

**Multiple proteins differing between laboratory stocks of mammalian orthoreoviruses affect both virus sensitivity to interferon and induction of interferon production during infection.**

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***Running title:*** Reovirus proteins affecting interferon sensitivity and induction

## Highlights

- Seven proteins differ between two reovirus stocks.
- Differences in sensitivity to interferon depends on both  $\mu 2$  and  $\lambda 2$  proteins.
- Differences in interferon induction depends on both  $\mu 2$  and  $\lambda 1$  proteins.
- Sensitivity to interferon and induction of interferon can be partly separated.
- Multiple reovirus proteins are involved in the control of the interferon response.

## **ABSTRACT**

In the course of previous works, it was observed that the virus laboratory stock (T3D<sup>S</sup>) differs in sequence from the virus encoded by the ten plasmids currently in use in many laboratories (T3D<sup>K</sup>), and derived from a different original virus stock. Seven proteins are affected by these sequence differences. In the present study, replication of T3D<sup>K</sup> was shown to be more sensitive to the antiviral effect of interferon. Infection by the T3D<sup>K</sup> virus was also shown to induce the production of higher amount of  $\beta$  and  $\alpha$ -interferons compared to T3D<sup>S</sup>. Two proteins, the  $\mu$ 2 and  $\lambda$ 2 proteins, were found to be responsible for increased sensitivity to interferon while both  $\mu$ 2 and  $\lambda$ 1 are responsible for increased interferon secretion. Altogether this supports the idea that multiple reovirus proteins are involved in the control of induction of interferon and virus sensitivity to the interferon-induced response. While interrelated, interferon induction and sensitivity can be separated by defined gene combinations. While both  $\mu$ 2 and  $\lambda$ 2 were previously suspected of a role in the control of the interferon response, other proteins are also likely involved, as first shown here for  $\lambda$ 1. This also further stresses that due caution should be exerted when comparing different virus isolates with different genetic background.

***Keywords:*** Reovirus; Interferon; Reverse genetics

## 1. Introduction

In the last few years, it has been observed that so-called wild-type reovirus can slightly vary in sequence from one laboratory stock to the other, even if they are all referred to as type 3 Dearing strain. This could result from random genetic drift or to different experimental conditions for virus growth. Isolation of single plaques, sometimes used as a routine procedure to avoid accumulation of non-infectious viral mutants upon virus propagation has also likely contributed to this situation. Variations between sequences of laboratory stocks are observed when comparing the few cases where complete sequences were reported from a given stock (**Kobayashi et al., 2007; van den Wollenberg et al., 2012; Chakrabarty et al., 2014; Sandekian and Lemay, 2015a**). Also, differences in phenotypic properties between virus stocks were observed in few cases (**Yin et al., 2004; Coffey et al., 2006; Nygaard et al., 2013; Berard et al., 2015**). These variations have led to the denomination of viral subtypes such as T3D<sup>C</sup>, T3D<sup>F</sup>, T3D<sup>H</sup>, T3D<sup>N</sup>, T3D<sup>K</sup>, T3D<sup>S</sup> and T3D<sup>W</sup> (**Sandekian and Lemay, 2015a**).

Previous work has shown that the viral stock encoded by the plasmids used in reverse genetics (herein referred to as T3D-Kobayashi, T3D<sup>K</sup>) do differ from the laboratory virus stock in 7 out of 11 proteins (**Sandekian and Lemay, 2015a**). In the course of this previous work the laboratory wild-type virus stock (herein referred to as T3D-Sandekian, T3D<sup>S</sup>) was reconstructed by site-directed mutagenesis and reverse genetics, in order to allow comparisons with viral mutants derived from this laboratory stock. Although this aspect was not specifically mentioned in the previous publication, it was noticed that the reverse genetics virus (T3D<sup>K</sup>) is significantly more sensitive to interferon than T3D<sup>S</sup>.

A plethora of viruses is presently considered as possible oncolytic viruses for cancer treatment as recently reviewed by many authors (**Ilkow et al., 2014; Miest and Cattaneo, 2014; Pikor et al., 2015; Turnbull et al., 2015**). Among these, reovirus is one of the most advanced in

clinical settings, being currently in phase III. It presents the advantage of exhibiting a natural tropism for cancer cells while being essentially nonpathogenic in adult humans (**Kelly et al., 2009; Harrington et al., 2010; Black and Morris, 2012; Clements et al., 2014; Chakrabarty et al., 2015**). The interferon response often plays a role in determining the ability of a virus to discriminate between cancer cells, frequently exhibiting a reduced interferon response, and normal cells (**Randall and Goodbourn, 2008; Naik and Russell, 2009; Kaufman et al., 2015**). However, examples abound where interferon can still contribute to limiting oncolytic activity, as recently reviewed (**Vaha-Koskela and Hinkkanen, 2014; Ebrahimi et al., 2017**). The original model of reovirus oncolytic activity postulated that a decreased in the interferon-induced protein kinase PKR was responsible for the increased ability of Ras-transformed cells to allow reovirus replication resulting in cell lysis (**Strong et al., 1998**). Further work indicated that the defective interferon secretion of Ras-transformed cells favors cell-to-cell viral propagation in these cells compared to normal cells (**Shmulevitz et al., 2010**). It thus appears to be essential to gain a further understanding of the viral determinants that control induction of the interferon response and the sensitivity of different viral isolates to this response. This could possibly lead to better optimization of viral strains toward oncolytic activity, as many investigators believe to be possible, and as recently reviewed (**Mohamed et al., 2015; Kemp et al., 2016**). This is especially envisaged since the advent of plasmid-based reverse genetics to manipulate the viral genome (**Lemay, 2011; van den Hengel et al., 2013; Stuart et al., 2017**). In the present study, reverse genetics was thus used to introduce each of the T3D<sup>K</sup> gene in the T3D<sup>S</sup> genetic background, either separately or in different combinations, in order to determine which protein(s) is responsible for this difference in interferon response.

While no single protein of T3D<sup>K</sup> in the T3D<sup>S</sup> background was sufficient to reconstitute the full phenotype of sensitivity observed in T3D<sup>K</sup>, a combination of  $\mu 2$  and  $\lambda 2$  was shown to be

both necessary and sufficient. Also, as somewhat expected from previous studies by others (Zurney et al., 2009; Irvin et al., 2012), the  $\mu 2$  protein was shown to be partly responsible for higher levels of interferon induction upon T3D<sup>K</sup> infection compared to T3D<sup>S</sup>. However, an unexpected finding is that  $\lambda 1$  is also responsible for the full level of induction observed. At least three virus proteins from thus appear to be involved in the interferon response in the context of reovirus infection.

## 2. Materials and methods

### 2.1. Cell lines and viruses

L929 mouse fibroblasts were originally obtained from the American type culture collection (ATCC® CCL-1™). The baby hamster kidney cell line (BHK) stably expressing the T7 RNA polymerase (BSR-T7 cells) has been described (Buchholz et al., 1999) and was a generous gift from the laboratory of Dr. John Hiscott (Lady Davis Research Institute, Montréal, Canada). Both cell lines were grown in minimal Eagle medium (MEM) with 5% fetal bovine serum.

Wild-type laboratory stock of reovirus type 3 (T3D<sup>S</sup>) was previously described (Sandekian and Lemay, 2015a,b) and was rescued by reverse genetics following introduction of the appropriate mutations in the plasmids encoding the wild-type virus from Dr. Terry Dermody's laboratory (T3D<sup>K</sup>). Other viruses, harboring various combinations of genes from T3D<sup>K</sup> in the T3D<sup>S</sup> background were obtained by reverse genetics, as described below.

All virus stocks were routinely grown on L929 cells and virus titers determined by TCID<sub>50</sub>, as described (**Danis and Lemay, 1993**). In addition, since some assays used in the course of the work rely on similar cell-killing and lysis ability of the different viruses, serial binary dilutions of each virus was used to infect L929 cells in single wells of a 96-well plates. Cells were incubated for 4–5 days before being fixed and remaining cells stained with methylene blue, as previously described (**Sandekian et al., 2013; Sandekian and Lemay, 2015a**).

### *2.2. Reovirus reverse genetics*

The plasmids corresponding to the 10 genes of reovirus serotype 3 Dearing, T3D<sup>K</sup>, under the transcriptional control of the T7 promoter were originally obtained from the laboratory of Dr. Terence Dermody (Vanderbilt University, Nashville, Tennessee) (**Kobayashi et al., 2007**). The recovery of the wild-type laboratory stock T3D<sup>S</sup> was achieved by first submitting each plasmid to site-directed mutagenesis for each of the gene segment differing between T3D<sup>S</sup> and T3D<sup>K</sup>. Plasmids were then used to recover infectious virus by the improved reverse genetics approach using transfection in BHK cells expressing the T7 RNA polymerase (**Kobayashi et al., 2010**). Rescued viruses were propagated as described before (**Brochu-Lafontaine and Lemay, 2012; Sandekian and Lemay, 2015a,b**).

### *2.3. Determination of interferon sensitivity*

Mouse type I  $\beta$ -interferon was obtained from PBL interferon source. Two different approaches were used to determine interferon sensitivity on mouse L929 cells. Decreased virus titer at near saturating concentration, 200 international units(IU)/ml, was measured by TCID<sub>50</sub>, as previously used. Similarly, interferon sensitivity to varying dilutions of interferon on L929 cells

was determined, also as before (**Sandekian and Lemay, 2015a**). Briefly, binary dilutions of interferon were prepared from 500 IU/ml in single wells of a 96-well plates seeded with L929 cells. Cells were infected with the different viruses at a MOI of 0.05, incubated for 4–5 days before being fixed and stained with methylene blue. Remaining cell-retained stain was solubilized and quantitated, essentially as described before (**Sandekian et al., 2013**), using a BioTEK microplate reader Elx800. Relative cell destruction was determined by comparison with mock-infected cells.

#### *2.4. Determination of interferon induction*

Enzyme-linked immunosorbent assay (ELISA) was performed on different dilutions of mock-infected or infected cell culture medium to determine the concentration of either  $\beta$ -interferon or all subtypes of  $\alpha$ -interferon (Verikine mouse interferon beta and alpha ELISA kit, PBL Assay Science). Results from mock-infected cells were always below detection level. Values were obtained using the microplate reader (BioTEK Elx800).

### **3. Results**

#### *3.1. Generation and characterization of T3D<sup>S</sup>/T3D<sup>K</sup> monoreassortants.*

The differences in sequence between the wild-type laboratory virus stock of reovirus serotype 3 Dearing T3D<sup>S</sup> (originally obtained from ATCC) and that of the serotype 3 Dearing recovered using the plasmid-based reverse genetics system, T3D<sup>K</sup>, were previously reported (**Sandekian and Lemay, 2015a**); for the sake of simplicity this information is repeated herein



(Table 1). A total of 24 nucleotide differences, 21 transitions and 3 transversions, were observed. These resulted in 12 amino acids changes in 7 different viral proteins.

In a first set of experiments, each of these seven T3D<sup>K</sup> genes were separately used to replace their homologous gene in the T3D<sup>S</sup> background. The seven monoreassortant viruses were recovered and propagated with similar final titers. In parallel, in order to avoid discrepancies due to possible differences in cell-killing ability at the same multiplicity of infection, cells were infected with serial binary dilutions of the different viruses, as described in Materials and methods (section 2.1). All viruses had similar replication phenotype, as measured by this approach (data not shown).

The infectious titers of the different viruses were then compared in the absence or presence of interferon at 200 IU/ml (Fig 1). Each virus exhibited resistance to interferon treatment similar to that of T3D<sup>S</sup>. Only viruses harboring the M1 gene (encoding the  $\mu$ 2 protein) and possibly the L2 gene (encoding the  $\lambda$ 2 protein) were slightly more sensitive to interferon but far from the high sensitivity observed for T3D<sup>K</sup>. The different viruses were also examined at various interferon concentrations, as described in Materials and methods (section 2.3). Again, no single gene can reconstitute the full T3D<sup>K</sup> sensitivity phenotype and only M1 had an effect in this assay (data not shown).

### *3.2. Introduction of different T3D<sup>K</sup> gene combinations in T3D<sup>S</sup> background*

The previous results indicated that interferon sensitivity of T3D<sup>K</sup> depends on a combination of more than one gene, and suggested that at least M1 (encoding  $\mu$ 2) could be partially but not solely responsible. Results also suggested that at least one of the L genes is involved. Viruses harboring different gene combinations of T3D<sup>K</sup> in the T3D<sup>S</sup> background were

thus obtained and their sensitivity to interferon first examined by the TCID<sub>50</sub> assay at 200 IU/ml of  $\beta$ -interferon (Fig. 2).

In a first virus, the M2 gene (encoding  $\mu$ 1) and the S1 gene (encoding both  $\sigma$ 1 and  $\sigma$ 1s) were left aside since they were the least likely to be involved in the previous experiment; as expected, the three L genes with M1 and M3 were sufficient to recover the full sensitivity. A series of 4, 3 or 2 gene combinations were then examined. It appears that neither the two M genes (M1+M3) or the three L genes can reconstitute the phenotype. In contrast a combination of the three L genes with M1 (but not M3) reconstitutes the full sensitivity. When examining each of the L genes, the L2 gene together with M1 appears both necessary and sufficient while M1+L3 had an effect but did not reconstitute the full phenotype.

The two most interesting combinations, namely M1+L2 and M1+L3, were thus further examined using various dilutions of interferon (Fig. 3), as described in Materials and methods. Again, in this assay, the M1+L2 combination showed a pattern of sensitivity very close to that of T3D<sup>K</sup>, thus confirming that these two genes are sufficient to reconstitute the full sensitivity phenotype. Interestingly, the M1+L3 combination did increase interferon sensitivity to a similar level at low interferon concentration but this effect was lost at the highest interferon concentrations, approaching that of T3D<sup>S</sup>. Furthermore, when cells were infected at low MOI in the presence of high interferon concentrations (125 IU/ml), the amount of infectious virus produced by the virus harboring the M1+L2 combination and T3D<sup>K</sup> were similarly affected. In contrast, the virus harboring the M1+L3 combination behave essentially as T3D<sup>S</sup> (data not shown). Altogether, the M1+L2 combination thus appears as the main determinant of interferon sensitivity of T3D<sup>K</sup> compared to T3D<sup>S</sup>. The contribution of L3 observed more easily at lower interferon concentrations will be further discussed below.

### *3.3. Induction of interferon by T3D<sup>K</sup> gene combinations in T3D<sup>S</sup> background*

To get a more complete understanding of the contribution of M1, L2 and L3 to the control of the interferon response, and to determine if their effect could be due to differences in induction of interferon, interferon induction following viral infection was next examined. This will also allow to verify if a direct correlation exists between induction of interferon consecutive to virus infection, and the sensitivity of the virus to interferon. Although this was not discussed in previous studies, a higher induction upon T3D<sup>K</sup> compared to T3D<sup>S</sup> infection was expected. In fact, amino acid 208 of T3D<sup>K</sup>  $\mu$ 2 protein is a serine (Table 1) previously shown to result in a decreased ability to control induction of interferon and of the interferon-induced response. In contrast, viruses with proline at this position, as in T3D<sup>S</sup>, were more able to repress the induction (Zurney et al., 2009; Stebbing et al., 2014).

In the present study, the production of interferon at the protein level was directly measured in the culture medium of infected cells using commercially available ELISA assays. This confirmed a close to thousandfold higher  $\beta$ -interferon secretion upon T3D<sup>K</sup> infection compared to T3D<sup>S</sup> (Fig. 4). This was most evident after 15 hours post-infection, although the effect was already detected after 8 hours. These results were confirmed with  $\alpha$ -interferon, although levels were reduced compared to those of  $\beta$ -interferon.

As expected, the monoreassortant harboring solely the T3D<sup>K</sup> M1 gene in a T3D<sup>S</sup> background induced a higher amount of interferon but not as much as the T3D<sup>K</sup> virus itself. Unexpectedly, the sole presence of T3D<sup>K</sup> L3 was able to increase significantly interferon induction and a combination of M1+L3 was both necessary and sufficient to allow full induction

potential as in T3D<sup>K</sup>. In contrast, the addition of L2 to M1 did not increase interferon levels compared to M1 alone.

It thus appears that, for induction of interferon, the M1+L3 combination is necessary and sufficient to reach the full level while L2 has no effect despite its role in sensitivity to exogenously added interferon. The strong combined effect of M1+L3 on interferon induction probably explains why this virus appears more sensitive to interferon. This was most evident at lower exogenous concentrations of interferon, the induced interferon contributing to the overall effect in this case.

#### **4. Discussion**

The importance of the interferon response in the early control of viral infection in various contexts appears to be well established. This is supported by the observations that a wide range of viruses have actually developed more than one protein, in order to control or limit this response within an acceptable limit (**Hoffman et al., 2015; Lopez et al., 2015; Weber-Gerlach and Weber, 2016**). Previous data have suggested that multiple reovirus proteins be involved in the control of induction of interferon or sensitivity to the interferon-induced response (**Imani and Jacobs, 1988; Beattie et al., 1995; Bergeron et al., 1998; Sherry et al., 1998; Zurney et al., 2009; Irvin et al., 2012; Stebbing et al., 2014; Sandekian and Lemay, 2015a**). In the present study, it was further established that at least three viral proteins, namely  $\mu 2$ ,  $\lambda 2$  and  $\lambda 1$  are involved in the control of either induction and/or resistance to interferon.

In previous works from the laboratory (**Rudd and Lemay 2005; Sandekian and Lemay, 2015a; Boudreault et al. 2016**), it was shown that reovirus interferon sensitivity can be

dissociated from interferon induction by a single amino acid substitution in one of the methyltransferase domains of the  $\lambda 2$  mRNA capping enzyme. In contrast, another group has shown that a single amino acid substitution in the viral  $\mu 2$  protein increases the interferon response, contributing to a concomitant increase in interferon sensitivity (**Zurney et al., 2009; Irvin et al., 2012**). In the present study, the  $\mu 2$  protein was further shown to affect the production of interferon and it was confirmed that this could affect interferon sensitivity. However, the higher interferon sensitivity of T3D<sup>K</sup> compared to T3D<sup>S</sup> is also due in part to a single amino acid difference in  $\lambda 2$ , while the higher induction of interferon also relies on a single amino acid substitution, but in  $\lambda 1$ . Altogether, these various observations further stress that, while both phenotypes are interrelated, it is still possible to partly separate induction of interferon production by reovirus infection from the sensitivity of the virus to the induced response. A role of both  $\mu 2$  and  $\lambda 2$  proteins in interferon sensitivity is also consistent with former classic genetics reassortment studies in which genes encoding both proteins were shown to be involved (**Sherry et al. 1998**).

The  $\mu 2$  protein was previously shown to affect interferon response by nuclear accumulation of interferon regulatory factor 9 (**Zurney et al., 2009**). In addition, this protein interacts with microtubules and affects the morphology of viral inclusions (**Mbisa et al., 2000; Parker et al., 2002; Miller et al., 2004; Yin et al., 2004**). More recently, it was established that this association affects virus assembly and modulates the efficiency of genome assembly, thus resulting in differences in the percentage of infectious virions produced upon infection (**Ooms et al., 2012; Shah et al., 2017**). It remains to be determined if this could also independently contribute to differences in host-cell response and virus sensitivity to interferon.

The difference in  $\lambda 2$  single amino acid difference between T3D<sup>K</sup> and T3D<sup>S</sup> is in the protein domain assigned to one of the protein's methyltransferase activity involved in mRNA capping (**Reinisch et al. 2000; Bujnicki and Rychlewski 2001**), as was also the case in the previously isolated interferon sensitive mutant (**Sandekian and Lemay, 2015a**). The amino acid substitutions respectively at positions 504 and 636 are in fact closely located in the crystallographic structure of the viral core (PDB1EJ6). In both cases, the substitution is located at the extreme outside end of the  $\lambda 2$  turret (Fig. 5); although this position is not in direct contact with putative catalytic sites, it is tempting to speculate that it indirectly affects either cap methylation or viral mRNA exit. As mentioned by others (**Mohamed et al. 2015**), it will clearly be of interest to further examine the nature of the 5'-end of the viral mRNAs produced by these different viruses. The importance of the 2'-O-methylation of the first mRNA nucleotide has been clearly established in the last few years. For many viruses, abolished or decreased 2'-O-methylation results in increased induction of the response, increased sensitivity to this response, or both (**Daffis et al., 2011; Zust et al., 2011; Szretter et al., 2012; Habjan et al., 2013; Kimura et al., 2013; Ma et al., 2014; Menachery et al., 2014; Chang et al., 2016; Devarkar et al., 2017**). The 2'-O-methylation appears as a major discriminatory factor between cellular and foreign mRNA as a target of innate immune response (**García-Sastre, 2011; Hyde and Diamond, 2015; Leung et al., 2016**).

The involvement of the  $\lambda 1$  protein in the level of interferon induction came as a surprise finding in the course of this study. It remains to be determined if the protein can repress interferon signaling in T3D<sup>S</sup>, a property that is lost in T3D<sup>K</sup>, or if the T3D<sup>K</sup> protein positively contribute to the interferon induction. Alternatively, a defect in the T3D<sup>K</sup> protein could affect the viral mRNA synthesis or cap structure, thus indirectly contributing to augmenting interferon

induction. Although the exact contribution of the two proteins to RNA synthesis and capping is not yet completely understood, purified  $\mu 2$  and  $\lambda 1$  share nucleotide and RNA triphosphatase activities (**Bisaillon and Lemay, 1997; Bisaillon et al., 1997; Kim et al., 2004**). This could suggest an impact of one or more of these activities in the production of viral mRNAs as interferon inducers. Interestingly, the single amino acid difference between T3D<sup>K</sup> and T3D<sup>S</sup> is among the 5 amino acids difference previously noted between T3D and serotype 1 T1L virus. These differences are affecting NTPase activity associated with the viral core and synthesis of the viral mRNA and/or cap structure (**Harrison et al., 1999**). Recently, viral-triggered ATP release by vesicular stomatitis virus was found to be associated with interferon induction (**Zhang et al., 2017**), the relative activity of a viral ATPase is thus potentially relevant in this context and deserves further study.

Altogether, the data reported herein demonstrate that different reovirus proteins, by themselves or in combinations, could affect either induction of interferon or sensitivity of a given virus to the interferon response. It should thus be possible to combine mutant forms of different viral proteins in order to modulate both the induction of interferon and/or interferon response, and sensitivity to the response. As previously mentioned, this could well be of importance to optimize oncolytic viral strains to different tumor cell types (**Randall and Goodbourn, 2008; Naik and Russell, 2009; Kaufman et al., 2015; Vaha-Koskela and Hinkkanen, 2014; Ebrahimi et al., 2017**). Virus replication in the absence of exogenously added interferon was also briefly examined. As somehow expected it was noticed that the T3D<sup>K</sup> virus has an approximately three to fivefold lower viral titers than T3D<sup>S</sup> at both 24 and 48-hour post-infection in conditions allowing viral propagation (Fig. S1). Interestingly both the interferon-sensitive and interferon-inducing reassortants exhibited essentially the same replicative phenotype as T3D<sup>K</sup>.

Further study will be needed to establish the importance of both phenotypes in viral replication in different cell types and *in vivo* using these viruses.

Overall, the results obtained in the present study also further stress that one should be very careful while interpreting previous data using virus stocks from different laboratories. The advent of plasmid-based reverse genetics (**Kobayashi et al. 2007, 2010**), and recent progress resulting in increased efficiency of the procedure (**Eaton et al. 2017; Kanai et al. 2017**) should now allow all laboratories to work with well genetically characterized viruses in order to avoid possible discrepancies due to unknown genetic changes in viruses used.

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## Figure Legends

**Fig. 1.** Interferon sensitivity of virus monoreassortants. Genes from reovirus reverse genetics virus T3D<sup>K</sup> were separately introduced in the genetic background of the T3D<sup>S</sup> laboratory virus stock using reverse genetics, as described in the text. Virus titers in the absence or presence of 200 IU/ml of  $\beta$ -interferon were compared, as described in the text. Fold reductions in titers due to interferon presence are presented on a logarithmic scale.

**Fig. 2.** Interferon sensitivity of multiple reassortants. Viruses harboring various gene combinations of T3D<sup>K</sup> in the T3D<sup>S</sup> background were compared with viruses harboring the full gene complement of either parental virus. Results are presented as fold decrease in the presence of interferon, on a logarithmic scale, as in figure 1. Error bars indicate the highest value obtained in cases where two independent experiments were performed.

**Fig.3.** Sensitivity of double reassortant viruses to different interferon concentrations. The double reassortant viruses harboring M1+L2 or M1+L3 of T3D<sup>K</sup> in the T3D<sup>S</sup> background were examined by the interferon dilution assay. The average of three independent experiments is presented as the relative cell destruction, compared to control mock-infected cells, determined by optical density of methylene blue stain, as described in Materials and methods (section 2.3).

**Fig. 4.** Interferon induction by viral reassortants. L929 cells were infected at a MOI of 20 TCID<sub>50</sub> units per cell. Infected cells media were recovered after 8 or after 15 hours post-infection and directly used in commercial ELISA assay, as described in Materials and methods, for either  $\beta$ -

interferon (left panel) or  $\alpha$ -interferon (right panel) quantitation. Results are presented as the average of two independent experiments with error bars representing the standard error of the mean.

**Fig.5.** Positions of  $\lambda$ 2 amino acid substitutions on the crystal structure (PDB1EJ6). The position of the previously described substitution in the interferon-sensitive P4L-12 virus (amino acid 636) is indicated as a green sphere while the amino acid difference between T3D<sup>S</sup> and T3D<sup>K</sup> (amino acid 504) is indicated as a red sphere. The methyltransferase domain (amino acids 434 to 691) is in cyan. The image, at left, shows a side view of the protein, the virion-anchored region being on the left. The image at right shows a top view of the molecule from the outside of the virion.

Images were obtained using the PyMOL Molecular Graphics System Version 2.0.0.

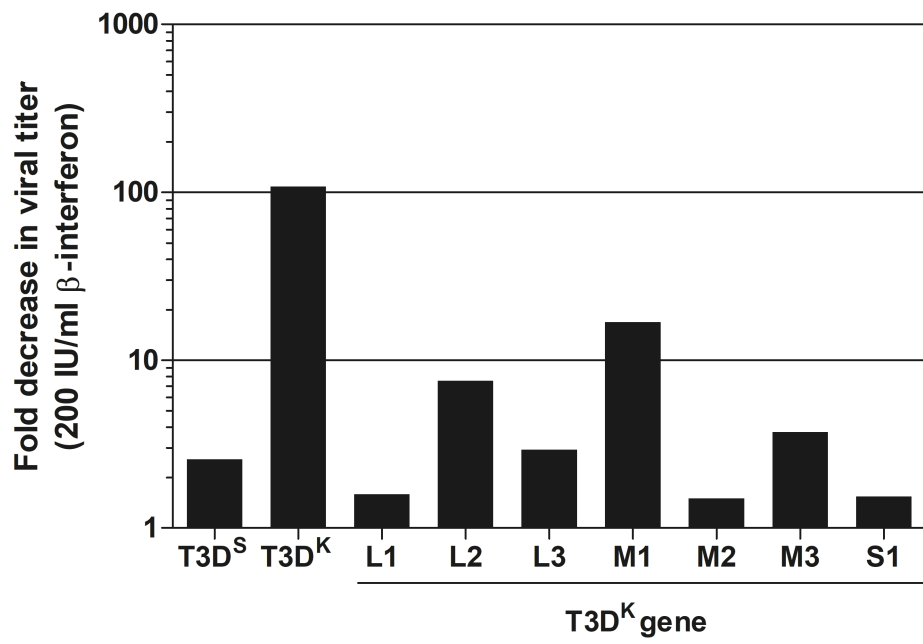
**Fig.S1.** Replicative ability of parental and reassortant viruses. The two parental viruses T3D<sup>S</sup> and T3D<sup>K</sup>, as well as the interferon-sensitive reassortant (harboring the M1+L2 gene of T3D<sup>K</sup> in the T3D<sup>S</sup> background) and interferon-inducing reassortant (harboring the M1+L3 gene of T3D<sup>K</sup> in the T3D<sup>S</sup> background), were examined for their replicative ability. Each virus was used to infect L929 cells at a multiplication of infection of 0.05 TCID<sub>50</sub> unit per cell and cells incubated for either 24 or 48 hours. Cells and tissue culture were recovered, submitted to three cycles of freeze-thaw (−80 °C to room temperature) and infectious titers determined by TCID<sub>50</sub> on L929 cells. Results are presented as the average of two experiments with error bars representing the standard error of the mean.

**Table 1**Sequence differences between T3D<sup>S</sup> and the reverse genetics virus T3D<sup>K</sup>

Gene (protein)	Nucleotides			Amino acids		
	T3D <sup>S</sup>	T3D <sup>K</sup>	Position	T3D <sup>S</sup>	T3D <sup>K</sup>	Position
L1 ( $\lambda$ 3)	A	G	1440	--	--	--
	G	A	2205	--	--	--
	G	A	2973	--	--	--
	G	A	3167	S	N	1048
	T	C	3594	--	--	--
L2 ( $\lambda$ 2)	A	G	1524	E	G	504
	T	C	3079	--	--	--
L3 ( $\lambda$ 1)	A	G	635	--	--	--
	G	T	1038	--	--	--
	C	T	1534	S	I	500
M1 ( $\mu$ 2)	C	T	635	P	S	208
	A	G	1038	Q	R	342
	G	A	1534	--	--	--
M2 ( $\mu$ 1)	T	C	943	V	A	305
	C	T	1173	--	--	--
	A	G	1374	T	A	449
M3 ( $\mu$ NS)	G	A	556	E	K	180
	A	C	1390	--	--	--
	T	C	2132	V	A	705
S1 ( $\sigma$ 1)	T	C	438	--	--	--
	T	C	758	I	T	249
	C	A	770	S	Y	253
	G	A	1234	A	T	408
S1 ( $\sigma$ 1s)	--	--	--	--	--	--
S2 ( $\sigma$ 2)	T	C	702	--	--	--
S3 ( $\sigma$ NS)	--	--	--	--	--	--
S4 ( $\sigma$ 3)	--	--	--	--	--	--

Accession numbers for T3D<sup>S</sup> are KP208804 to KP208813; accession number for T3D<sup>K</sup> are EF494435 to EF494444.

### T3D<sup>S</sup> Background



### T3D<sup>S</sup> Background

