Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia

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A highly pathogenic avian influenza virus, H5N1, caused disease outbreaks in poultry in China and seven other east Asian countries between late 2003 and early 2004; the same virus was fatal to humans in Thailand and Vietnam[1]. Here we demonstrate a series of genetic reassortment events traceable to the precursor of the H5N1 viruses that caused the initial human outbreak in Hong Kong in 1997 (refs 2–4) and subsequent avian outbreaks in 2001 and 2002 (refs 5, 6). These events gave rise to a dominant H5N1 genotype (*Z*) in chickens and ducks that was responsible for the regional outbreak in 2003–04. Our findings indicate that domestic ducks in southern China had a central role in the generation and maintenance of this virus, and that wild birds may have contributed to the increasingly wide spread of the virus in Asia. Our results suggest that H5N1 viruses with pandemic potential have become endemic in the region and are not easily eradicable. These developments pose a threat to public and veterinary health in the region and potentially the world, and suggest that long-term control measures are required.

The Asian outbreak of highly pathogenic avian influenza H5N1 disease in poultry in 2003 and 2004 was unprecedented in its geographical extent, and its transmission to humans was an ominous sign[1]. To trace the ecological and genetic origins of these outbreaks, we compared H5N1 viruses recently isolated from poultry in Indonesia, Thailand and Vietnam as well as from humans in Thailand and Vietnam with 253 H5N1 isolates obtained during prospective surveillance of live poultry markets in Hong Kong and in Guangdong, Hunan and Yunnan provinces, China, from 2000 to 2004 (Fig. 1). Results of this surveillance are summarized in Table 1. Since 2001, H5N1 viruses have continued to circulate in mainland China with a seasonal pattern, peaking from October to March, when the mean temperature is below 20 °C (Fig. 2). The survival and viability of influenza A virus are known to increase at lower environmental temperatures[7]. H5N1 viruses were isolated exclusively from aquatic poultry during 2000; however, from 2001 onwards they were isolated from both aquatic and terrestrial poultry, although the rate of isolation remained greatest in ducks.

The genes of the virus isolates that encode the surface antigens haemagglutinin (HA) and neuraminidase (NA) were derived from the Goose/Guangdong/1/96 (*Gs/Gd*)-like lineage. Six genes, which encode internal viral proteins, arose from many other sources through reassortment and served as the basis for assignment to different genotypes (Fig. 3). By 2001, six H5N1 reassortants (genotypes *A*, *B*, *C*, *D*, *E* and X_0) had been isolated from aquatic and, for the first time since 1997, terrestrial poultry[4,5]. From 2002 onwards, eight new H5N1 genotypes (*V*, *W*, X_1, X_2, X_3, *Y*, *Z* and Z^+) were detected. Genotypes *A*, *C*, *D* and *E* and their common precursor *Gs/Gd* were no longer found, suggesting that later genotypes had acquired a survival advantage by means of adaptation. At least nine genotypes of H5N1 viruses continued to circulate in southern China in 2002. All genotypes, except for *Gs/Gd* and X_0–X_3, had a five-amino-acid deletion (position 80–84) in the NS1 protein. Similarly, viruses isolated in 2002 and later, except for genotypes *B*, *W* and Z^+, had a 20-amino-acid deletion in the stalk of the NA molecule (position 49–68). Deletion in the NA stalk may be associated with adaptation of influenza viruses to land-based poultry[8].

Since January 2002, genotype *Z*, which contains both the NA and NS1 deletions, has become the dominant H5N1 virus in southern China (Table 1 and Fig. 3). In February 2003, human H5N1 disease was diagnosed for the first time since December 1997. The human isolates (A/HK/212/03 and A/HK/213/03) had the same gene constellation as genotype *Z* but lacked the NA stalk deletion, and were designated genotype Z^+ (Fig. 3)[6]. Sixty-two H5N1 virus isolates from 2003 were genetically sequenced and 60 of them belonged to genotype *Z* (Table 1). All of the viruses that caused outbreaks in Indonesia, Thailand and Vietnam in late 2003 and early 2004 (refs 1, 9) were genotype *Z* viruses (Fig. 4).

Although all viruses tested derived their HA genes from *Gs/Gd*-like viruses, the *Z* and Z^+ genotypes, as well as the single known isolate of genotype *V* (Ck/ST/4231/03), formed a distinct sublineage (2002/04-*V*, *Z*, Z^+; 81% bootstrap support) that included viruses from Hong Kong; Guangdong, Hunan and Yunnan provinces of mainland China; Indonesia; Thailand; and Vietnam (Fig. 4a). Within this sublineage, viruses isolated from humans and poultry in Thailand and Vietnam formed a separate group (96% bootstrap) most closely related to H5N1 viruses isolated from poultry and wild birds in Hong Kong (genotypes *Z* and Z^+). Viruses isolated in Indonesia formed a separate group related to those isolated in Yunnan (Fig. 4a).

Phylogenetic relationships of the other seven gene segments were broadly consistent with those of the HA gene (Fig. 4b; see also Supplementary Figs 1 and 2). The *M* and *NS* genes were closest to those of influenza viruses of diverse subtypes isolated from ducks in southern China, suggesting that aquatic avian viruses were the gene

Figure 1 Map of China showing Hong Kong and Guangdong, Hunan and Yunnan provinces, where influenza surveillance was conducted.

donors for these new reassortants (Fig. 4b; see also Supplementary Fig. 2). Bi-directional transmission of influenza virus between terrestrial and aquatic poultry gave rise to new H9N2 reassortants[10], and a similar mechanism may generate novel H5N1 reassortants.

It is notable that in the short time since its emergence in 2002, genotype *Z* has replaced genotypes *A*–*E*, *X* and *Y* to become dominant in both aquatic and terrestrial poultry in this region (Table 1). To define the genetic stability of this new gene constellation, we analysed the rates of non-synonymous (K_a) and synonymous (K_s) nucleotide substitutions in six internal gene segments of genotype *Z* viruses isolated in 2002–04. A K_a/K_s ratio >1 suggests evidence of positive natural selection[11]. Of these internal genes, the *M2* gene was under positive selection pressure in late 2002 to early 2003 but under less selection pressure in late 2003 to early 2004. The *NS1* and *NS2* genes, acquired in late 2000, were also under positive selection pressure (Supplementary Table 1). These findings suggest that the gene constellation of genotype *Z* viruses has not yet fully adapted to poultry, and this raises the possibility that they may continue to evolve through mutation or reassortment to achieve greater viral fitness[12].

The presence of residue Asp 31 in the M2 protein invariably confers resistance to the amantadines[13], a group of antiviral drugs used for treatment of human influenza. Sequence analysis revealed that Asp 31 was present in all avian and human genotype *Z* viruses isolated in Thailand and Vietnam, but in only one of six viruses isolated from poultry in Indonesia. Asp 31 was also observed in some genotype *B*, *Y* and Z^+ viruses. The distribution of this mutation in different virus genotypes suggests that it was independently acquired rather than having descended from a single lineage of amantadine-resistant *M2* genes (Fig. 4b).

Mutations Ser64Ala and Glu66Ala in the M2 protein were also observed in genotype *Z* and Z^+ viruses from highly pathogenic avian influenza H5N1 outbreaks in Kowloon and Penfold parks in Hong Kong in December 2002, from viruses isolated from humans in Hong Kong in February 2003, and from viruses isolated in Thailand and Vietnam in 2003–04. The remaining genotype *Z* viruses, including those isolated from Indonesia and mainland China in 2003–04, maintained Ser 64 in the M2 protein. Residue Ser 64 is the predominant site of post-translational phosphorylation in the M2 protein[14,15]. The mutation Ser64Ala induces no significant change in ion-channel activity[14], and phosphorylation of the cytoplasmic tail does not affect intracellular transport of M2 protein or viral assembly[15]. Therefore, the biological role of phosphorylation at position 64 of M2 is still unclear. The biological significance of residue 66 of the M2 ion channel has not been reported.

The molecular determinants of H5N1 transmission to humans in Vietnam and Thailand in 2004 are unclear. In H5N1 viruses recently isolated from humans and poultry in Thailand and Vietnam, amino acid residues at the receptor-binding pocket of HA1—that is, positions Gln 222 and Gly 224 (positions 226 and 228 for H3 influenza numbering)—retain configurations (2,3-NeuAcGal linkages) predicted to have affinity for avian cell-surface receptors[16]. The substitution Ser227Asn, identified in viruses isolated from two patients with H5N1 influenza after visiting Fujian Province, China, in 2003 (ref. 6), was not seen in any other H5N1 viruses. Other amino acid residues relevant to receptor binding (residues 91, 130–134, 149, 151, 179, 186, 190–191, 220–225) were identical to

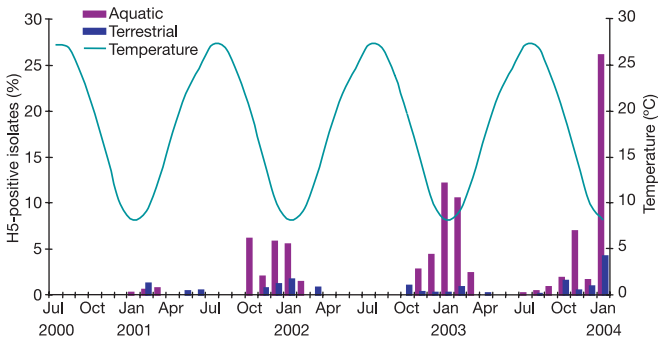

Figure 2 Seasonality of the isolation of avian H5N1 viruses from domestic poultry in mainland China during July 2000 to January 2004 (see Table 1). The mean monthly temperature in southern China (approximated from the monthly average temperatures of the cities Changsha, Kunming and Xiamen) is shown for reference.

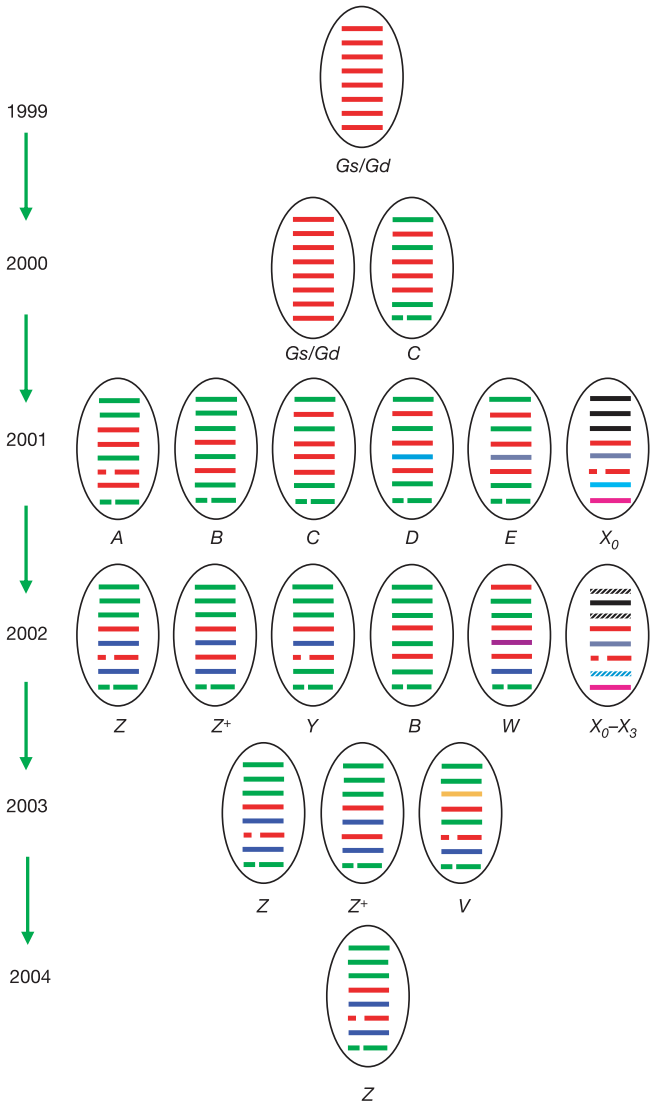

Figure 3 The genotypes of H5N1 influenza virus reassortants from eastern Asia. The eight gene segments are (horizontal bars starting at the top downwards): *PB2*, *PB1*, *PA*, *HA*, *NP*, *NA*, *M* and *NS*. Each colour represents a virus lineage (red indicates origin from Gs/Gd/1/96). Genotypes (indicated by letters) were defined by gene phylogeny: a distinct phylogenetic lineage with bootstrap support ≥70% (≥50% for *M*, *NP* and *PA* genes) indicated a common origin. Genotypes *A*, *B* and *C* were reassortants of Gs/Gd/1/96 and one or more aquatic avian viruses. Genotype *D* was created when the *NP* gene of genotype *C* was replaced by that of a Dk/HK/Y280/97-like virus (H9N2 subtype). Genotype *E* was created when the *NP* gene of genotype *C* was replaced by that of another avian virus. Further reassortment of genotype *E* with other aquatic avian influenza viruses gave rise to the genotypes X_0–X_3, distinguished by the sources of their *PB2*, *PA* and *NS* genes. Genotype *W* differs from genotype *B* only in its *PB2*, *NP* and *M* genes. Further reassortment of genotype *A* or *B* with other aquatic avian viruses gave rise to genotypes *V*, *Y*, *Z* and Z^+. Alternatively, genotype *V* may have resulted from reassortment of genotype *Z* with other aquatic avian viruses.

those of A/HK/156/97 and *Gs/Gd*-like viruses[3], with the exception of A/Vietnam/3046/04, which had an Ala134Val mutation.

The HA molecules of most genotype *Z* viruses isolated since late 2002 in Hong Kong, of two out of six isolates from Indonesia, and of all isolates from Thailand, Vietnam and Yunnan Province in late 2003 and early 2004 had acquired a potential *N*-linked glycosylation site at positions 154–156. Glycosylation at this site, adjacent to the receptor-binding[3] and antigenic sites[17] at the globular tip of the H5 influenza HA molecule (Supplementary Fig. 3), is capable of altering the receptor-binding profile[18] and may help the virus to evade the host antibody response.

Lys 627 in the PB2 protein has been associated with increased virulence of H5N1 viruses in mice[19] and of H7N7 viruses in humans[20]. Three out of four H5N1 human virus isolates from Vietnam had this mutation, but the human virus from Thailand did not, nor did any other avian influenza viruses tested. No recent H5N1 viruses characterized in this study had Glu 92 in the NS1 protein, which is reportedly associated with increased virulence in pigs[21].

The apparently simultaneous occurrence of H5N1 outbreaks across eastern Asia remains unexplained, but the presence of H5N1 viruses in dead migratory birds suggests that wild bird populations may be involved. In Hong Kong between late 2002 and the time of this report, genotype Z^+ H5N1 virus was isolated from a dead little egret (*Egretta garzetta*), and genotype *Z* viruses were isolated from two dead grey herons (*Ardea cinerea*), a black-headed gull (*Larus ridibundus*), a feral pigeon (*Columba livia*), a tree sparrow (*Passer montanus*) and a peregrine falcon (*Falco peregrinus*). In the gene phylogenies, the H5N1 viruses isolated from wild birds have either an out-group or sister-group relation to recent Thailand and Vietnam H5N1 isolates (Fig. 4; see also Supplementary Figs 1 and 2). The timing and distribution of the H5N1 infection in poultry in China from 2001 onwards (Fig. 2) coincides with the general period of winter bird migration to southern China; however, it is not known whether the H5N1 virus has become established in wild bird populations. The potential role of wild birds in the maintenance and spread of H5N1 viruses must be considered in strategies for regional control.

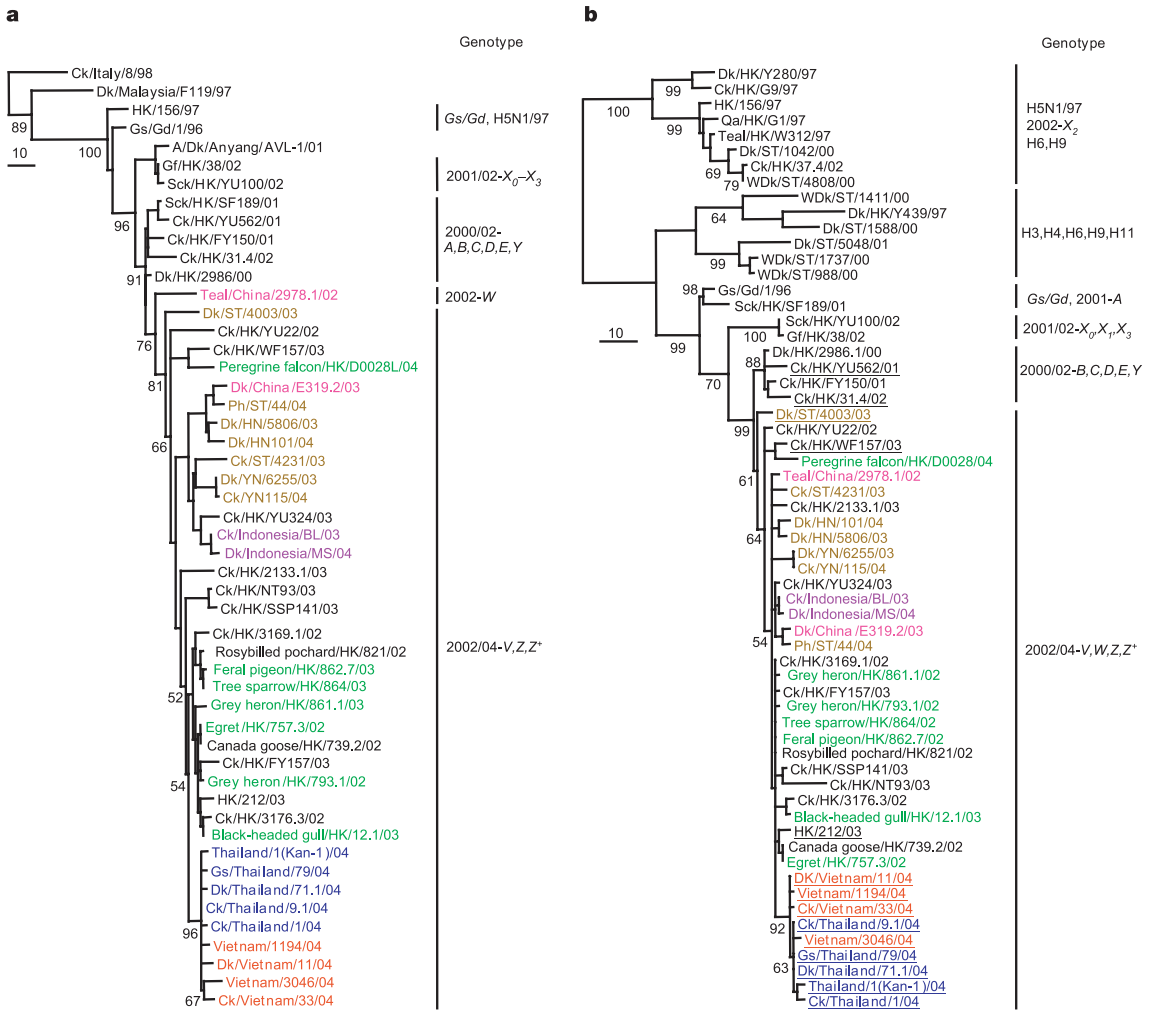

Figure 4 Phylogenetic relationships of the haemagglutinin (**a**) and matrix protein (**b**) genes of representative influenza A viruses isolated in southeastern Asia, including 2 of 6 from Indonesia, 5 of 8 from Thailand and 4 of 12 from Vietnam. Trees were generated by using maximum parsimony in the PAUP* program[25] (neighbour-joining analysis with the Tamura–Nei γ-model, implemented in the MEGA program[26], revealed the same relationships). Numbers below branches indicate bootstrap values from 1,000 replicates. Only bootstrap values that define important groups have been included owing to space constraints. Analysis was based on nucleotides 1–1012 (1,012 bp) of the *HA* gene and 90–945 (856 bp) of the *M* gene. The HA tree was rooted to A/tern/South Africa/61 and the M tree to A/equine/Prague/1/56. Scale bar, 10 nucleotide changes. Green text indicates viruses isolated from wild birds in Hong Kong; pink text indicates viruses from smuggled birds in China; and other colours show the country of origin of isolates from the late 2003 to early 2004 H5N1 outbreak. Underlined viruses have the amantadine-resistance mutation (Ser31Asn) in the M2 ion channel. Ck, chicken; Dk, duck; Gd, Guangdong; Gf, Guinea fowl; Gs, goose, HK; Hong Kong; HN, Hunan; Qa, quail; SCk, silky chicken; ST, Shantou; WDk, wild duck; YN, Yunnan.

Table 1 **Analysis of H5N1 influenza viruses isolated from poultry in Hong Kong and mainland China during 2000–04**

	2000		2001		2002		2003		2004	
	Aquatic	Terrestrial	Aquatic	Terrestrial	Aquatic	Terrestrial	Aquatic	Terrestrial	Aquatic	Terrestrial
Sampling										
Hong Kong										
H5N1-positive/total tested	33/533	0/8,256	37/606	36/16,116	14/578	323/14,691	4/3,694	112/12,146	1/756	0/1,807
Non-H5N1 isolates	16	715	11	983	10	369	12	288	0	20
Mainland China										
H5N1-positive/ total tested	0/445	0/1,891	38/2,579	10/3,197	73/4,539	8/4,059	297/7,209	50/10,308	152/1152	26/1,673
Non-H5N1 isolates	122	143	468	290	507	297	301	361	22	75
Total number sampled	11,125		22,498		23,867		33,357		5,388	
Genetic analysis										
Hong Kong										
Number of viruses analysed (genotypes detected)	11 (9*Gs/Gd*; 2*C*)	–	13 (4*B*, 9*C*)	24 (13*A*, 4*B*, 4*C*, 1*D*, 2*E*)	12 (1*B*, 8*Z*, 3Z^+)	100 (7*B*, 8X_0, 2X_1, 3X_2, 1X_3, 10*Y*, 69*Z*)	4 (4*Z*)	38 (37*Z*, 1Z^+)	1 (1*Z*)	–
Mainland China										
Number of viruses analysed (genotypes detected)	–	–	7 (2*B*, 5X_0)	1 (1X_0)	10 (3*B*, 5*Z*, 2*W*)	7 (7*Z*)	11 (11*Z*)	9 (8*Z*, 1*V*)	3 (3*Z*)	2 (2*Z*)
Number of genotypes	2		6		9		3		1	

Faecal droppings from apparently healthy poultry in live poultry markets in Hong Kong (2000–04), Guangdong (2000–04) and Hunan and Yunnan (2002–04) provinces were sampled monthly for influenza virus isolation. For each month that H5N1 virus was identified, one isolate was selected from each type of infected poultry for sequencing. During H5N1 disease outbreaks, additional isolates were sequenced. Of a total of 96,235 samples, 253 H5N1 virus isolates were genetically sequenced and analysed.

H5N1 virus is now endemic in poultry in Asia (Table 1) and has gained an entrenched ecological niche from which to present a long-term pandemic threat to humans. At present, these viruses are poorly transmitted from poultry to humans, and there is no conclusive evidence of human-to-human transmission. However, continued, extensive exposure of the human population to H5N1 viruses increases the likelihood that the viruses will acquire the necessary characteristics for efficient human-to-human transmission through genetic mutation or reassortment with a prevailing human influenza A virus. Furthermore, contemporary human H3N2 influenza viruses are now endemic in pigs in southern China[22] and can reassort with avian H5N1 viruses in this 'intermediate host'. Therefore, it is imperative that outbreaks of H5N1 disease in poultry in Asia are rapidly and sustainably controlled. The seasonality of the disease in poultry, together with the control measures already implemented, are likely to reduce temporarily the frequency of H5N1 influenza outbreaks and the probability of human infection. However, complacency would be unwise. Governments in the region face an endemic and recurrent problem that presents a serious threat to human health. Although other countries in the region have been affected, Hong Kong has remained remarkably free of H5N1 outbreaks in poultry in 2004, thanks to preventive measures implemented over the past few years[23]. □

Methods

Surveillance, virus isolation and characterization

Faecal droppings from apparently healthy poultry in live poultry markets in Hong Kong and in Guangdong, Hunan and Yunnan provinces were sampled monthly for influenza virus isolation. Methods used for virus isolation and characterization have been previously described[24]. In addition to systematic surveillance in the poultry markets, sick or dead poultry from markets and farms in Hong Kong were similarly studied. This analysis included representative H5N1 viruses isolated from birds in Vietnam ($n = 8$), Thailand ($n = 7$) and Indonesia ($n = 6$), and from humans in Vietnam ($n = 4$) and Thailand ($n = 1$) during the 2003–04 H5N1 outbreak.

Genetic analysis

All sequences were edited with the Staden software package and aligned with ClustalX. Phylogenetic trees were generated using PAUP* version 4.0 (ref. 25) and MEGA version 2.1 (ref. 26). K_a/K_s analysis was conducted by the Pamilo–Bianchi–Li method as implemented in MEGA.

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Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf

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Specific adaptors regulate the activation of initiator caspases; for example, FADD and Apaf-1 engage caspases 8 and 9, respectively[1]. The adaptors ASC, Ipaf and RIP2 have each been proposed to regulate caspase-1 (also called interleukin (IL)-1 converting enzyme), which is activated within the 'inflammasome', a complex comprising several adaptors[2]. Here we show the impact of ASC-, Ipaf- or RIP2-deficiency on inflammasome function. ASC was essential for extracellular ATP-driven activation of caspase-1 in toll-like receptor (TLR)-stimulated macrophages. Accordingly, ASC-deficient macrophages exhibited defective maturation of IL-1β and IL-18, and ASC-null mice were resistant to lipopolysaccharide-induced endotoxic shock. Furthermore, activation of caspase-1 in response to an intracellular pathogen (*Salmonella typhimurium*) was abrogated severely in ASC-null macrophages. Unexpectedly, Ipaf-deficient macrophages activated caspase-1 in response to TLR plus ATP stimulation but not *S. typhimurium*. Caspase-1 activation was not compromised by loss of RIP2. These data show that whereas ASC is key to caspase-1 activation within the inflammasome, Ipaf provides a special conduit to the inflammasome for signals triggered by intracellular pathogens. Notably, cell death triggered by stimuli that engage caspase-1 was ablated in macrophages lacking either ASC or Ipaf, suggesting a coupling between the inflammatory and cell death pathways.

Excessive levels of the proinflammatory cytokines IL-1β and IL-18 are associated with septic shock and autoimmune syndromes[3]. Activated monocytes and macrophages use caspase-1 to cleave pro-IL-1β and pro-IL-18 to the mature cytokines. Recent studies have suggested that caspase-1 is activated within a multiple adaptor complex termed the inflammasome[4]. ASC (apoptosis-associated speck-like protein containing a CARD)[5], also known as Pycard[4] or TMS1 (ref. 6), is one putative component of the inflammasome. A caspase activation and recruitment domain (CARD) within ASC binds to the caspase-1 prodomain, and overexpression studies have reported that ASC both promotes and inhibits activation of caspase-1 (refs 4, 7–9). To establish whether ASC is a positive or negative regulator of caspase-1 in a physiological setting, we generated ASC-deficient mice by gene targeting (Supplementary Fig. S1).

We investigated the role of ASC in caspase-1-dependent IL-1β release *ex vivo* using thioglycollate-elicited peritoneal macrophages. Synthesis, processing and release of mature IL-1β by macrophages *ex vivo* seem to require two distinct stimuli. An inflammatory stimulus such as lipopolysaccharide (LPS) primes cells to transcribe and synthesize pro-IL-1β, and then a second stimulus—such as ATP,

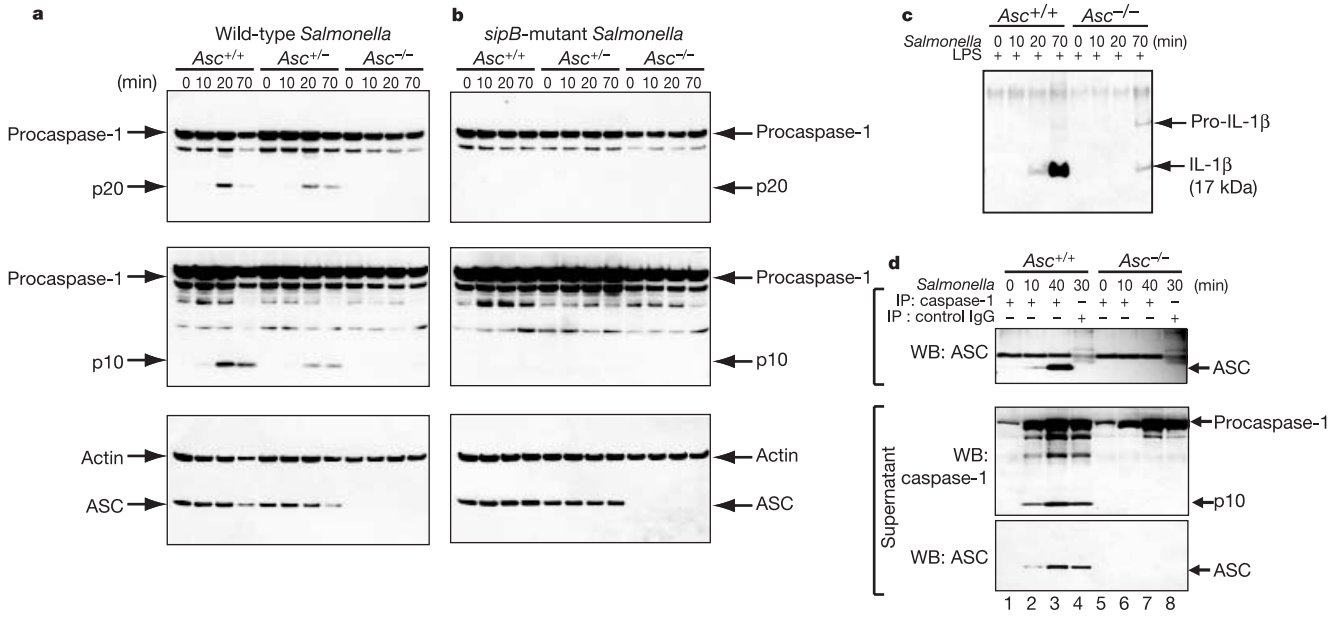
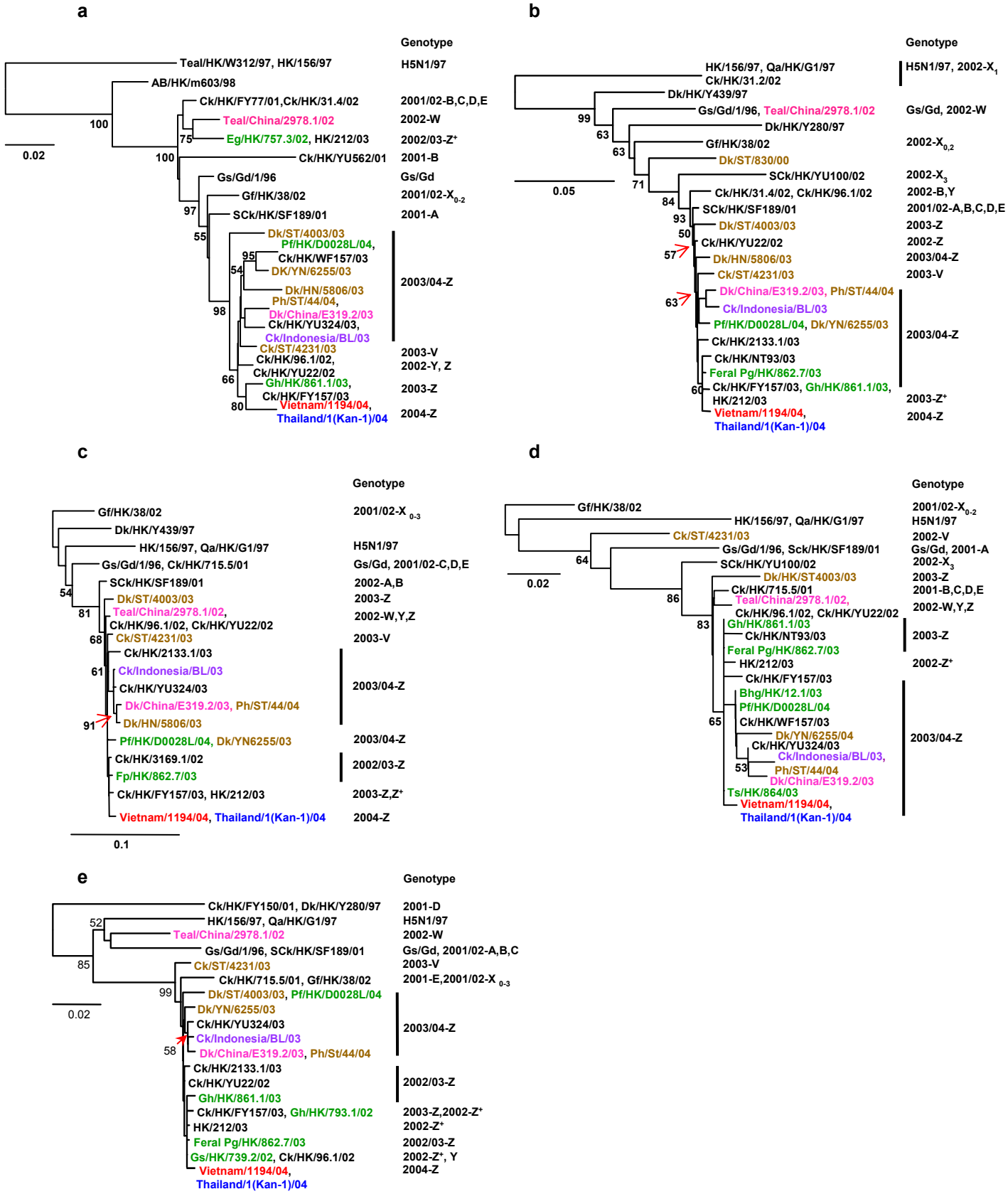
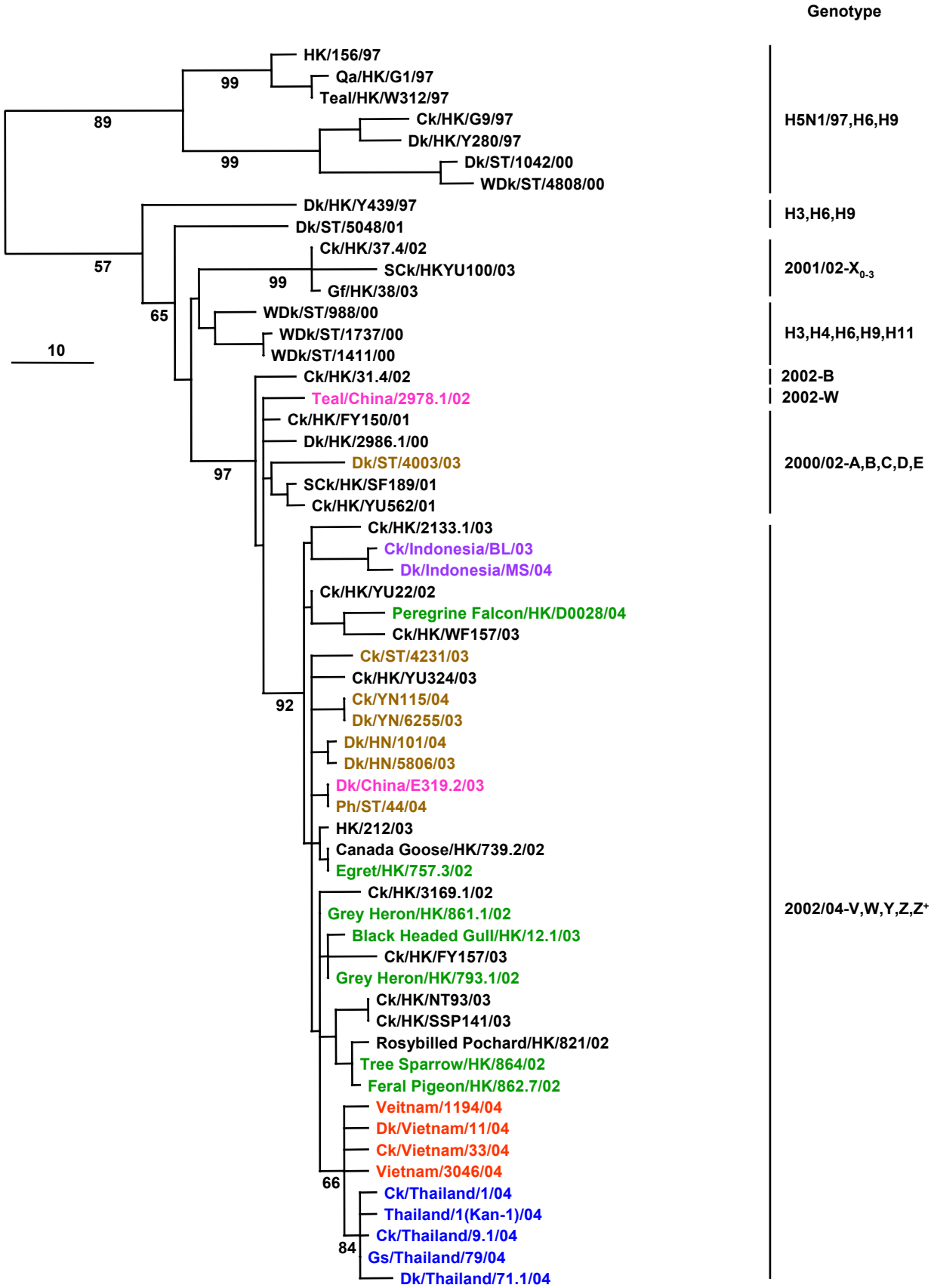


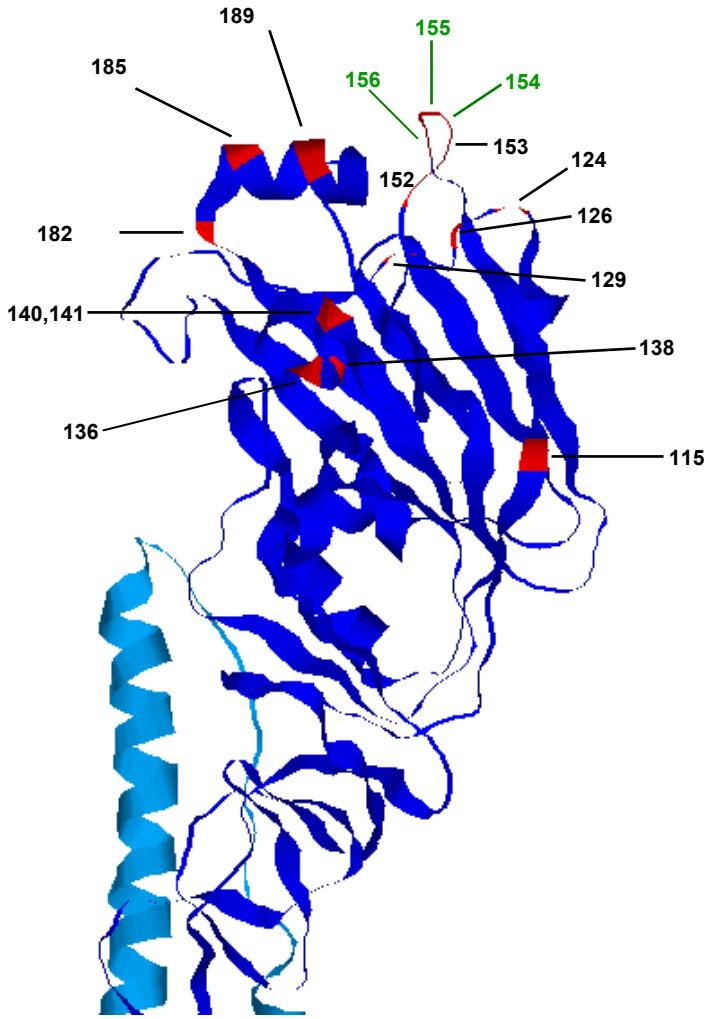
Figure 1 ASC is essential for *S. typhimurium*-induced caspase-1 activation. LPS-stimulated macrophages were infected with either wild-type SL1344 (**a**, **c**, **d**) or a *sipB*-mutant *S. typhimurium* (**b**) for the times indicated. **a**, **b**, Cell lysates were immunoblotted with antibodies against the p10 and p20 subunits of caspase-1, ASC and actin. **c**, Pro-IL-1β and mature IL-1β in culture supernatants were detected by immunoprecipitation and western blotting. **d**, Caspase-1 present in culture supernatants was immunoprecipitated with antibodies to the caspase-1 p10 subunit and co-precipitating ASC was detected by western blotting.



Supplementary Figure 1 Phylogenetic relationships of **a**, NA (pos 1-1100), **b**, PB2 (1055-2110), **c**, PB1 (1-1232), **d**, PA (1410-2127) and **e**, NP (1-980) genes. Neighbour-joining (NJ) analysis conducted using the Tamura-Nei (gamma) model, as implemented in MEGA2, revealed the same relationships as the parsimony analysis. Trees were originally constructed from larger datasets and branches were then collapsed using the Compress/Expand Subtree tool in MEGA2[16]. Each terminal branch was then labeled with a single representative virus from the collapsed group. Numbers at nodes indicate bootstrap values from 1000 replicates. NA gene tree rooted to A/Parrot/Ulster/73, PB2 and NP to A/Equine/Prague/1/56, PB1 to B/Lee/40 and PA to A/Ann Arbor/6/60. Scale bars represent genetic distance. Abbreviations used: Bhg = Black headed gull, Ck = Chicken, Dk = Duck, Feral Pg = Feral pigeon, Gd = Guangdong Gf = Guinea fowl, Gh = Grey heron, Gs = Goose, HK = Hong Kong, HN = Hunan, Pf = Peregrine falcon, SCk = Silky chicken, ST = Shantou, Ts = tree sparrow, YN = Yunnan. Green text indicates viruses isolated from wild birds in Hong Kong, while pink text indicates viruses from smuggled birds from China. Other text colours highlight the country of origin of virus isolates from the H5N1 outbreak that occurred in late 2003 to early 2004.



Supplementary Figure 2 Phylogenetic relationships of influenza A virus non-structural (NS1) gene from East Asia. Tree was generated using maximum parsimony in PAUP. Numbers below branches indicate bootstrap values from 1000 replicates. Neighbour-joining (NJ) analysis conducted using the Tamura-Nei (gamma) model, as implemented in MEGA2[16], revealed the same relationships as parsimony analysis. Nucleotides 1-689 (689 bp) were used in the analysis. The tree was rooted to A/Gs/Gd/1/96. Scale bar represents 10 nucleotide changes. Abbreviations used: Ck = Chicken, Dk = Duck, Gd = Guangdong, Gf = Guinea fowl, Gs = Goose, HK = Hong Kong, HN = Hunan, Qa = Quail, SCk = Silky chicken, ST = Shantou, WDk = Wild duck, YN = Yunnan. Green text indicates viruses isolated from wild birds in Hong Kong, while pink text indicates viruses from smuggled birds from China. Other text colours highlight the country of origin of virus isolates from the H5N1 outbreak that occurred in late 2003 to early 2004.



Supplementary Figure 3 Location of an additional potential glycosylation site (amino acid residue positions 154-156) and its relationship to H5 escape mutant positions. Amino acid residue positions have been imposed upon the 3D structure of hemagluttinin from the Protein Data Bank (1JSM). The dark blue ribbons represent the HA1 chain and the light blue represents the HA2 chain of the molecule. RasMol v2.7.2.1 was used to visualize the molecule.

Supplementary Table 1 **Rates of non-synonymous (Ka) and synonymous (Ks) nucleotide substitution in genotype Z viruses**

	Late 2002/Early 2003						**Late 2003/Realy 2004**					
	Aquatic			**Terrestrial**			**Aquatic**			**Terrestrial**		
	Ka	**Ks**	**Ka/Ks**	**Ka**	**Ks**	**Ka/Ks**	**Ka**	**Ks**	**Ka/Ks**	**Ka**	**Ks**	**Ka/Ks**
M1	0.000	0.016	0.000	0.000	0.005	0.000	0.000	0.026	0.000	0.000	0.010	0.000
M2	0.005	0.000	**>1**	0.008	0.000	**>1**	0.012	0.022	0.557	0.000	0.004	0.000
NP	0.001	0.028	0.035	0.002	0.010	0.181	0.001	0.027	0.024	0.0000	0.004	0.000
NS1	0.003	0.009	0.267	0.003	0.000	**>1**	0.016	0.017	0.925	0.008	0.002	**4.75**
NS2	0.004	0.006	0.715	0.003	0.000	**>1**	0.016	0.019	0.851	0.004	0.000	**>1**
PA	0.003	0.023	0.121	0.002	0.017	0.017	0.003	0.065	0.045	0.002	0.005	0.315
PB1	0.003	0.034	0.091	0.001	0.015	0.015	0.003	0.031	0.103	0.000	0.015	0.000
PB2	0.002	0.024	0.090	0.002	0.012	0.191	0.005	0.038	0.125	0.001	0.008	0.078

Ka and Ks values were calculated using the Pamillo-Bianchi-Lee method in MEGA[26]. Pair-wise comparisons of the sequences of five viruses from each group were analysed with the mean value is presented above. Nucleotides analysed for each gene were: M1, M2, NS1, NS2: complete gene; NP: 1-968, PA: 1405-2150, PB1: 1-1434, PB2: 940-2084.