 627 domain, named after the prominent host-specific amino acid residue 627, as well as for the NLS domain (Fig. 1) (Kuzuhara et al. 2009a, b; Tarendeau et al. 2007, 2008; Yamada et al. 2010). Residue 627 is typically lysine in mammalian adapted influenza viruses, while in avian influenza viruses glutamic acid predominates. The replacement of the lysine with glutamic acid has little effect on the overall fold of the 627 domain. However, it disrupts a prominent basic patch on the protein surface. The PB2 of the 2009 pandemic H1N1 influenza viruses which unusually had a glutamic acid at this position acquired suppressor mutations at nearby sites (Fig. 2) (Mehle and Doudna 2009). The serine at position 590 and arginine at 591 were proposed to neutralise the glutamic acid at position 627 and partially restore the positively charged surface of the 627 domain. The function of the 627 domain remains obscure, although it has been implicated in RNA binding as well as in modulating the accessibility of the NLS domain (Tarendeau et al. 2007, 2008). The C-terminal bipartite NLS mediates nuclear import of PB2 via the importin- $\alpha/\beta_1$  pathway (Gabriel et al. 2008; Tarendeau et al. 2007). The NLS domain has been co-crystallised with the cellular factor importin- $\alpha 5$ . The structure shows that the extreme C-terminus containing the NLS assumes an extended conformation that positions the bipartite NLS into a superhelical groove on importin  $\alpha 5$ . However, in the absence of importin- $\alpha$ , the C-terminal extremity is tethered to the core of the domain. This interaction is mediated by a salt bridge between amino acid residues D701 and R753. PB2 D701N is another important host adaptive mutation observed upon avian influenza virus adaptation to mammals (Fig. 3). It was proposed that PB2 D701N mutation would disrupt the salt bridge between D701 and R753 and thus expose the NLS and lead to enhanced importin- $\alpha$  binding (Gabriel et al. 2008; Tarendeau et al. 2007). Individually expressed PB2 efficiently accumulates in the nucleus where it assembles with the PB1-PA dimer into a heterotrimer (Deng et al. 2006). However, it has been proposed that PB2 can also enter the nucleus as part of a PB1-PB2 dimer (Naito et al. 2007).

Structural analysis demonstrates that the PA subunit consists of two domains, the N-terminal endonuclease domain and a C-terminal domain that mediates the interaction with PB1 (Fig. 1) (Dias et al. 2009; He et al. 2008; Obayashi et al. 2008; Yuan et al. 2009). The two domains are separated by a flexible linker sequence. The N-terminal endonuclease domain is involved in the cleavage of cellular pre-mRNAs generating 5' capped RNA fragments for priming transcription. The C-terminal



**Fig. 2** Key adaptive mutations in the PB2 627-NLS domain of the influenza A virus RNA polymerase. Surface representation of the PB2 627-NLS domain of a human H5N1 isolate with PB2 627K mammalian signature (*panel A*) and of a 2009 pandemic H1N1 influenza virus with PB2 590S/591R mammalian signatures (*panel B*) (Yamada et al. 2010). Positions of key adaptive residues are highlighted. These are coloured according to the electrostatic surface potential calculated using APBS in PyMOL (Baker et al. 2001). Red and blue indicate negative and positive regions, respectively. Structures were generated using the following PDB accession numbers: 3KC6 for H5N1 and 3KHW for H1N1



**Fig. 3** Conformational change in the PB2 NLS domain. The C-terminus of PB2 shows two conformations. In the open form, the NLS at the extreme end is exposed to allow interaction with importin- $\alpha$  (*left side*). In the closed form the NLS is tethered to the main body of the domain through a salt bridge between R753 and D701 (*right side*). PB2 D701N adaptive mutation would facilitate exposure of the NLS. Structures were generated with PyMOL using published PDB accession number 2GMO and RCSB (Tarendeau et al. 2007). Modified from (Gabriel et al. 2013)

domain of PA that assumes a “dragon head” shape interacts with the N-terminus of PB1 through its “jaw” (He et al. 2008; Obayashi et al. 2008).

NP is an essential component of the influenza virus transcriptional machinery. It is a highly basic protein that consists of two major domains, the head and body domains (Ng et al. 2008; Ye et al. 2006). The two domains are separated by a negatively charged groove that is involved in RNA binding. Within the RNP, the NP occurs as a homo-oligomer; a tail loop of one NP molecule is inserted into a cavity in the body domain of a neighbouring NP (Chan et al. 2010). NP is responsible for maintaining the double helical structures of vRNPs and cRNAs through NP–NP interactions (Arranz et al. 2012; Moeller et al. 2012; York et al. 2013).

Several auxiliary viral proteins encoded by the polymerase gene segments have been identified, i.e. PB1-F2, PB1-N40, and the more recently described PA-X, PA-N155 and PA-N182 polypeptides which play a role in influenza virus pathogenesis (Chen et al. 2001; Jagger et al. 2012; Muramoto et al. 2013; Shi et al. 2012; Wise et al. 2009).

### 3 Host Adaptive Mutations in the Viral Polymerase Complex

After the first report of an H5N1 highly pathogenic avian influenza virus (HPAIV) transmission from infected poultry to humans in 1997 in Hong Kong, evidence steadily increased that the viral polymerase is a major determinant of interspecies transmission and pathogenesis (Subbarao et al. 1998). Many subsequent *in vitro* and *in vivo* studies identified host adaptive signatures in the viral polymerase complex and NP which are associated with enhanced polymerase activity, pathogenicity and transmission in mammalian species.

Most notably, the PB2 627K mammalian signature is highly prevalent in isolates obtained from humans infected with H5N1, H7N7, H9N2, H7N9 and recently H10N8 where it often correlates with fatal outcome (Chen et al. 2013, 2014; de Jong et al. 2006; Fouchier et al. 2004; Kageyama et al. 2013; Peiris et al. 1999; Subbarao et al. 1998). However, some isolates obtained from H5N1 and H7N9 avian influenza virus infected patients have maintained the avian signature PB2 627E but acquired PB2 701N as another mammalian signature which also correlates with severe disease in humans (Chen et al. 2013; de Jong et al. 2006; Kageyama et al. 2013; Le et al. 2009). In contrast, 2009 pandemic H1N1 strains do neither contain PB2 627K nor 701N (Garten et al. 2009). Instead, they have acquired another alternative signature in PB2 590S/591R which is absent from seasonal H1N1 viruses that circulated before 2009 (Mehle and Doudna 2009).

Besides these PB2 adaptive signatures, several other mutations in PB1, PA, NP and NEP were implicated in enhanced viral replication and pathogenesis in mammalian species (Table 1) (reviewed in (Cauldwell et al. 2014; Gabriel et al. 2013;

**Table 1** Host adaptive signatures in the viral polymerase complex

vRNP	Host adaptive mutations	Function and pathogenicity in mammals	Reference
PB2	D9N	Mitochondrial PB2 localisation ↓ IFN-β expression ↑ virulence in mice	(Graef et al. 2010; Kim et al. 2010)
	L89V, G309D, T33K, R477G, I495V, A676T	↑ virulence of H5 in mice	(Li et al. 2009)
	E158G	↑ virulence of H1 and H5 in mice	(Zhou et al. 2013a, b)
	D253N	↑ polymerase activity of H9	(Mok et al. 2011)
	T271A	↑ polymerase activity of H1	(Bussey et al. 2010)
	I504V, PA I550L	Cellular RNA-polymerase II degradation ↑ pathogenicity of H1 in mice	(Llompert et al. 2014)
	T588I	↑ MAVS binding ↓ IFN-β expression	(Foeglein et al. 2011; Zhao et al. 2013)
	S590R, G591Q	Observed in human H1 isolates ↑ polymerase activity of H1	(Mehle and Doudna 2009)
	Q591K	↑ polymerase activity of H5 and H9 ↑ virulence of H5 and H9 in mice	(Mok et al. 2011; Yamada et al. 2010)
	E627K	Observed in human H1, H3, H5, H7, H9 and H10 isolates ↑ polymerase activity of H1, H3, H5, H7 and H9 ↑ virus replication requires NP from same viral background ↑ polymerase mobility in the nuclei ↑ binding of vRNPs to importin-α1 and -α7; ↓ reduced polymerase activity in importin-α1 and -α7 silenced cells; ↓ reduced viral replication in importin-α1 and -α7 silenced cells	(Bogs et al. 2011; Bortz et al. 2011; Chen et al. 2013, 2014; de Jong et al. 2006; Foeglein et al. 2011; Fouchier et al. 2004; Gabriel et al. 2005, 2008, 2011; Gao et al. 2009; Hatta et al. 2001, 2007; Herfst et al. 2010, 2012; Hudjetz and Gabriel 2012; Jagger et al. 2012; Kageyama et al. 2013; Labadie et al. 2007; Li et al. 2009; Mok et al. 2011; Paterson et al. 2014; Peiris et al. 1999; Shinya et al. 2004, 2009; Steel et al. 2009; Subbarao et al. 1993, 1998; Yamada et al. 2010)
		↑ binding to NP ↑ recruitment of RNA promoter	

(continued)



**Table 1** (continued)

vRNP	Host adaptive mutations	Function and pathogenicity in mammals	Reference
		<p>↑ virulence in mice; ↓ virulence in importin-<math>\alpha 7^{-/-}</math> mice</p> <p>↑ contact and airborne transmission of H1, H3 and H5 in guinea pigs</p> <p>↑ airborne transmission of H5 in ferrets</p>	
	D701N	<p>Observed in human H5 and H7 isolates</p> <p>↑ polymerase activity of H1, H3, H5 and H7</p> <p>↑ polymerase mobility in the nuclei</p> <p>↑ binding to importin-<math>\alpha</math>; ↓ reduced viral replication in importin-<math>\alpha 1</math> and -<math>\alpha 7</math> silenced cells</p> <p>↑ virulence in mice; ↓ virulence in importin-<math>\alpha 7^{-/-}</math> mice</p> <p>↑ contact and airborne transmission of H1, H3 and H5 in guinea pigs</p> <p>↑ airborne transmission of H1 in ferrets</p>	(Boivin and Hart 2011; Bortz et al. 2011; de Jong et al. 2006; Foeglein et al. 2011; Gabriel et al. 2005, 2008, 2011; Gao et al. 2009; Herfst et al. 2010; Kageyama et al. 2013; Resa-Infante et al. 2008; Shinya et al. 2009; Steel et al. 2009; Tarendeau et al. 2007; Zhou et al. 2013a, b)
PB1	L472V, L598P	↑ polymerase activity of H1	(Xu et al. 2012)
NP	A150R	<p>↑ polymerase activity of H5</p> <p>↑ binding to NP</p>	(Ng et al. 2012)
	N319K	<p>↑ polymerase activity of H7</p> <p>↑ virulence of H7 in mice</p> <p>↑ binding to importin-<math>\alpha</math></p>	(Gabriel et al. 2005, 2008, 2011)
NEP	S7L, Y41C, E75G, X161M	↑ virus replication in the absence of PB2 627K	(Manz et al. 2012)

Effect of host adaptive mutations on viral polymerase activity, pathogenicity and transmission in mammals. ↑ enhancing effect; ↓ reducing effect

Manz et al. 2013)). Here, we will summarise our current knowledge on the key PB2 adaptive sites at positions 627, 701 and 590/591 and discuss their implications for viral replication, pathogenesis and transmission in mammals.

### ***3.1 Polymerase Activity in Mammalian Cells***

The PB2 E627K mutation was shown to mediate enhanced viral polymerase activity in a broad range of mammalian, including human cells. This ability generally correlated with avian-mammalian adaptation and pathogenesis in mice (Gabriel et al. 2005; Hatta et al. 2001). Interestingly, the enhancing effect of this host adaptive mutation on polymerase activity was not observed in avian cells (Hudjetz and Gabriel 2012; Labadie et al. 2007; Mehle and Doudna 2008; Paterson et al. 2014). Several studies have proposed potential mechanisms for how the PB2 E627K mutation might result in enhanced viral polymerase activity in mammalian cells. Avian influenza viruses preferentially replicate in the avian intestine where the temperature is around 40 °C (Murakami et al. 1988; Murphy et al. 1982a, b). In contrast, human influenza viruses have a replication optimum at lower temperatures (33–37 °C). This cold-sensitivity of avian influenza viruses is believed to restrict their ability to replicate efficiently in the upper respiratory tract of humans where the medium temperature is around 33 °C. The PB2 E627K mutation was shown to promote replication of H5N1 HPAIV in the upper respiratory tract of humans and mice (Hatta et al. 2007; Le et al. 2009; Massin et al. 2001). However, the mechanism by which the E627K mutation would promote higher polymerase activity at lower temperature remains unclear. Several studies proposed that the PB2 E627K mutation increased polymerase activity by promoting vRNP assembly in mammalian cells by increasing the affinity of PB2 for NP (Labadie et al. 2007; Mehle and Doudna 2008; Ng et al. 2012; Rameix-Welti et al. 2009). However, recent studies challenged this idea by suggesting that the observed apparent increase in PB2-NP affinity is the result of increased accumulation of vRNPs due to increased polymerase activity (Cauldwell et al. 2013; Paterson et al. 2014). In fact, more recently the PB2 627E mediated restriction of the viral polymerase was shown to be independent of NP but dependent on the length of the viral RNA template. Moreover, the restricted polymerase activity of PB2 627E complexes could be rescued by the introduction of specific mutations into the promoter region of viral RNAs (Crescenzo-Chaigne et al. 2002; Paterson et al. 2014). Therefore, it was proposed that PB2 627 might affect the recruitment of the viral RNA promoter by the viral RNA polymerase in mammalian cells. It was also hypothesised that an inhibitory factor must exist in human cells that restricts PB2 627E polymerase activity (Mehle and Doudna 2008). This concept was opposed by another study suggesting that instead, a positive factor is present in human cells which promotes PB2 627K polymerase activity (Moncorge et al. 2010). In agreement with the existence of a host factor that interacts with the viral polymerase and modulates its activity, it was found

that the identity of 627 also affects the intranuclear mobility of the viral polymerase (Foeglein et al. 2011). In particular, low polymerase activity of PB2 627E correlated with significantly slower diffusion in human but not avian nuclei suggesting an interaction with a relatively large soluble inhibitory cellular factor and/or with an insoluble “static structure”. Several host factors have been identified that differentially regulate H5N1 polymerase according to the identity of the PB2 627 residue, including the DEAD box RNA helicase DDX17/p72 that facilitated efficient transcription and replication of a human isolate of an H5N1 virus (627K) in mammalian cells (Bortz et al. 2011). In addition, importin- $\alpha$  isoforms show differential interaction with the viral polymerase and vRNPs depending on the identity of PB2 627 (see below). Several compensatory mutations in the viral polymerase subunits NP and NEP have been described that result in increased activity of avian influenza virus derived polymerases in mammalian cells (Table 1). The 2009 swine origin pandemic H1N1 virus acquired suppressor mutations at positions 590/591 of PB2 (Mehle and Doudna 2009), while adaptive mutations in the viral NEP were able to enhance polymerase activity in mammalian cells of an H5N1 polymerase containing the PB2 627E avian signature (Manz et al. 2012).

The host adaptive mutation PB2 D701N also mediates enhanced viral polymerase activity in mammalian, including human cells, and furthermore, correlates with avian-mammalian adaptation and pathogenesis in mice (Gabriel et al. 2005). Originally, this mutation was observed upon adaptation of H3N2 and H7N7 HPAIV to mice (Brown et al. 2001; Gabriel et al. 2005). The polymerase activity increasing effect of the PB2 D701N mutation was observed at a broad range of temperatures from 33 to 39 °C (Gabriel et al. 2005; Zhou et al. 2013a). Sequencing analysis of isolates obtained from humans infected with H5N1 revealed that PB2 701N, similar to 627K, contributes to efficient virus propagation in the human upper respiratory tract (Le et al. 2009).

It was suggested that PB2 590S/591R mammalian signatures specific for 2009 pandemic H1N1 influenza viruses contribute to elevated polymerase activity compared to seasonal H1N1 strains in which PB2 590G/591Q are present. The PB2 S590G/R591Q mutation reduced 2009 pandemic H1N1 viral polymerase activity in mammalian cells (Mehle and Doudna 2009). Interestingly, introduction of PB2 627K or 701N mammalian signatures into PB2 of 2009 pandemic H1N1 strains further elevated viral polymerase activity in human cells (Herfst et al. 2010; Song et al. 2011; Yamada et al. 2010).

In general, enhanced polymerase activity in mammalian cells seems to be predictive of elevated pathogenicity in mammalian species. However, it should be noted that none of these mammalian signatures (PB2 672K, 701N and 590S/591R) occur in combination in naturally circulating influenza virus strains. These observations suggest that during viral adaptation influenza viruses can acquire alternative mutations that enhance replicative fitness in mammalian cells.

### ***3.2 Pathogenicity in Mammals***

Both key PB2 627K and 701N mammalian signatures were shown to enhance not only viral polymerase activity but also pathogenicity of numerous influenza virus subtypes and strains in mice (Bogs et al. 2011; Fornek et al. 2009; Gabriel et al. 2005; Hatta et al. 2001; Salomon et al. 2006; Shinya et al. 2004). Furthermore, both signatures contributed individually to H5N1 virus propagation in the human upper respiratory tract (Le et al. 2009). In H5N1 infected patients in Vietnam, the presence of either PB2 627K or 701N mammalian signatures correlated with a high virus load in the blood. Disseminated virus replication and hypercytokinemia were hallmarks of fatal H5N1 and H7N9 infection in humans (de Jong et al. 2006; Zhou et al. 2013b). Interestingly, the presence of viral RNA in the blood was only observed together with very high pharyngeal virus loads. This suggests that after primary infection of the human respiratory tract, H5N1 HPAIV containing PB2 627K or 701N mammalian signatures may disseminate to other organs when a certain threshold in the lung is reached. Other studies performed in chickens further support the observation that these mammalian signatures specifically enhance pathogenicity in mammals. In contrast to mammalian species, PB2 E627K mutation did not change virulence in chickens (Bogs et al. 2011).

Whether the polymerase activity elevating effect of PB2 G590S/Q591R mutations (Mehle and Doudna 2009) contributes to pathogenicity in mammals remains unclear. However, a PB2 Q591K mutation was shown to enhance not only H5N1 polymerase activity, but also replication and virulence in mice (Yamada et al. 2010). On the other hand, introduction of PB2 627K or 701N into 2009 pandemic H1N1 influenza viruses did enhance viral polymerase activity but not virus replication or virulence in mice (Herfst et al. 2010; Song et al. 2011; Yamada et al. 2010). Future studies will be required to understand the role of PB2 590S/591R in 2009 pandemic H1N1 influenza virus pathogenicity in mammals.

### ***3.3 Transmissibility in Mammals***

There is ample evidence that mammalian signatures in the PB2 subunit of the viral polymerase are a prerequisite for the acquisition of sustained inter-host transmission. Host adaptive mutations in PB2 E627K or D701N have been repeatedly shown not only to enhance pathogenicity in mammals but also transmission of H3N2 or H5N1 influenza viruses between mammalian hosts (Gao et al. 2009; Steel et al. 2009). PB2 K627E mutation in human H3N2 or avian H5N1 strains reduced contact and aerosol transmission in guinea pigs, whereas introduction of PB2 701N was sufficient to recover transmissibility (Steel et al. 2009). However, the presence of these single mammalian signatures, while required, is generally not sufficient to confer sustained mammalian-to-mammalian transmission. Introduction of PB2 627K or 701N into various avian H5N1 virus strains showed that the presence of either of these signatures is a prerequisite for efficient contact transmission in

guinea pigs but required additional mammalian signatures in the hemagglutinin (Gao et al. 2009; Steel et al. 2009). Studies performed in ferrets further highlighted that airborne transmissibility of H5 influenza viruses occurred when mammalian signatures in the hemagglutinin as well as in PB2, such as 627K or 590S/591R were present (Herfst et al. 2012; Imai et al. 2012). This is in agreement with observations that recent H7N9 strains which contain the mammalian signatures PB2 627K or 701N show limited airborne transmissibility in ferrets (Richard et al. 2013). Thus, the presence of mammalian signatures seems to be required but not sufficient for sustained airborne transmission in mammals.

The 2009 pandemic H1N1 strains showed airborne transmission properties in the human population as well as in the ferret model. However, discrepancies were reported when their transmission efficiencies were compared to seasonal H1N1 influenza viruses. While some studies report that 2009 pandemic H1N1 influenza viruses have an increased potential for airborne transmission compared to seasonal H1N1 strains (Munster et al. 2009), others show decreased transmissibility (Maines et al. 2009) in the ferret model. Furthermore, it was also reported that PB2 R591Q reduced airborne transmission in ferrets suggesting that PB2 591R is required for replicative advantage and aerosol transmission in mammals (Yamada et al. 2010). On the other hand, introduction of neither PB2 627K nor 701N mammalian signatures into 2009 pandemic H1N1 influenza viruses enhanced airborne transmission in ferrets (Herfst et al. 2010; Yamada et al. 2010). However, a recent study reports that introduction of PB2 701N into a distinct 2009 pandemic H1N1 lineage was able to elevate transmissibility in ferrets (Zhou et al. 2013a).

Thus, transmission is a polygenic trait and the presence of key mammalian signatures in PB2 seems to be necessary but not always sufficient for sustained mammalian-to-mammalian transmission.

## 4 Cellular Interaction Partners of the RNA Polymerase

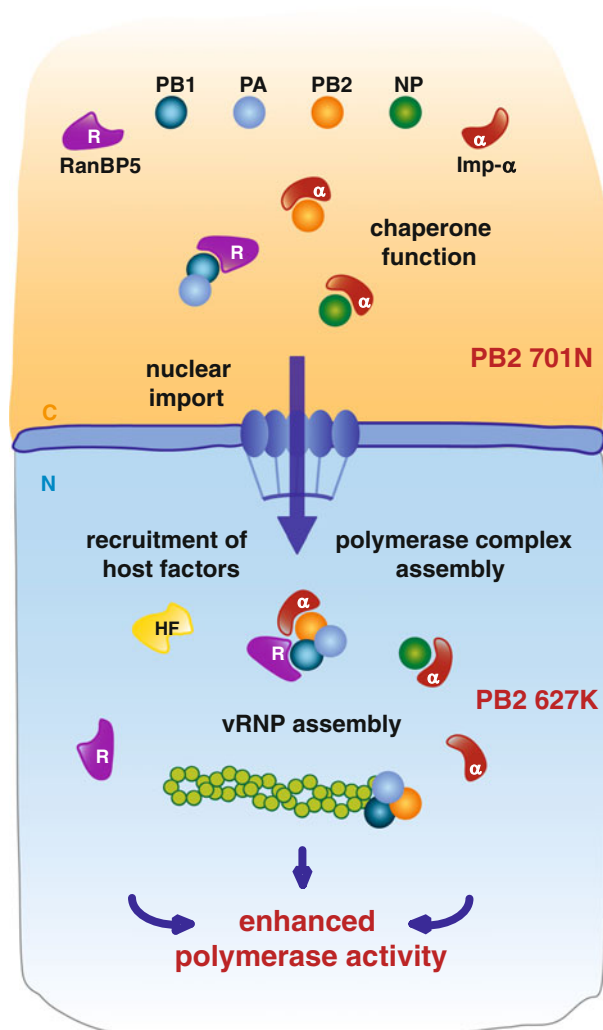
Genome-wide RNA interference (RNAi) screens (Brass et al. 2009; Hao et al. 2008; Karlas et al. 2010; König et al. 2010; Shapira et al. 2009; Sui et al. 2009) as well as proteomic analyses (Bradel-Tretheway et al. 2011; Jorba et al. 2008; Mayer et al. 2007) have identified numerous cellular factors involved in influenza virus replication. Thereby, cellular networks were identified which are involved in various steps of the viral life cycle, such as virus entry, nuclear import, transcription and replication, cell signalling and nuclear export (reviewed in (Gabriel et al. 2013; Shaw 2011; Watanabe et al. 2010)). Substantial body of evidence emerged in the last years that components of the cellular import machinery play a major role in influenza virus interspecies transmission and pathogenesis. Therefore, here we will focus on the role of the nuclear import factors, namely importin- $\alpha$  proteins, and discuss their implication in influenza virus replication and avian-mammalian host adaptation. Furthermore, we will highlight novel insights into the interactions of the viral polymerase with innate immune pathways and cellular

RNA polymerase II that were recently found to contribute to influenza virus pathogenicity in the mammalian host.

#### ***4.1 Importin- $\alpha$ Mediated Host Adaptation and Pathogenesis***

Influenza virus vRNPs as well as the polymerase subunits utilise differential nuclear transport pathways to enter the nucleus of the host cell (reviewed in (Hutchinson and Fodor 2013; Resa-Infante and Gabriel 2013)). Transport of vRNPs is mediated by the classical importin- $\alpha/\beta_1$  pathway (O'Neill et al. 1995; O'Neill and Palese 1995). Thus, NP binds directly to the adaptor protein importin- $\alpha$  which then triggers the nuclear import of vRNPs together with the receptor protein importin- $\beta_1$ . The monomeric PB2 and NP proteins are also believed to be transported into the nucleus by the classical, importin- $\alpha$  dependent, pathway (Gabriel et al. 2008; Tarendeau et al. 2007). In the nucleus, PB2 assembles with PB1 and PA which are transported separately as PB1-PA dimers by the non-classical, importin- $\alpha$  independent, nuclear import pathway by direct interaction with RanBP5 (Fig. 4) (Deng et al. 2006). In addition, Hsp90 was proposed to be involved in the nuclear import of PB1-PB2 or PB1-PA heterodimers (Naito et al. 2007).

In recent years, evidence increased that key mammalian signatures, such as PB2 627K and 701N, utilise importin- $\alpha$  isoforms to promote viral replication in mammalian cells. It was proposed that divergence between avian and human homologues of importin- $\alpha$  isoforms ( $\alpha 1$ -  $\alpha 7$ ) (Gabriel et al. 2011) but not RanBP5 might be sufficient to restrict virus replication in a host-dependent manner. Accordingly, interaction studies revealed that PB2 proteins from human influenza virus strains have higher binding affinities to human importin- $\alpha$  isoforms than PB2 proteins from avian strains (Resa-Infante et al. 2008). PB2 D701N as well as NP N319K mutations were shown to enhance PB2 and NP binding affinities to human but not avian importin- $\alpha$  isoforms. This correlated with enhanced nuclear localisation of PB2 and NP in mammalian cells (Boivin and Hart 2011; Gabriel et al. 2008). In contrast, the E627K mutation did neither affect monomeric PB2 binding to human importin- $\alpha$  isoforms nor alter PB2 nuclear localisation. In the context of vRNPs on the other hand, the PB2 E627K mutation led to enhanced binding of vRNPs to human importin- $\alpha 1$ , - $\alpha 5$  and - $\alpha 7$  isoforms without affecting subcellular distribution of the viral polymerase subunits. This enhanced vRNP binding to these importin- $\alpha$  isoforms was proposed to be mediated by NP (Hudjetz and Gabriel 2012). This is in agreement with previous findings suggesting that importin- $\alpha$  isoforms may have additional, yet unknown, functions beyond nuclear transport (Resa-Infante et al. 2008). It should also be noted that single cargo proteins might interact with several importin- $\alpha$  isoforms but may show distinct specificities for certain isoforms when complexed with other proteins due to competition for endogenous importin- $\alpha$  availability (Friedrich et al. 2006). Moreover, importin- $\alpha 1$  and - $\alpha 7$  were proposed to act as positive regulatory host factors for polymerases containing PB2 627K mammalian signatures (Hudjetz and Gabriel 2012). These differential importin- $\alpha$



**Fig. 4** Potential cytoplasmic and nuclear functions of importin- $\alpha$  proteins and their effect on influenza virus polymerase activity. Chaperone activities were proposed for RanBP5 and importin- $\alpha$  which might inhibit aggregation of PB1 and NP. Importin- $\alpha$  may also participate in the assembly of the viral polymerase complex contributing to the formation of functional vRNP complexes. Following nuclear transport, importin- $\alpha$  might be additionally required for viral transcription and/or replication. Moreover, importin- $\alpha$  might indirectly recruit other cellular host factors that additionally affect viral polymerase activity. All of these functions might contribute to enhanced polymerase activity mediated by host adaptive signatures in PB2. While PB2 D701N facilitates nuclear localisation determined by enhanced importin- $\alpha$  binding in the cytoplasm, PB2 E627K containing vRNPs utilise importin- $\alpha$  for promoting viral polymerase activity in the nucleus. *C* Cytoplasm; *N* Nucleoplasm; *R* RanBP5;  $\alpha$  Importin- $\alpha$  proteins; *HF* Host factors. Modified from (Resa-Infante and Gabriel 2013)

interaction patterns with PB2 containing 701N and 627K mammalian signatures are consistent with structural reports that PB2 D701N but not E627K would modulate NLS exposure and accessibility for importin- $\alpha$  binding (Tarendeau et al. 2007, 2008). Whether this is linked to the exposed “basic patch” on PB2 627K and its potential binding to acidic importin- $\alpha$  isoforms needs further elucidation.

In the viral context, replication of H7N7 HPAIV with avian signatures in PB2 and NP (PB2 627E, PB2 701D, NP 319N) depended on importin- $\alpha$ 1 and - $\alpha$ 3, whereas replication of H7N7, H5N1 and H3N2 influenza viruses with mammalian signatures in PB2 and NP (PB2 627K, PB2 701N, NP 319K) depended on importin- $\alpha$ 1 and - $\alpha$ 7 (Gabriel et al. 2011). Thus, it was proposed that a switch from importin- $\alpha$ 3 to - $\alpha$ 7 dependency is required for efficient virus replication in mammalian cells. In contrast to importin- $\alpha$ 1 and - $\alpha$ 7, importin- $\alpha$ 3 negatively regulated the activity of both PB2 627K as well as 627E polymerases in mammalian cells (Hudjetz and Gabriel 2012). However, the restricting activity of importin- $\alpha$ 3 was not observed during replication of mammalian influenza viruses containing mammalian signatures (PB2 627K or 701N) suggesting that its restrictive activity is overcome at later stages during viral replication and that other viral and/or cellular factors affect importin- $\alpha$  specificity. The preference of avian influenza viruses for importin- $\alpha$ 3 was particularly interesting due to the high homology (99 % amino acid identity) of this isoform in birds and humans. It has been proposed that interaction with importin- $\alpha$ 3 might play an important role during the initial stage of interspecies transmission of avian influenza viruses by paving the way for adaptation to the mammalian host.

With importin- $\alpha$  knockout ( $\alpha^{-/-}$ ) mice becoming available, it became possible to confirm that animals lacking the importin- $\alpha$ 7 gene ( $\alpha 7^{-/-}$ ) were less susceptible to mammalian including human H1N1, H5N1 and H7N7 influenza virus infections compared to wildtype (WT),  $\alpha 4^{-/-}$  or  $\alpha 5^{-/-}$  mice. Reduced pathogenicity in  $\alpha 7^{-/-}$  mice correlated with a restricted pulmonary infection compared to disseminated virus replication in WT animals (Gabriel et al. 2011). Interestingly, 2009 pandemic H1N1 influenza viruses with PB2 590S/591R mammalian signatures were exceptional in displaying importin- $\alpha$ 3 as well as - $\alpha$ 7 dependencies for their replication in human cells. This highlights that adaptive mutations other than PB2 E627K and D701N might additionally affect importin- $\alpha$  specificities.

Future studies will be required to understand how importin- $\alpha$  isoforms are utilised beyond nuclear transport and thereby contribute to enhanced viral replication and pathogenesis in the mammalian host.

## ***4.2 Interactions of the Viral Polymerase with Innate Immune Pathways***

The outcome of influenza virus infections is greatly influenced by the cellular innate immune responses, dominated by the type I interferon system. Influenza virus infection triggers the activation of innate immune responses through the recognition of pathogen associated molecular patterns by pathogen recognition



receptors (reviewed in (Iwasaki and Peiris 2013)). The RNA helicase, RIG-I, is a key sensor of influenza virus RNA (Rehwinkel et al. 2010). Upon activation, RIG-I signals through the mitochondrial antiviral signalling (MAVS) protein that in turn activates kinases leading to the phosphorylation of transcription factors required for the expression type I IFN and other proinflammatory cytokines.

Influenza virus has evolved several strategies to counteract these host immune responses. The viral non-structural protein 1 (NS1) is an IFN antagonist that inhibits the expression of IFN by several independent mechanisms that include the specific inhibition of RIG-I activation. NS1 is also known to inhibit CPSF30, a cellular mRNA polyadenylation factor, leading to the general inhibition of host gene expression, including of antiviral genes (reviewed in (Hale et al. 2008; Krug and Garcia-Sastre 2013)). In addition to NS1, several other influenza virus proteins possess activities that lead to the inhibition of IFN expression. A systematic mapping of physical and regulatory interactions between influenza virus and the host cell resulted in the discovery of an important role of the RNA polymerase subunits and NP in regulating antiviral host responses (Shapira et al. 2009). Importantly, over-expression of PB1, PB2 and NP, individually or in combination, was found to inhibit cellular interferon responses to either vRNA transfection or viral infection, implicating non-NS1 viral proteins in modulating host responses. Further studies highlighted the viral polymerase subunit PB2 as a major player in mediating NS1-independent inhibition of type I interferon expression by associating with and inhibiting MAVS (Graef et al. 2010; Iwai et al. 2010). Although most PB2 in infected cells localises to the nucleus where transcription and replication of the viral genome occurs, PB2 of certain influenza viruses can also be detected at the mitochondria (Carr et al. 2006; Woodfin and Kazim 1993). Only PB2 proteins of seasonal H1N1 (pre-2009), H2N2 and H3N3 human influenza viruses associate with mitochondria while PB2 proteins of the 2009 H1N1 pandemic and avian influenza viruses, including H5N1 HPAIV, do not (Graef et al. 2010). A single amino acid polymorphism at amino acid residue 9 within the N-terminal mitochondrial targeting signal was found to be responsible for this differential localisation. An asparagine residue at position 9 in seasonal human influenza viruses results in mitochondrial localisation, while an aspartic acid in avian influenza viruses leads to non-mitochondrial localisation. Compared to a wild-type virus encoding mitochondrial PB2, an influenza virus expressing a non-mitochondrial PB2 N9D mutant induced higher levels of IFN- $\beta$  in cell culture and it was attenuated in mice (Graef et al. 2010). In contrast, the D9N mutation in the PB2 protein of an avian H5N1 virus that allows mitochondrial localisation resulted in increased virulence in mice (Kim et al. 2010). Taken together, these studies suggest that the mitochondrial localisation of PB2 and consequently, its ability to inhibit IFN- $\beta$  expression, are important determinants of virulence.

This conclusion has been reinforced by a more recent study reporting that a T588I mutation in PB2 that has been identified in swine isolates of the 2009 H1N1 pandemic virus enhanced their virulence in the mouse model. The T588I mutation was shown to enhance PB2 binding to MAVS and as a result, exacerbate PB2-mediated inhibition of IFN- $\beta$  expression (Zhao et al. 2013).

It has been hypothesised that the non-mitochondrial PB2 expressed by highly pathogenic H5N1 viruses might, at least in part, be responsible for hypercytokinemia induced by these viruses in human hosts. In agreement with this, an H5N1 avian influenza virus PB2 was found to promote type I IFN inducing properties of a swine influenza virus strain in porcine dendritic cells (Ocana-Macchi et al. 2012). In particular, a reassortant swine H1N1 influenza virus with a PB2 segment derived from an H5N1 HPAIV induced higher levels of INF- $\beta$  expression compared to the wild-type swine virus. Both the avian and swine virus PB2 proteins contain an aspartic acid at position 9, characteristic of non-mitochondrial PB2 proteins, suggesting that other differences between these two proteins, which differ at 17 amino acid positions, are responsible for their differential effects on INF- $\beta$  expression.

Thus, PB2 might act as a determinant of pathogenicity not only by contributing to polymerase activity as part of the trimeric viral polymerase complex, but also by acting independently of PB1 and PA as a regulator of innate immune responses.

### ***4.3 Interactions of the Viral Polymerase with Cellular RNA Polymerase II***

The influenza virus polymerase is also likely to contribute to the inhibition of cellular responses to viral infection through a mitochondria-independent mechanism. The trimeric influenza virus polymerase is known to bind to the C-terminal domain of the large subunit of cellular RNA polymerase II. It has been proposed that this binding inhibits elongation by Pol II during cellular transcription and it also triggers the proteolytic degradation of the large subunit of Pol II (reviewed in (Vreede and Fodor 2010)). General inhibition of host gene expression appears to be an efficient way of counteracting antiviral host mechanisms.

At early stages during infection, the influenza virus RNA polymerase associates with the cellular Pol II transcriptional machinery, presumably in order to facilitate access to 5' capped RNA primers required for viral transcription (Engelhardt et al. 2005). However, later in infection, this interaction might serve as a means to induce the degradation of the large subunit of Pol II in order to prevent the expression of antiviral genes (Rodriguez et al. 2007; Vreede et al. 2010). The ability of influenza viruses to induce Pol II degradation has been correlated with virulence, and it has been proposed that this property might be an important factor in determining influenza virus pathogenicity (Rodriguez et al. 2009). In particular, amino acid residues 504 in PB2 and 550 in PA were found to be important for Pol II degradation and the pathogenicity of H1N1 as well as 2009 H1N1 pandemic influenza viruses in mice (Llompарт et al. 2014).

These studies suggest that the viral polymerase might be involved in determining pathogenicity also by binding to cellular Pol II and inhibiting cellular gene expression. However, further studies are required to correlate the ability of influenza viruses to inhibit and degrade Pol II and elevated pathogenicity. Moreover,

it remains unclear to what extent the binding of the viral polymerase to Pol II contributes to changes in general cellular gene expression and how this affects pathogenicity.

## 5 Conclusion and Future Perspectives

The viral polymerase complex is a major determinant of influenza virus inter-species transmission and pathogenesis in the mammalian host. However, viral pathogenesis is a polygenic trait which involves the interaction of multiple viral proteins with large cellular networks. Virus–host interactions may change during viral adaptation in order to ensure efficient virus replication in the new host cell. Thereby, viral polymerase proteins might switch from the use of one cellular factor to another to promote various steps in the viral life cycle such as nuclear import and transcription/replication of the viral genome.

We are still far from understanding the impact of these interactions on viral replication in the host cell. Thus, it remains a challenge for the future to elucidate pathways which affect interspecies adaptation, pathogenesis and transmission in mammals.

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