In vivo evasion of MxA by avian influenza viruses requires human signature in the viral nucleoprotein

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Zoonotic transmission of influenza A viruses can give rise to devastating pandemics, but currently it is impossible to predict the pandemic potential of circulating avian influenza viruses. Here, we describe a new mouse model suitable for such risk assessment, based on the observation that the innate restriction factor MxA represents an effective species barrier that must be overcome by zoonotic viruses. Our mouse lacks functional endogenous *Mx* genes but instead carries the human *MX1* locus as a transgene. Such transgenic mice were largely resistant to highly pathogenic avian H5 and H7 influenza A viruses, but were almost as susceptible to infection with influenza viruses of human origin as nontransgenic littermates. Influenza A viruses that successfully established stable lineages in humans have acquired adaptive mutations which allow partial MxA escape. Accordingly, an engineered avian H7N7 influenza virus carrying a nucleoprotein with signature mutations typically found in human virus isolates was more virulent in transgenic mice than parental virus, demonstrating that a few amino acid changes in the viral target protein can mediate escape from MxA restriction in vivo. Similar mutations probably need to be acquired by emerging influenza A viruses before they can spread in the human population.

INTRODUCTION

Influenza A viruses pose a constant threat to humans because zoonotic viral transmissions can cause severe disease and give rise to devastating pandemics (Watanabe et al., 2014; Yoon et al., 2014; Neumann and Kawaoka, 2015). Innate restriction factors represent effective species barriers that need to be overcome by viruses to invade the human host (Cauldwell et al., 2014; Iwasaki and Pillai, 2014). In mice, innate immune control of influenza viruses is mediated by the IFN-regulated *Mx1* gene (Haller et al., 2015). In man, the *MX1* ortholog (encoding MxA protein) may play a similar role, as it provides broad resistance to influenza and other viruses in cell culture systems (Pavlovic et al., 1990; Haller et al., 2015). However, formal genetic proof that MxA helps protect humans from influenza virus–induced disease has so far been missing, as none of the known minor *MX1* gene variants has to date been linked to enhanced influenza virus susceptibility (Ciancanelli et al., 2016).

MxA belongs to the dynamin superfamily of multidomain large GTPases, and the MxA coding sequence is highly conserved in humans and other primates (Mitchell et al., 2013; Haller et al., 2015). Sequence comparison revealed that loop L4 is the most divergent part of the otherwise conserved molecule, and mutational analysis showed that the L4

domain acts as an autonomous antiviral module that determines the antiviral specificities in different species (Mitchell et al., 2012; Patzina et al., 2014). L4 binds to the influenza viral nucleoprotein (Patzina et al., 2014), which leads to viral inhibition by a yet to be defined mechanism that requires GTP hydrolysis and oligomerization of MxA (Mitchell et al., 2013; Haller et al., 2015; Nigg and Pavlovic, 2015).

When major restriction factors are at work, viruses usually evolve to acquire mutations that help them to evade such host defense strategies (Simon et al., 2015). Recent studies suggest that MxA is targeting the nucleoprotein of influenza A viruses which encapsidates the viral genome, a process that is pivotal to virus replication (Dittmann et al., 2008; Zimmermann et al., 2011). Indeed, more recent studies indicate that influenza A viruses that successfully established stable lineages in humans acquired adaptive mutations in the nucleoprotein that allow partial MxA escape (Mänz et al., 2013; Götz et al., 2016). It remained unclear, however, whether these cell culture experiments accurately reflect the in vivo situation.

A suitable animal model is required to answer this question. Early attempts to constitutively express MxA cDNA in mice yielded disappointing results, as the transgene was not expressed well in the lungs (Pavlovic et al., 1995). We now generated a new mouse line that carries the complete
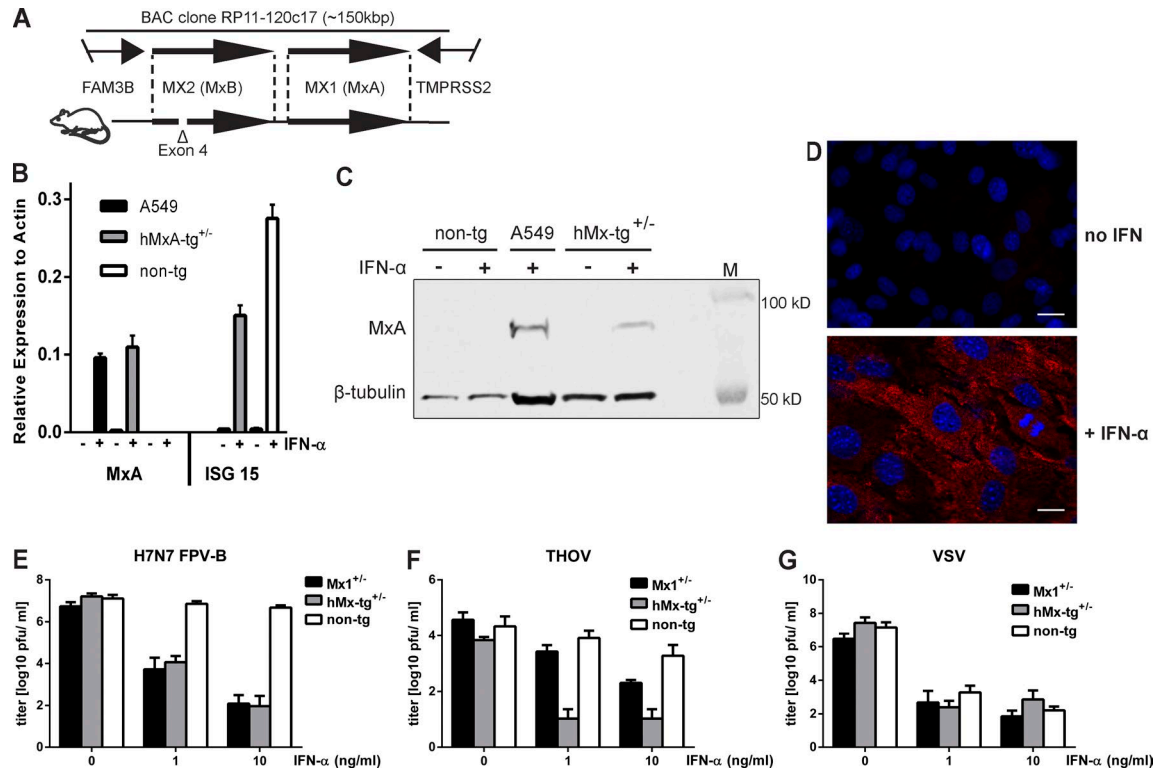
Figure 1. **Cells from transgenic mice carrying the human *MX* locus exhibit IFN-dependent resistance to influenza A virus and THOV.** (A) Schematic drawing showing the fragment of human chromosome 21 present in BAC clone Rp11-120c17 (top) and transgenic mice (bottom). Note that the *MX2* gene is defective in our transgenic mice as a result of a deletion that includes coding exon 4. (B and C) MEFs prepared from transgenic (hMx-tg$^{+/-}$) or nontransgenic (non-tg) littermates were treated with 10 ng per ml of IFN-α for 18 h (+) or were left untreated (–) before analysis of the cellular MxA content by RT-qPCR (B) or Western blot (C). IFN-treated human A549 cells served as positive control. ISG15 served as IFN treatment control in MEFs. Western blots were simultaneously probed with an antibody recognizing β-tubulin to demonstrate similar loading of the gel. RT-qPCR ΔCT values were calculated relative to actin gene expression from technical triplicates; SEM is shown. (D) MEFs from hMx-tg$^{+/-}$ mice were treated with 10 ng per ml of IFN-α for 18 h before visualization of MxA expression (red) by indirect immunofluorescence. Untreated cells served as controls. Nuclei were stained with DAPI (blue). Bars, 10 µm. (E) MEFs from hMx-tg$^{+/-}$ and non-tg littermates were treated for 18 h with plain medium (0) or medium containing 1 or 10 ng per ml of IFN-α before infection (MOI, 0.01) with H7N7 avian influenza A virus strain FPV-B. Culture supernatants were harvested at 24 h after infection and virus titers were determined. Cells from mouse embryos carrying one functional allele of the endogenous *Mx1* gene (Mx1$^{+/-}$) were used as controls. Mean values with standard error of means of three independent experiments are shown. (F and G) MEFs from hMx-tg$^{+/-}$ and non-tg littermates were treated for 18 h with plain medium (0) or medium containing 1 or 10 ng per ml of IFN-α before infection (MOI = 0.1) with THOV (F) or VSV (G). Culture supernatants were harvested at 24 h (VSV) or 72 h (THOV) after infection and virus titers were determined. Mx1$^{+/-}$ MEFs were used as controls. Mean values with SEM of three independent experiments are shown.

human *MX1* locus as a transgene and readily expresses MxA in response to IFN exposure in all major organs. Using this mouse model, we provide first in vivo evidence that a few surface-exposed amino acids on the nucleoprotein are indeed responsible for MxA evasion and play a key role in the adaptation of avian influenza A viruses to humans, as previously indicated by biochemical and cell culture experiments (Mänz et al., 2013; Götz et al., 2016). These results indicate that MxA is a decisive factor that confers influenza virus resistance in humans. They provide strong evidence that newly emerging influenza viruses need to acquire the ability to evade MxA restriction to spread efficiently in the human population.

RESULTS AND DISCUSSION
Cells from transgenic mice carrying the human *Mx* locus exhibit IFN-dependent resistance to influenza A virus
To assess the role of MxA in vivo, we developed a transgenic mouse that carries a bacterial artificial chromosome that includes the entire human *MX* locus (Fig. 1 A). Repeated backcrossing of founder animals to C57BL/6 mice lacking functional endogenous *Mx* genes (Staeheli et al., 1988) yielded a new transgenic mouse line, designated hMx-tg. cDNA sequencing revealed that the *MX2* gene is defective in hMx-tg mice as a result of an accidental deletion that includes exon 4 (Fig. 1 A). Thus, *MX1* is the only intact human gene in our hMx-tg mouse strain.
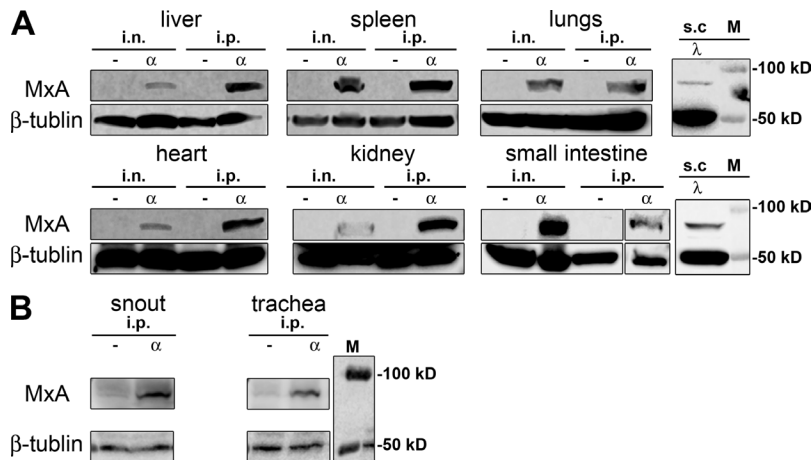
Figure 2. **IFN-induced expression of the MxA-encoding transgene in vivo.** (A) hMx-tg mice were either left untreated (–) or treated with 1 µg per animal of either IFN-α (α) or IFN-λ (λ) by i.n., i.p., or s.c. routes 18 h before the indicated organs were collected and analyzed for MxA content by Western blot. Blots were simultaneously probed with an antibody recognizing β-tubulin to demonstrate similar loading of the gel. (B) Mice were left untreated (-) or were treated for 18 h with 1 µg of IFN-α per animal before homogenates from trachea and snouts were prepared. Western blot analysis was done as described for A.

Cultured mouse embryonic fibroblasts (MEFs) prepared from hMx-tg mice expressed the transgene when stimulated with IFN-α, but not under standard culture conditions, and maintained normal regulation of other IFN-stimulated genes such as ISG15 (Fig. 1 B). MxA protein levels in IFN-treated transgenic MEFs were slightly lower than in IFN-treated human A549 cells (Fig. 1 C). MxA accumulated in the cytoplasm of IFN-treated hMx-tg MEFs (Fig. 1 D) as expected from earlier studies of human cells (Staeheli and Haller, 1985). When pretreated with IFN-α and challenged with a mouse-adapted H7N7 influenza A virus (Israël, 1979), MEFs from hMx-tg mice but not from nontransgenic littermates exhibited a high degree of resistance that was comparable to cells from mice carrying one functional copy of the endogenous *Mx1* resistance gene (Fig. 1 E). Furthermore, IFN-treated MEFs from hMx-tg but not from nontransgenic mice were highly resistant to Thogoto virus (THOV) known to be extremely sensitive to MxA (Kochs and Haller, 1999; Fig. 1 F). In contrast, IFN-induced resistance to vesicular stomatitis virus (VSV) was independent of either endogenous Mx1 or transgenically expressed MxA (Fig. 1 G), confirming other studies that additional IFN-induced restriction factors contribute to inhibition of VSV (Sadler and Williams, 2008).

hMx-tg mice are highly resistant to infection with avian influenza A viruses and THOV

The transgene was expressed in all organs tested when the animals were treated with IFN-α by either the i.n. or the i.p. route (Fig. 2). Detectable levels of MxA were also induced by IFN-λ in lungs and small intestine, whereas transgene expression was not detected in organs of untreated mice (Fig. 2 A). We additionally checked MxA expression in the upper respiratory tract (trachea and snout) for relevance to influenza virus infection. As expected, MxA was induced upon IFN-α treatment in the upper respiratory tract (Fig. 2 B). These results demonstrated that our transgenic mice carry a copy of the human *MX1* gene that is functional and expressed in response to type I and type III IFN, similar to the authentic gene in human tissue (Holzinger et al., 2007).

To assess influenza virus susceptibility, transgenic mice were challenged with 10–30 median lethal doses 50 (LD$_{50}$) of highly pathogenic H5 and H7 influenza A viruses of avian origin. All transgenic mice survived such infections, whereas the nontransgenic littermates developed severe disease and most of them had to be sacrificed for animal welfare reasons before day 9 after infection (Fig. 3, A–D). Resistance of hMx-tg mice to these avian viruses was remarkably robust: all transgenic

Table 1. **Approximate LD$_{50}$ values of influenza A viruses of avian or human origin in C57BL/6 mice carrying functional endogenous *Mx1* genes (Mx1), lacking functional endogenous *Mx1* (non-tg) or carrying the human MxA transgene (hMx-tg)**

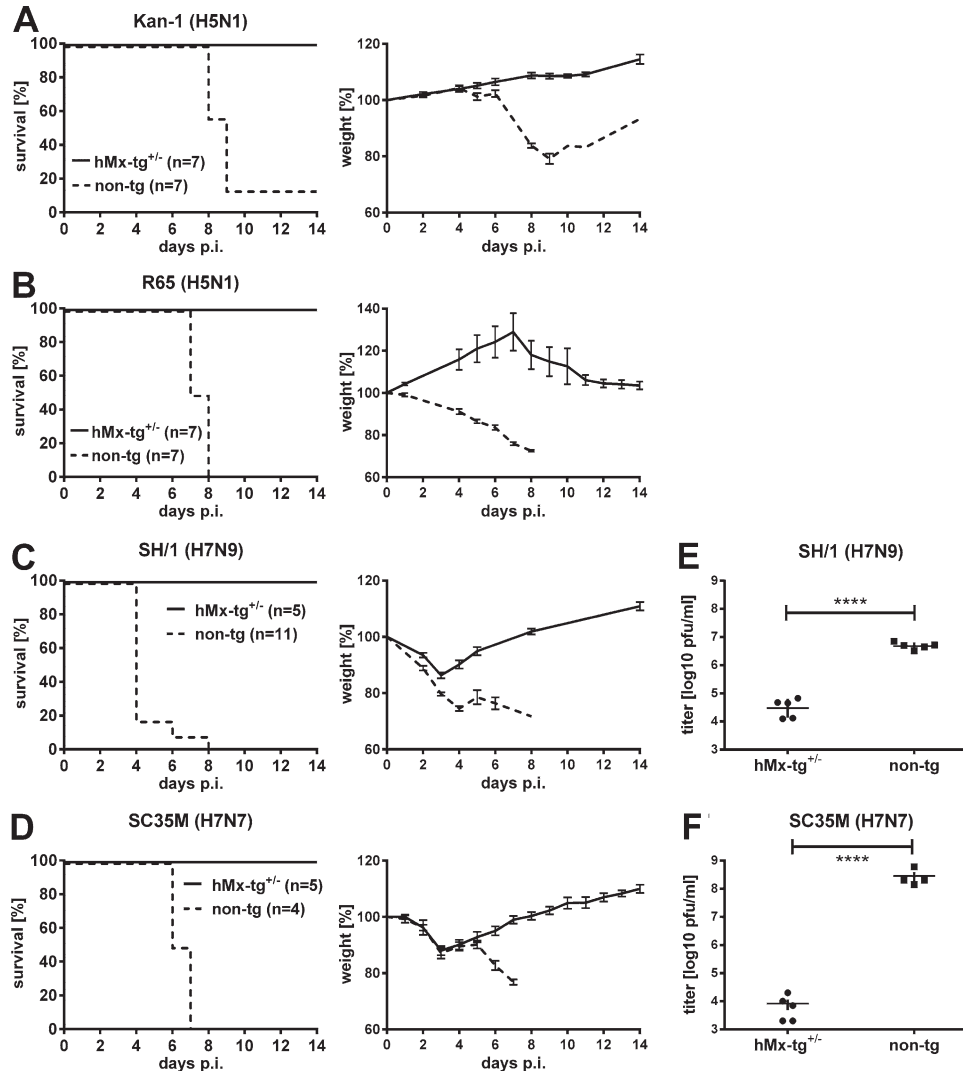| Influenza strain	LD$_{50}$ [pfu]		
	hMx-tg	non-tg	Mx1
Avian			
KAN-1 (H5N1)	$>10^6$	5×10^0	$>10^6$
R65 (H5N1)	8×10^4	5×10^0	3×10^5
SH/1 (H7N9)	3×10^4	1×10^3	$>10^5$
SC35M (H7N7)	2×10^4	8×10^2	1×10^5
Human			
PR8 (H1N1)	2×10^3	1×10^3	$>10^6$
WSN (H1N1)	2×10^3	5×10^2	$>4 \times 10^5$
HK68 (H3N2)	5×10^1	1×10^1	4×10^4

Figure 3. **hMx–tg mice are highly resistant to infection with influenza A viruses of avian origin.** Groups of hMx-tg^{+/-} and non-tg mice were challenged by the i.n. route with 10–30 LD$_{50}$ (values refer to non-tg mice) of (A) the avian influenza A virus strains KAN-1 (1.5 × 10^2 pfu; seven animals each), (B) R65 (2 × 10^2 pfu; seven animals each), (C) SH/1 (10^4 pfu; five hMx-tg^{+/-} and eleven non-tg), or (D) SC35M (5 × 10^3 pfu; five hMx-tg^{+/-} and four non-tg). Weight loss was monitored daily; animals were sacrificed and scored dead when weight loss reached 25% of the initial weight on the day of infection. (E and F) Other groups of transgenic and nontransgenic mice were challenged through the i.n. route with (E) SH/1 (10^4 pfu; five animals each) or (F) SC35M (5 × 10^3 pfu; five hMx-tg^{+/-} and four non-tg). Viral titers in the lungs at day 5 after infection are shown. ****, P < 0.0001, Student's t test.

animals exhibited only very mild body weight losses and survived challenge infections with >10^4 LD$_{50}$ (Table 1) of the highly pathogenic H5N1 influenza A virus strains A/KAN-1 (Fig. 3 A) and A/R65 (Fig. 3 B). Resistance of hMx-tg mice toward the H7N9 strain A/SH/1 (Fig. 3 C) and the H7N7 strain A/SC35M (Fig. 3 D) was similarly pronounced, and the LD$_{50}$ values of these viruses in hMx-tg mice were ∼30-fold higher than in nontransgenic controls (Table 1). Enhanced survival of transgenic mice infected with SH/1 and SC35M correlated with strongly reduced virus titers in the lungs compared with nontransgenic controls (Fig. 3, E and F). Earlier work indicated that avian H7N9 influenza A viruses, which recently emerged in China, can partially evade restriction by

MxA in cell culture (Riegger et al., 2015). In contrast, our infection studies with transgenic mice clearly demonstrated that MxA is able to control the A/SH1 human H7N9 isolate very efficiently in the context of the whole organism. Thus, the MxA restriction factor is protective against a broad range of avian influenza viruses and represents a veritable barrier that limits zoonotic virus transmissions.

The Mx1 protein of mice confers a high degree of resistance to infection by members of the *Thogotoviridae* family, such as THOV and Dhori virus (DHOV), whereas human MxA selectively inhibits THOV (Frese et al., 1995; Haller et al., 1995). Our hMxA-tg mice exhibited robust resistance to challenges with THOV (Fig. 4 A), but were highly susceptible
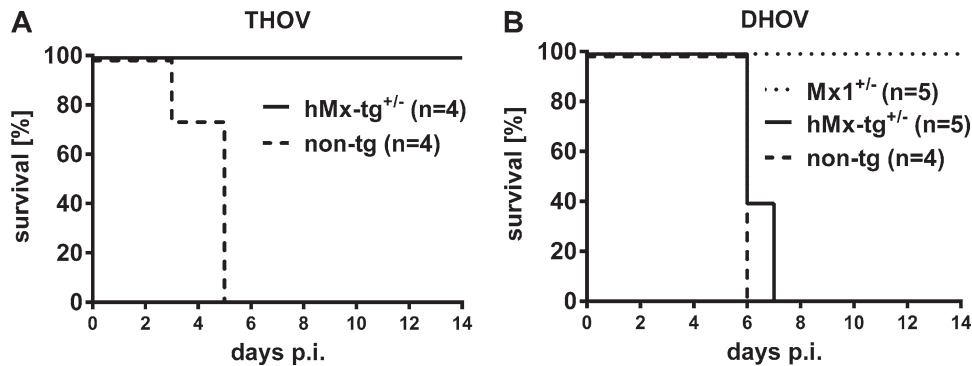
Figure 4. **hMx–tg mice are resistant to infection with THOV but susceptible to DHOV.** hMx-tg^{+/−} and non-tg mice were challenged by i.p. infection with (A) 10^3 pfu of THOV (four animals each) or (B) 10^2 pfu of DHOV (five hMx-tg^{+/−} and four non-tg). Survival was monitored for 14 d. Mice carrying one functional allele of the endogenous *Mx1* gene (Mx1^{+/−}, five animals) served as positive control for the DHOV challenge experiment. Animals were sacrificed and scored dead when weight loss reached the 25% limit.

to DHOV (Fig. 4 B), demonstrating that the antiviral specific-ity of MxA is maintained in our transgenic mice. These find-ings are in agreement with earlier findings from studies with a transgenic mouse line that constitutively expressed low levels of MxA and confirmed that these two related viruses dif-fer drastically with regard to MxA sensitivity (Thimme et al., 1995). Collectively, these results demonstrated that our trans-genic mice behaved as predicted from previous studies with cultured human cells (Haller et al., 2015), and that they repro-duced the innate anti-influenza immune response of humans.

hMx–tg mice exhibit only moderate resistance to influenza A viruses of human origin

When challenged with ~10 LD$_{50}$ of mouse-adapted A/WSN (H1N1), A/PR8 (H1N1), or A/HK68 (H3N2), most of the transgenic mice developed severe disease symptoms with only slightly delayed kinetics compared with nontransgenic littermates (Fig. 5 A). These challenge conditions were not particularly stringent as control mice carrying a functional endogenous *Mx1* gene survived such virus doses (Table 1) without developing severe disease symptoms. Nevertheless, the transgene conferred some protection which became obvious only when viral titers in the lungs were measured (Fig. 5 C) or when the challenge virus dose was reduced to ~3 LD$_{50}$ (Fig. 5 B). Under such nonstringent conditions, most of the transgenic mice lost <25% of their initial body weight and survived the infection, whereas >50% of non-transgenic littermates had to be sacrificed.

A similar picture emerged when influenza virus strains with no passage history in mice, such as A/Udorn/72 and A/HH/2009, were used for challenge experiments. These nonadapted viruses, which do not induce disease in mice replicated to significantly higher titer in the lungs of non-transgenic mice compared with transgenic animals, but the differences were moderate, as in the case of mouse-adapted WSN (Fig. 5 C), indicating that mouse adaptation does not alter the MxA resistance phenotype of human influenza virus isolates. It should be noted that influenza viruses of both avian and human origin were all restricted very well in mice that express endogenous *Mx1* genes (Table 1), indicating that human influenza viruses evolved to specifically evade re-striction by human MxA but not mouse Mx1. This finding is most likely caused by the fact that the mouse does not serve as a natural host for influenza viruses, and mouse Mx1 does therefore not exert any selective pressure on influenza virus evolution. In contrast, human *MX1* and influenza virus genes are apparently genes in conflict in an evolutionary arms race (Mitchell et al., 2013). Accordingly, our in vivo findings clearly demonstrate that influenza A viruses of human origin are rather poorly restricted by MxA, confirming and extend-ing previous results from experiments with human cell cul-ture systems (Dittmann et al., 2008). Collectively, the present animal experiments provide first solid in vivo evidence that MxA is indeed a major influenza restriction factor in humans.

Avian influenza virus with adaptive mutations in the nucleoprotein gains virulence in hMx–tg mice

Partial escape of human influenza A viruses from restriction by MxA in a cell culture system was recently linked to three signature mutations in the viral nucleoprotein (Mänz et al., 2013). The vast majority of avian influenza viruses possess nucleoproteins with arginine at position 100, leucine at po-sition 283, and phenylalanine at position 313, whereas most influenza A viruses of human origin possess nucleoproteins with isoleucine or valine at position 100, proline at position 283, and tyrosine or valine at position 313. Avian influenza A viruses with engineered nucleoproteins carrying all three human signature mutations are genetically instable (Mänz et al., 2013), suggesting that MxA escape has a substantial fitness cost. Due to this technical difficulty, conclusive experimen-tal proof that these adaptive mutations play a decisive role in MxA evasion during viral infections was not available.

Recently, a variant of the avian H7N7 influenza A virus strain SC35M was described that carries all three human
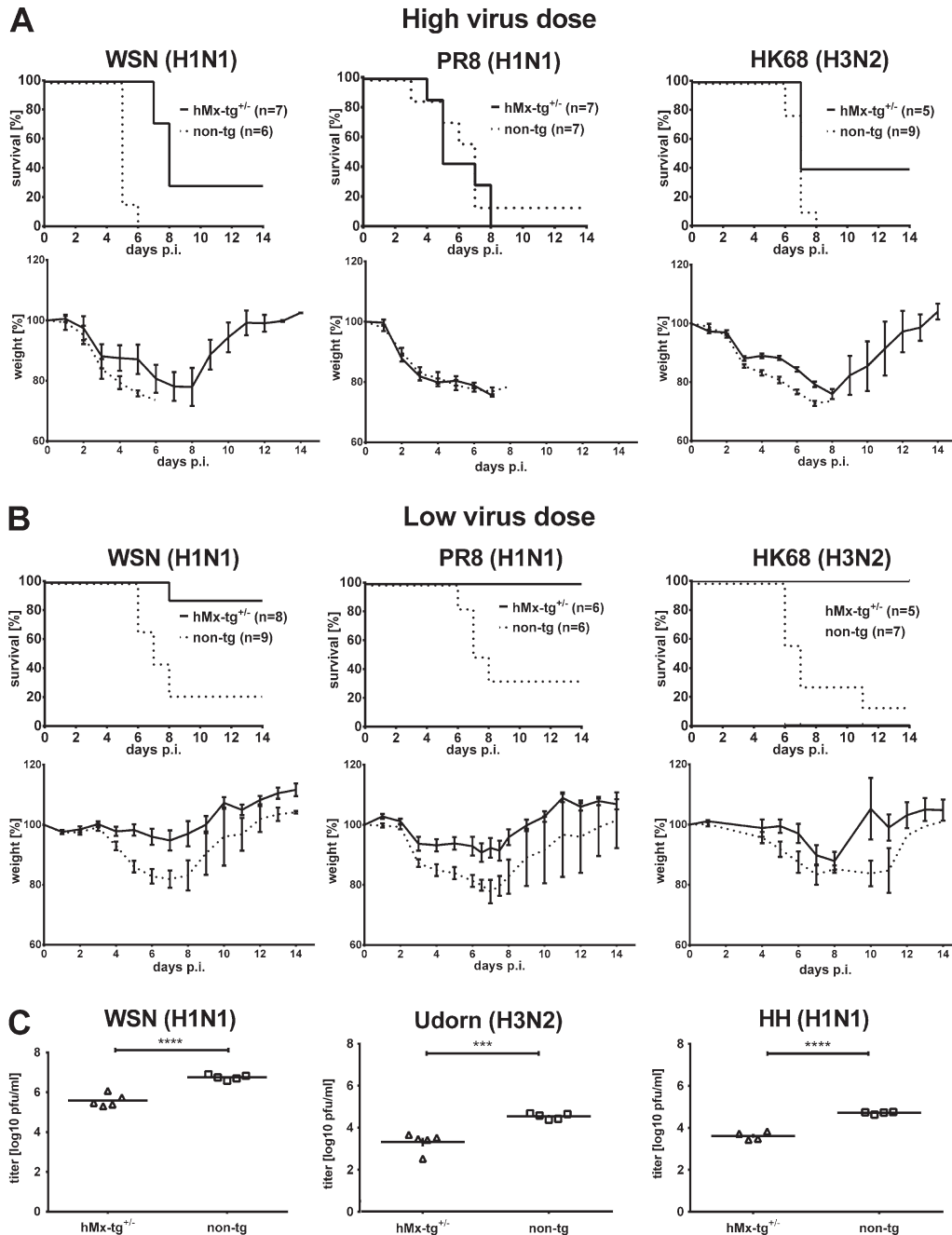
Figure 5. **hMx-tg mice exhibit only moderate resistance to influenza A viruses of human origin.** (A) Groups of hMx-tg^{+/−} and non-tg mice were challenged by the i.n. route with ~10 LD_{50} (high dose; values refer to non-tg mice) of mouse-adapted influenza A virus strains of human origin, namely A/WSN (5×10^3 pfu; seven hMx-tg^{+/−} and six non-tg), A/PR8 (10^4 pfu; seven animals each) or A/HK68 (100 pfu; five hMx-tg^{+/−} and nine non-tg). (B) Other groups of transgenic and nontransgenic mice were challenged with 2–3 LD_{50} (low dose) of A/WSN (1.3×10^3 pfu; eight hMx-tg^{+/−} and nine non-tg), A/PR8 (3×10^3 pfu; six animals each) or A/HK68 (30 pfu; five hMx-tg^{+/−} and seven non-tg). Weight loss was monitored daily; animals were sacrificed and scored dead when weight loss reached the 25% limit. (C) Groups of hMx-tg^{+/−} and non-tg mice were infected with 10^4 pfu of human influenza virus strains WSN, Udorn or HH. Viral titers in the lungs at day 4 after infection are shown. ***, P < 0.0005; ****, P < 0.0001, Student's *t* test.

signature mutations (100V, 283P and 313Y) along with one additional glycine to aspartic acid mutation at position 16 in the nucleoprotein (Götz et al., 2016). This SC35M variant carrying four mutations in the nucleoprotein, designated

SC35M-4x, was genetically stable and showed no detectable growth retardation in MDCK cells. Importantly, SC35M-4x replicated to higher titers than parental SC35M in human A549 cells constitutively expressing MxA (Götz et al., 2016),

indicating that SC35M-4x may represent a suitable tool for testing the hypothesis that viruses carrying nucleoproteins with a human signature are far more virulent in the face of a functional MxA-driven innate immune response than viruses carrying nucleoproteins with avian signature.

In hMx-tg mice, SC35M-4x induced slightly more weight loss and replicated to significantly higher titers in the lungs of such animals than parental SC35M (Fig. 6 A). The difference in viral lung titers was most pronounced on day 5 after infection, indicating that SC35M-4x continued to replicate even when the transgene is expected to be expressed well in the infected lungs as a result of virus-induced IFN synthesis. To verify this exceptional property of SC35M-4x more directly and to exclude the possibility that SC35M-4x had simply outpaced the innate immune response, we preactivated MxA transgene expression by treating the animals with IFN-α one day before virus challenge. Under such experimental conditions, which are expected to result in high levels of MxA in the lung tissue within 18 h (Fig. 2), high doses of parental SC35M no longer caused detectable weight loss in our transgenic mice (Figs. 6, B and C). In contrast, SC35M-4x still induced pronounced weight loss (Fig. 6 B), and six of seven IFN-treated transgenic animals that received 10^6 pfu of SC35M-4x had to be sacrificed (Fig. 6 C). Titers of SC35M-4x in the lungs of IFN-treated transgenic mice were ~10-fold higher than parental SC35M on day 3 after infection (Fig. 6 B). Of note, in IFN-treated mice lacking the MxA transgene, lung titers of SC35M and SC35M-4x did not differ significantly at day 3 after infection (Fig. 6 D), demonstrating that the IFN-mediated resistance in transgenic mice is strongly dependent on MxA and that enhanced virulence of SC35M-4x is caused by successful evasion of MxA-mediated growth restriction by this virus.

Collectively, our new transgenic mouse shows fine-tuned expression of the human influenza virus restriction factor MxA in all major organs, including the upper and the lower respiratory tract. These mice thus represent the first small animal model that accurately mimics one important aspect of the innate immune response of humans toward influenza virus. Unlike all other currently available mouse models, our MxA-transgenic mouse can readily distinguish between MxA-sensitive influenza virus strains and virus strains which can evade MxA restriction and, consequently, possess a high pandemic potential in humans. Our MxA-transgenic mice will facilitate future basic research aimed at understanding the molecular details of influenza virus restriction by the innate immune system. For example, our current work revealed that H5N1 viruses are inhibited far more strongly by MxA than the H7 avian influenza viruses. We suspect that the high MxA susceptibility of H5 viruses is multifactorial, but further work is required to understand the molecular basis of this phenomenon. Our MxA-transgenic mouse might also be used to rapidly assess the pathogenic potential and overall fitness of emerging influenza A viruses in humans. Such functional analysis could complement current risk assessment strategies of emerging influenza viruses, including viral genome sequencing and screening for alterations in known viral virulence genes. Moreover, our transgenic mice might be useful in assessing the in vivo relevance of polymorphic MxA mutations that occur naturally in the human population. Such mutations could easily be introduced into the transgene of our mouse by CRISPR/Cas9 technology. Further, our transgenic mice might be used to analyze the in vivo importance of MxA cofactors.

MATERIALS AND METHODS
Transgenic mice
Human male bacterial artificial chromosome library clone RP11-120C17 (Cheung et al., 2001) was used to generate transgenic mice. This clone contains the complete human *MX1* and *MX2* genes, as well as fragments of the adjacent genes *FAM3B* and *TMPRSS2*. Microinjection of DNA into the pronucleus of fertilized eggs from superovulated B6.SJL females and transfer of manipulated eggs into pseudo-pregnant Swiss Webster female mice was performed by a commercial service (Taconic). Integration of the transgene into the genome of resulting offspring was verified by PCR using the following targeted primer sets: *MX1*, 5′-GCCTTT AATCAGGACATCACTGCTCTCATG-3′, 5′-CTGAAC CCACAATATCTCTGAGGTATGCC-3′; *FAM3B*, 5′-TGA ATTCCTTTCTGCAGACTTAGG-3′, 5′-GCAATTTGG GAGGATTGAGG-3′; *TMPRSS2*, 5′-GAGGTTAGGACT GCACTTGAGG-3′, 5′-TCCAGGCTTGTGGATCAGC-3′. Founder animals harboring the transgene were repeatedly backcrossed to C57BL/6 mice. Heterozygous males and females from backcrosses 5–12 (designated hMx-tg) were used for the experiments described in this study. Nontransgenic littermates served as controls.

Sequencing the *MX* gene transcripts
MEFs of hMx-tg and nontransgenic littermates were stimulated with human chimeric IFN-$\alpha_{B/D}$ (10 ng/ml; Horisberger and de Staritzky, 1987) for 18 h. RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's protocol and reverse transcribed into cDNA with the QuantiTect Reverse Transcription kit (QIAGEN). *MX1* and *MX2* genes were amplified by PCR. Products were purified with peqGOLD Cycle-Pure kit (PEQLAB) according to the manufacturer's protocol before sequencing. Primers for *MX1* were as follows: 5′-ATGGTTGTT TCCGAAGTGGAC-3′, 5′-TGTCAAGTGCCGGGGCCA GC-3′; primers for *MX2* were as follows: 5′-ATGTCTAAG GCCCACAAGCCTTGGC-3′, 5′-TTCAGTGGATCTCTT TGCTGGAGAATTG-3′.

Viruses
For infection of mice, the following influenza A virus strains were used: A/Swan/Germany/R65/2006 (H5N1) designated R65, A/Thailand/1(KAN-1)/2004 (H5N1) designated KAN-1, A/Shanghai/1/2013 (H7N9) designated SH/1, A/Seal/ Massachusetts/1/1980 (H7N7) designated SC35M, A/PR/ 8/1934 (H1N1) designated PR8, A/WSN/1933 (H1N1)
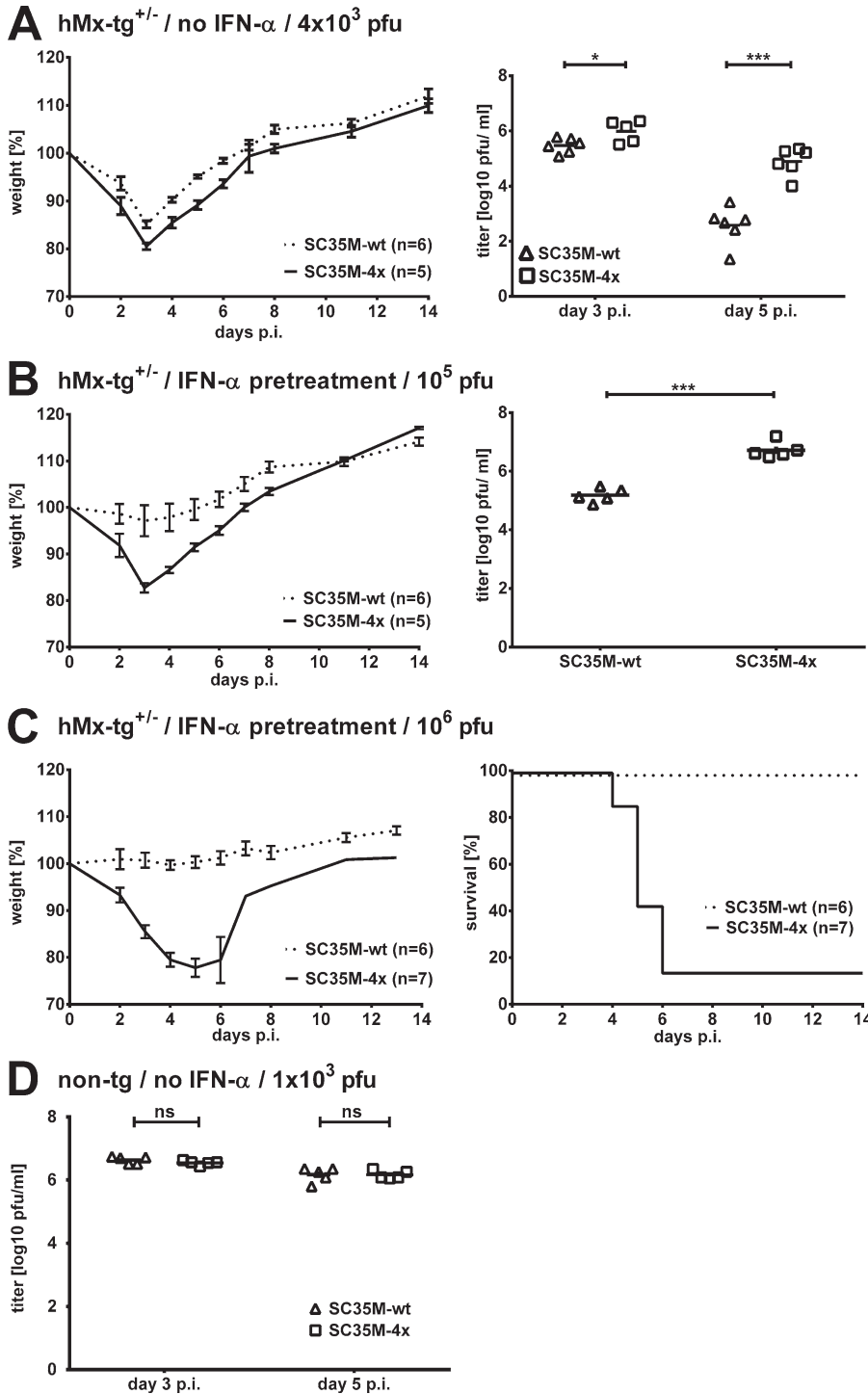
Figure 6. **SC35M–4x exhibits enhanced MxA resistance in transgenic mice.** (A) hMx-tg^{+/−} mice were infected with 4×10^3 pfu of either parental SC35M-wt or SC35M-4x (six and five animals, respectively). Body weight changes with SEM and lung titers on days 3 and 5 after infection are shown. (B and C) hMx-tg^{+/−} were pretreated with 10 µg of IFN-α by s.c. injection 1 d before infection with (B) 10^5 pfu (six animals with SC35M-wt and five animals with SC35M-4x) or (C) 10^6 pfu of either parental SC35M-wt or SC35M-4x (six animals SC35M-wt and five animals SC35M-4x). Kinetics of body weight changes, survival, and lung titers at day 3 (six animals SC35M-wt and five animals SC35M-4x) after infection are depicted. Weight loss was monitored daily and SEM depicted; animals were sacrificed and scored dead when body weight loss reached the 25% limit. (D) Groups ($n = 5$) of nontransgenic mice were infected with 10^3 pfu either parental SC35M-wt or SC35M-4x, and viral lung titers were determined on days 3 and 5 after infection. *, $P < 0.05$; ***, $P < 0.0001$.

designated WSN, A/Hong Kong/8/68 (H3N2) designated HK68, A/Udorn/72 (H3N2) designated Udorn, and A/HH/05/2009 (H1N1) designated HH. R65 and SH/1 are avian isolates with no passage history in mammals. The avian influenza virus strain KAN-1 was isolated from a diseased boy and has since been passaged in MDCK cells. SC35M represents a mouse-adapted variant of an avian H7N7 virus that

was originally isolated from a diseased seal. PR8, WSN, and HK68 are mouse-adapted viruses of human origin, whereas the human virus isolates Udorn and HH have no passage history in mice. SC35M-4x differs from parental SC35M by four amino acid substitutions in the nucleoprotein, namely G16D, R100V, L293P, and F313Y (Götz et al., 2016). Two different THOVs were used for infection studies in mice: Thogoto

virus strain SiAr126 lacking a functional ML gene (designated THOV; Albanese et al., 1972) and DHOV strain India1313/61 (designated DHOV; Anderson and Casals, 1973). The L cell–adapted variant of A/Fowl plague virus/Dobson (H7N7), designated FPV-B (Israël, 1979), and vesicular stomatitis virus serotype New Jersey (VSV) were used for infection of MEFs.

Cells

To generate MEFs, 14 d post coitum embryos of hMx-tg and nontransgenic littermates were mechanically and enzymatically homogenized before released cells were collected by centrifugation and resuspended in cell culture medium. Madin-Darby canine kidney epithelial cells (MDCK), human lung epithelial cells (A549), and baby hamster kidney cells (BHK-21) were originally obtained from the ATCC. All cells were cultivated in DMEM supplemented with 10% fetal bovine serum, 526 mg/liter glutamine, 20,000 U/liter penicillin and 40 mg/liter streptomycin at 37°C, 95% relative humidity and 5% CO_2.

Immunofluorescence microscopy

MEFs were stimulated with human chimeric IFN-$\alpha_{B/D}$ (Horisberger and de Staritzky, 1987; 10 ng per ml for 18 h) or were mock treated before fixing with 3% formaldehyde. Cells were permeabilized for 10 min using PBS containing 5% normal donkey serum and 0.1% Triton X-100 before they were incubated with a rabbit anti-Mx serum (Meier et al., 1990) in PBS containing 3% normal donkey serum at 4°C overnight. Goat α-rabbit Cy3 (Jackson ImmunoResearch Laboratories) served as a secondary antibody. Slides were mounted in DAPI-containing IS Mounting Medium (Dianova). Digital images were taken with an ApoTome fluorescence microscope (ZEISS) using AxioVision software.

Virus infection of cell cultures

MEFs were seeded, stimulated the next day with different doses of human chimeric IFN-$\alpha_{B/D}$ (Horisberger and de Staritzky, 1987) for 18 h, and subsequently infected with a MOI of 0.1 of VSV or THOV for 24 and 72 h, respectively. Infections with the influenza A virus strain FPV-B were performed at a MOI of 0.01, and supernatants were harvested at 24 h after infection. Viral titers were determined by plaque assay.

IFN treatment of mice

Human chimeric IFN-$\alpha_{B/D}$ (Horisberger and de Staritzky, 1987) or mouse IFN-λ (IL-28A; PeproTech) were applied i.n. in a volume of 40 μl to ketamin/xylazine-anesthetized animals. Where indicated, IFN was administered in 100 μl into the peritoneal cavity or in 120 μl s.c. on the back of the animals.

RT–qPCR

Reverse transcription was performed using the Revert Aid First Strand cDNA Synthesis kit (Fermentas) as recommended by the manufacturer. QuantiTect SYBR Green PCR kit and commercially available primer pairs (QIAGEN) were used for comparative analysis. ΔCT values were used

to characterize expression of target genes compared with housekeeping genes.

Western blot

Protein samples from homogenized cells or tissues were incubated at 95°C in Lämmli buffer and loaded on SDS-PAGE gels. Proteins were blotted on a methanol-activated PVDF membrane (PerfectBlue Semi-Dry electro blotter; PEQLAB). Membranes were blocked in PBS containing 1% milk powder before incubation with primary antibodies (mouse anti-MxA; Flohr et al., 1999) and mouse anti–tubulin-β (Sigma-Aldrich) for 1 h, followed by incubation with horseradish peroxidase–coupled secondary antibody (goat anti–mouse IgG; Jackson ImmunoResearch Laboratories) for 1 h. Bound antibodies were visualized using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Mouse infection experiments

All mice used in the study were bred locally in our facility and handled in accordance with guidelines of the Federation for Laboratory Animal Science Associations and the national animal welfare body. Animal experiments were performed in compliance with the German animal protection law and approved by the local animal welfare committee (Regierungspräsidium Freiburg; permit G-12/46). Virus stocks were diluted in PBS. Infection of ketamin/xylazine-anesthetized animals with influenza viruses was performed i.n. with 40 μl inoculum. For infection with THOVs, 100 μl of the virus dilutions were administered into the peritoneal cavity. Mice were monitored daily. If severe illness or weight loss of >25% occurred, mice were sacrificed by cervical dislocation. If required, organs were flash frozen in liquid nitrogen and stored at −80°C until further processing.

Plaque assay

Serial dilutions of cell culture supernatants or homogenized organ samples were placed on MDCK cells for 1 h at room temperature. After inoculum removal, cells were incubated with medium containing 1.5% Avicel for three days at 37°C. Cells were fixed with 4% formaldehyde and plaques were stained using crystal violet.

LD$_{50}$ determination

The 50% median lethal dose (LD$_{50}$) values were calculated as described previously (Riegger et al., 2015) by using three to five animals for each virus dilution.

Statistical analysis

Virus titration data of cell culture supernatants are from at least three independent experiments with three technical replicates. Geometric mean values of virus titers in lungs of at least four individual mice were determined. Where indicated, two tailed Student's t test was used to determine if a difference in virus titers between two groups was significant. Values of $P < 0.05$ were considered to indicate a significant difference. Statistical analyses were conducted with GraphPad Prism v6.2.

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