

PB1-F2 expedition from the whole protein through the domain to aa residue function

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Summary. – More than decade ago during systematic search for alternative reading frame derived peptides encoded by influenza A virus recognized by CD8⁺ T cells, PB1-F2 protein was discovered serendipitously by Chen *et al.* (2001). Since that time, an increasing body of evidence has continued to highlight the multifunctional meaning of this unusual influenza A protein. After twelve years of intensive research with 56 pubmed records for PB1-F2 in the title there is still a lot yet to explore. Is it a proapoptotic “explosive” protein that suppresses the mechanisms of early innate immune response or does it function as an NS1 antagonist? What is the root of its strain and cell specificity? What is the relationship between PB1-F2 and pathogenicity or secondary bacterial infection? Here we attempt to “take a trip” from the whole protein level through domains and regions to very particular aminoacid residues in correlation with its function in different virus isolates, cell type or animal model.

Keywords: PB1-F2; domain; pathogenicity; virulence; influenza A virus

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1. Introduction

Among human health treating viruses, influenza A virus (IAV) occupies eminent space since few other viruses including HIV or smallpox have been observed to possess such worldwide spreading potential (Fouchier *et al.*, 2012; Moszynski, 2012). ssRNA segmented genome of the IAV code to date 16 protein products, some of which are expressed by alternative splicing (NS2, M2, M3), some by ribosomal frame shift (PB1-F2, N40, PA-X) and some by different ATG in frame translation initiation (PA-N155 and PA-N182) (Muramoto *et al.*, 2012). There are several features predetermining IAV to be such a successful virus. Multiple host species including birds and mammals make

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Abbreviations: IAV = influenza A virus; HK97 = A/Hong Kong/156/1997(H5N1); 1918 = A/Brevig Mission/1/1918 (H1N1); HK68 = A/Hong Kong/1/1968 (H3N2); VH95 = A/Wuhan/359/1995 (H3N2) non-inflammatory IAV strain; HPAI = highly pathogenic avian influenza virus; LPAI = low pathogenic avian influenza virus; pdm = pandemic virus; VDAC = voltage dependent anion channel; ANT = adenosine nucleotide transporter

it impossible for IAV to be globally eradicated. Antigenic shift and drift as the major driving force of IAV variability enable virus to overcome pre-existing immunity in population mediated mainly by virus neutralizing antibody (Hoper *et al.*, 2012). The “hit and run” strategy of rapid replication and spreading to further victims ensures that host immune system is not capable of establishing an antiviral state soon enough to hinder/block infection. Another important factor affecting virus long term spreading is its ability to suppress immune response (Friesenhagen *et al.*, 2012), virus replication kinetics (Hatta *et al.*, 2001; Ozawa *et al.*, 2011; Smith *et al.*, 2011) and cleavability of HA glycoprotein (Kido *et al.*, 2012). The aforementioned IAV features resemble the overall devastating effects on host organisms termed pathogenesis. Multiple proteins of IAV have been shown to contribute to the pathogenetic effects of influenza A virus including IFN antagonist NS1 (Ehrhardt *et al.*, 2010). HA possessing multi-basic cleavage site (Kido *et al.*, 2012) or M2 and its relation to inflammasome induction (Ichinohe *et al.*, 2010).

PB1-F2 discovered by Chen *et al.* (2001) represents another widely accepted viral factor of pathogenicity. This small protein with a molecular weight of 10,5 kDa was shown to target mitochondrial membrane (Gibbs *et al.*, 2003; Yamada *et al.*, 2004), to induce cell death via interaction with VDAC3 and ANT1 mitochondrial membrane proteins (Zamarin *et al.*, 2005), impact virulence and pathogenesis in mouse model (Zamarin *et al.*, 2006) by disrupting alveolar macrophages

(Coleman, 2007) and increase secondary bacterial infection (McAuley *et al.*, 2007). It has been shown more recently that single mutation N66S contribute to increased virulence of HPAI H5N1 (HK97) and 1918 IAV (Conenello *et al.*, 2007). It is not yet clear what the pathogenicity determinants of the PB1-F2 are but it is probable that interaction with viral polymerase subunit PB1 (Kosik *et al.*, 2011; Mazur *et al.*, 2008) affecting catalytic activity contributes to the pathogenicity of IAV. Despite some discrepancy surrounded in relation to IFN type I response (Le Goffic *et al.*, 2010), it is reasonable to think that PB1-F2 plays an important role in IFN type I response modulation (Conenello *et al.*, 2011; Dudek *et al.*, 2011; Le Goffic *et al.*, 2010; Varga *et al.*, 2011). Sequence variability of PB1-F2 from different strain mirrors distinct structural signatures (Solbak *et al.*, 2012). Sequence alignment of the PB1 proteins of five distinct viruses (ABA55039 A/Brevig Mission/1/1918(H1N1), NP_040985 A/Puerto Rico/8/1934(H1N1), ACU79879 A/Hong Kong/1/1968(H3N2), YP_308665 A/Goose/Guangdong/1/96(H5N1), ADA72001 A/swine/4/Mexico/2009(H1N1)) revealed that there was a 92,5% sequence position identity while alignment of the PB1-F2 proteins of the same viral isolates showed just 39,6% sequence position identity (Fig. 1). This discrepancy is caused by degeneracy of the genetic code as third nucleotides of PB1 codons equals second nucleotides of PB1-F2 codons. Since PB1-F2 is one of the most variable IAV proteins it is not surprising

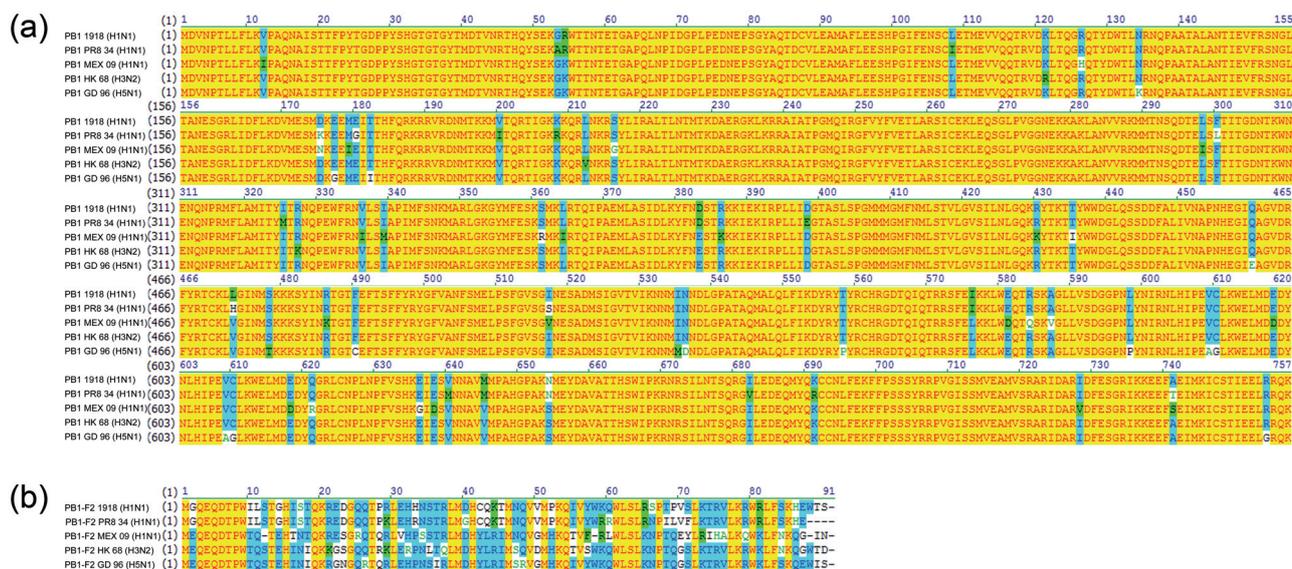


Fig. 1

VNTI advanced 11.5.0 based alignment of the PB1 and PB1-F2 aminoacid sequences of the five distinct IAV isolates

a) PB1 proteins of the A/Brevig Mission/1/1918(H1N1) [1918], A/Puerto Rico/8/1934(H1N1) [PR8 34], A/Hong Kong/1/1968(H3N2) [HK68], A/Goose/Guangdong/1/96(H5N1) [GD96], A/swine/4/Mexico/2009(H1N1) [MEX 09]. b) PB1-F2 proteins of the identical isolates shown variability of the PB1-F2 ORF and a high degree of conservancy of the PB1 protein sharing the same RNA segment. Identical aa displayed yellow-red, synonymous green-black, prevalent blue-blue, minor-non-synonymous black color.

that the effects of the PB1-F2 are strain, cell type and host specific (Hai *et al.*, 2010; Chen *et al.*, 2010; Marjuki *et al.*, 2010; McAuley *et al.*, 2010a,b; Pena *et al.*, 2012a; Schmolke *et al.*, 2011) During the last pandemics in 2009 it was speculated that the introduction of the stop codons in the PB1-F2 ORF after 11aa residue could be responsible for the mild course of infection (Schnitzler and Schnitzler, 2009). Subsequently, several groups showed the mild effect of reconstitution of the PB1-F2 ORF on virulence and pathogenicity of pdm H1N1 2009 flu (Hai *et al.*, 2010; Ozawa *et al.*, 2011; Pena *et al.*, 2012b). The overall importance of the PB1-F2 for virus seems to be very complex, influenced by multiple factors including its aminoacid sequence variability, other viral protein cooperation, host cell protein interactions as well as host species background. Here we provide a global overview on the role of PB1-F2 at different structural levels including whole protein, domain, region and very particular amino acids residues in relation to the above mentioned properties.

2. Influenza viruses encode either full length, truncated or no PB1-F2 at all

PB1-F2 represents a very unstable protein since proteasome has been shown to be involved in its rapid degradation (Chen *et al.*, 2001; Schmolke *et al.*, 2011). Degradation is probably one of the most important properties of the PB1-F2 as the level of the protein in general affects its functions. Expression is affected by a complex mode of infection. During *in vitro* infection of the A549 epithelial cell line with A/WSN/33 H1N1, PB1-F2 is detectable first in 6HPI, and in 24HPI it was not detectable at all (Le Goffic *et al.*, 2010). Interestingly, under natural infection *in vivo* 48HPI is first detected in PB1-F2 in lung tissue. Later post infection from A/WSN/33 72HPI and 96HPI it was still possible to detect PB1-F2 expressed by the same strain A/WSN/33 in the lungs of C57/BL/6 mice (Le Goffic *et al.*, 2011). It is important to note that the inability to detect PB1-F2 could be caused by the formation of amyloid fibers (Chevalier *et al.*, 2010). Comparison of the growth kinetics between recombinant virus USSR/90/77 expressing C-terminally truncated form of the PB1-F2 protein 57aa long and its PB1-F2 deletion variant has shown that despite minor differences, a delay in PB1-F2 deficient virus growth using MDCK cell line for titration was observed. Growth properties of another recombinant virus based on the same virus background expressing N66S PB1-F2 gene from 1918 Spanish flu virus did not differ significantly in comparison with C-terminally truncated PB1-F2 expressing virus. (Meunier and von Messling, 2012). In agreement with our observations, N terminus is responsible for increased PB1 and other viral protein expression (Kosik *et al.*, 2011) as well as overall replication kinetics (Smith *et al.*, 2011). Such observation of MDCK cell is important

since this cell line has been shown to replicate IAV very efficiently (Ueda *et al.*, 2008) and the contribution of PB1-F2 to replication kinetics is more significantly visible in the A549 cell line. Reconstitution of the PB1-F2 from the pdm H1N1 2009 IAV on the background of PR8 elevated virus growth in comparison to 11aa WT PB1-F2 from the same isolate. Interestingly, despite truncated PB1-F2 had produced twice as large plaques than full length recombinant whereas application of the same PFU on the cells had produced almost twice the plaque count after full length PB1-F2 virus infection (Chen *et al.*, 2010). As both of the viruses possess the same HA, equal adsorption on infected cell surface is probable. Thus an increased plaque count suggests more effective defence against cell antiviral response (IFN type I) to be established in full length PB1-F2 pdm H1N1 2009 expressing virus infected cells. Using *ex vivo* ferret and macaque, lung cultures displayed no differences in replication kinetics 24 and 48, neither for HPI USSR/90/77 PB1-F2 (57aa) nor PB1-F2 knock out for PB1-F2 1918 viruses. On the other hand, PB1-F2 1918 expressing recombinant virus developed more severe clinical symptoms in ferret model (Meunier and von Messling, 2012). In another epidemiologically important host swine, infection with A/California/04/09 with restored ORF of full length PB1-F2 resulted in increased replication and histopathology in the infected nasal turbinates, trachea and proximal lung 24HPI (Pena *et al.*, 2012b). Similar effects were observed *ex* as well as *in vivo*. Common reservoir of the IAV is generally accepted by waterfowl. Since more than 95% of avian IAV isolates preserved PB1-F2 ORF 90 aa long it is reasonable to think that PB1-F2 plays an important role in this species. In agreement with this hypothesis, deletion of PB1-F2 ORF from HPAI H5N1 slows progression of clinical signs and sickness and even leads to decreased mortality in comparison with PB1-F2 expressing virus. PB1-F2 seems to play a role in organ dissemination as its expression leads to increased viral titers in spleen, colon, lung, liver and kidney 1DPI. Later post infection viral titers are equal, suggesting the early infection phase role of PB1-F2 in ducks. Deletion or N66S polymorphism of the PB1-F2 has no effect on HPAI to LPAI flip (Schmolke *et al.*, 2011).

3. Inflammation, pathogenic effects of PB1-F2 and secondary bacterial infection

Inflammation of IAV infected tissue is an important factor of pathogenicity for example in the case of 1918 IAV or HK97 (Alymova *et al.*, 2011; Conenello *et al.*, 2007). Pathogenicity of the IAV could be divided into virus determined and host immune system determined. Overall pathogenesis of the IAV is a consequence of the cooperation of the aforementioned factors. PB1-F2 possesses both of these factors. Its relation to virus RNA dependent RNA polymerase (vRdRp) fits the cri-

teria for virus determined pathogenicity contribution (Kosik *et al.*, 2011; Mazur *et al.*, 2008; McAuley *et al.*, 2010b). In the terms of host immune system contribution, modulation of the cytokines response, selective immune cell pro-apoptosis (Coleman, 2007) and more recently identified neutrophil chemoattraction has been described for PB1-F2 functions (Conenello *et al.*, 2011; Le Goffic *et al.*, 2011). Capacity to enhance the inflammatory response seems to be a general feature of PB1-F2 proteins encoded by PB1 genes that are direct introductions from the avian gene pool. Notably, PB1 segment has been newly introduced for all pandemics of the 20th century. Exposition of human lung epithelial cells A549 or mouse macrophage cell line RAW264.7 to panel of the C-terminal peptides of the PB1-F2 caused cell death only in the case of PR8 and 1918 viruses for RAW264.7 and PR8 for A549 cell line respectively. No significant cell death was observed for any other H2N2 1957, H3N2 1968, H3N2 1995, and H5N1 2004 PB1-F2 derived C-terminal peptides. Exposition of mice lungs to the same panel of peptides caused an increased dendritic cells, T cell, macrophage and neutrophil count for all subjected peptides (McAuley *et al.*, 2010a). Similarly increased bronchoalveolar cellularity was observed in another study with HK97 S66 PB1-F2 variant (Conenello *et al.*, 2011). An infection study with recombinant viruses expressing various PB1-F2 confirmed peptide based observations for PR8 and 1918 PB1-F2 which are the only ones with cell death induction potential. One difference is that while cell death induction capable peptides derived from PB1-F2 of the PR8 and 1918 act on the RAW264.7 cell line only, infection with PR8 viruses expressing PB1-F2 or the PR8 and 1918 induced cell death solely on the A549 cell line. In general, PR8 PB1-F2 is the most potent cell death inductor. This observation means that cell death is a strain specific factor, which does not affect pathogenesis of the IAV in common (McAuley *et al.*, 2010a). High throughput PB1-F2 interaction screening of human leukocyte cDNA library in two hybrid systems revealed macrophage migration inhibitory factor (MIF) as an interaction partner of the PB1-F2 (Guan *et al.*, 2012). Since MIF is responsible for the preservation of inflammatory states by preventing untimely apoptosis of the macrophages and monocytes during infection, this interaction could affect functions of this multitropic cytokine (Mitchell *et al.*, 2002) and mediate pro-inflammatory function of the PB1-F2 during IAV infection by recently unknown mechanism.

3.1 PB1-F2 with residues L62, R75, R79 and L82 display inflammatory activity while PB1-F2 with P62, H75, Q79 and S82 display antimicrobial activity

H3N2 viruses have evolutionarily expressed full length PB1-F2 with inflammatory (A/Hong Kong/1/1968 (HK68)) or its non-inflammatory descendant (A/Wuhan/359/1995

(WH95)). PB1-F2 from these two related viruses differ in 5 aa of the C-terminal part (61 to 90 aa) of the molecule. Administration of 26aa long peptide variants with altered aminoacids in 62, 75, 79, and 82 to mice lung managed to completely revert pro/non-inflammatory phenotype measured as body weight change and mortality. The most significant effect was observed in the case of HK68 derived peptide and quadruple mutation WH95 L62,R75, R79, L82 which was characterized by increased TNF- α level, extensive lung consolidation with dense neutrophil monocyte and macrophage infiltrates. Interestingly, while secondary infection with *S. pneumoniae* was increased 70 fold following treatment of mice with HK68 H3N2 derived pro-inflammatory peptide in comparison to PBS treated group, titre of bacteria was significantly lower in the group of mice treated with WH95 non-inflammatory peptide in comparison to the negative control group suggesting the antibacterial potential of P62, H75, Q79, and S82 conferring peptide. The bactericidal effect of these residues was confirmed by exposing *S. pneumoniae* to non-inflammatory peptide derived from WH95 virus (Alyмова *et al.*, 2011).

3.2 PB1-F2 mutations T51M, V56A and E87G may decrease lethality

These three mutations in PB1-F2 ORF of the HPAI H5N1 A/Vietnam/1203/2004 have been established to decrease lethality in mallard ducks. Despite the fact that viruses replicate in mallard ducks to a similar 3DPI level regardless of aminoacids in 51, 56 or 87 positions respectively, primer extension assay clearly displayed that T, V and E aminoacids in C-terminal end of the PB1-F2 increase accumulation of viral RNA during the infection cycle. Additionally, these aminoacids affect the activity of vRdRp (Marjuki *et al.*, 2010).

3.3 PB1-F2 N66S polymorphism and the complex role of this aa residue

The rare polymorphism of PB1-F2 in position 66 was described as a factor affecting virulence and pathogenicity of IAV. Serine in this position was identified in 1918 IAV and HPAI HK97 to increase pathogenicity in mouse model. Recombinant virus expressing S66 replicated 100-fold higher than N66 isogenic variant (Conenello *et al.*, 2007). Expression of the HK97 derived S66 form of PB1-F2 in mouse lung epithelial adenoma cell line (LA-4) 24HPI was almost undetectable in comparison to infection with isogenic virus with PB1-F2 N66. It is surprising that despite inability to detect PB1-F2 S66, such virus exhibit 10-fold higher virus titre on LA-4 cell line than WT virus expressing a high level of PB1-F2 24HPI (Schmolke *et al.*, 2011). This is in agreement with a report by Varga *et al.* (2011) which showed decreased IFN- β induction despite a lower amount of S66 PB1-F2 PR8 being

produced. Neither WT nor S66 variant of PB1-F2 co-localize with inner mitochondrial membrane protein prohibitin, but rather diffuse cytoplasm and nucleus distribution. In mouse model C57/BL/6/A2G-Mx1, expressing Mx1 protein it was shown that S66 PB1-F2 mutation increase 10-fold LD₅₀ in comparison to N66 PB1-F2 variant. Neurological symptoms were observed for S66 PB1-F2 from the HK97 in mice model while deletion mutant and N66 WT form of the PB1-F2 did not exhibit such features. Additionally, infection of only S66 PB1-F2 expressing virus had spread to the brain 8DPI in 6 of 7 C57/BL/6/A2G-Mx1 mice, while no virus was detected in the spleen or lung on day 8 post infection. Given that LPAI H5N1 lacking HA multibasic cleavage site does not differ in lethality regardless of amino acid in position 66 of the PB1-F2 suggests that tissue tropism determines the impact of the PB1-F2 (Schmolke *et al.*, 2011). In duck fibroblast cell line S66 variant of the PB1-F2 is expressed in a lower amount than N66 form. The serine variant had slightly enhanced disease progression in ducks. The course of infection with S66 or N66 PB1-F2 isogenic viruses A/WSN/33 with PB1 segment derived from HK97 in transgenic adaptive immune system deficient mouse model (RAG1^{-/-}, CD4^{-/-}, CD8^{-/-} or IFN- γ ^{-/-}) compared to infection of the WT mouse model exhibits similar decreased body weight (0 to 7/11DPI) in S66 PB1-F2 virus. Authors had concluded none of the CD8⁺ CD4⁺ or B cell to be essential for pathogenesis mechanism (Conenello *et al.*, 2011). Since virus specific CD8⁺ cells are initially detectable 5DPI with peaks between 9,5DPI to 11DPI and IgM serum level peaks 8DPI to 10 DPI while virus titre peaks 2 to 3DPI, with complete viral clearance by day 11 (Miao *et al.*, 2010), it is not surprising that adaptive response did not affect morbidity in these experiments. However it would be interesting to study PB1-F2 in the same experimental mice models with secondary infection by homologous and heterologous viruses. Transcriptional profiling of mouse lungs infected with S66 and N66 PB1-F2 HK97 have clearly shown that S66 variant induces a lower level of the RIG-1, IFN- β , Mx1, and STAT-1 1DPI, but higher levels of IFN regulated response 3DPI in comparison with N66 PB1-F2. Thus serine 66 in PB1-F2 protein of the HK97 caused a delay in early interferon response in mice (Conenello *et al.*, 2011). Uncontrolled virus replication during the first days post infection may result in an increased count of infected lung cells which in turn could cause higher cytokine production and increased cellularity of immune cells to lungs. This hypothesis is supported by increased levels of multiple cytokines in lungs 3DPI and later post infection including IFN- β , IL-6, KC (monocyte trafficking to lungs), M-CSF (macrophage activation and maturation), TNF- α , MCP-1, RANTES, IFN- γ . Increased monocytes, neutrophils, and dendritic cells have also been reported for S66 PB1-F2 of the HK97 (Conenello *et al.*, 2011). Similar observations were made for reconstituted S66 PB1-F2 from

pdm 2009 H1N1 strain (Hai *et al.*, 2010). As effects of PB1-F2 variants differ significantly in relation to time post infection, it would be interesting to compare kinetics of the PB1-F2 expression to kinetics of expression of the IFN- β , - α and the other cytokines. Such an experiment could contribute to resolving the PB1-F2 \leftrightarrow cytokines enigma. Despite PB1-F2 seemingly inhibiting IFN- β induction in MAVS dependent manner (Varga *et al.*, 2011), the additional indirect impact of the PB1-F2 on cytokine expression should not be excluded. Increased expression of the NS1 and other viral proteins in the presence of the N-terminal part of PB1-F2 (Kosik *et al.*, 2011; Mazur *et al.*, 2008) as well as the virus co-infection dependent effect of the PB1-F2 on cytokines (Le Goffic *et al.*, 2010) support this assumption. IFN- β antagonism seems to be strain independent and S66 enhanced. PR8 derived PB1-F2 protein with N66S mutation exhibit fold increased IFN-I inhibition compared to WT PB1-F2. Additionally, enhanced IFN-I suppression in murine lungs epithelial cell line LA-4 as well as murine and human dendritic cells was also observed supporting IFN antagonism of the PB1-F2 as general feature (Varga *et al.*, 2011). Reconstitution of the PB1-F2 ORF in pdm H1N1 2009 with serine in 66 position leads to higher viral titers on A549 human lung epithelial cells than N66 variant. However both of these viruses produced similar size plaques on MDCK cell line. In the Balb/c mouse model there was no difference in replication kinetics of S66 or N66 PB1-F2 expressing viruses. In the DBA/2 mouse model, both viruses replicated to a similar level (Hai *et al.*, 2010). It has recently been shown that DBA/2 mice are more susceptible to IAV infection than Balb/c (Boon *et al.*, 2009). Thus overall effects of PB1-F2 might be overplayed. Secondary bacterial infection of the *S. pneumoniae* revealed a 5-fold increase of this respiratory pathogen in the group infected with S66 PB1-F2 IAV in comparison to 11aa PB1-F2 expressing IAV. N66 PB1-F2 IAV caused a 2-fold increase in *S. pneumoniae* titer. Thus neither PB1-F2 from pdm H1N1 2009 nor S66, N66 variants of the PB1-F2 significantly affect secondary bacterial infection. In ferrets, only slightly more weight loss with similar virus titers in nasal wash was associated with S66 PB1-F2 expression in comparison with N66 or 11aa ORF expressing IAV (Hai *et al.*, 2010).

4. PB1-F2 modulates type I interferon and other cytokine responses

To restrict virus proliferation, IAV-infected cells mount a strong antiviral response. IFN- β is the most potent antiviral cytokine and is massively produced during IAV infection after pathogen associated molecule recognition by TLR3, RIG-1, NLR, and MDA-5 (Guillot *et al.*, 2005; Le Goffic *et al.*, 2007; Varga *et al.*, 2011). Upon recognition of viral RNA species, RIG-1 interacts with mitochondrial antiviral protein

(MAVS also known as VISA, CARDIF or IPS-1). This leads to secretion of IFN and auto/paracrine activation of JAK/STAT pathway which in turn leads to the formation of ISG3 transcription complex formation and expression of protein kinase R (PKR), Mx GTPase and other antiviral proteins (Varga *et al.*, 2011). In the human epithelial cell line A549, infection with IAV Δ PB1-F2 caused reduced IFN- β level 24HPI, while 8HPI showed no difference in IFN- β level regardless of the presence or absence of the PB1-F2 suggesting that PB1-F2 of amyloid fibrils can exacerbate IFN- β response. This effect seems to be restricted to epithelial cell line as infection of immune cell line Jurkat, U937 or mouse alveolar macrophages induces a similar level of IFN- β in response to PB1-F2 presence or absence. Importantly, induction of IFN- β by PB1-F2 from several distinct IAV strains is fully dependent on co-infection with Δ PB1-F2 virus (Le Goffic *et al.*, 2010). Thus PB1-F2, not solely but probably in cooperation with other IAV expressed proteins contribute to IFN- β response during the late stage of epithelial cell line IAV infection. IAV viruses with alteration in ATG start codon of the PB1-F2 could express N-terminally truncated form of the PB1 protein termed N40 because of PB1 and PB1-F2 share the same genome segment but not the same ORF (Wise *et al.*, 2009). However this protein was shown to have no effect on IFN- β (Le Goffic *et al.*, 2011), thus proving that PB1-F2 protein along with PB1, PB2, PA (Graef *et al.*, 2010; Iwai *et al.*, 2010), and NS1 is involved in the IFN- β response modulation. The same scientific group has shown induction of the NF- κ B, major pro-inflammatory transcription factor, during A/WSN/33 H1N1 infection. Additionally, while higher infection dose exhibits no differences, lower infection dose (5×10^4 PFU) of the Balb/c mice with A/WSN/33 H1N1 WT or Δ PB1-F2 differs in IFN- β induction. WT IAV induced a higher level of IFN β mRNA. Detection of pro-inflammatory cytokines IFN- γ , IL-6, CXCL1/KC (major neutrophil chemoattractant) in bronchoalveolar fluid by ELISA revealed that infection of Balb/c mice with A/WSN/33 H1N1 virus induces a higher amount of these pro-inflammatory molecules than Δ PB1-F2 virus (Le Goffic *et al.*, 2011). This suggests a pro-inflammatory role and potential immunopathology effects of PB1-F2. Importantly, virus independent treatment of J774 macrophage cell line with 26aa long peptides derived from HK68 H3N2 PB1-F2 resulted in a 20-fold increase of TNF- α secretion which was shown for mice lung infected with HK97 H5N1 as well (Conenello *et al.*, 2011), while reconstituted PB1-F2 from pdm 2009 H1N1 did not alter TNF- α level at all (Hai *et al.*, 2010). However, the involvement of C-terminal aminoacids L62, R75, R79 and L82 responsible for increased inflammation and TNF- α level (Alyмова *et al.*, 2011) post infection by HK68 H3N2 were recently identified. Reconstituted PB1-F2 from pdm 2009 H1N1 harbours non-inflammatory residues (P,H,Q) in positions 62, 75, 79 identical to non-inflammato-

ry HK68 H3N2 descendant WH95. In contrast to Le Goffic studies (Le Goffic *et al.*, 2010; Le Goffic *et al.*, 2011) other groups have shown the opposite function of the PB1-F2 on IFN- β . In the lung explantates of ferrets and macaques it was established that infection with USSR/90/77 PB1-F2 (57aa) or PB1-F2 knock out or PB1-F2 1918 viruses 24HPI and 48HPI slightly upregulates IFN- β , IL-6, and IL-8 cytokines with lower induction by USSR/90/77 PB1-F2 (57aa). Similar experiments on ferret blood derived macrophages resulted in mild and delayed IL-1 β , IL-6, and IL-8 mRNA transcription in the case of USSR/90/77 PB1-F2 (57aa) or USSR/90/77 PB1-F2 1918 while PB1-F2 lacking virus infection resulted in upregulation of mentioned cytokines (Meunier and von Messling, 2012). This observation could suggest that the N-terminal half of the PB1-F2 is responsible for the suppression of pro-inflammatory cytokines transcription in immune competent cells. Despite Varga *et al.* (2011) having shown that the C-terminal domain of PR8 derived PB1-F2 mediates IFN antagonism activity, C-terminal domain in the noted study includes aa fragment corresponding to 38 to 87aa. Overlapping peptide 19aa long could be involved in IFN modulation activity. In ferret nasal washes of animals infected with USSR/90/77 PB1-F2 1918, the level of IFN- α mRNA 1DPI was 10-fold lower than USSR/90/77 PB1-F2 (57aa) or PB1-F2 knock out viruses while there were no differences in IFN- β mRNA level suggesting that the C-terminal part of the PB1-F2 1918 could also influence type I IFN response. Early after infection of pigs with recombinant pdm 2009 H1N1 virus conferring complete PB1-F2 ORF (A/California/04/09 PB1-F2), a higher level of IFN- α and IL-1 β was induced (Pena *et al.*, 2012b). Infection of the mouse epithelial cell line LA-4 as well as bone marrow derived macrophages and dendritic cell with HPAI HK97 PB1-F2 N66/S66 viruses did not significantly alter the mRNA level of type I and II IFN. However absence of the PB1-F2 leads to higher expression of the IL-1 β and IL-6. S66 PB1-F2 induces the lowest level of IFN induced response of all (Schmolke *et al.*, 2011). Reconstituted PB1-F2 derived from pdm 2009 H1N1 strain of the IAV induces a similar level of several cytokines including IFN- γ (type II IFN), IL-1 β , and TNF- α (pdm H1N1 2009 PB1-F2 contains non-inflammatory P,H, Q motive mentioned above) in Balb/c mouse model while MCP-1, MIP-1 β and RANTES were significantly higher in mouse infected with S66 PB1-F2 IAV. In the DBA/2 mouse model S66, PB1-F2 induces no detectable level of IL-1 β , while RANTES and MIP-1 β (the same as for Balb/c) are increased in comparison to 11aa long PB1-F2 in pdm 2009 H1N1 (Hai *et al.*, 2010). Effects outlined of the PB1-F2 are strain specific, however interferon suppression seems to be a common property as H5N1 HK97 as well as PR8 strain both exhibit IFN- β antagonism (Varga *et al.*, 2011). On the other hand, host cell factors could even revert this feature since PB1-F2 in A549 human lung epithelial cells, ferrets and

macaques lung explantates have been shown to exacerbate IFN- β and other cytokines levels (Le Goffic *et al.*, 2010, 2011; Meunier and von Messling, 2012).

5. Intracellular localization of PB1-F2 is critical for its ability to enhance vRdRp activity

Usually, minigenome polymerase reconstitution system contains in addition to expression plasmids for PB1, PB2, PA, and NP, an extra plasmid for PB1-F2. The rationale for this lies in basal differences in PB1-F2 translation initiation between viral segment based and plasmid based PB1-F2 expression. While Kozak sequences surrounding PB1 in IAV RNA segment are suboptimal, favouring Kozak optimal PB1-F2 translation initiation, in plasmid based expression PB1 ATG is surrounded by optimal Kozak sequences inevitably leading to the favouring of translation of PB1 protein. In our previous work concerning DNA vaccination with PB1 expressing plasmid, we were not able to detect expression of the PB1-F2 after transfection with PB1 expressing plasmid. Additionally, while we were able to detect anti PB1 antibodies in sera of pPB1 DNA vaccinated mice, we were not able to detect anti PB1-F2 antibodies in the same sera suggesting none or trace expression of PB1-F2 from PB1 expression plasmid (Kosik *et al.*, 2012). Pena *et al.* (2012b) used the vRdRp activity luciferase reporter system employing expressing plasmid derived from the A/California/04/09 in which PB1 gene contains PB1-F2 ORF. Even employing PB1-F2 expression thereby disadvantaging the reporter system revealed slightly increased the activity of vRdRp (Pena *et al.*, 2012b). It is a matter of speculation as to whether or not independent plasmid for PB1-F2 could provide even higher effects on vRdRp. Furthermore, infection of luciferase reporter plasmid transfected cells with the same virus confirmed the increased activity of vRdRp in the functional PB1-F2 ORF environment. Three aminoacid changes in HPAI H5N1 A/Vietnam/1203/2004 PB1-F2 namely T51M, V56A, and E87G lead to decreased activity of the vRdRp in chicken fibroblast DF-1 or human 293T cell line. Similar to Pena *et al.* (2012), Marjuki *et al.* (2010), and McAuley *et al.* (2010) used pPB1-F2 less luciferase reporter system. M51, A56, and G87 caused a 20-50% decrease in polymerase activity (Marjuki *et al.*, 2010). The author's conclusion is in contrast with the results of our group identifying the N-terminal half of the PB1-F2 as the major mediator of PB1-F2 to PB1 relation (Kosik *et al.*, 2011). However the minor role of the C terminal part of PB1-F2 cannot be excluded since PB1-F2 effects on vRdRp and the replication cycle could be a multistep process involving several PB1-F2 regions in ordered sequence. Another report confirmed the importance of PB1-F2 for vRdRp activity where stop mutation preventing expression of PB1-F2 had decreased polymerase activity by nearly 35%

in the 293T cells transfected with reporter system harbouring HPAI HK97 derived PB1-F2 (Chen *et al.*, 2010). A similar effect was observed for another H5N1 derived PB1-F2 from A/Vietnam/1203/04 where blocking PB1-F2 expression led to a more than 50% decrease of polymerase activity measured in 293T as well as A549 cell lines (McAuley *et al.*, 2010b). In the study mentioned, the effect was strain and cell type dependent since absence of PB1-F2 during infection of PR8 Δ PB1-F2 had even increased activity of vRdRp on A549 cell line while vRdRp activity was decreased on 293T cells in comparison with WT PB1-F2 expressing virus. WH95 derived PB1-F2 has no measurable effect on vRdRp activity. Subcellular localization preference of the PB1-F2 might dramatically affect functional output of this multifunctional protein. There are at least two possible scenarios for PB1-F2 behaviour during the infection cycle. One is for cytosol and nuclear targeted PB1-F2 affecting most probably the activity of vRdRp. The second is for mitochondrial targeted PB1-F2 thus modifying cytokines response (MAVS) and pro-apoptosis effects. The rate of one pathway to a second should then be determined by PB1-F2 aminoacid composition and host cell factors available *in situ* at the time. This hypothesis of the "disbalanced" behavior of PB1-F2 is in agreement with the observation that viruses expressing PB1-F2 with suboptimal MTS are prevalently localized to cytoplasm and nucleus and their mitochondrial targeting sequence (MTS) optimal mutants with increased mitochondrial localization have dramatically decreased vRdRp activity (Chen *et al.*, 2010).

6. Subcellular localization of PB1-F2 and its relation to apoptosis

A pioneering report describing PB1-F2 discovery outlined mitochondrial localization of the PB1-F2 protein (Chen *et al.*, 2001), which was confirmed by taq fusion studies identifying MTS to region 69 to 82 as minimal MTS and 65 to 87 as optimal MTS (Gibbs *et al.*, 2003). Some discrepancies surrounded precise mapping of MTS since using different a fusion flag resulted in the identification of 46 to 75 as minimal MTS. In the case of the infection of mouse lung epithelial cells LA-4 and the infection of duck fibroblasts PB1-F2 derived from HPAI HK97 localized mainly to the cytoplasm and nucleus, no significant co-localization with mitochondrial membrane protein prohibitin is displayed regardless of the presence or absence of serine residue in position 66 (Schmolke *et al.*, 2011). Comparison of subcellular localization of the different strain derived PB1-F2 exhibit variation. PB1-F2 from A/Taiwan/3355/1997 H1N1 and commonly used laboratory strain PR8 share subcellular distribution with mitochondrial preferences despite more than six decades of evolution. Surprisingly, PB1-F2 derived from the HK97 and H7N7 A/Netherlands/219/2003 does not

specifically localize to mitochondria, but a cytoplasmic and nuclear pattern is typical for this isolate. Since most protein variation is localized to C-terminal domain as well as minimal mitochondrial targeting sequence (Chen *et al.*, 2010), it is not surprising given the variability of mitochondrial localization. The purpose of such inconsistency most probably lies in the advantage of nonessential nature of the PB1-F2 for IAV. Observed cell, strain and host determined feature variability of PB1-F2 could provide a crucial advantage to the virus during novel host adaptation. Thus while in avian host it might be more important for PB1-F2 to function in nucleus as observed for HK97 PB1-F2, in mammalian host mitochondrial localization with pro-apoptosis effects on immune cells might be desired as in the case of PR8 PB1-F2. This is in agreement with no effect of HK97 PB1-F2 expression having been observed at the apoptosis induction level on human monocytic cell line U937, but increased vRdRp activity and virus growth (Chen *et al.*, 2010). Recently discovered interaction of PB1-F2 with MIF protein (Guan *et al.*, 2012) will hopefully bring some light to relation to PB1-F2 and apoptosis as MIF prevents apoptosis in macrophage cells (Mitchell *et al.*, 2002) thus preserving inflammation. It will be interesting to compare the conservancy and presence of MIF interaction responsible aminoacid residues of the different strain derived PB1-F2 with its ability to induce apoptosis in immune competent cells.

6.1 Leucines, lysines, and arginines aa of mitochondrial targeting sequence

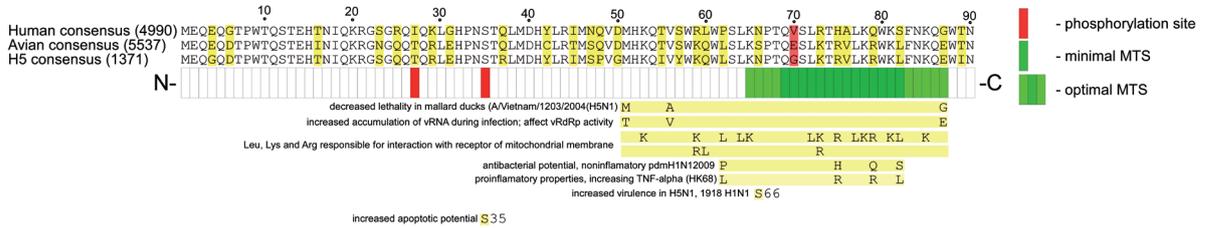
Effective mitochondrial targeting has been shown not to be restricted to defined aa in defined position, but rather formation of amphipathic helix determines the ability of PB1-F2 and other virus proteins (HTLV-I p13) to target mitochondrion (Gibbs *et al.*, 2003; Chen *et al.*, 2010; Yamada *et al.*, 2004). Leucines, lysines and arginines play cardinal roles in amphipathic helix formation. Moreover, leucine rich domains were considered to be responsible for interaction with hydrophobic binding groove on TOM20 import receptor on mitochondrial membrane (Pfanner, 2000). Comparison of the PB1-F2 protein sequence from PR8 (predominantly localized to mitochondria) and HK97 (diffusely localized to cytoplasm and nucleus) revealed that while only three L in positions 72, 77, and 82 are presented in HK97 PB1-F2, four L residues are presented in positions 69, 72, 78, and 82 of the PB1-F2 from the PR8 virus (Chen *et al.*, 2010). Both of these proteins confer six K or R basic residues in the same region 65-87aa which exceed five basic residues reported to be needed for efficient amphipathic helix formation (Gibbs *et al.*, 2003). Changing the Q69 and H75 of HK97 PB1-F2 to L increases mitochondrial localization of protein suggesting the involvement of this particular residue in MTS (Chen *et al.*, 2010). Notably, mutation of conservative L in positions

72 and 77 did not abolish mitochondrial localization (Gibbs *et al.*, 2003) in the presence of L69 and R75, thus while Gibbs *et al.* showed that even loss of three L in position 72, 75, and 77 do not lead to the loss of mitochondrial localization, Chen *et al.* (2010) presented that both L in position 69 and 75 are needed for increased mitochondrial direction. It is probable that basic R in position 75 partially compensates L function in this site.

7. PB1-F2 serine 35 and threonine 27 phosphorylation acceptor sites and their role in apoptosis

Identification of the MTS in PB1-F2 and co-localization with mitochondria (Gibbs *et al.*, 2003; Chen *et al.*, 2001; Yamada *et al.*, 2004) lead researchers inevitably to the assumption that PB1-F2 is connected to programmed cell death pathways. This was later enhanced by identification of interaction of PB1-F2 with voltage dependent anion channel (VDAC1), adenosine nucleotide transporter (ANT3), proteins of outer and inner mitochondrial membrane (Zamarin *et al.*, 2005) as well as the intrinsic ability of PB1-F2 to create pores in membranes (Chanturiya *et al.*, 2004). Unfortunately, it is still not known which particular residue of PB1-F2 are responsible for interaction with VDAC1 and ANT3 which could bring some light to understanding the importance of this relation. Proapoptotic effect was observed in cell dependent manner with obvious preferences for immune competent cells (Coleman, 2007). It has been shown more recently that proapoptosis is not general property of the PB1-F2 but is strain dependent particularly for commonly used laboratory strain PR8 and 1918 viruses (McAuley *et al.*, 2010a). A necessary condition for proapoptotic function of PB1-F2 appears to be mitochondrial localization, thus it is not surprising that isolates with altered MTS crucial residues L69, L72, L/R75, K78 and L82 (Gibbs *et al.*, 2003; Chen *et al.*, 2010; Yamada *et al.*, 2004) are not able to effectively induce cell death (McAuley *et al.*, 2010a). Another factor affecting caspase 3 activation has been reported for PB1-F2 molecule. Extensive study identified the phosphorylation site of PB1-F2 serine residue in position 35 as preferential. Negligible phosphorylation of the threonine 27 is due to closely localized proline residue in position 28, which caused spatial hindrance to phosphorylation. Interaction of the PB1-F2 with protein kinase C alpha (PKC α) by yeast two hybrid system has also been proven. Additionally, while inhibitors of the PKC α (bisindolylmaleimide, staurosporine) decreased overall in the amount of phosphorylated PB1-F2, activators of PKC α (phorbol 12-myristate 13-acetate) increased phosphorylation two fold. Serine 35 mutant virus exhibited slightly impaired propagation in comparison with WT PB1-F2 virus on A549 or MDCK cells. However later, replication on human primary monocytes 36HPI revealed the decreased

(a) PB1-F2



(b)

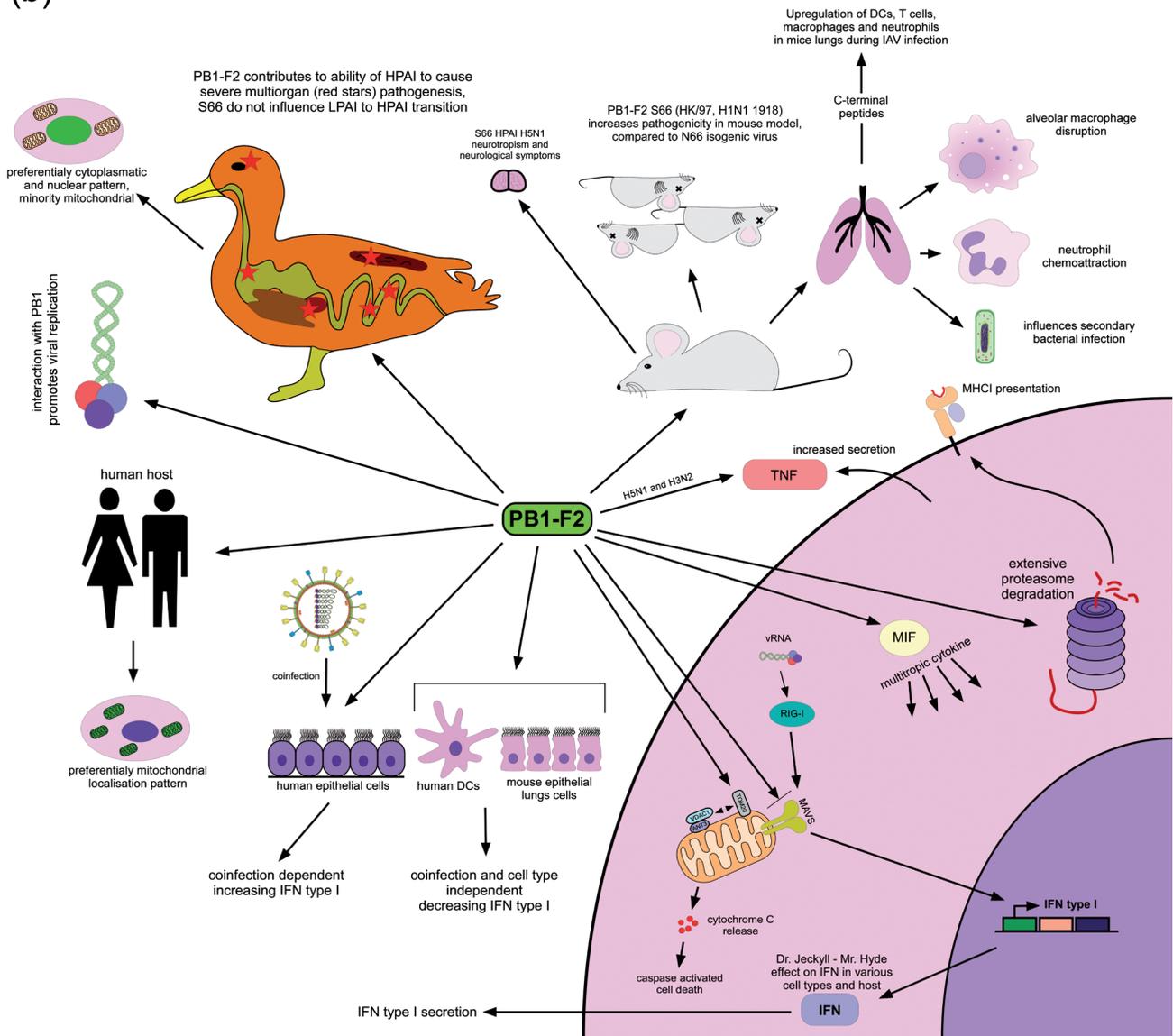


Fig. 2

Structural-functional map summarizing the complex behaviour of the PB1-F2

a) Alignment of the PB1-F2 consensus aminoacid sequences of human, avian and avian H5 subtype isolates representing described to date functionally important and most conserved aa residues created on www.fludb.org. Unhighlighted fields represent identical aa in given position, bright yellow represents non-identical aa in one consensus sequence and bright red represents various aa for all consensus sequences. b) Documented PB1-F2 impact on different cellular and viral protein functions, cell and host species type.

growth of phosphorylation deficient mutant PB1-F2 S35L virus independently of IFN-I induction measured by MxA level. Importantly, WT virus possessing S35 PB1-F2 induces dramatically higher activity of apoptotic marker caspase 3 (Mitzner *et al.*, 2009).

8. Conclusion

PB1-F2 protein differs significantly in amino acid sequence as well as in almost all described functions, features and relations depending on strain, cell type and host species (Fig. 2). The significance of this protein is thus an enigma, although the high degree of presence of the PB1-F2 ORF in avian isolates and preservation of more than half a molecule (57aa) in swine and human isolates highly support the role of this protein for these hosts at least. Sequential variance resembling functional variability makes PB1-F2 a very versatile tool offering virus diverse opportunity to survive in different biological environments. Such functional complexity could serve as adaptation-evolutionary “punch” keeping IAV a continual danger to human health.

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