The dynamic proteome of influenza A virus infection identifies M segment splicing as a host range determinant

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Pandemic influenza A virus (IAV) outbreaks occur when strains from animal reservoirs acquire the ability to infect and spread among humans. The molecular basis of this species barrier is incompletely understood. Here we combine metabolic pulse labeling and quantitative proteomics to monitor protein synthesis upon infection of human cells with a human- and a bird-adapted IAV strain and observe striking differences in viral protein synthesis. Most importantly, the matrix protein M1 is inefficiently produced by the bird-adapted strain. We show that impaired production of M1 from bird-adapted strains is caused by increased splicing of the M segment RNA to alternative isoforms. Strain-specific M segment splicing is controlled by the 3′ splice site and functionally important for permissive infection. In silico and biochemical evidence shows that avian-adapted M segments have evolved different conserved RNA structure features than human-adapted sequences. Thus, we identify M segment RNA splicing as a viral host range determinant.
Influenza A viruses (IAVs) are negative-sense, single-stranded RNA viruses with a segmented genome. IAV infection causes seasonal epidemics and sporadically pandemic outbreaks in the human population with significant morbidity, mortality, and economic burden. IAVs can infect both mammals (e.g., humans, pigs, horses) and birds (e.g., chicken, waterfowl). However, strains that are replicating in birds typically do not infect mammals and vice versa. Pandemics occur when influenza strains of avian origin with novel antigenicity acquire the ability to transmit among humans. Understanding the molecular basis of host specificity is therefore of high medical relevance.

The species barriers that hinder most avian IAVs from successfully infecting humans are effective at several stages in the viral life cycle. For example, the avian virus receptor hemagglutinin (HA) recognizes oligosaccharides containing terminal sialic acid (SA) that are linked to galactose by α2,3. In the human upper respiratory airway epithelium, the dominant linkage is of α2,6 type, to which human-adapted HA binds. Despite these differences in receptor binding, many avian viruses are internalized by human cells and initiate expression of the viral genome. Such infections typically lead to an abortive, nonproductive outcome in mammalian cell lines. Our understanding of this intracellular restriction is still incomplete. One well-established factor is the influenza RNA-dependent RNA polymerase (RdRP): this enzyme catalyzes replication of the viral genome and transcription of viral messenger RNAs (mRNAs). Polyribosomes from avian strains are considerably less active in mammalian cells than their counterparts from mammalian-adapted strains. A wealth of experimental data described adaptive mutations that alter receptor specificity or fusion activity of HA (reviewed in ref. 9) and polymerase activity (reviewed in ref. 9). However, relatively little is known about the contribution of other IAV genes for permissive vs. non-permissive infection.

A crucial aspect for permissive infection is the correct timing of viral gene expression: IAV proteins are produced at the specific phase of infection when they are needed. One example is the M gene, which encodes predominantly two polypeptides: the larger protein, M1, is produced from a collinear transcript. The smaller one, M2, is encoded by a differentially spliced transcript. M1 is the matrix protein with multiple functions that encapsulates the viral genome, and also mediates nuclear export. M2 is a proton-selective channel that is an integral part of the viral envelope. The ratio of spliced to unspliced products increases during infection, which reflects the changing demands required for optimal viral replication.

Systems-level approaches have provided important insights into the molecular details of host–virus interaction. For example, RNA interference (RNAi) screens identified host factors required for IAV replication. Also, interaction proteomics experiments identified many cellular binding partners of IAV proteins. A number of studies also quantified changes in protein abundance. However, these steady-state measurements cannot reveal the dynamic changes in protein synthesis during different phases of infection. Early studies used radioactive pulse labeling to monitor protein synthesis in IAV-infected cells. However, radioactive pulse labeling cannot provide kinetic profiles for individual proteins. More recently, stable isotope labeling by amino acids in cell culture (SILAC) emerged as a powerful means to study the dynamic proteome. SILAC-based pulse labeling methods such as pulse SILAC (pSILAC) and dynamic SILAC can quantify protein synthesis and degradation on a proteome-wide scale. Moreover, metabolic incorporation of bioorthogonal amino acids such as azidohomoalanine (AHA) provides a means to biochemically enrich for newly synthesized proteins. In combination with SILAC, AHA labeling can be used to quantify proteome dynamics with high temporal resolution.

Here, we use metabolic pulse labeling and quantitative mass spectrometry to compare proteome dynamics upon infection of human cells with a human-adapted and a bird-adapted IAV strain. We find that host proteins behave surprisingly similar, but observe striking differences in the production of viral proteins, especially for the matrix protein M1. Follow-up experiments with reporter constructs, in silico studies, and reverse genetics identify an evolutionarily conserved cis-regulatory element in the M segment as a host range determinant.

Results
Quantifying the dynamic proteome of IAV infection. To assess species specificity of IAVs, we used a model system comparing a low-pathogenic avian H3N2 IAV (A/Mallard/439/2004—Mail) to a seasonal human IAV isolate of the same subtype (A/Panama/2007/1999—Pan). While the avian virus is not adapted to efficient growth in cultured human cells and causes a non-permissive infection, the seasonal human virus replicates efficiently. We demonstrated previously that the Pan virus produces >1000-fold more infectious viral progeny than the non-adapted virus, even though both strains efficiently enter human cells and initiate their gene expression program.

We reasoned that comparing the kinetics of protein synthesis upon infection with both strains might reveal determinants of species specificity. To this end, we performed proteome-wide comparative pulse-labeling experiments by combining labeling with AHA and SILAC (Fig. 1a): cells incorporate AHA instead of methionine into newly synthesized proteins when the cell culture medium is supplemented with this bioorthogonal amino acid. AHA contains an azido group, which can be used to covalently couple AHA-containing proteins to alkynyl beads via click chemistry. In this manner, newly synthesized proteins can be selectively enriched from the total cellular proteome. Combining AHA labeling with SILAC reveals the kinetics of protein synthesis with high temporal resolution. First, we fully labeled human lung adenocarcinoma cells (A549) using SILAC. Second, individual cell populations were infected with either Pan or Mal virus or left uninfected. Third, all cells were pulse labeled with AHA for 4 h during different time intervals post infection (0–4, 4–8, 8–12, and 12–16 h). The three cell populations for every time interval were then lysed, combined, and AHA-containing proteins were enriched from the mixed lysate using click chemistry (Fig. 1b). After on-bead digestion, peptide samples were analyzed by high-resolution shotgun proteomics.

We quantified proteins using two readouts: (i) SILAC-based relative quantification to assess differences in de novo protein synthesis and (ii) intensity-based absolute quantification (iBAQ) to quantify absolute amounts of newly synthesized proteins. Our data thus provides kinetic profiles for relative and absolute differences in de novo protein synthesis across the course of infection (Supplementary Data 1). In total, we identified 7729 host and 10 viral proteins and quantified 6029 proteins in at least two biological replicates with overall good reproducibility (Supplementary Fig. 1).

The dynamic host proteome. It is well established that IAV induces a global reduction in the production of host proteins. This host shutoff was attributed to a plethora of viral effector functions. To assess the host shutoff in our proteomic data, we investigated iBAQ values for viral and host proteins. As expected, viral proteins were potently induced, while the production of host proteins decreased over time (Fig. 1c). The difference between host and viral protein synthesis reached several orders of magnitude and was highest during the 8–12 h pulse interval. Moreover, the total cellular protein output dropped to ~24% (Pan) or...
Fig. 1 A strategy to quantify protein de novo synthesis proteome-wide. **a** SILAC-L/M/H-labeled A549 cells were infected with the human seasonal H3N2 IAV isolate Panama (Pan) or the avian H3N2 isolate Mallard (Mal) or left uninfected. The methionine analogous AHA was given to the methionine-depleted medium in different 4 h intervals. **b** After lysis and enrichment for proteins that incorporated AHA, samples were subjected to shotgun proteomics. Absolute and relative protein synthesis profiles were quantified for host and viral proteins. **c** iBAQ-based quantification of protein synthesis levels for host and viral proteins in cells infected with either Pan or Mal virus, as indicated. Median, 25th and 75th percentile of the respective populations are given. **d** Quantification of the total newly synthesized protein mass for host and viral proteins with either Pan or Mal infection as indicated. Data were normalized to the 0–4 h time period. All data are based on the mean of n = 2 biological replicates.
modulation of translation efficiency, which argues against the idea that modulation of translation efficiency affects species specificity.

**Dysregulated synthesis of viral proteins.** Since the observed differences in host protein synthesis were surprisingly subtle, we focused our attention to the dynamics of viral protein synthesis. Production of most viral proteins peaked in the 8–12 h period (~33% (Mal) at later stages of infection, of which ~20–40% was of viral origin (Fig. 1d)). At this level of detail, we observed no major differences between both strains. Thus, both strains initiate viral protein synthesis and induce the shutoff of host protein synthesis to an overall similar extent.

Next, we investigated the profiles of individual host proteins across the course of infection. For this, we directly looked at SILAC ratios comparing infected and non-infected cells (Fig. 2a, b). As expected, synthesis of the vast majority of host proteins markedly decreased over time. However, some proteins were less affected by the host shutoff and displayed only a mildly decreased or even increased production. To assess this observation more systematically, we selected the proteins that were least affected by the shutoff at different pulse periods and performed gene ontology (GO) analysis. The heatmap of enriched GO terms provides a global overview of biological processes as the infection progresses (Fig. 2c and Supplementary Data 2). For example, many well-known interferon-induced antiviral defense proteins (e.g., MX1, several IFIT proteins, several oligoadenylate synthase proteins) were relatively strongly produced at late stages of infection. Also, many ribosomal proteins (GO term "peptide chain elongation") largely escaped the host shutoff. Interestingly, we also observed significant enrichment of proteins involved in steroid metabolism and mitochondrial proteins (mito-ribosomal, respiratory chain proteins) at early and intermediate stages of infection, respectively. Cellular responses to infection with the Pan and Mal strain were overall similar. To assess potential differences between permissive and non-permissive infection, we compared protein log 2 fold changes between both viruses directly (Fig. 2d). Interestingly, type I interferon response proteins were first preferentially produced during non-permissive infection. At later stages, however, infection with the Pan virus elicited a stronger interferon response.

Several different hypotheses were made to explain the IAV-induced host shutoff. This included mechanisms at the transcriptional\(^{39}\), post-transcriptional\(^{40,41}\), and translational\(^{42}\) level. To study the relationship of mRNA and protein levels, we quantified mRNA levels at 8 h post infection by RNA-sequencing (RNA-seq). mRNA level differences at this time point showed good correlation with corresponding differences in de novo protein synthesis, particularly during the subsequent 8–12 h period (Fig. 2e–g). Thus, mRNA level changes play an important role for the shutoff of individual mRNAs, corroborating the view that the host shutoff is mainly due to reduced host mRNA levels\(^{41}\). The correlation was higher for the Mal than for the Pan strain, suggesting that infection with the latter strain involves additional post-transcriptional processes.

Traditionally, IAV is thought to prioritize the translation of viral over host mRNAs\(^{42}\), but more recent experimental and computational analyses challenge this view\(^{41,43}\). We investigated this question by calculating protein synthesis efficiencies (i.e., the amount of protein made per mRNA). To this end, we divided iBAQ values by corresponding RPKM (Reads Per Kilobase Million) values (Fig. 2h). Infection with both strains reduced host protein synthesis efficiencies compared to uninfected controls. Importantly, we did not observe preferential translation of viral transcripts. This suggests that mRNAs from human and avian influenza virus strains access the translational machinery with comparable efficiency, which argues against the idea that modulation of translation efficiency affects species specificity.

**Increased M segment RNA splicing.** We next sought to investigate the mechanism for the impaired M1 production. To this end, we first quantified the levels of viral mRNAs from our RNA-seq data. In total, the avian virus produced ~2/3 of the mRNA of the human strain with the single largest difference observed for M1 (Fig. 4a). The strain-specific differences in M1 mRNA levels were very similar to the observed differences in M1 protein production (Fig. 4b). Hence, the impaired M1 protein production during non-permissive infection can largely be explained by reduced M1 mRNA levels.

M1 is encoded on segment 7 (i.e., the M segment), which is the most conserved segment between Pan and Mal (~89% nucleotide identity). The M1 protein is produced from a colinear transcript that can be alternatively spliced into three additional isoforms, which all use a common 3′ splice site\(^{45}\). The M2 mRNA, which encodes the ion channel M212, RNA 3, which is not known to encode a peptide, and M4 mRNA, which is proposed to be translated to an isoform of the M2 ion channel in certain strains\(^{38}\). We investigated the relative proportion of these isoforms to viral origin (Fig. 1d). At this level of detail, we observed no major differences between both strains. Thus, both strains initiate viral protein synthesis and induce the shutoff of host protein synthesis to an overall similar extent.
isoforms in the RNA-seq data via splice junction reads. We detected all known isoforms plus a yet undescribed transcript of the avian M segment, which we call RNA 5. This transcript results from splicing at 5’ donor GG site (pos 520/521) and the common 3’ acceptor site and contains an open reading frame in-frame with M1 with a missing internal region (Fig. 4c).

While only a few percent (2–3%) of the M1 mRNA was alternatively spliced during permissive infection, ~27% was spliced upon infection with the avian strain (Fig. 4d). Thus, the reduced level of M1 mRNA during non-permissive infection is at least partially due to increased splicing of the M1 mRNA to alternative isoforms. In contrast, M2 mRNA levels were rather...
We found that the Mal strain produced considerably more M1 in chicken cells than in human cells (Fig. 4f). Hence, the reduced M1 production of avian viruses in human cells appears to reflect poor adaptation to the mammalian splicing environment.

In principle, the increased splicing of the M segment RNA by bird-adapted strains may reflect an evolutionary adaptation to different needs of the corresponding viral proteins in avian cells. In this case, avian-adapted strains would produce less M1 in both human and avian cells. Alternatively, the optimal balance of viral proteins could be constant and independent of the host species. In this case, avian-adapted strains would produce less M1 in both human and avian cells. Alternatively, the optimal balance of viral proteins could be constant and independent of the host species. In this case, avian-adapted strains would produce more M1 in avian than in human cells. To investigate this experimentally, we infected both human and chicken cells with the Mal virus and monitored the production of viral proteins using pSILAC (Fig. 4e).

We found that the Mal strain produced considerably more M1 in chicken cells than in human cells (Fig. 4f). Hence, the reduced M1 production of avian viruses in human cells appears to reflect poor adaptation to the mammalian splicing environment.

**A cis-regulatory element controls M segment RNA splicing.**

The differences in M1 mRNA splicing can be due to (i) cis-regulatory elements (i.e., specific signals encoded in the M segment), (ii) trans-acting factors (i.e., other viral or host factors that interact with M1 mRNA), or (iii) a combination of both. To assess whether cis-regulatory elements are involved, we sought to investigate M segment RNA splicing outside the context of infection. We therefore designed a splicing reporter system (Fig. 5a, b). To this end, we cloned the coding region of the M segment into a eukaryotic expression vector and fused it to an N-terminal Flag/HA tag. Importantly, this construct avoids the strong 5′ splice site of RNA 3′ and enabled us to assess the relative levels of M1 to M2 proteins. When we transfected human A549 cells with these reporter constructs, we found that M2 was produced to high levels with the construct containing the Mal M sequence, but was barely detectable when the Pan M sequence was transfected (Fig. 5c). Thus, our reporter system recapitulates splicing differences observed during infection. We conclude that cis-regulatory elements in the M segment cause excessive splicing of the avian variant.

**Fig. 3 Viral protein synthesis is dysregulated during non-permissive infection.**

**a** SILAC protein synthesis profiles of human vs. avian-adapted IAV proteins based on shared peptides. No shared peptides were identified for NS1 and M2. The average of n = 2 biological replicates is shown. **b** MS1 spectra of individual precursor (M1—top, HA—middle, NP—lower) peptides in M and H SILAC channels. **c** Cells were infected with the indicated viruses at MOI = 1 (FFU/cell) and analyzed by immunofluorescence microscopy for NP trafficking. Nuclei were counterstained with DAPI. Scale bars: 10 μm. **d** For quantification, NP staining pattern was categorized as predominantly cytosolic, nuclear or both. At least 140 cells were counted per condition.
To determine the sequence responsible for the strain-specific splicing, we made chimeric reporter constructs (Fig. 5b). When swapping the entire intron sequence of the M2 splice variant (nucleotides 52–739, corresponding to ~70% of the coding sequence), we did not observe major changes in the relative amount of M1 to M2. In contrast, integrating the human 3′ splice site region into the avian construct, we observed a strong increase in splicing, similar to the avian wild-type strain. Conversely, when we integrated the human 3′ splice site region into the human construct, we observed strong impaired splicing down to the levels of the avian wild-type construct. Thus, the splice site region alone is sufficient to switch the species-specific splicing phenotype in reporter systems using both viral and host RNA polymerases. Interestingly, this region has been reported to contain an RNA secondary structure and a binding site for the splicing factor SRSF1. We conclude that a cis-regulatory element in the splice site region determines the strain-specific splicing pattern.

Evolutionary conserved RNA structure elements in M segments. We next wanted to assess whether our findings are also relevant for other human- or bird-adapted IAVs. Specifically, we sought to identify functionally relevant RNA secondary structures that have been conserved during evolution of avian- and human-adapted IAVs. To this end, we analyzed multiple sequence alignments (MSAs) from hundreds of recent human H3N2 and avian isolates using the RNA structure prediction program RNA-Decoder. We identify functionally relevant RNA secondary structures that have been conserved during evolution of avian- and human-adapted IAVs. To this end, we analyzed multiple sequence alignments (MSAs) from hundreds of recent human H3N2 and avian isolates using the RNA structure prediction program RNA-Decoder. We conclude that a cis-regulatory element in the splice site region determines the strain-specific splicing pattern.
This program is capable of disentangling overlapping evolutionary constraints due to encoded amino acids and RNA structure features and has been shown to successfully identify evolutionarily conserved RNA structures overlapping protein-coding regions, for example, in viral genomes such as hepatitis C and HIV\textsuperscript{52,53}. Importantly, RNA-Decoder captures evidence on conserved RNA structure based on the evolutionary signals encoded in the sequences of the input alignment. This is a key finding.
advantage over computational methods that identify RNA structures based on their thermodynamic stability in vitro, as these methods assume that the RNA has no interactions with other molecules (e.g., proteins and other RNAs) in vivo. Also, RNA-Decoder employs a probabilistic framework, which is capable of estimating the reliability of its predictions.

The RNA secondary structure that is best supported by the evolutionary signals in the two M5s (the so-called maximum-likelihood structure) markedly differs between human and avian strains, particularly in the region around the 3′ splice site (Fig. 5e): the avian region encodes a hairpin-like structure (Fig. 5f) overlapping the 3′ splice site, which is absent from the human-adapted sequences. This structure is similar to a hairpin reported by Moss et al. for four sequences, but differs in details (Supplementary Fig. 3). Most importantly, the evolutionary conserved structure reported here leaves the GC motif immediately downstream of the AG consensus at the 3′ splice site unpaired, making it potentially more accessible to splicing. In human-adapted sequences, this region is involved in a different conserved secondary structure (Fig. 5e) that is mutually exclusive with this hairpin. We conclude that the M segment of avian and human-adapted isolates contain evolutionarily conserved RNA secondary structures that markedly differ in exactly the region that is critical for strain-specific splicing. Importantly, the consensus sequences of avian and human isolates diverge by five nucleotides in the region of interest (pos: 745, 754, 760, 766, and 772), all of which are retained with the two strains that we investigated here (Supplementary Fig. 4A). Thus, the Pan and Mal strains are by and large representative for human and avian strains in this region.

In addition to these computational analyses, we also wanted to assess RNA secondary structures experimentally. To this end, we in vitro transcribed the region of the M segment containing evolutionarily conserved secondary structure (nucleotides 707–825) from both strains, purified the RNA, and analyzed it on native and denaturing agarose gels (see Fig. 5g). We observed different migration behavior for Pan and Mal under native, but not denaturing conditions, consistent with different secondary structures (lanes 1 and 2). Importantly, when substituting eight nucleotides in the region 707–779 (termed 3′ splice site region in our manuscript) from Mal into the Pan backbone (lane 3), we observed the same migration pattern as for the Mal wild-type RNA. Conversely, when substituting eight nucleotides from the Pan into Mal backbone (lane 4), we observed the same migration pattern as for the Pan wild-type RNA.

To investigate the global significance of these findings, we extended our analyses to other IAV isolates. The M segment of the seasonal Pan strain originates from the M segment of the A/Brevig Mission/1/1918 (p1918) virus, which is at the evolutionary root of human strains and caused the 1918 “Spanish flu” pandemic. Therefore, we cloned the M segment of p1918 into the seasonal Pan strain (lane 5). Collectively, these findings support the global significance of the strain-specific splicing patterns with respect to host range.

The 3′ splice site region is a host range determinant. The experiments with reporter constructs described above are advantageous because they allow us to study the impact of M segment sequence features in isolation. Nevertheless, it is also important to assess the relevance of these findings during infection. We therefore mutated eight nucleotides in the splice site region of the Pan wild-type strain to the corresponding nucleotides in the Mal strain using reverse genetics (“Pan-Av” for a Pan strain with an avian splice site region, see Fig. 6a and Supplementary Data 3).

We first compared the kinetics of viral protein synthesis upon infection of A549 cells with both strains using pSILAC31. M1 synthesis was selectively impaired during Pan-Av infection during both the 6–12 and 12–18 hours post infection (hpi) time intervals. At later stages, the Pan-Av strain also showed impaired production of other essential viral proteins (Fig. 6b), suggesting that viral replication is also impaired. Next, we quantified M1 and M2 protein (Fig. 6c) and mRNA levels (Fig. 6d, e). The Pan-Av strain displayed decreased M1 protein and mRNA levels, mimicking the behavior of the Mal strain (compare to Fig. 4).

To assess the impact of M segment splicing on IAV replication in human cells, we assessed the growth characteristics of the different viruses (Fig. 6f). As expected, the Pan strain reached ~1000-fold higher titers than the Mal strain. Exchanging the entire M segment of the Pan strain with the M segment of the Mal strain (Pan + Mal M) reduced titers about 10-fold. Importantly, a similar ~10-fold attenuation was also seen in the Pan-Av strain, which only differs from the Pan strain by eight nucleotides. We conclude that the 3′ splice site of the IAV M segment is indeed an important host range determinant.

Discussion

Advances in high-throughput sequencing have provided insights into the extraordinary diversity of viruses and their genomic determinants of host adaptation. However, the mechanism how these adaptive mutations enable replication in a given host is less understood. Our proteomic pulse labeling data allowed us to take an unbiased look at protein synthesis upon permissive and non-permissive infection. This is advantageous since it allows us to investigate changes in protein synthesis with high temporal resolution, which provides complementary information to more classical steady-state measurements. We found that the synthesis profiles of host cell proteins were remarkably similar. Hence, the outcome of infection does not appear to depend on a specific host response. In contrast, we observed striking differences in the synthesis profiles of viral proteins. Particularly, the matrix protein M1 was inefficiently produced during non-permissive infection. Our follow-up experiments showed that this depends—at least partially—on excessive splicing of the avian M1 mRNA to alternative transcripts. Systematic computational analysis of the RNA structure of the M segment revealed characteristic and evolutionarily conserved differences in the splicing site regions between human and bird-adapted strains. Exchanging eight nucleotides in the 3′ splice site region from the human-adapted strain to corresponding sequences in the bird-adapted strain markedly impaired replication. Thus, our proteomic analysis of IAV infection identifies M segment splicing as a host range determinant. A hypothetical model for the influence of M segment splicing on the IAV host range is presented in Fig. 7.
The cell culture-based infection model and splicing reporter system employed here are advantageous because they enable experiments under well-controlled conditions. Having said this, it is important to keep in mind that these model systems do not represent the full complexity of IAV infections in vivo. Also, the Pan and Mal strain employed here do not represent the full diversity of human- and bird-adapted IAVs. However, our computational analyses show that differences in RNA secondary structure of the 3′ splice site are widely conserved in human- and bird-adapted IAVs. Moreover, recent results from the Steel lab show that avian M segments restrict growth and transmission of mammalian-adapted IAV strains in a guinea pig model.56

**Fig. 6 The 3′ splice site is a host range determinant.** a (Top) Eight nucleotide polymorphisms of Mal virus at the M segment 3′ splice site were integrated into wild-type Pan virus generating “Pan-Av” mutant. a (Bottom) Design of pulsed SILAC experiment. A549 cells were infected with wild-type or mutant virus at an MOI of 4 (FFU/cell) and viral gene expression was assessed by pulse labeling during two time intervals. b pSILAC-based quantification of viral protein production comparatively for Pan and Pan-Av virus. One replicate (n = 1) is depicted. c Kinetics of M1/M2 protein levels as detected by immunoblotting. d RNA-seq-based quantification of viral mRNA levels at 8 hpi (MOI = 1FFU/cell) comparing Pan and Pan-AV infection. The average of n = 2 biological replicates is depicted. e Quantification of M segment-derived RNAs based on splice junction reads. The area of the pies scales with the total number of splice junction reads. The average of n = 2 biological replicates is depicted. f Multicycle replication curve of the indicated viruses at an MOI of 0.05 FFU/cell on A549 cells. Means and standard deviations of biological triplicates (n = 3) are shown along with significance estimates based on two-sided paired t tests for the 16–48 h time points (n.s.: non-significant, **p < 0.01, ***p < 0.001).
Together, these findings strongly suggest that our results are also relevant outside the specific experimental model system employed here.

Our global assessment of protein de novo synthesis upon infection revealed a global reduction in overall protein output during infection with both strains, probably reflecting a global stress response. Also, we observed the well-known shutoff of host protein synthesis\textsuperscript{38,40,41,57}. Specific classes such as interferon-related, ribosomal and mitochondrial proteins escaped the shut-off. We observed that the amount of protein synthesis upon infection primarily depends on mRNA levels. Thus, altered translation does not play a major role for the host shut-off, consistent with recent findings\textsuperscript{41}. Surprisingly, we also found that viral transcripts were not more efficiently translated than host transcripts. This contrasts with early studies based on reporter systems\textsuperscript{37}, but corroborates recent ribosome profiling data\textsuperscript{41}. Our finding is also consistent with the fact that the codon usage of IAV genes is not optimized to reflect the codon usage of the host\textsuperscript{42}. It is also interesting that the translation efficiency of the bird- and human-adapted strains was similarly poor. Thus, adaptation towards high translational efficiency does not seem to be required for crossing the species barrier.

Our unbiased proteomic analysis indicates that the differences between permissive and non-permissive infection depend on differences in viral rather than host protein synthesis. However, the orchestrated synthesis of the viral proteome appears to be critically important for permissive infection. This supports the emerging view that modulation of viral protein synthesis underpins host adaptation\textsuperscript{58}. Specifically, we find that the strain-specific differences in M1 protein synthesis critically depend on a conserved cis-regulatory element, which controls M-segment mRNA splicing. M1 is particularly important for the nuclear export of the viral genome to the cytoplasm\textsuperscript{10,11}. Consistently, we observed that the genome of the bird-adapted strain was inefficiently exported (Fig. 3c). We were not able to directly assess the role of M1 for nuclear export because the level of ectopically expressed M1 is much lower than the level reached during infection. Interestingly, we also observed differences in HA and NA expression between both strains (Fig. 3a), which may be relevant in this context.

We found that exchanging only eight nucleotides of the human-adapted M segment to the bird-adapted sequences markedly impaired viral replication. Hence, the cis-regulatory element described here plays an important role for host adaptation. However, it is critical to also emphasize that this is not the only relevant factor for IAV host range. For example, despite the overall similar host response, we and others have previously described host factors affecting human and avian virus infections\textsuperscript{15–18,20,27}. It is also well-established that the RdRP of avian-adapted strains is less active in human cells\textsuperscript{14,44}. Moreover, differences in the binding specificity of viral HAs are known to play an important role for host adaptation\textsuperscript{5}. Lastly, M-segment splicing does not only depend on cis-regulatory elements but also on trans-acting factors, such as NS1, RdRP, NS1-BP, or HNRNPK\textsuperscript{49,59–62}. Indeed, while M1 production was clearly impaired in our mutant strain (Fig. 6b), the wild-type bird-adapted strain produced even less (Fig. 3a). Also, the increased production of avian M1 in chicken cells relative to human cells indicates that host factors play a role (Fig. 4f). It is therefore important to interpret our findings in the broader context of viral and host factors that jointly determine the success of IAV replication.

We are living in a pandemic era of IAV infections that began at around 1918\textsuperscript{63}. At this time, a virus of ultimately avian origin acquired the ability to spread among humans and later on contributed its genetic material to other pandemic viruses until present. The M gene of this p1918 virus is in some regions similar to bird-adapted sequences, but shows important signatures of mammalian adaptation, especially at the 3′ splice site region\textsuperscript{64}. Our results suggest that mammalian adaptation at the 3′ splice site was linked to modulating M segment RNA splicing, which may have been relevant for the emergence of the 1918 pandemics in humans.

**Methods**

**Cells and viruses.** A549 (ATCC CCL-185) and HEK293T (ATCC CRL-11268) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, and antibiotics. Madin–Darby canine kidney (MDCK) type II cells (ATCC CRL-2936) were grown in minimal essential medium supplemented with 2 mM l-glutamine and 10% (v/v) fetal bovine serum. DF-1 (ATCC CRL-12203) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 4 mM l-glutamine. All cells were maintained at 37 °C and 5% CO\textsubscript{2}. Stocks of the avian influenza virus A/Mallard/439/2004 (H3N2) (Mal) (GISAID accession numbers EPI859640-EPI859647) were grown in the allantoic cavities of 10-day-old embryonated chicken eggs for 2 days at 37 °C. A/Panama/2007/1999 (H3N2) (Pan) (NCBI accession numbers: DQ487333–DQ487340), Pan + Mal M reassortant and Pan-Av mutant viruses were grown in MDCK type II cells. Virus stocks were titrated on MDCK type II cells by measuring plaque-forming units (PFUs) or fluorescence-forming units (FFUs). For the latter, cells were infected with different dilutions of virus stock for 5 h. Then, cells were harvested by trypsinization, fixed, and permeabilized by incubation in 75% ethanol for at least 12 h at 4 °C and stained with specific antibody against NP antigen (clone AASH, Bio-Rad/Serotec, 1:1000, #MCA4400). An Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (Invitrogen) was...
used as secondary reagent (1:1000). Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva software package.

Cloning and mutagenesis. Gene fragments containing the coding sequence (nucleotides 29–1007) of segment 7 of A/chicken/Rostock/45/1934 (GenBank accession: CY774423), A/Vietnam/1203/2004 (GenBank accession: HM006762.1), A/swine/Netherlands/25/1980 (GenBank accession: Z26862.1), A/BrevigMission/1/1972 (GenBank accession: AY130766, “p19B”), a mutated A/BrevigMission/1/1978 (with the following point mutations: G712A, A714G, G740A, C745T, A725G, G740A, G745T, G754A, G760A, “p19B/ w/ Mal 707–779”), and a mutated version of A/chicken/Rostock/45/1934 (with the following point mutations: G712A, A714G, G740A, C745T, A725G, G740A, G745T, G754A, “A/chicken/Rostock/45/1934 w/ Pan 707–779”) fused to attB1 and attB2 sites by PCR using the following primers:

fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAGCCTTCTAGCTGA
rev: CAAATG

This was cloned using Gateway Technology (Invitrogen) according to the manufacturer’s protocol. Entry clones were generated from pDONR221 vector, expression clones from pDEST26-Flag/HA Destination vector.

All constructs were confirmed by cycle sequencing.

Reverse genetics. Recombinant IAVs derived from the A/Panama/2007/99 backbone were generated using an eight plasmid system for this strain based on pHW2000-Pan M plasmid: A/California/07/1963 (A/CA/63) was used as reference strain.

In vitro transcription. First, the M segment region of interest (707–825) was amplified by PCR using a position 2 subclone wasenson on phCH1807 of human HEK293T cells, followed by passage on MDCK type II cells. For the rescue of the Pan-Mal M reassortant virus, we used pHW2000-Mal M, whereas the Pan-AV virus was generated with pHW2000-Pan-M plasmid.

In vitro transcription (first). The M segment region of interest (707–825) was amplified by PCR using a position 2 subclone wasenson on phCH1807 of human HEK293T cells, followed by passage on MDCK type II cells. For the rescue of the Pan-Mal M reassortant virus, we used pHW2000-Mal M, whereas the Pan-AV virus was generated with pHW2000-Pan-M plasmid:

fw: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAGCCTTCTAGCTGA
rev: CAAATG

This was cloned using Gateway Technology (Invitrogen) according to the manufacturer’s protocol. Entry clones were generated from pDONR221 vector, expression clones from pDEST26-Flag/HA Destination vector.

All constructs were confirmed by cycle sequencing.
Data analysis. Raw files of the pAHA-SILAC were analyzed with MaxQuant software version 1.6.0.1. Default settings were kept, except that "quantify" option was turned on. Label-free quantification via iBAQ calculation was enabled. Lys4/Arg6 and Lys8/Arg10 were set as labels and oxidation of methionines, n-terminal acetylation, and deamidation of asparagine and glutamine residues were defined as variable modifications. The in silico digests of the human Uniprot database (downloaded January 2018), the protein sequences of 12 Pan and Mal Influenza virus proteins and a database containing common contaminants were done with Trypsin/P. The false discovery rate was set to 1% at both the peptide and protein level and was assessed by parallel searching a database containing the reverted sequences from the Uniprot database. The resulting text files were filtered to exclude SILAC protein ratios were averaged. For pSILAC, ratios for viral proteins by site (i.e., protein identifications that are only explained by a modified peptide). Plotting and statistics were done using R and figures were compiled in Illustrator (Adobe). Raw files for pSILAC were analyzed as described above, except that MaxQuant software version 1.5.2.8 was used. For the pSILAC experiment comparing mock infection in human and avian cells, extra were searched against a database additionally containing the uniprot entries of gallsus gallus. For the PSLAC experiment comparing Pan and Pan-AV, quantification option was set to off.

Proteomic data processing. Two MaxQuant output files were used: proteinGroups.txt and evidence.txt. iBAQ values from infected samples were extracted from proteinGroups.txt. iBAQ values were first normalized by scaling to the iBAQ protein median across all mock-infected samples. This assumes that there are no differences in overall protein synthesis for different mock-infected samples. The iBAQ values were averaged for the corresponding label-swap replicates and proteins were categorized as host or viral. For estimating the newly synthesized protein mass, intensity values of H and M SILAC channels were divided by the summed up intensities of the light channel (mock infected). Data were then averaged for label-swap replicates and summed up for viral and host proteins independently. Finally, data were normalized to the 0–4 h time period.

SILAC ratios of host proteins were processed by first transforming them into log2 space. The median SILAC-H/L and SILAC-M/L ratios from the input samples were used to estimate the mixing ratio of the input and the H/L and M/L ratios after the replicate measurements were averaged. Proteins that were quantified in only one replicate were excluded. Later the top 2% of proteins (highest log2 fold-change) of either Pan/Mock or Mal/Mock condition were selected and multi-set GO enrichments were searched using Metascape tool.

Protein level data was matched to RNA level data based on the HGNC official gene symbol. Protein synthesis efficiencies were calculated by subtracting log10(RPKM) from log10(iBAQ) values.

For quantification of pAHA-SILAC viral protein expression kinetics, we extracted all quantitative-iBAQ-level evidences for each individual replicate from evidence.txt. Median log2 ratios were then normalized to 0 for individual replicates. Comparative viral protein expression kinetics were based on Pan/Mal shared peptides. For each time period, replicate, and viral protein, the Pan/Mal SILAC protein ratio was calculated as the median of all SILAC peptide level ratios. Replicate SILAC protein ratios were averaged. For pSILAC, ratios for viral proteins were extracted from proteinGroups.txt. Non-normalized and log2-transformed ratios were used.

RNA-sequencing and data processing. Total RNAs from A549 cells infected with Pan or Mal strain (infection conditions as described in the pAHA-SILAC experiment) were extracted using TRIzol reagent (Life Technologies) following the manufacturer’s protocol. Truseq Stranded mRNA sequencing libraries were prepared with 500 ng total RNA according to the manufacturer’s protocol (Illumina). The libraries were sequenced on HiSeq 2000 platform (Illumina) and yielded in total 186 million 101nt single-end reads. The sequencing reads were first subjected to adapter removal using flexbar with the following parameters: -x -y 5 u -m 28 -ae RIGHT -at 2 -ao 1 n -4 -2 GP5. Reads mapped to the reference sequences of rRNA, tRNA, snRNA, snoRNA and mRNAs (available from Ensembl and RepeatMasker annotation) using Bowtie2 (version 2.1.0) with default parameters in --end-to-end -&– sensitive mode) were excluded. The remaining reads were then mapped to the human and Pan/Mal influenza A reference genome using TopHat (v2.0.10) with parameters: -N 3 -read-gap-length 2 -read-edit-dist 3 –mismatch 2 –library-type fr-firststrand –proper-pairs –no-multiples. Note that each sample is uniquely ungapped, perfectly aligned, and is checked for the existence of secondary structures in the two input alignments (the so-called maximum-likelihood structure). The alignments were processed with Bowtie2 and SAMtools (v1.2.1) as described above. The resulting alignments were then used as input to RNA-Decoder [57]. We used RNA-Decoder to identify the RNA secondary structure that best supported by the evolutionary signals contained in the two input alignments (the so-called maximum-likelihood structure). The predictions by RNA-Decoder also included the posterior base-pairing probabilities for each base pair of the predicted RNA structure. Predicted base pairs with a base-pairing probability smaller than 25% were omitted from the RNA structure. The multiple sequence alignment was generated by RNA-Decoder in one chunk, that is, without partitioning it artificially into sub-alignments.
Finally, the predicted RNA structure element nucleotides 733–766 was plotted with the sequence of the M segment using VARNA tool26 and the RNA structures predicted for the two alignments of avian- and human-adapted sequences was visualized using R-chie69, including information on the pairing probability of each base pair.

Nucleotide-level conservation. To retrieve M segment consensus sequences for human and avian-adapted strains, we used the NIAMD IRD70 through the website at http://www.fluhdb.org (downloaded 10 July 2018). The following options were applied to retrieve avian-adapted consensus sequences: date range: 1999–2011, complete genome, minimum length M segment sequence 1027, and exclude duplicated and laboratory strains. The following options were used to retrieve the human consensus: date range: 1999–2011, complete genome, minimum length M segment sequence 1002, and exclude duplicated and laboratory strains.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Proteomic data relating to the pAHA-SILAC and pSilAC experiments was uploaded to ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013211 (pAHA-SILAC) and PXD015475, PXD015474 (pSilAC) RNA-sequencing data is publicly available under PRJNA45615 [https://www.ncbi.nlm.nih.gov/geo?query=PRJNA45615]. The source data underlyings Figs. 1c, d, 2a–d, h, 3a, 4a, b, e, f, g, 6b–f and Supplementary Figs. 2 and 4a–c are available as Source Data file.

Received: 8 October 2018; Accepted: 12 November 2019; Published online: 04 December 2019.

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Acknowledgements

This work was supported by the German Ministry of Education and Research (Virosign grant 0316180B). We would like to acknowledge the excellent technical assistance of Gudrun Heins. We thank Katrina Meyer for providing the pDEST26-Flag/HA plasmid and Koshi Imami for setting up the nLC system using monolithic columns. We also thank Marina Chekulaeva for in vitro transcription constructs and Sascha Sauer of the MDC Scientific Genomics Platform for RNA-sequencing.

Author contributions

B.B. performed most of the experiments and data analyses. K.E. established the AHA pulse labeling technology and generated pAHA-SILAC data together with B.B. A.S. and K.P. performed the infection experiments for proteomic analysis supervised by T.W. X.W. and J.H. generated and analyzed RNA-seq data supervised by W.C. I.H. performed qRT-PCR analysis supervised by L.W. and B.B. M.H., L.W., and B.V. contributed to cloning and transfection of reporter constructs. I.M.M. performed the RNA structure analyses. M.B. conducted reverse genetic experiments. T.W. and M.S. conceived and supervised the study. B.B., L.W., T.W., and M.S. interpreted the data. B.B. and M.S. wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-13520-8.

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Peek review information *Nature Communications* thanks Aleksandra Nita-Lazar and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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