Apobec3 encodes *Rfv3*, a gene influencing neutralizing antibody control of retrovirus infection

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Abstract

Recovery from Friend Virus 3 (*Rfv3*) is a single autosomal gene encoding a resistance trait that influences retroviral neutralizing antibody responses and viremia. Despite extensive research for 30 years, the molecular identity of *Rfv3* has remained elusive. Here we demonstrate that *Rfv3* is encoded by *Apobec3*. *Apobec3* maps to the same chromosome region as *Rfv3* and has broad inhibitory activity against retroviruses including HIV. Not only did genetic inactivation of *Apobec3* convert *Rfv3*-resistant mice to a susceptible phenotype, but *Apobec3* was found to be naturally disabled by aberrant mRNA splicing in *Rfv3*-susceptible strains. The link between *Apobec3* and neutralizing antibody responses highlights an *Apobec3*-dependent mechanism of host protection that might extend to HIV and other human retroviral infections.

The study of viral resistance factors has provided important insights into the evolutionary strategies of defense utilized by mammalian hosts (1–5). *R*ecovery from *F*riend *v*irus (FV) gene 3 (*Rfv3*) was first identified as a resistance trait in 1978 (6,7) and later genetic studies showed that the phenotypes of decreased viremia and FV-specific neutralizing antibody responses segregated as a single gene (8). Since the generation of neutralizing antibodies is critical for recovery from FV infection (1,9) and a desired but often unrealized outcome in various retroviral infections including HIV-1, we have focused our efforts on identifying the gene encoding *Rfv3*. The *Rfv3* locus maps to a 0.83 centimorgan region of chromosome 15 (Fig. S1A) (10–12), which contains at least 61 annotated genes (table S1), one of which is murine *Apobec3* (*mA3*), a member of a family of deoxycytidine deaminases with antiretroviral and anti-retroelement activity [as reviewed in (13)]. This fact, along with the presence of substantial polymorphism in *mA3* (table S1), led us to consider *mA3* as a prime candidate for *Rfv3*.

Since *Rfv3* has no described *in vitro* phenotype, our investigation required the generation of *mA3*-deficient mice (14). First, an inactivated *mA3* gene (Fig. S1B) was introduced into the *Rfv3*$^{r/r}$ C57BL/6 (B6) background to test its ability to act as a defective *Rfv3* allele in matings with *Rfv3*$^{s/s}$ mice (Table 1). Since the *Rfv3* resistance trait is dominant over susceptibility (7), *Rfv3*$^{r/s}$ F$_1$ offspring should control viremia and mount effective neutralizing antibody

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responses. Conversely, if *mA3* encodes *Rfv3*, then the gene from a *mA3*$^{-/-}$ parent will be null, and the resultant F$_1$ offspring with an *Rfv3*$^{-/s}$ genotype are predicted to exhibit higher levels of viremia and weaker neutralizing antibody responses. To test this possibility, B6 × BALB/c F$_1$ offspring were infected with FV and plasma viremia levels were measured. At 7 days post infection (dpi), the F$_1$ mice containing an inactivated *mA3* gene exhibited 15-fold higher levels of viremia than their congenic partners carrying the wild-type *mA3* allele (Fig. 1A). These high viral loads in *mA3*$^-$ F$_1$ mice were comparable to FV levels found in fully susceptible *Rfv3*$^{s/s}$ BALB/c parental mice. Thus, *mA3* is a restriction factor contributing to the early control of FV infection in adult immunocompetent mice.

Rfv3-mediated recovery from FV infection correlates strongly with FV-specific neutralizing antibody responses at 28 dpi (7). Thus, *mA3*$^+$ and *mA3*$^-$ F$_1$ congenic strains were monitored for up to one month following FV infection. However, F$_1$ mice lacking the B6 *mA3* allele suffered a markedly higher rate of FV-induced death (Fig. 1B) indicating that like *Rfv3*^{s} susceptibility, *mA3* inactivation compromised recovery from FV disease. Compared to *mA3*$^+$ F$_1$ mice, the three surviving *mA3*$^-$ F$_1$ mice exhibited 14-fold higher mean viremia (Fig. S2A) and low FV-specific neutralizing antibody titers at 28 dpi (Fig. S2B), but the small number of surviving animals precluded obtaining statistically significant data. Therefore, separate cohorts of mice were studied for FV-specific antibodies at 14 dpi, prior to the steep decline in survival of *mA3*$^-$ F$_1$ mice. The *mA3*$^-$ F$_1$ mice exhibited significantly less FV binding antibody than *mA3*$^+$ F$_1$ mice and the low levels of FV antibodies in *mA3*$^-$ F$_1$ mice proved comparable to levels detected in the parental *Rfv3*$^{s/s}$ BALB/c mice (Fig. 1C). These findings indicated that *mA3* influenced FV-specific antibody responses.

To better assess FV-specific neutralizing antibody responses in mice expressing or lacking *mA3*, these studies were repeated in high-recovery B6 × A.BY F$_1$ mice, which generally survive more than 1 month after FV infection due to protective cell-mediated immune responses associated with the *H-2*$^{b/b}$ haplotype (Table 1) (5,15). Plasma samples obtained at 28 dpi revealed significantly lower FV-specific neutralizing antibody titers in *mA3*$^-$ F$_1$ mice compared to *mA3*$^+$ F$_1$ mice (Fig. 1D). These findings confirmed that *mA3* influenced FV-specific neutralizing antibody responses, and demonstrated that this effect operated independently of *H-2*, a known property of *Rfv3* (7).

Purebred B6 mice are highly resistant to FV infection (Table 1), but their resistance can be overcome by inoculating aged mice with high doses of FV (16) or by using immunodeficient mice, including those that fail to produce specific antibodies (9). Genetic inactivation of *mA3* in B6 mice might therefore recapitulate the *Rfv3*^{s} susceptible phenotype without a requirement for outcrossing to susceptible strains. To test this possibility, >16 week-old B6 *mA3*$^{+/+}$ and *mA3*$^{-/-}$ mice were infected with FV. Plasma viremia was 6.2-fold higher in *mA3*$^{-/-}$ mice than in *mA3*$^{+/+}$ mice at 8 dpi (Fig. 2A). Furthermore, *mA3*$^{-/-}$ mice exhibited significantly lower neutralizing antibody titers than wild-type mice by 28 dpi (Fig. 2B). Thus, *mA3* inactivation was sufficient both to enhance viremia and to diminish neutralizing antibody production even in the highly resistant B6 genetic background. These results were confirmed in a second highly resistant strain of mice, 129/Ola (Table 1, Supp. Text and Fig. 2C). Together, these findings demonstrate that genetic inactivation of *mA3* recapitulates the *Rfv3*^{s} phenotype and indicate that *Rfv3* is encoded by *mA3*.

Both resistant and susceptible mouse strains contain the *mA3* gene and express *mA3* mRNA. Cloning of splenocyte *mA3* mRNA from the *Rfv3*$^{r/r}$ 129/Ola strain revealed the predominant expression of a full-length *mA3* transcript, while most mRNA transcripts from *Rfv3*$^{r/r}$ B6 mice lacked exon 5 sequences (Fig. S3 and Fig. S4) (17). *mA3* transcripts from both *Rfv3*$^{s/s}$ strains BALB/c and A.BY were distinguished by the absence of exon 2 sequences (Fig. 3A and Fig. S4). Quantitative RT-PCR revealed similar levels of total *mA3* mRNA in both the *Rfv3*$^{r/r}$ and

$Rfv3^{s/s}$ strains but a 17-fold lower level of Exon2-containing transcripts in both $Rfv3^{s/s}$ mouse strains (Fig. 3B). Thus, the presence of an alternatively spliced *mA3* mRNA lacking exon 2 correlated with the $Rfv3^s$-susceptible phenotype.

Translational read-through of the *mA3* mRNA lacking exon 2 predicts two possible outcomes: no mA3 protein expression, or a truncated mA3 protein (Fig. 3A and Fig. S5). We investigated the antiviral activity of this Δexon 2 mA3 protein by cloning it as a fusion with an N-terminal triple FLAG epitope tag. NIH3T3 cells were co-transfected with the expression construct and an FV molecular clone (pLRB302) (18) to test the infectivity of the resulting FV virions. For comparison, co-transfections were performed with a FLAG-tagged expression construct containing the *mA3* cDNA from B6 mice lacking exon 5. When standardized for relative mA3 expression levels, the BALB/c Δexon 2 mA3 protein was at least 3 to 5-fold less potent at inhibiting FV infectivity than the B6 Δexon 5 mA3 protein (Fig. 3C). In control experiments, the full-length mA3 protein from 129/Ola also potently inhibited FV (Fig. S6). These data indicated that even if a truncated mA3 protein was expressed in $Rfv3^{s/s}$ mice, its antiviral activity would be significantly impaired.

The involvement of *mA3* in the control of FV viremia before the onset of adaptive anti-FV immune responses confirms its stature as a bona fide innate immune factor *in vivo*. In addition, *mA3* influences the development of virus-specific neutralizing antibody responses, perhaps by: (1) limiting the early viral antigenic load and evading a form of "high-zone tolerance" (19–21), or (2) by inhibiting early FV-induced injury of critical cell types, such as B cells and antigen-presenting cells, required for the development of FV-specific humoral immunity. However, mA3 is expressed in B cells and is evolutionarily related to activation-induced deaminase, an enzyme that controls somatic hypermutation and class-switch recombination in these cells (22). Thus, mA3 may also be involved in shaping the antibody repertoire.

The human *Apobec3* family has been implicated in the control of HIV-1 infection, but HIV-1 encodes Vif, which thwarts the actions of Apobec3G (A3G) and Apobec3F (A3F) (23–26). Compromised A3G/A3F antiviral activity may therefore contribute to the generally poor neutralizing antibody response observed in HIV-1 infection (27). Vif antagonists, if and when they are available, may enhance the generation of effective humoral immune responses against HIV-1. Finally, studies exploring the apparent intrinsic resistance of individuals who are extensively exposed to HIV-1 yet remain uninfected, have genetically mapped this phenotype to chromosome 22q12–13 (12), a location distinguished by a tandem array of the seven human *Apobec3* family members. Genome-wide studies of the entire human *Apobec3* locus, with particular emphasis on functional differences induced by alternative splicing, are clearly merited to fully explore the potential contribution of this gene family to HIV resistance, neutralizing antibody production, and disease progression.
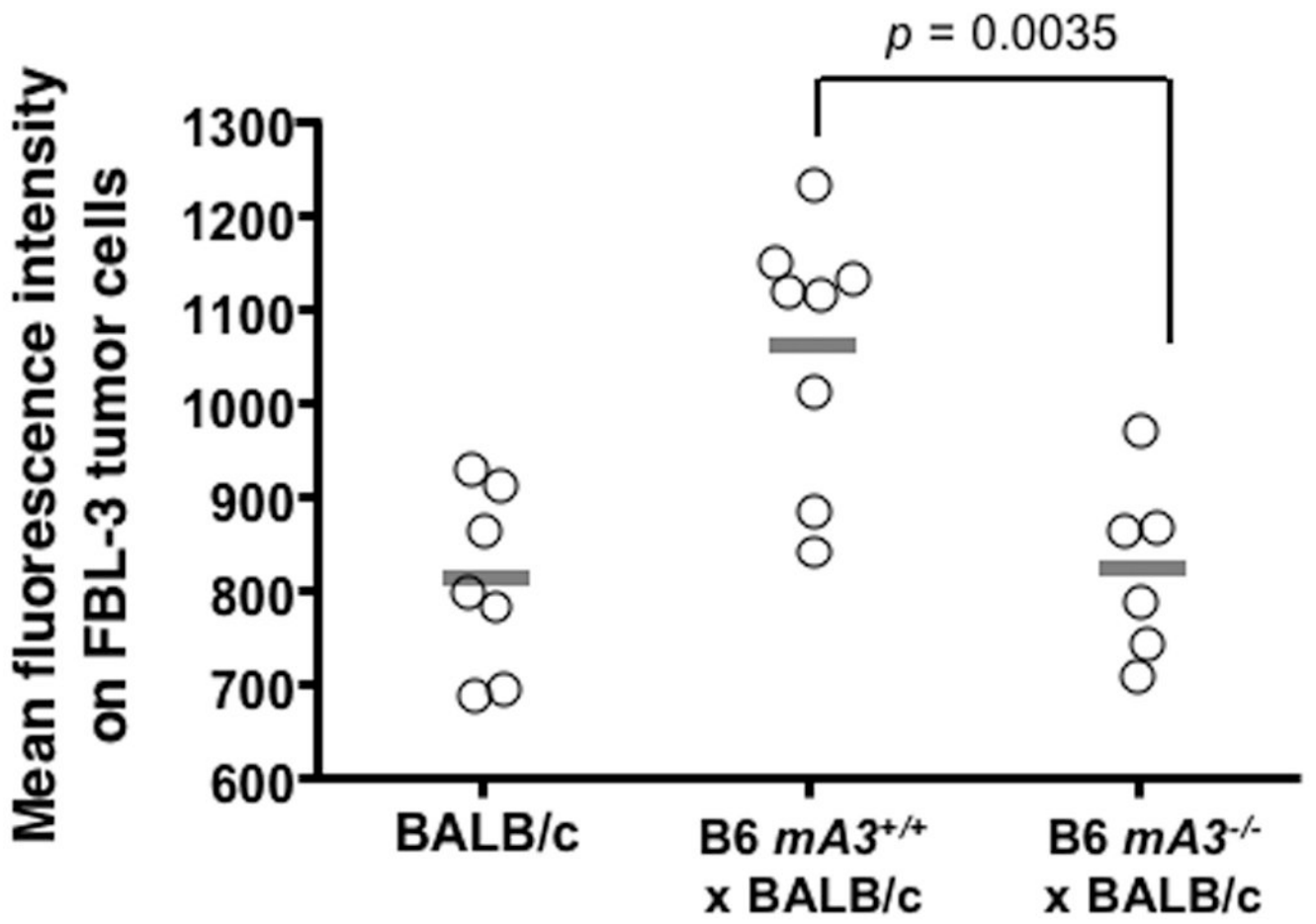
Supplementary Material

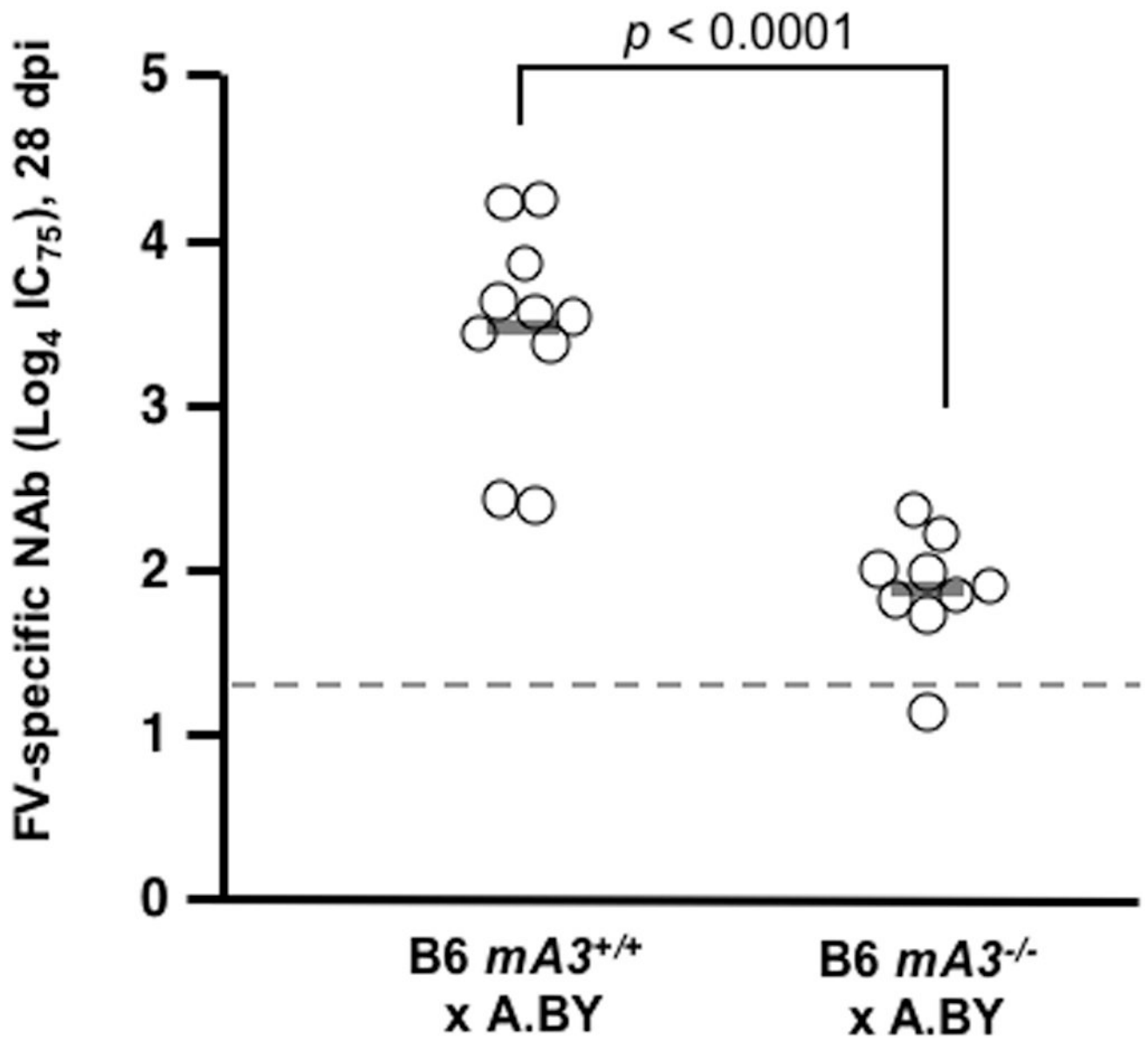Refer to Web version on PubMed Central for supplementary material.

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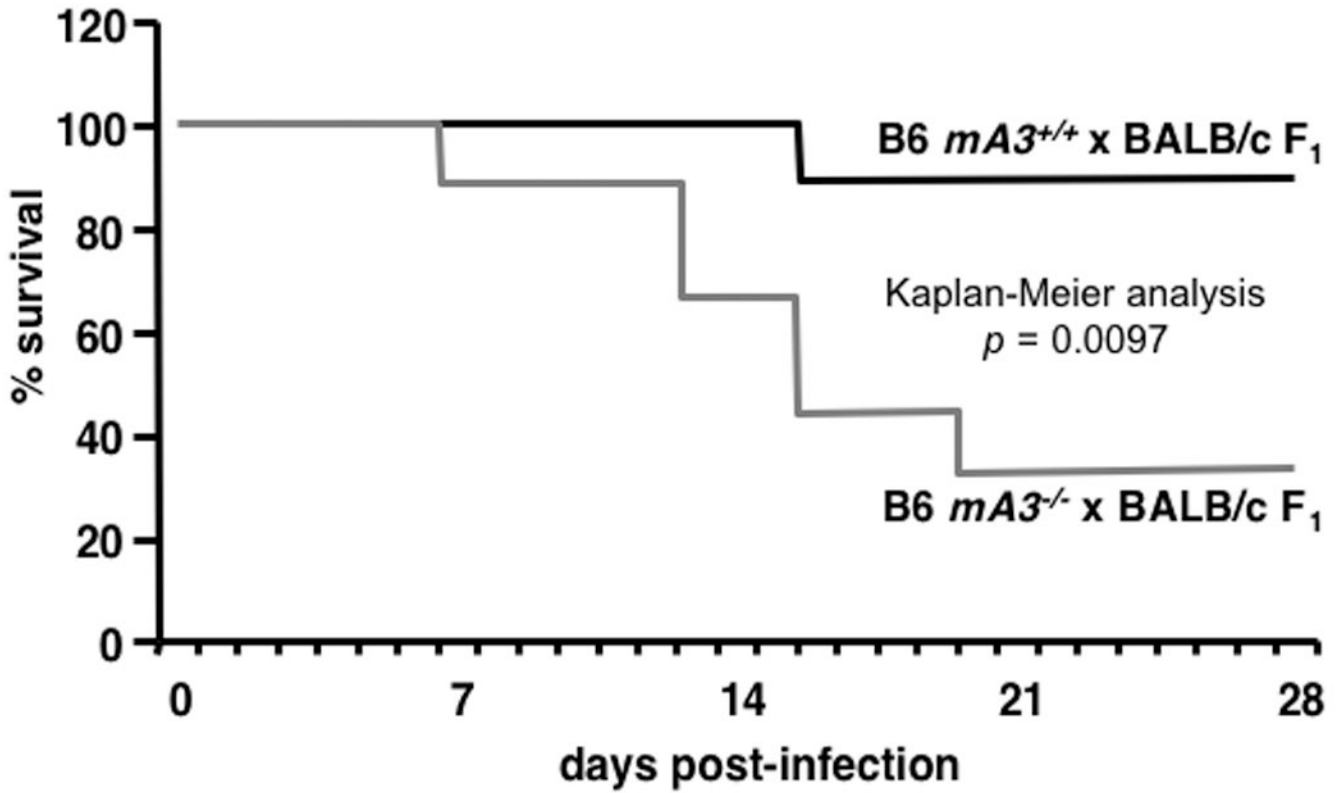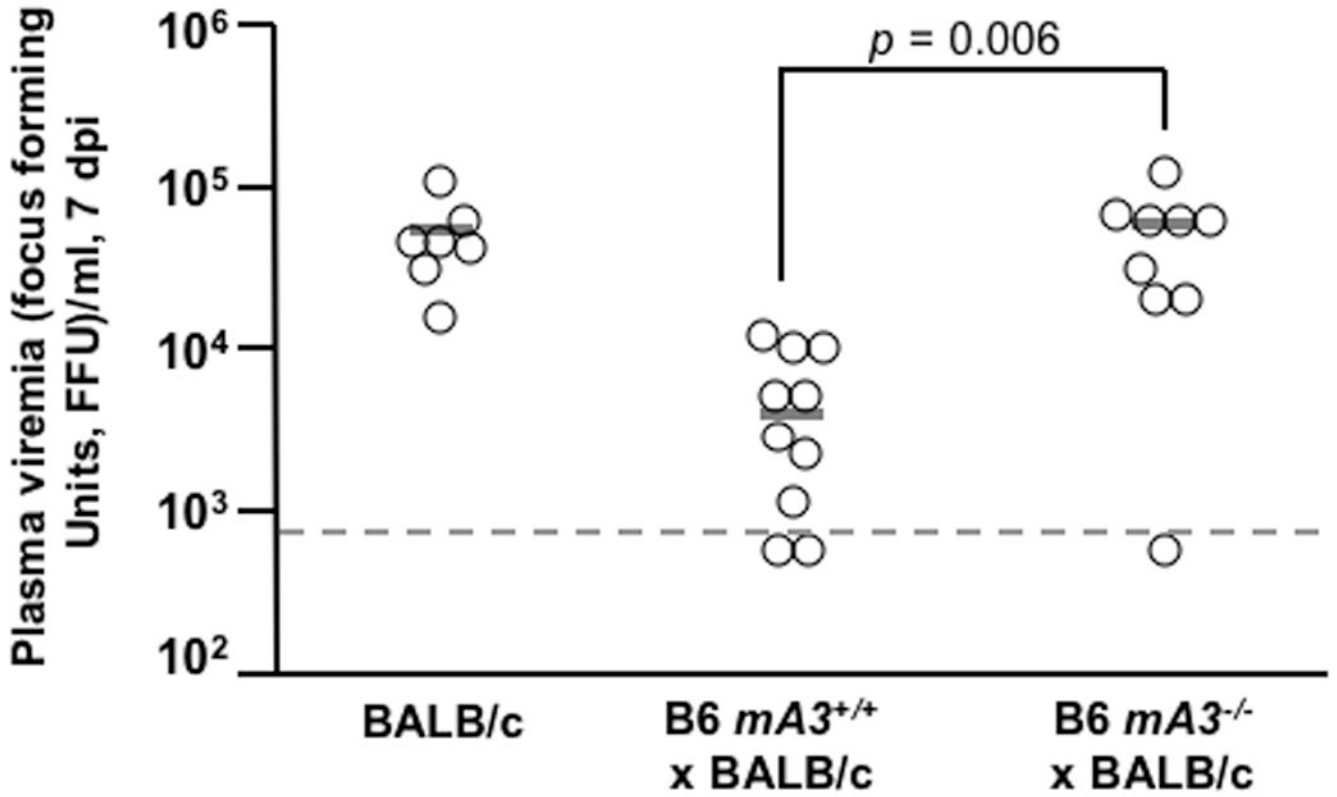
Fig. 1.
The *Rfv3* genetic restriction is mediated by *mA3 in vivo*. (A–C) *mA3* confers the *Rfv3* phenotype in low-recovery *H-2^{b/d}* mice. Congenic *mA3^+* and *mA3^−* (B6 × BALB/c) F_1 mice were infected with 140 spleen focus-forming units (SFFU) of FV. *A*, *mA3* is required for viremic control at an early timepoint (7 dpi); *B*, *mA3* inactivation compromises *Rfv3*-associated survival from FV-induced disease; *C*, *mA3* influences FV-specific IgG production 14 dpi. FV binding IgG was measured by flow cytometry using FV antigen-expressing FBL-3 cells. (D) *mA3* confers the *Rfv3* phenotype in high-recovery *H-2^{b/b}* mice. Congenic (A.BY × BALB/c)F_1 mice were infected with 1400 SFFU of FV. Mean 28 dpi neutralizing antibody (NAb) titers (75% inhibitory concentration, IC_{75}) are significantly lower in *mA3^−* F_1 mice. Open circles indicate individual mice data, gray bars indicate means and dashed lines refer to the assay detection limit. Statistical analyses were performed with two-tailed Student's *t* test.
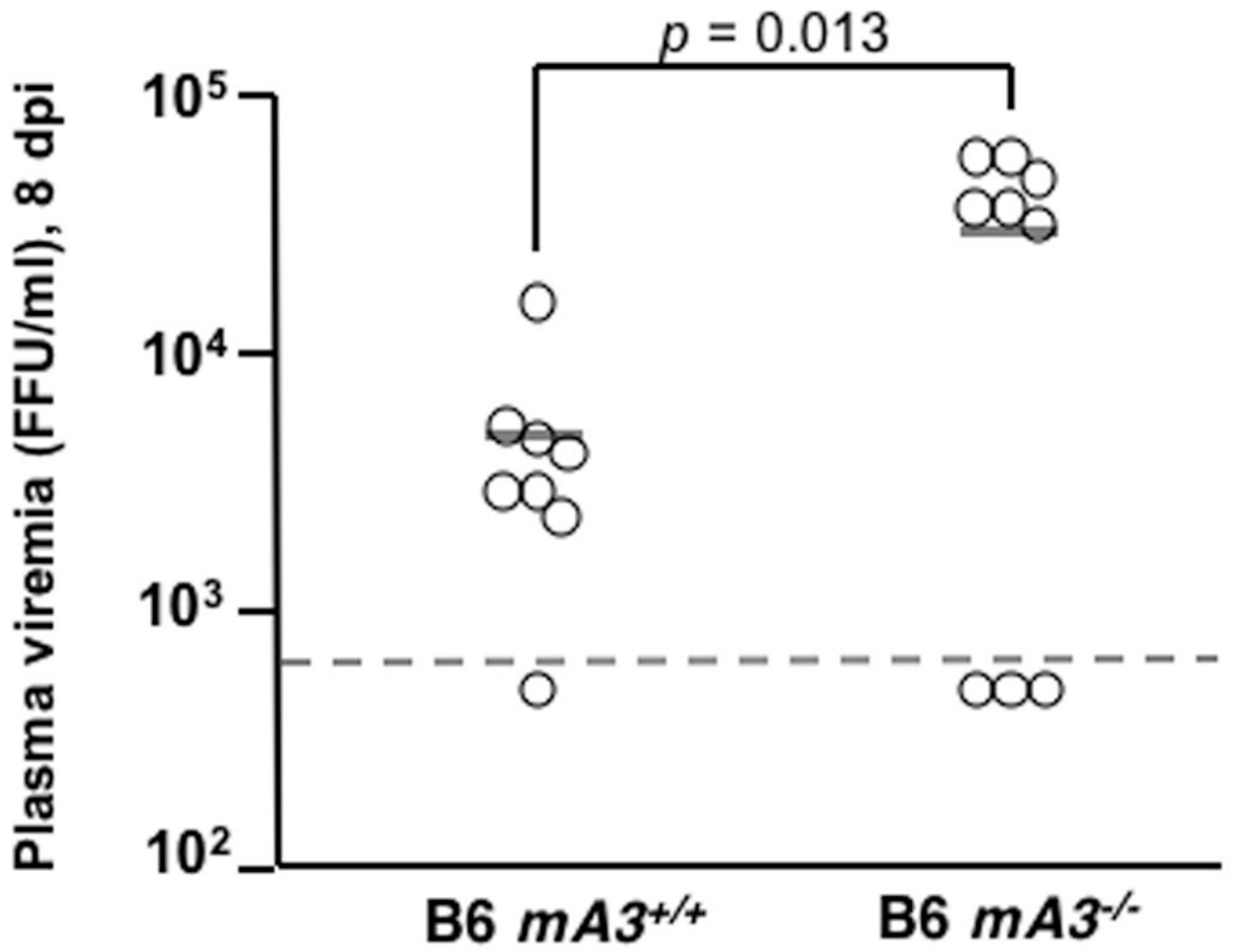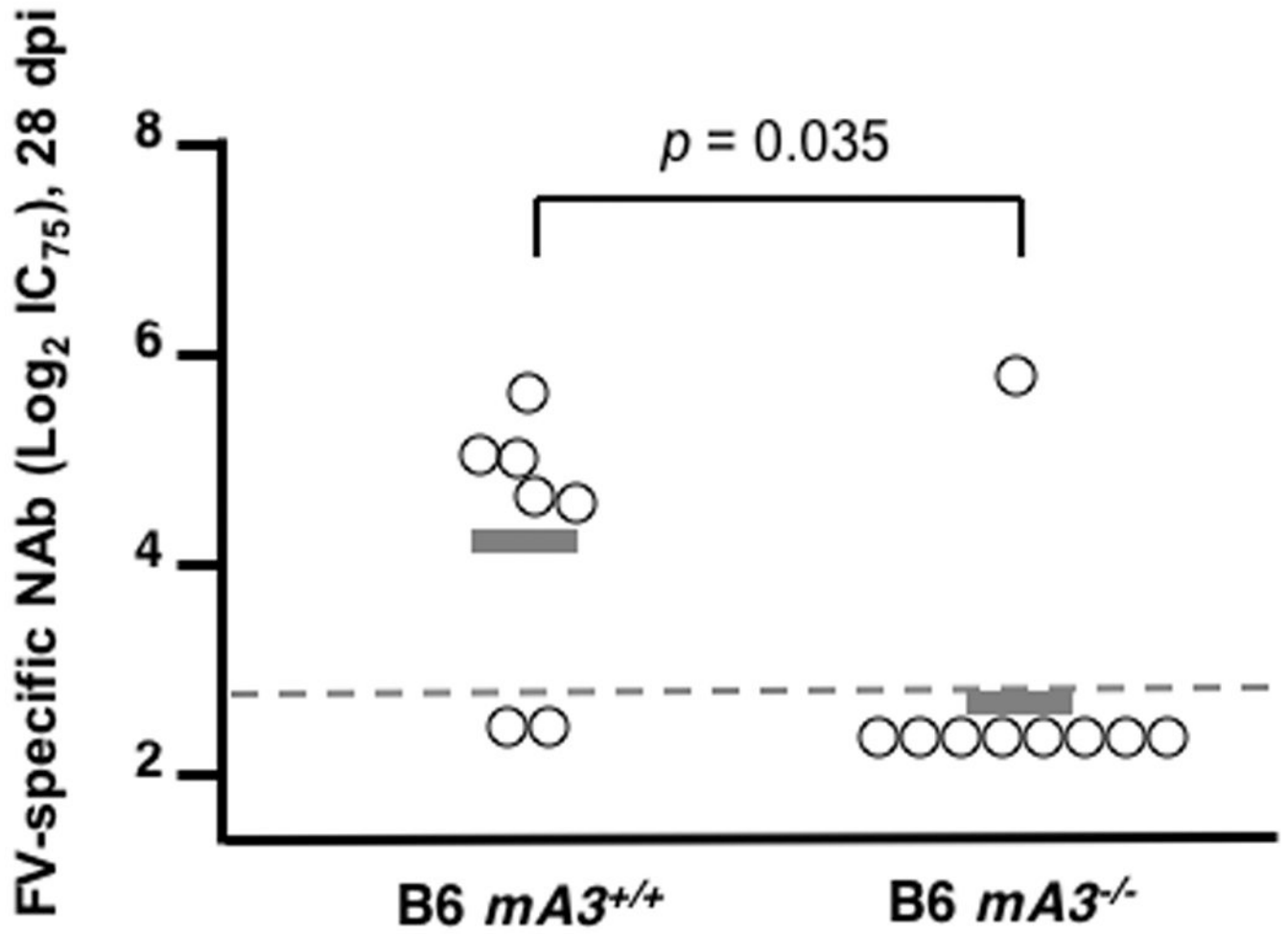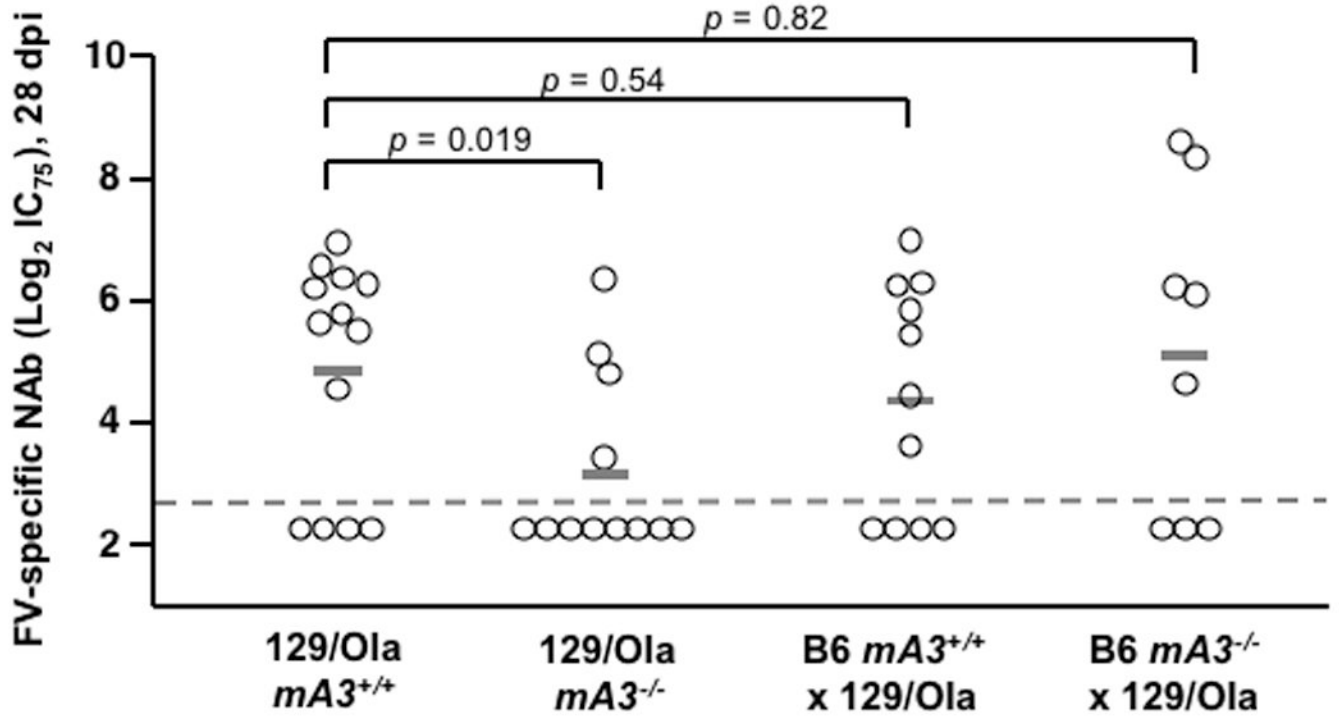
Fig. 2.
mA3 influences FV-specific neutralizing antibody responses in highly resistant (*Fv2$^{r/r}$*) mice.
(A–B) *mA3$^{+/+}$* and *mA3$^{−/−}$* B6 mice (>16 weeks old) were infected with 5000 SFFU of FV.
A, mA3 influences early viremic control at 7 dpi; *B, mA3* is required for FV-specific neutralizing
antibody production 28 dpi. (C) *mA3* influences neutralizing antibody responses in 129/Ola
mice. *mA3$^{+/+}$* and *mA3$^{−/−}$* 129/Ola mice were crossed with *mA3$^{+/+}$* and *mA3$^{−/−}$* B6 mice to
generate F$_1$ offspring. FV-specific neutralizing antibody titers (IC$_{75}$) at 28 dpi with 5000 SFFU
of FV are shown. Additional information is found in the Supplementary Text. Statistical
analysis was performed using a two-tailed Student's *t* test.
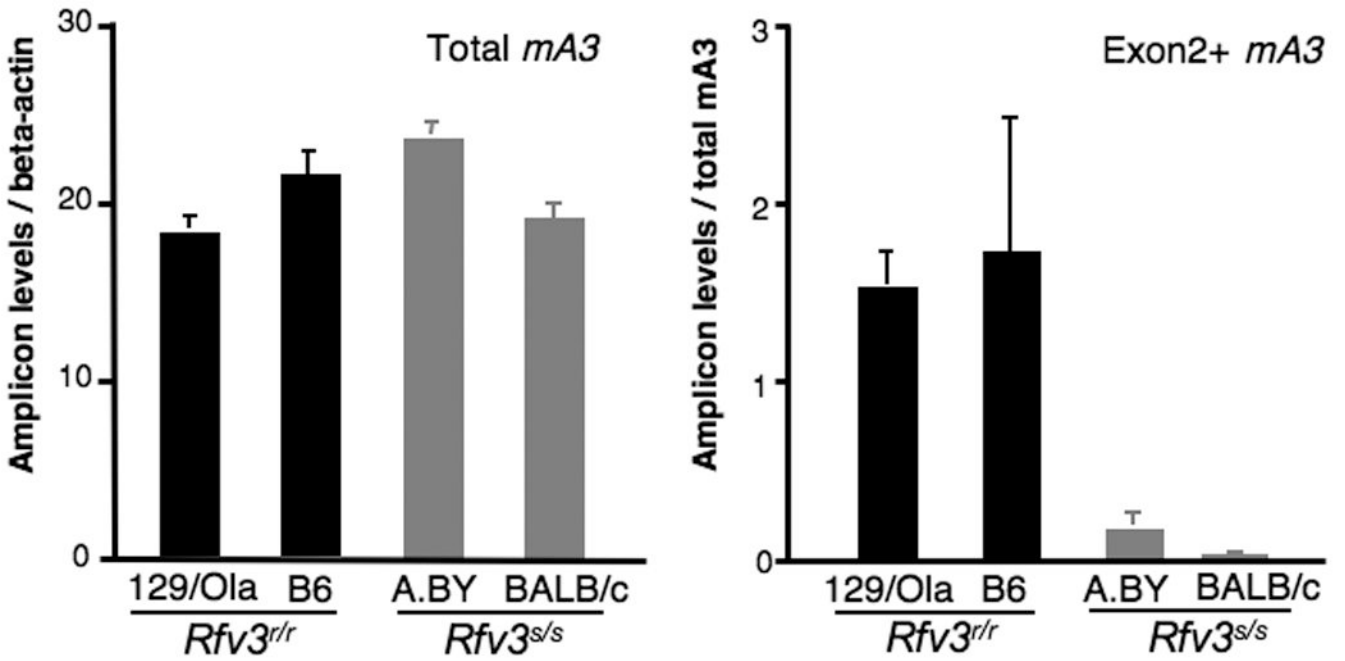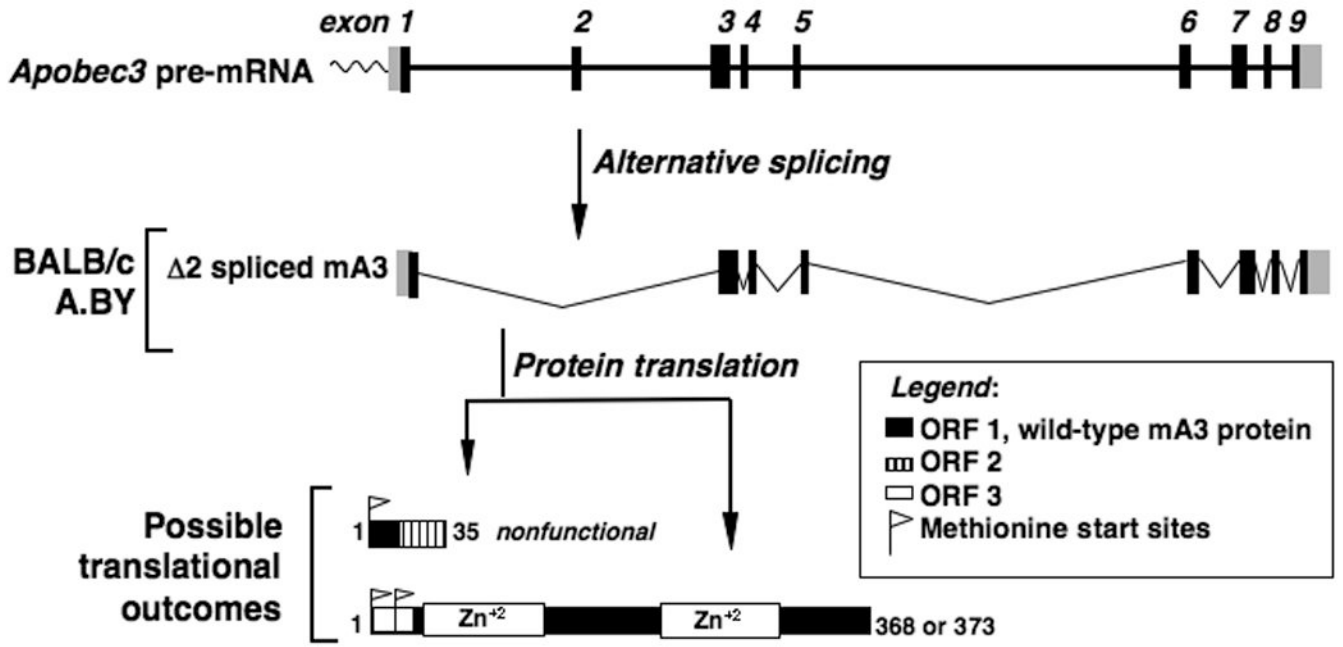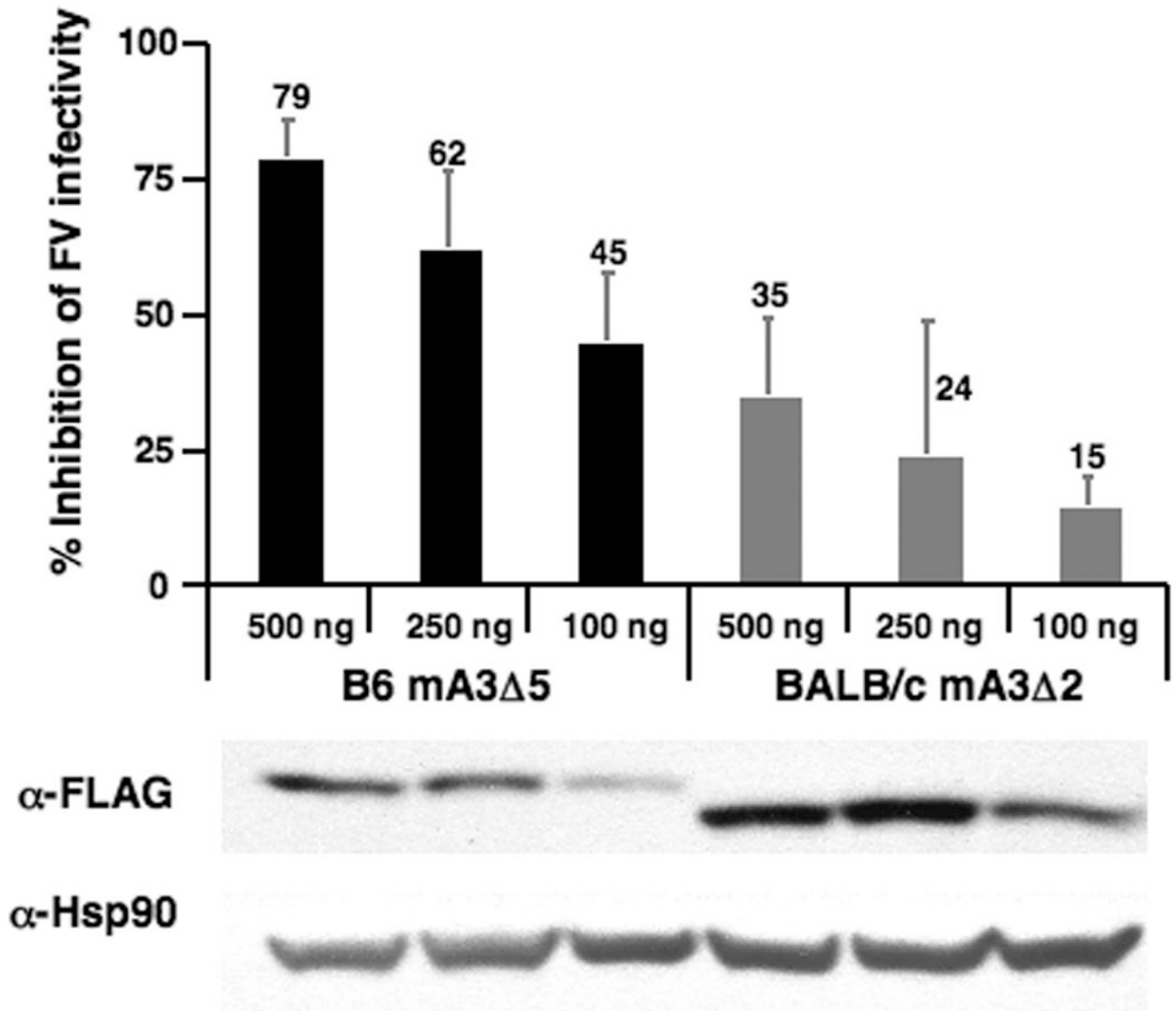
Fig. 3.
Molecular basis of *Rfv3^s* susceptibility. (A) Aberrant *mA3* exon 2 splicing in *Rfv3$^{s/s}$* mice. If the wild-type mA3 open reading frame (ORF1) is used, frameshift-induced translational termination will result in a nonfunctional peptide. However, two start sites in an alternative reading frame (ORF3) may be utilized to translate a mutant mA3 protein with a novel N-terminus and a 56 amino acid deletion. (B) Decreased Exon2$^+$, but not total *mA3* transcripts, in *Rfv3$^{s/s}$* mice. Quantitative RT-PCR of total (left) and Exon2$^+$ (right) *mA3* splenocyte transcripts in *Rfv3$^{r/r}$* and *Rfv3$^{s/s}$* mouse strains were performed. Amplification levels were normalized to beta-actin (left) or total *mA3* (right). (C) Decreased antiviral activity of the mA3 Δexon2 relative to the mA3 Δexon5 spliceform. An FV molecular clone was co-transfected in NIH3T3 cells with FLAG-tagged mA3 constructs. The infectivity of harvested virions were assayed in *Mus dunni* cells and normalized against reverse transcriptase activity. Co-transfections with FLAG vector alone were at 0% inhibition (not shown). Expression of mA3 was assessed by anti-FLAG immunoblotting. Error bars correspond to SD from triplicate experiments.

Table 1

FV infection characteristics of various mouse strains used in this study.

Type	Strain	General FV susceptibility	Viremia	Rfv3	Neutralizing Antibody	H-2[*]	Cell-mediated immunity	Fv2[#]	Splenomegaly induction
Wild-type	C57BL/6 (B6)	resistant	resistant	r/r	high	b/b	high	r/r	no
	BALB/c	susceptible	chronic	s/s	low	d/d	very low	s/s	yes
	A.BY	susceptible	chronic	s/s	low	b/b	high	s/s	yes
	129/Ola[°]	resistant	resistant	r/r	high	b/b	high	r/r	no
F$_1$ hybrids	B6 × BALB/c	susceptible	acute	r/s	high	b/d	low	r/s	yes
	B6 × A.BY	susceptible	acute	r/s	high	b/b	high	r/s	yes

[*] H-2 is the murine major histocompatibility complex (MHC), which dictates cell-mediated immunity against FV (5,15).

[#] Fv2 is a dominant FV susceptibility gene that facilitates splenomegaly induction through aberrant signaling in erythroblasts (4).

[°] FV susceptibility data on 129/Ola were based on results from this study (see Suppl. Text and Fig. 2C). The cell-mediated immune response of this strain was inferred from its H-2 haplotype.