Molecular Therapy
Methods & Clinical Development
Original Article

Characterization of the function of Adenovirus L4 gene products and their impact on AAV vector production

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Efficient manufacturing of recombinant adenovirus-associated virus (AAV) vectors is critical to the successful development of genomic medicines. We attempted to optimize AAV vector production in a producer cell line platform. In this system, helper functions required for AAV replication and production are provided via infection with a replication-competent wild-type Adenovirus. To evaluate strategies for the reduction of replication and packaging of adenovirus and to understand the interplay of recombinant AAV and the helper virus during AAV vector production, wild-type adenovirus was compared to a mutant (Ad5ts149) containing a temperature-sensitive mutation in the DNA polymerase gene. Infection of a producer cell line with Ad5ts149 at the restrictive temperature reduced recombinant AAV titer and altered the pattern of AAV protein expression. Further investigation revealed that the adenoviral late L4-22K/33K gene products regulated both AAV *rep/cap* gene transcription and splicing of the *rep/cap* transcripts. Furthermore, the L4-33K gene products were found to impact AAV production in both the producer cell line and transient transfection platforms. Optimization of Adenovirus L4-22K/33K expression to facilitate efficient expression and splicing of AAV *rep/cap* transcripts therefore represents a unique opportunity to optimize AAV vector production.

INTRODUCTION

Viral vectors based on adeno-associated virus (AAV) have been employed in over 130 *in vivo* gene therapy clinical trials.[1,2] Recombinant AAV vectors (rAAV) have found widespread use due to their safety (e.g., low immunogenicity, lack of pathogenicity) and their ability to promote robust and persistent gene expression in a variety of tissues. AAV vectors are commonly manufactured via transient transfection of HEK293 cells with two or three plasmids containing the AAV *rep/cap* expression cassette, the vector genome including an expression cassette encoding the gene of interest, and the adenoviral helper genes.[3,4] Alternative manufacturing platforms rely on co-infection of insect or mammalian cells with recombinant baculoviruses or herpes simplex viruses, respectively. AAV producer cell lines (PCLs) which incorporate the AAV (*rep* and *cap* genes) and vector sequences (AAV2 inverted terminal repeats and transgene expression cassette) required to support AAV vector manufacturing consistently produce high-quality vectors and are a scalable, low-cost, highly productive platform.[5] Therefore, they represent an effective alternative to these commonly used systems.

Production of AAV in the PCL platform is accomplished via infection with wild-type (WT) human Adenovirus 5 (wtAd5) helper virus. E1A, E1B, E2A, E4orf6, and the viral associated (VA) RNAs comprise the minimal set of adenoviral genes required for rAAV replication and packaging.[6,7] The E1A proteins control expression of the Rep78/68 proteins by transcriptionally activating the AAV p5 promoter,[8–10] while the complex composed of the E1B55K protein and the E4orf6 gene product plays a role in both AAV mRNA transport[11,12] and inhibition of the cellular DNA damage response pathway.[13] Both E4orf6 and the E2A single-stranded DNA-binding protein (DBP) support AAV DNA replication.[14–16] The VA RNAs promote AAV protein synthesis by inhibiting the activity of double-stranded RNA-activated kinase (PKR) and interfering with PKR-mediated shutdown of cellular translation, a feature of the host cell defense against viral infection.[17,18] The VA RNAs have also been reported to work in conjunction with the DBP to promote AAV capsid protein synthesis.[19]

One drawback of the PCL platform is that during the manufacturing process, replication and packaging of the helper wtAd5 occurs alongside that of the rAAV, leading to potential competition for viral factors and cellular resources. Furthermore, the presence of wtAd5 as a process impurity poses a safety risk requiring implementation of robust operations for viral clearance. Ad5ts149, an adenovirus mutant that harbors a temperature-sensitive mutation within the E2B gene encoding the DNA polymerase,[20,21] may be used to provide helper functions in place of wtAd5. At the non-permissive temperature (39°C–40°C), Ad5ts149 is deficient in viral DNA replication and synthesis of late viral proteins.[20] However, because the minimal

Received 14 June 2024; accepted 31 October 2024;
https://doi.org/10.1016/j.omtm.2024.101370.

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adenoviral helper functions are thought to be provided by early gene products, replication of the helper adenovirus may not be required to support production of rAAV.[22] Indeed, early co-infection experiments suggested that Ad5ts149 could support WT AAV2 viral replication and packaging in KB cells.[20,21] In addition, it has been reported that in the context of an A549-based PCL, Ad5ts149 infection at the semi-permissive temperature of 37°C can support the production of rAAV.[23] These studies suggest that use of Ad5ts149 can reduce the production of the helper adenovirus in the context of the PCL platform.

Recent publications, however, have reported that proteins encoded by adenoviral late genes also play a role in AAV vector production. In an AAV packaging cell line, the adenovirus L4-22K/33K gene products were shown to be essential for amplification of the AAV *rep* and *cap* genes and, consequently, for optimal Rep and Cap protein expression.[24] Adsero *et al.* observed that the L4 region overlaps with the E2A promoter sequence, a component of the adenoviral helper plasmid used in HEK293/transfection-based production systems.[25] The authors demonstrated that in the transfection platform, the L4-22K protein is required for rAAV production, and the L4-33K gene product contributes to rAAV yield. The L4-22K/33K proteins have been reported to play a role in various aspects of the adenoviral life cycle, including regulation of early and late gene expression,[26–29] splicing of viral mRNAs,[30–32] and packaging of the viral genome.[33,34] Considering the above evidence, we sought to explore the possibility of using Ad5ts149 in the PCL context and to understand the role of the L4 gene products in AAV production.

Here, we report a significant reduction in AAV vector production at the non-permissive temperature when Ad5ts149 is used as a helper in the context of a model PCL. Lower titers were accompanied by a reduction in the levels of both the Cap protein and the Rep proteins derived from spliced mRNAs (Rep68 and Rep40). Additional analyses confirmed a defect in AAV transcript splicing. At the restrictive temperature, Ad5ts149 infection resulted in the expression of the minimal essential adenoviral helper genes at levels similar to wtAd5. In contrast, expression of the adenoviral late gene products was decreased. By utilizing dicer-substrate small interfering RNA (DsiRNA) knockdown and overexpression of the L4 genes, we confirmed that the L4-22K/33K gene products play a novel role in the regulation of AAV *rep/cap* gene expression and are required for efficient production of rAAV in the AAV PCL platform.

RESULTS

Suppression of adenovirus replication with the adenovirus variant Ad5ts149 reduces AAV vector production

The AAV PCL platform requires infection with wtAd5 to induce rAAV production, a process in which the adenoviral helper is co-replicated and produces viral progeny in conjunction with the AAV vector. In this study, we evaluated the effect on production of using Ad5ts149, a temperature-sensitive mutant, to provide helper function in place of wtAd5. PCL cells were infected with wtAd5 or Ad5ts149 at either the semi-permissive (37°C) or restrictive (39°C) temperature. Production of rAAV and adenovirus was quantified as nuclease-resistant vector genome copies per milliliter using culture samples treated with nuclease as the template for qPCR as described in materials and methods. Replication of rAAV and adenovirus were measured as total viral genome copies per milliliter in a similar manner, except nuclease treatment was omitted in the sample processing procedure. Results from different conditions were normalized to that of wtAd5 infection with an MOI of 5 at 37°C. As expected, at the semi-permissive temperature, replication and production of Ad5ts149 was reduced by 70%–80% relative to wtAd5, while at the restrictive temperature, Ad5ts149 replication and production was 2- to 3-logs lower than at 37°C (Figures 1A and 1B; Table S1). Increasing the MOI at 39°C resulted in an apparent dose-dependent increase in both replication and production of Ad5ts149. In contrast, with wtAd5 infection, only a slight decrease in replication and production was observed at 39°C relative to 37°C.

Ad5ts149 supports rAAV production at a level comparable to wtAd5 at 37°C, despite the 4- to 5-fold reduction in adenoviral production and replication at the semi-permissive temperature (Figures 1C and 1D; Table S1). However, at the restrictive temperature of 39°C, production of rAAV with Ad5ts149 was significantly reduced. At an MOI of 4, rAAV titer was 70-fold lower than that of wtAd5 infection at 37°C (Figure 1C). As was observed with the Ad5ts149 helper virus, at 39°C rAAV exhibits an apparent dose-dependent increase in production with increasing MOI of Ad5ts149. In contrast, we observed only a 4-fold reduction in replication of the AAV vector genome at the lowest MOI of Ad5ts149 at 39°C (Figure 1D). Overall, these results suggest that rAAV vector production from producer cells is dependent upon efficient replication and production of the helper virus. Notably, however, when replication of the helper virus was limited (e.g., Ad5ts149, MOI of 4 at 39°C), rAAV genome replication was largely maintained. Therefore, other features of vector production, such as AAV capsid expression and/or genome packaging, likely explain the drop in rAAV production under these conditions.

Suppression of Adenovirus replication and production altered AAV *rep/cap* transcription, splicing and protein expression

To address why rAAV production was reduced with Ad5ts149 infection at the restrictive temperature, we evaluated AAV Rep and Cap protein expression. While Cap protein expression in wtAd5-infected cells was similar at 37°C and 39°C, Ad5ts149 infection at 39°C resulted in significantly lower expression of Cap protein (Figures 2A and 2B). Similarly, while Rep expression remained similar overall for the wtAd5 infection at the two temperatures, a different pattern of Rep expression emerged following infection with Ad5ts149 (Figures 2C–2E and S1). At 37°C, we observed an increase in expression of both Rep78 and Rep68 compared to wtAd5, with the increase in Rep68 being particularly notable. When we compared Rep protein expression in Ad5ts149-infected cells at the two temperatures, we observed a clear reduction in expression of the Rep68 and Rep40 proteins (which are derived from spliced transcripts) at 39°C in addition to a slight increase

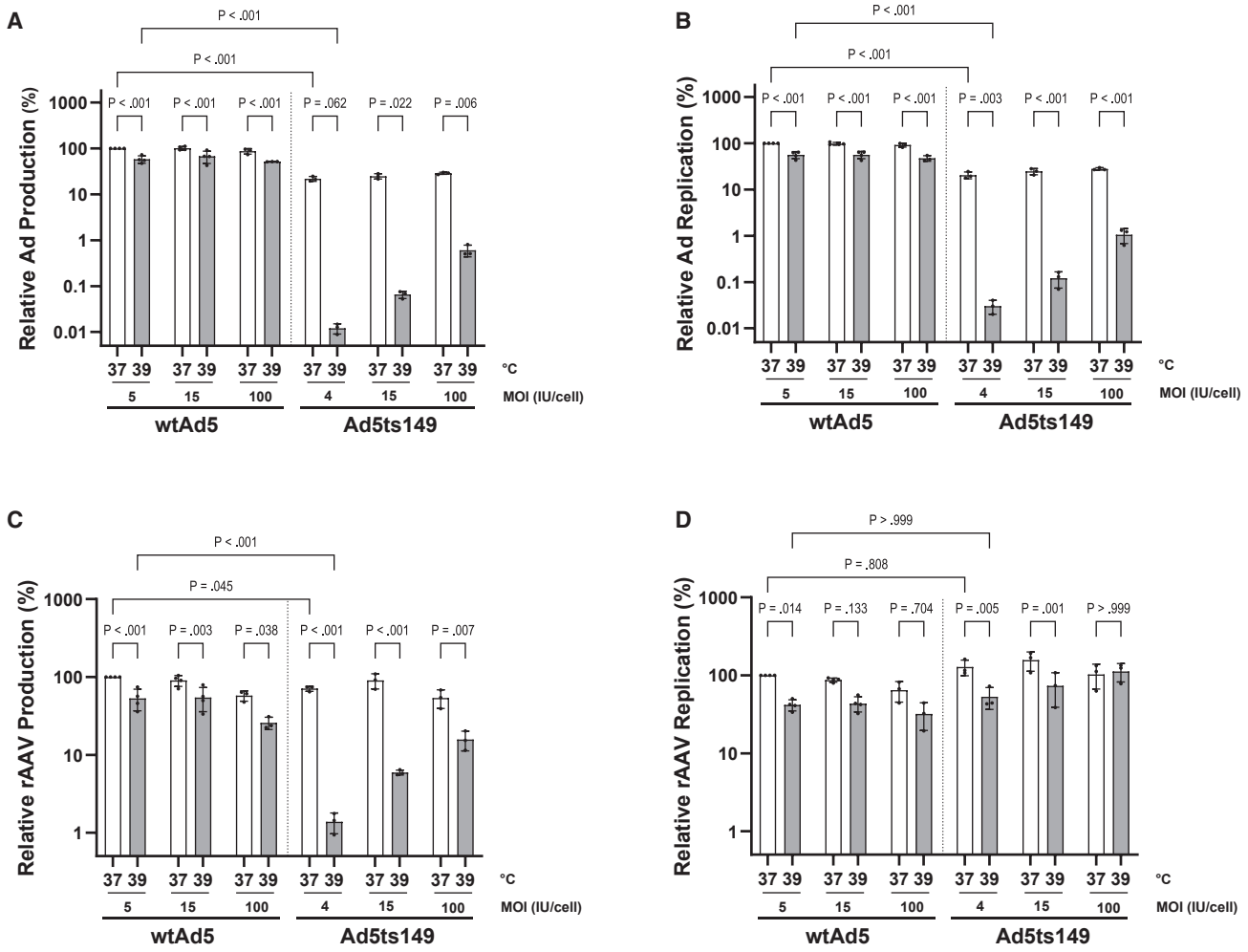


Figure 1. Comparison of wtAd5 and Ad5ts149 for rAAV and adenovirus replication and production in AAV producer cells

A model PCL was infected with wtAd5 or Ad5ts149 at MOIs indicated and harvested at 48 hours post infection (hpi) for analyses. Relative adenovirus production (A), adenovirus replication (B), rAAV production (C), and rAAV replication (D) were quantified and normalized to that of the wtAd5 MOI of 5 infection at 37°C. Blank bars represent results from 37°C infections, and gray bars represent results from 39°C infection. Data are shown as mean ± SD of three or four biological replicates and analyzed using two-way ANOVA, followed by Šídák's test in GraphPad Prism version 10.2.3. Data used for plotting are listed in Table S1.

in the expression of the Rep78 and Rep52 proteins (which are derived from unspliced transcripts). Increasing the MOI of Ad5ts149 at 39°C enhanced Cap, Rep68, and Rep40 protein expression in a dose-dependent manner. Taken together, these results provide evidence for a correlation between expression of the AAV Rep68/40 and Cap proteins and AAV vector production in the context of an Ad5ts149 infection at the restrictive temperature. The data suggest a dependence of Rep68/40 and Cap protein expression on adenoviral factors that are lacking in the absence of viral replication and production, presumably the adenoviral late gene products.

Amplification of the AAV *rep* and *cap* genes is critical for efficient AAV production from stable PCLs.[35–38] We assessed *rep* gene amplification by digital PCR (dPCR) (Figure 2F) and found that *rep* gene copy numbers were largely comparable for Ad5ts149 and wtAd5 at both temperatures. Similar results were obtained for the *cap* gene (Table S3). However, with wtAd5 infection at an MOI of 100, *rep/cap* copy numbers seemed to be reduced compared to infections at lower MOIs. This may be a result of competition from adenoviral replication at the high MOI, but the specific mechanism requires further investigation. These results imply that the reduction in rAAV vector production from Ad5ts149 at the restrictive temperature is not linked to a decrease in *rep/cap* amplification. We next assessed *rep/cap* gene expression at the transcriptional level by northern blot (Figure 3A). The pattern of AAV transcripts was uniform at 37°C and 39°C for wtAd5. Ad5ts149 infection at 37°C showed a similar pattern, but at the restrictive temperature of 39°C, the overall *rep/cap* transcription level and the relative abundance of the six AAV transcripts were altered. The northern blot revealed an increase in the level of the

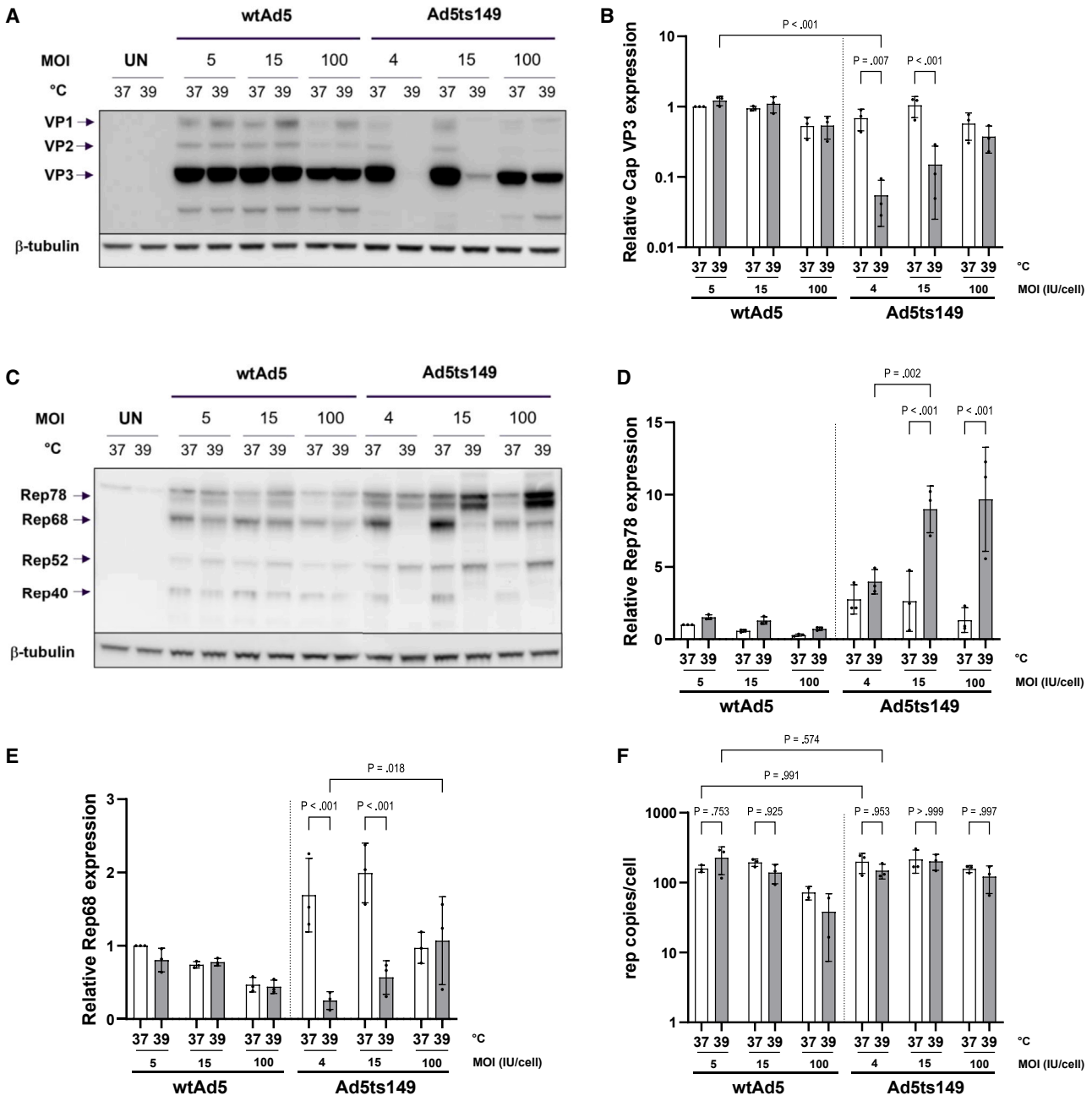


Figure 2. Suppression of adenovirus replication and production with Ad5ts149 at 39°C alters AAV Rep/Cap protein expression

(A) Representative AAV Cap western blot run using total cell lysates prepared from cells harvested from experiments described in Figure 1. β-Tubulin was used as the loading control. (B) Densitometric analysis of Cap VP3 expression in (A), normalized to tubulin and presented as relative ratio to wtAd5 infection of MOI of 5 at 37°C. (C) Representative Rep expression detected by western blot. (D and E) Densitometric analysis of Rep78 (D) and Rep68 (E) from (C) were presented as relative fold-change normalized to tubulin and then wtAd5 infection of MOI of 5 at 37°C. (F) Rep gene copies/cell quantified via dPCR using extrachromosomal DNA extracted from samples collected 48 hpi. Data are shown as mean ± SD of three biological replicates, except two for wtAd5 100 IU/cell condition and analyzed using two-way ANOVA, followed by Šídák's test in GraphPad Prism version 10.2.3.

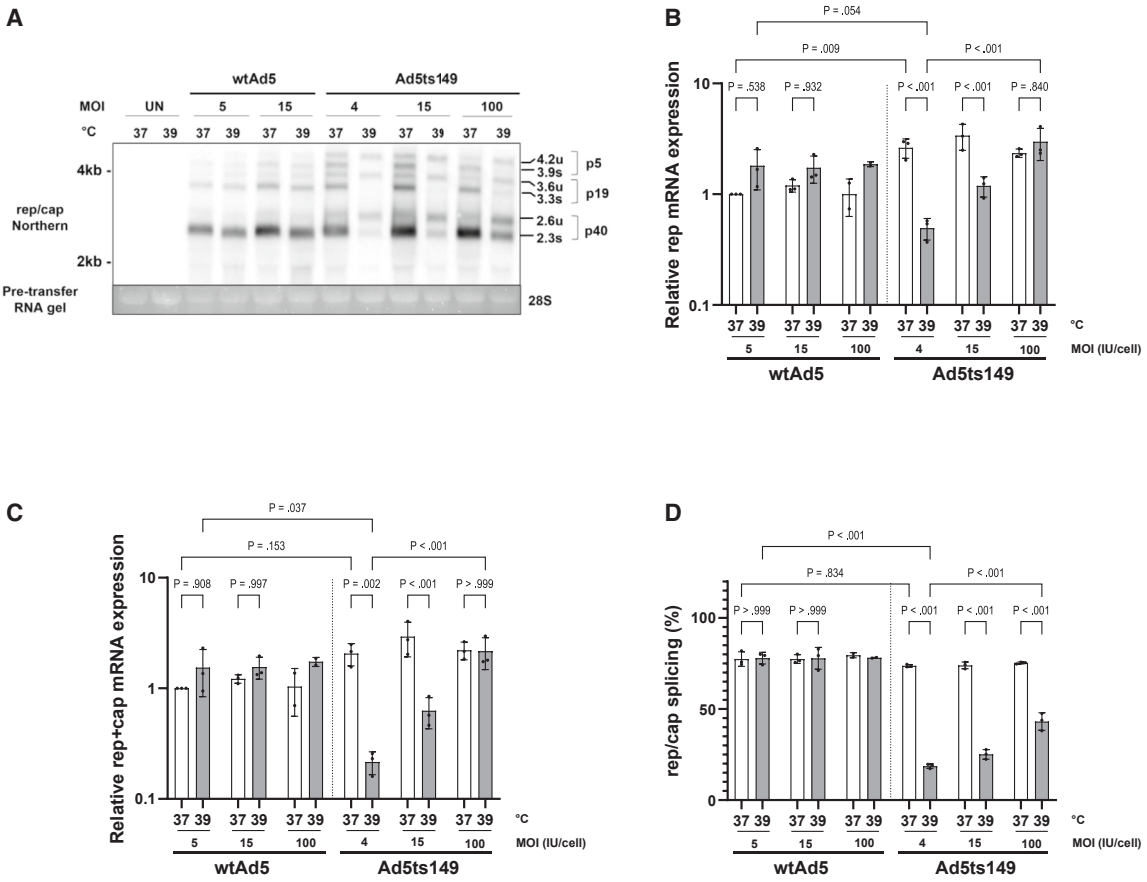


Figure 3. Suppression of adenovirus replication and production with Ad5ts149 at 39°C alters AAV *rep/cap* transcription and splicing

(A) Northern blot analysis of *rep/cap* transcripts from total RNA isolated from 48 hpi samples. The northern blot probe is located in the *cap* region but targets all 6 transcripts from the p5, p19, and p40 promoters. Each of the AAV transcripts is labeled with the expected size in kilobases and u (unspliced) or s (spliced). UN, uninfected. Bottom: ethidium bromide-stained gel was used to demonstrate total RNA loading. (B and C) Relative *rep* (B) and total *rep+cap* (C) mRNA levels were quantified by multiplex RT-dPCR using total RNA isolated from 48-hpi samples. As the producer cell line expression system has the wild-type AAV *rep* and *cap* gene arrangement, *rep* and *rep+cap* transcripts were assessed and presented instead of *rep* and *cap* separately due to the use of the same polyA signal. (D) AAV *rep/cap* splicing percentage evaluated from multiplex RT-dPCR. Data are shown as mean ± SD of three biological replicates, except two for wtAd5 100 IU/cell condition and analyzed using two-way ANOVA followed by Šídák's test in GraphPad Prism version 10.2.3.

unspliced p5 (4.2 kb) and p19 (3.6 kb) *rep* gene transcripts as well as the 2.6 kb unspliced p40 transcript. This increase was accompanied by a reduction in the corresponding spliced transcripts. This altered pattern of AAV transcript abundance is consistent with the reduction of expression of the AAV Rep68, Rep40, and Cap proteins, which are translated from spliced transcripts (Figures 2A and 2C).

To confirm the northern blot results, we quantified AAV transcripts by reverse-transcription digital PCR (RT-dPCR). We found that the level of *rep* mRNAs trended higher for Ad5ts149 at 37°C compared to wtAd5 (Figures 3B and 3C). This result is consistent with the results of the northern blot and with the observed increase in the Rep (especially Rep68) protein level at 37°C (Figures 2C and 3A). At 39°C, the mRNA levels of *rep* and total *rep/cap* in Ad5ts149-infected cells exhibited a dose-dependent pattern; at an MOI of 100, the levels were similar to those at 37°C, but at an MOI of 4, the abundance was reduced 5- to 10-fold (Figures 3B and 3C). This RT-dPCR result is also consistent with the pattern observed on the northern blot (Figure 3A). To assess the efficiency of splicing of AAV *rep* and *cap* transcripts, we performed RT-dPCR using specific primers and probes capable of differentiating the spliced and unspliced transcripts (Figure S1). For wtAd5, approximately 75% of the total *rep/cap* transcripts were spliced, regardless of the infection temperature (Figure 3D). Ad5ts149 infection at 37°C produced similar levels of splicing. However, we observed a 2- to 4-fold reduction in splicing with Adts149 infection at 39°C. Consistent with Rep and Cap protein expression and AAV vector production at 39°C, AAV splicing showed a trend toward a dose-dependent increase with MOI, suggesting that a common limiting factor associated with Ad5ts149 infection at the restrictive temperature might explain the altered AAV transcript splicing pattern and subsequent deficit in rAAV production with this helper virus.

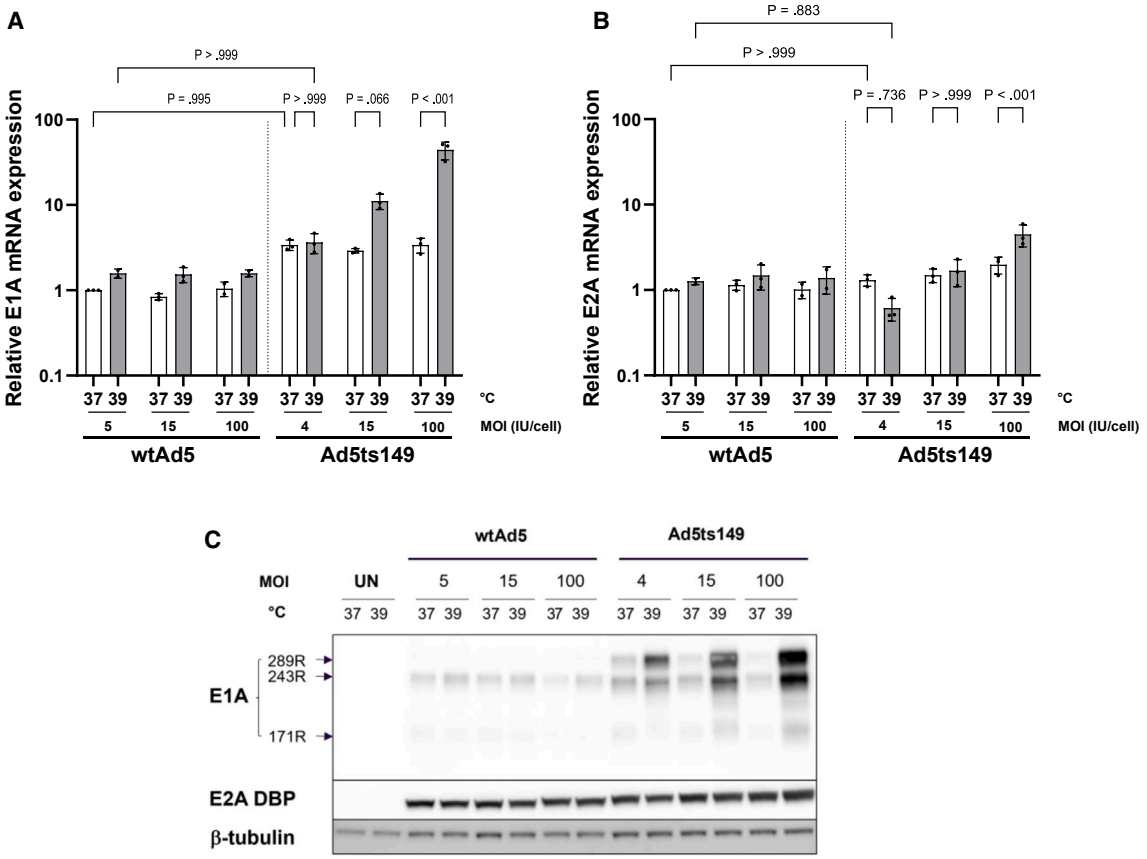


Figure 4. Adenovirus gene expression profiling shows comparable or higher early gene expression at 39°C with Ad5ts149

(A and B) Relative mRNA levels for adenovirus early genes E1A (A) and E2A (B) at 48 hpi quantified via RT-dPCR. (C) Representative adenovirus E1A and E2A western blot analysis at 48 hpi from adenovirus-infected PCL cells. β-Tubulin was used as the loading control. Data are shown as mean ± SD of three biological replicates, except two for wtAd5 100 IU/cell condition and analyzed using two-way ANOVA, followed by Šídák's test in GraphPad Prism version 10.2.3.

Adenovirus late gene expression is limited with Ad5ts149 infection at the restrictive temperature

To identify the viral factors that might be involved in the splicing of AAV transcripts, we analyzed the expression of adenoviral helper genes by RT-dPCR. The levels of mRNAs derived from the adenoviral early genes E1A and E2A with Ad5ts149 infection were comparable to or higher than wtAd5 at both 37°C and 39°C (Figures 4A and 4B; results from analysis of additional early genes are shown in Figure S3). Analysis of E1A and E2A protein expression via western blot (Figure 4C) was consistent with the RT-dPCR results, showing equivalent (E2A) or higher (E1A) protein expression from Ad5ts149 at 39°C compared to wtAd5. In contrast, but as expected, hexon mRNA was significantly reduced with Ad5ts149 infection at the restrictive temperature (Figure 5A). Using an anti-Ad5 capsid antibody, we also evaluated the expression of late structural proteins via western blot (Figure 5B). We observed a significant reduction in expression of the late gene products with Ad5ts149 infection at 39°C compared to 37°C or to wtAd5 infection at either temperature; however, there was a dose-dependent increase in these products with Ad5ts149 infection at 39°C. In summary, expression of late gene products is compromised with Ad5ts149 infection at the restrictive temperature, while early gene products are unaffected, suggesting that AAV splicing is likely regulated by adenoviral late, and not early gene products.

The adenovirus major late transcription unit encodes multiple late proteins from five regions, L1, L2, L3, L4, and L5 (Figure 5C). Since the L4-22K/33K gene products have been reported to regulate the splicing of adenoviral transcripts,[26,30–32] we speculated that they might also regulate AAV splicing. Using RT-dPCR, we compared L4-100K/22K/33K gene expression following infection with wtAd5 or Ad5ts149 (Figures 5D–5F). When compared to infection at 37°C, infection of Ad5ts149 at 39°C resulted in a significant reduction in the expression of the L4 genes encoding the 100K, 22K, and 33K proteins. Expression showed a dose-dependent increase with increasing MOI. At 39°C with an MOI of 4, the abundance of L4 mRNAs was reduced 10- to 100-fold relative to 37°C, whereas at an MOI of 100, levels were comparable at the two temperatures.

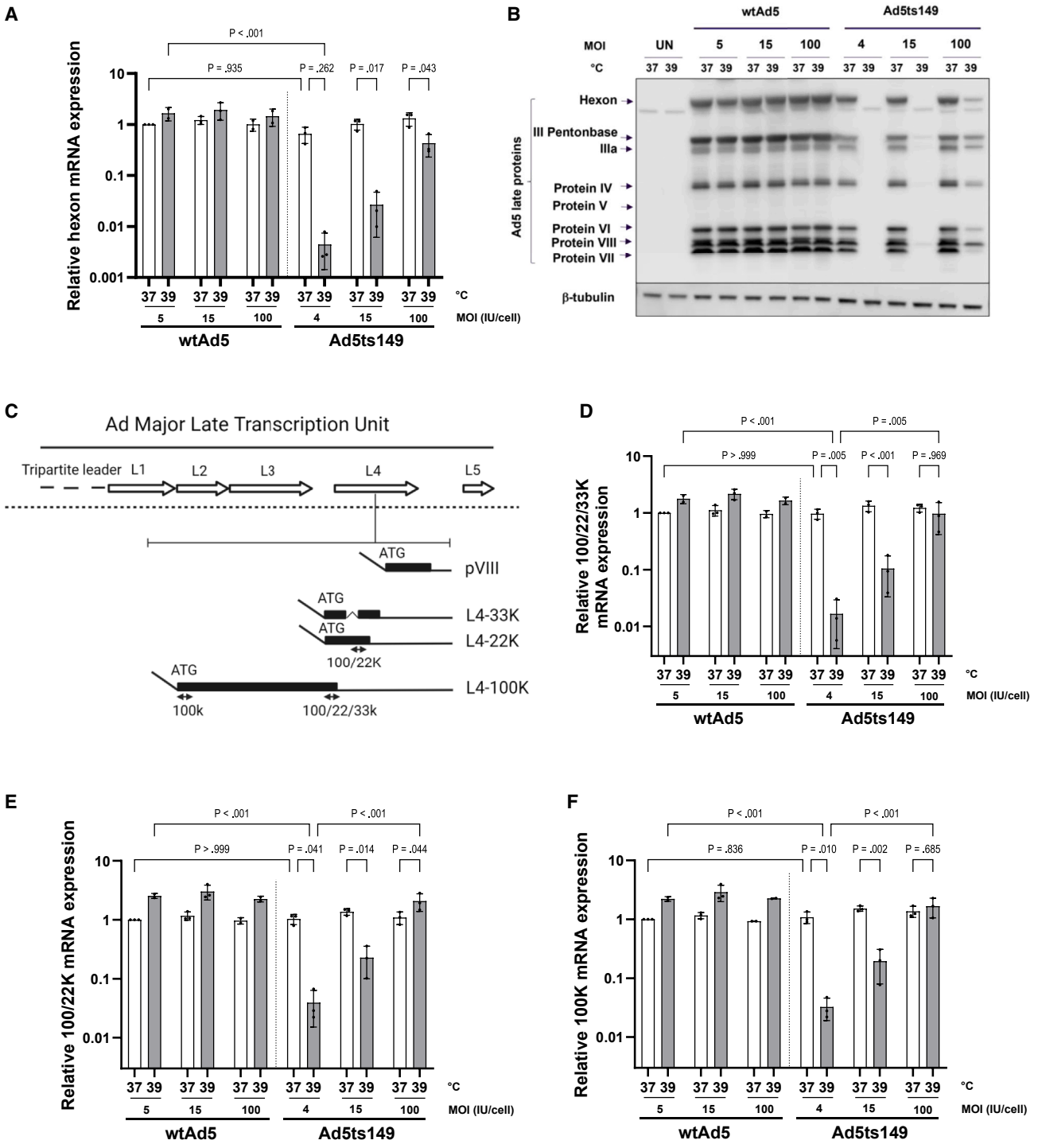


Figure 5. Adenovirus late gene expression is reduced at 39°C with Ad5ts149

(A) Relative adenovirus hexon mRNA levels quantified via RT-dPCR. (B) Adenovirus late structural gene expression assessed via western blot using anti-Ad5 capsid antibody and anti- β-tubulin as a loading control. (C) Schematic of adenovirus major late transcription unit illustrating the L4 mRNA transcripts and locations of the dPCR primers

(legend continued on next page)

Adenovirus L4 genes regulate *rep/cap* splicing and expression

To assess whether reduced L4 gene expression might explain the deficit in AAV splicing observed with Ad5ts149 infection at 39°C, we used DsiRNAs to knock down L4 gene expression (Figure S4A; as described by Su et al.[24]) in a PCL infected with wtAd5. Greater than 70% knockdown of L4-100K/22K/33K gene expression was achieved using the 100K-specific DsiRNAs, while 22K/33K and L4 A-targeted DsiRNAs achieved over 90% knockdown (Figure 6A). This level of knockdown efficiency likely reflects the fact that the 100K A/B DsiRNAs are specific for the 100K gene, while the 22K/33K and L4-A DsiRNAs target all three L4 genes (Figures S4B and S4C). Knockdown of L4 gene expression reduced AAV production significantly (Figure 6B). The L4-22K/33K DsiRNA had the greatest impact, reducing AAV production by 50- to 100-fold compared to the DsiRNA negative control. Knockdown of these genes also reduced *rep* DNA copies by 20-fold (Figure 6C; Table S4), while treatment with the L4-100K-targeted DsiRNAs resulted in a 10-fold reduction. In addition, AAV *rep/cap* mRNAs (Figure 6D) were reduced by approximately 50-fold with the L4-22K/33K and L4 A DsiRNAs and by approximately 3-fold with L4-100K knockdown. The RT-dPCR results were consistent with the pattern observed in the northern blot analysis (Figure 6E). Densitometric quantitation of the *cap* transcripts revealed that the ratio of the 2.3-kb spliced to the 2.6-kb unspliced transcript decreased from approximately 7 in the negative control to 2 for all L4 DsiRNAs (Figure 6F). Furthermore, RT-dPCR confirmed a reduction in AAV transcript splicing from 75% to approximately 50% by all L4 DsiRNAs (Figure 6G), supporting the hypothesis that L4 gene products play a role in AAV splicing. These results are supported by the western blot analysis, which showed that the L4-22K/33K and L4 A DsiRNAs reduced levels of both Rep and Cap proteins (Figure 6H). Overall, *rep/cap* gene amplification, transcription, and protein expression consistently reflected the level of AAV production observed upon knockdown of each of the L4 genes. These results suggest that the adenovirus L4 gene products play an essential role in AAV gene expression and virus production, as they regulate not only *rep* and *cap* gene amplification as previously reported[24] but also the synthesis and splicing of AAV transcripts.

Supplementation of the L4-33K gene improves AAV splicing and vector production

To further establish the role of the adenoviral L4 genes in the regulation of AAV gene expression, we supplemented the L4 genes in the context of an Ad5ts149 infection at 39°C. We hypothesized that enhancing L4-22K/33K gene expression would result in improved *rep/cap* splicing and gene expression. Plasmid constructs expressing the individual L4 genes, 100K, 22K, 33K, or pVIII from the EF1α promoter were transfected into producer cells. The next day, the cells were infected with Ad5ts149 at 39°C. AAV gene expression, *rep/cap* transcript splicing, and vector production were subsequently analyzed. Supplementation of either the L4-22K or L4-33K gene but not the 100K or pVIII genes enhanced AAV production by 2-fold (Figure 7A). To validate our hypothesis, we quantified the levels of *rep/cap* mRNA by RT-dPCR. Supplementation of L4-22K was associated with an increase in AAV *rep/cap* mRNA relative to the control (Figure 7B). However, only supplementation of the L4-33K gene increased splicing efficiency relative to the negative control (from ∼26% to ∼38%; Figure 7C). Consistent with this result, the abundance of AAV Cap and the Rep68/Rep40 proteins also showed an increase with supplementation of L4-33K (Figure 7D). As expected, *rep/cap* gene amplification was not affected by supplementation of any of the L4 genes (Figure S5; Table S5). Supplementation of 100K or pVIII via transfection of the EF1α-100K or pVIII constructs did not impact either splicing or mRNA levels, although DsiRNA-mediated knockdown of the 100K gene resulted in a reduction in the level of AAV splicing in the context of a wtAd5 infection (Figures 6F and 6G).

These results indicate that Adenovirus L4 gene products regulate Rep/Cap expression and AAV production at multiple levels. Adenovirus L4-33K regulates splicing of AAV transcripts, whereas L4-22K seems to regulate transcript abundance. L4-22K is a transcriptional regulator that is essential for the transition from early to late adenoviral gene expression.[26–28] Therefore, the L4-22K gene product may directly or indirectly regulate the transcription of AAV genes. The observed reduction in AAV splicing from 100K knockdown could result from an indirect effect on expression of the L4-22K and L4-33K proteins since 100K is required for efficient adenoviral late mRNA translation and deletion of 100K significantly reduced expression of some late genes.[39,40] Full restoration of AAV productivity from Ad5ts149 infection at 39°C may require near WT levels of L4-33K as well as the other L4 genes.

L4-33K regulates AAV *rep/cap* splicing and impacts AAV vector production in HEK293 cells

Given the role of the L4-22K/33K gene products in AAV gene expression and vector production in the context of producer cells, we evaluated supplementation of the L4 genes in the HEK293 transient transfection platform. Supplementation of L4-33K, in addition to the intrinsic L4 gene expression from the pAdhelper plasmid, led to a productivity increase for four different rAAV vectors comprising different capsids and vector genomes. The capsids tested include one natural serotype and three capsid variants derived via capsid engineering approaches.[41] The vector genomes contain transgene cassettes in which gene expression is driven by either a strong constitutive promoter such as chicken β-actin or a tissue-specific promoter (Figure 8A). Analysis of AAV transcript splicing for Cap_dGene_4 revealed that similar to the effect in the PCL, supplementation of the L4-33K gene resulted in an increase in splicing efficiency from ∼50% to ∼75% (Figure 8B). These results suggest that

(double arrow lines) used for quantitation of adenovirus L4 gene expression (diagram not drawn to scale). Open reading frame (ORF) for each gene product is highlighted in black rectangle. The illustration was adapted from Biasiotto and Akusjärvi[32] and Su et al.[24] and created with BioRender.com. (D–F) Relative mRNA levels for adenovirus L4 gene products were quantified via RT-dPCR from total RNA using dPCR primers described in (C) and Table S2. Data are shown as mean ± SD of triple biological replicates, except two biological replicates for wtAd5 100 IU/cell condition and analyzed using two-way ANOVA followed by Šídák's test in GraphPad Prism version 10.2.3.

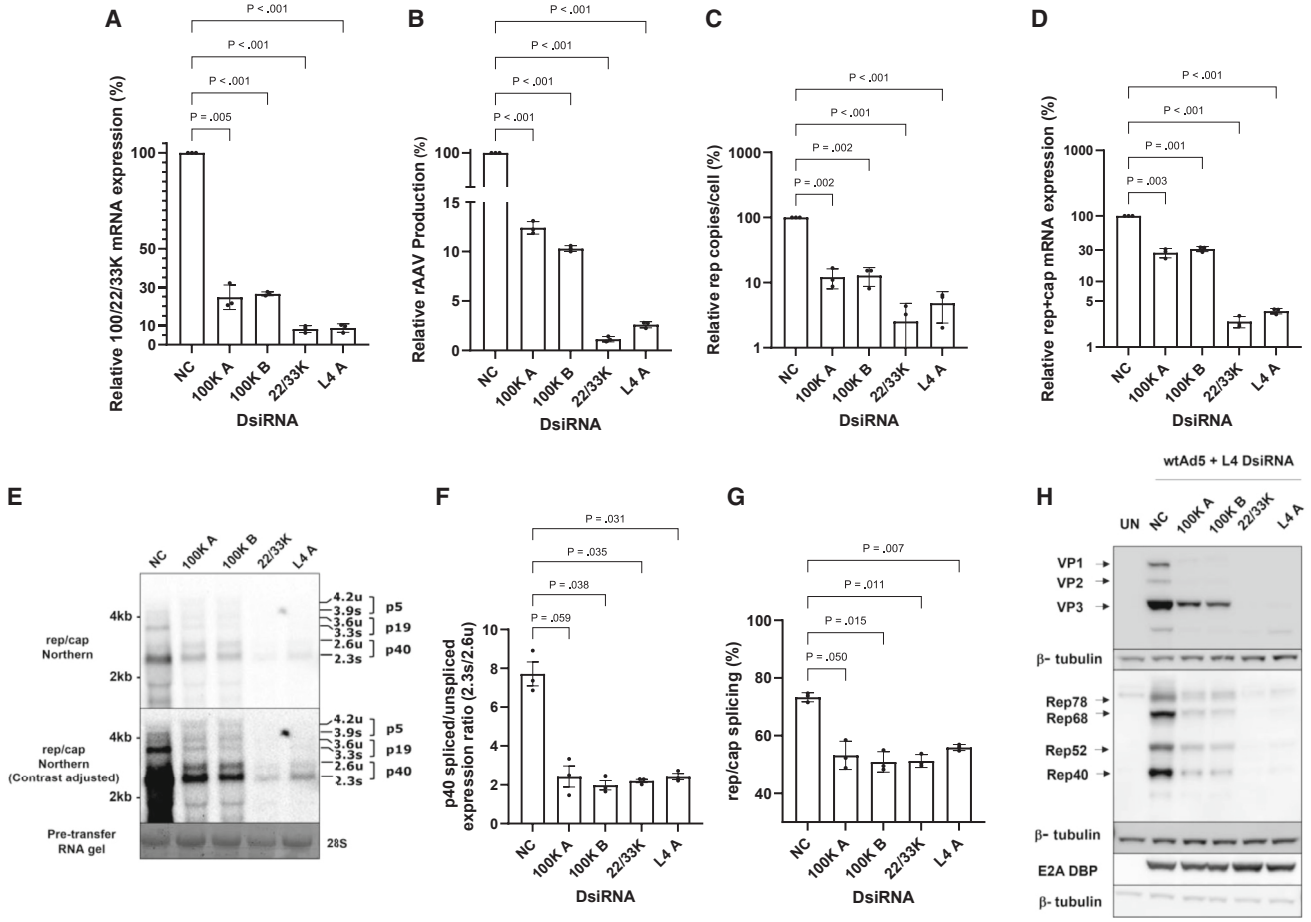


Figure 6. Knockdown of adenovirus L4 gene expression reduces *rep/cap* transcription, splicing, and expression

PCL cells were transfected with the indicated DsiRNAs followed by wtAd5 infection at 5 IU/cell immediately after the DsiRNA nucleofection. Analyses were performed with samples harvested at 72 hpi. (A) Relative adenovirus L4 gene expression evaluated by RT-dPCR. (B) Relative rAAV volumetric productivity from L4 DsiRNA compared to non-targeting DsiRNA negative control (NC). (C) Relative *rep* copies determined from extrachromosomal DNA with dPCR. (D) Relative total *rep* and *cap* mRNA level quantified from total RNA by multiplex RT-dPCR. (E) Top: northern blot analyzing *rep/cap* transcripts with a probe located within *cap* sequence. Center: contrast-adjusted northern blot image to visualize weaker bands. Bottom: ethidium bromide-stained gel demonstrating total RNA loading. (F) Relative ratio of spliced 2.3s and unspliced 2.6u p40 transcripts from densitometric analysis of northern blot in (E). (G) AAV splicing evaluated from multiplex RT-dPCR upon L4 gene knockdown via DsiRNA. (H) AAV Rep, Cap, and adenovirus E2A protein expression analyzed by western blot, with β-tubulin as a loading control. NC, non-targeting negative DsiRNA control. Data are shown as mean ± SD of three biological replicates and analyzed by one-way ANOVA, followed by Dunnett tests in GraphPad Prism version 10.2.3.

supplementation of L4-33K might be a broadly applicable strategy to improve vector production in the HEK293-based transfection system.

DISCUSSION

Generation of recombinant AAV in an AAV producer cell involves a complex interaction between the helper adenovirus and the viral vector. For example, the five adenoviral helper factors, E1A, E1B, E2A, E4orf6, and VA RNAs, which are necessary to support AAV gene expression and viral genome replication, are also required to support these processes for wtAd5, leading to competition between the helper and the AAV. In addition, the wtAd5 helper and AAV vie for the availability of cellular resources such as the nucleotides, amino acids and energy required for virus production. To mitigate competition and promote its own proliferation, AAV has evolved strategies, such as Rep-mediated repression of adenoviral gene expression, that interfere with the replication of the helper virus.[42] This repression is accomplished directly via the binding of Rep to viral promoters,[43,44] or indirectly via the inhibition of the cyclic AMP-dependent protein kinase A pathway.[45]

In this study, we evaluated the use of Ad5ts149, an adenovirus mutant with diminished replication due to a temperature-sensitive defect in the DNA polymerase gene, as a helper virus in the context of a model AAV PCL. Our objective was to reduce the replication and production of adenovirus in the producer platform and to understand more fully the interplay between the helper adenovirus

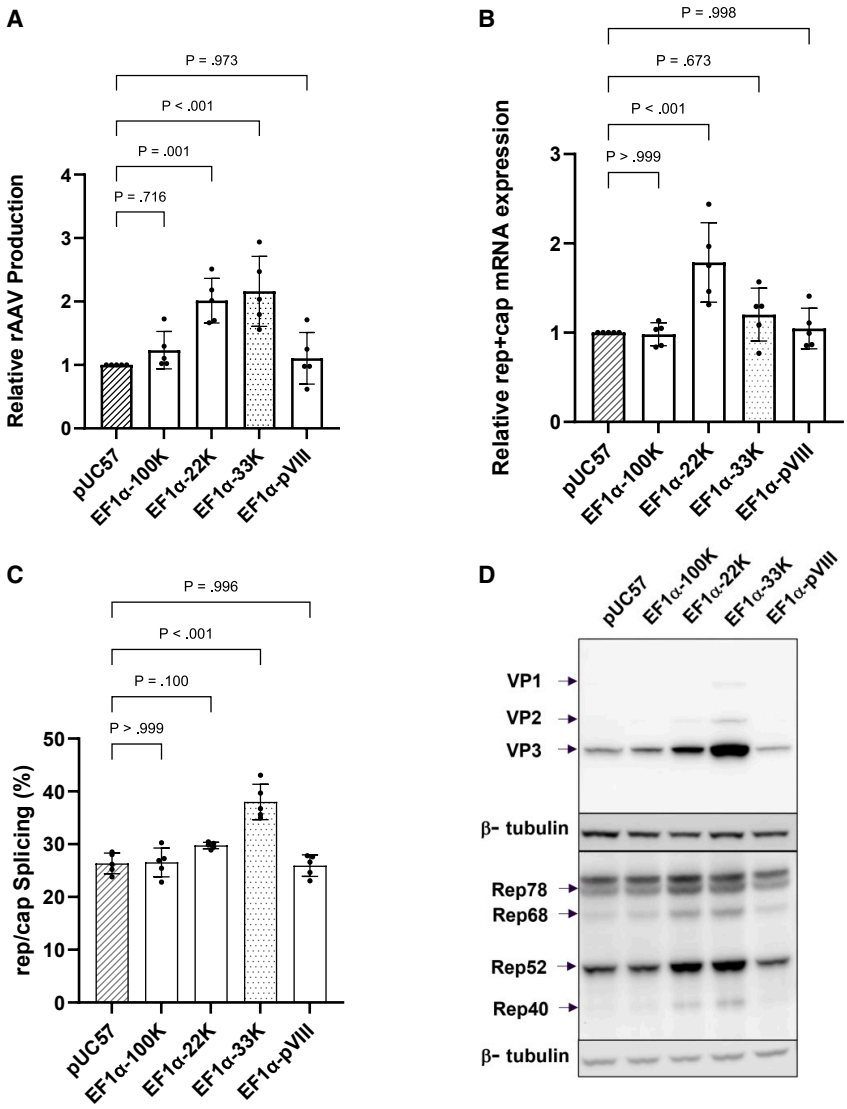


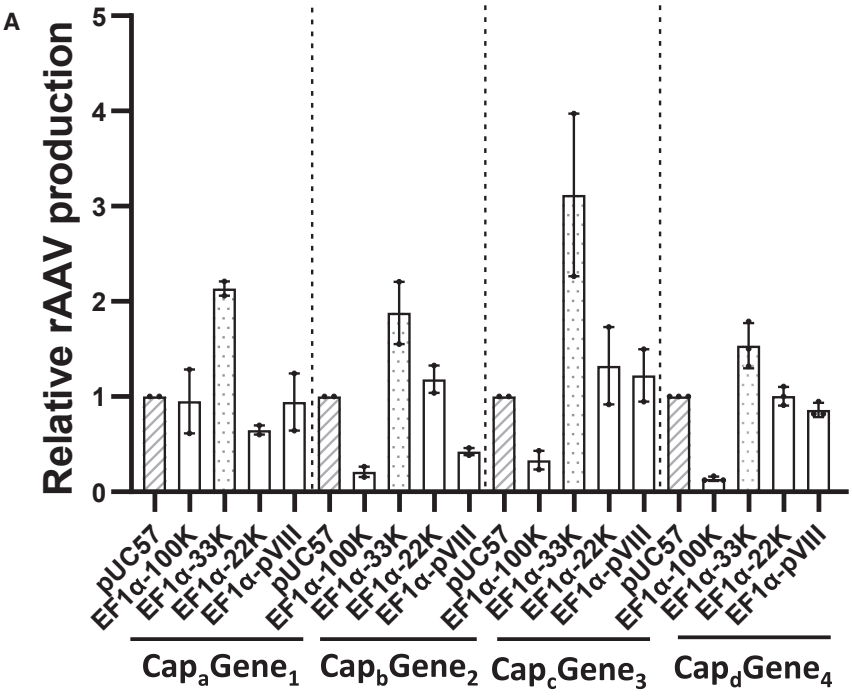
Figure 7. Supplementation of adenovirus L4-33K improves *rep/cap* splicing, expression, and AAV production in the context of Ad5ts149 infection at 39°C

AAV producer cells were transfected with L4 constructs expressing individual genes L4-100K, 22K, 33K, or pVIII driven by the EF1α promoter. Transfected cells were infected at 24 h post-transfection with Ad5ts149 at 15 IU/cell and incubated at 39°C. Cells were harvested for downstream analysis at 48 hpi. (A) Relative rAAV titer normalized to the pUC57 control. (B) Relative total *rep* and *cap* mRNA levels evaluated with RT-dPCR analysis. The fold change was calculated compared to the pUC57 control. (C) AAV *rep/cap* splicing evaluated from multiplex RT-dPCR. (D) Representative AAV Rep and Cap protein expression in whole-cell lysates analyzed via western blot. β-Tubulin was used as the loading control. Bar graph data are shown as mean ± SD of five biological replicates and analyzed by one-way ANOVA, followed by Dunnett tests in GraphPad Prism version 10.2.3.

and AAV during vector production. Utilizing this model PCL as an example, our results showed that at the semi-permissive temperature of 37°C, Ad5ts149 titer was reduced by about 70%–80% relative to wtAd5. However, at this temperature, Ad5ts149 infection supported AAV production at a level comparable to wtAd5 (Figure 1). This finding is consistent with the results of Farson et al.,[23] who observed a half to a log reduction in Ad5ts149 titer at 37°C following the infection of an A549-based AAV PCL. At this temperature, production of AAV with Ad5ts149 was similar to that observed with Ad*dl*309, a replication-competent E3-deleted virus.

At the restrictive temperature of 39°C, where Ad5ts149 replication was reduced by 2- to 3-logs, AAV vector production was significantly impaired. Further investigation revealed that under these conditions, AAV Cap protein expression was significantly reduced. This was accompanied by a selective decrease in the expression of the Rep68 and Rep40 proteins and consequently, an alteration of the pattern of Rep protein expression (Figures 2A and 2B). Notably, an increase in the MOI from 4 to 100 increased replication of Ad5ts149 at 39°C and coincided with a dose-dependent increase in the expression of the Rep68/40 and Cap proteins and AAV productivity (Figures 3B–3D). Ad5ts149 infection at the low MOI reduced AAV vector genome replication only slightly (~2-fold; Figure 1D). The maintenance of AAV vector genome replication at the restrictive temperature is likely explained by the expression of Rep78, which is equal to or greater than the expression at 37°C (Figures 2C and 2D). Continued expression of Rep78 also likely explains the maintenance of *rep/cap* amplification with Ad5ts149 infection at 39°C. Like Rep78, expression of E1A was also enhanced at 39°C in Ad5ts149-infected cells (Figure 4C). Since E1A activates the AAV p5 promoter,[10] this enhanced expression may explain the high level of Rep78 protein expression that is observed at the restrictive temperature.

Splicing of AAV transcripts is required for the expression of Rep68 and Rep40 and for the expression of the capsid proteins VP1, VP2, and VP3. Use of alternate splice acceptor sites located at nucleotide 2,201 (minor splice acceptor) and 2,228 (major splice acceptor) in the AAV2 genome helps to establish the correct stoichiometry of the three capsid proteins.[8,46–48] Ad5ts149 infection at 39°C causes a reduction in Rep68, Rep40, and all three capsid proteins (Figures 2A–2E and S1). This pattern correlates with a potential



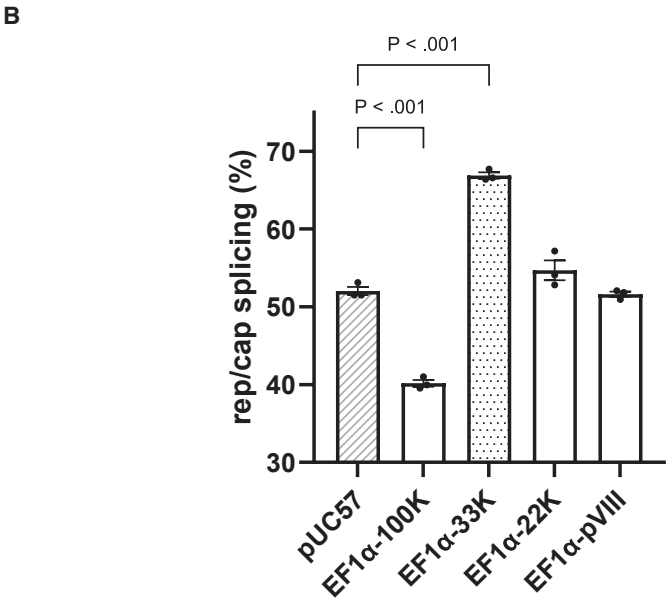


Figure 8. Adenovirus L4 supplementation enhances AAV vector production in HEK293-transient transfection system

Plasmids containing *rep*/*cap*/transgene, pAdhelper, and the EF1α promoter-driven L4 construct or vehicle control pUC57 were co-transfected into HEK293 cells at a molar ratio of 1:1:1. Cultures were harvested 72 h post-transfection for downstream analysis. Cap$_{a/b/c/d}$ refer to four different capsids, including one natural serotype and three engineered capsid variants derived from different serotypes. Gene$_{1/2/3/4}$ refer to four different transgenes in the AAV vector genome; vector genome sizes range from approximately 3.7–4.6 kb. (A) Relative rAAV titer from each L4 gene co-transfection normalized to the pUC57 control. Data are shown as mean ± SD of two or three biological replicates. (B) AAV splicing analyzed from Cap$_d$Gene$_4$ transfection production, as previously described. Data are shown as mean ± SD of three biological replicates and analyzed by one-way ANOVA followed by Dunnett tests in GraphPad Prism version 10.2.3.

splicing defect at this temperature, which was confirmed by northern blot analysis and RT-dPCR (Figures 3A and 3D). Consistent with previous reports,[49] at 37°C, spliced transcripts (2.3 kb) made up the majority of the p40 transcripts from either wtAd5 or Adt5s149 infection; spliced p5 and p19 transcripts were also present at a higher level than unspliced transcripts. The AAV transcript patterns are similar for wtAd5 at 37°C and 39°C and Ad5ts149 at 37°C, but Ad5ts149 infection at 39°C leads to a general shift toward more unspliced transcripts. Quantitation via RT-dPCR was consistent with northern blot analysis; ∼75% of transcripts were spliced with wtAd5 infection at 37°C, but we observed a relative reduction in splicing from 75% with wtAd5 to 20%–40% with Ad5ts149 at 39°C (Figure 3D). The percentage of spliced transcripts for wtAd5 (∼75%) is consistent with Mouw and Pintel[49] but higher than that reported by Stutika et al.[50] Different analytical methods and splicing variant coverage likely contribute to this difference. Overall, these results suggest that in the absence of efficient replication of the helper viral genome, one or more adenoviral factors are likely limiting, resulting in the observed AAV splicing defect in the PCL platform. Adenovirus infection is known to stimulate splicing of AAV transcripts[49]; however, the adenoviral gene products involved in this process have yet to be identified.

The dramatic effect on Rep and Cap protein expression and splicing of AAV transcripts resulting from defective replication of the Ad5ts149 helper prompted us to hypothesize that an adenoviral late gene product(s) was required for replication and packaging of the rAAV. As expected, adenoviral late gene expression was reduced at 39°C (Figure 5), and abundance of the adenoviral structural proteins and transcripts derived from the L4 region correlated with the level of replication of Ad5ts149. DsiRNA-mediated knockdown of the adenoviral late genes L4-22K and L4-33K confirmed that products of both genes are required for vector production in the PCL.

Although it was previously thought that the minimal set of adenoviral helper functions were largely early genes, novel roles for the L4-22K and L4-33K gene products in AAV vector production have recently been described.[25] L4 genes are expressed early in the late phase from the L4 promoter (L4P),[26] but due to negative feedback regulation of L4P by L4-22K and L4-33K, transcription during the late phase ultimately comes under the control of the stronger major late promoter (MLP).[51] L4-22K and L4-33K are related proteins that share an N-terminal sequence; however, because the L4-33K gene product is translated from a spliced transcript, the proteins differ in their carboxyl-terminal domains.[32] The L4-22K and L4-33K gene products are multifunctional and have been shown to play a role in adenoviral genome packaging and in regulation of the transition from early to late gene expression.[26,28,29,52] L4-33K acts as a virally encoded splicing factor to regulate alternative splicing of the adenoviral major late transcripts.[30,52,53] In this role, L4-33K specifically promotes the use of weak 3′ acceptors that are believed to bind the splicing factor U2AF inefficiently due to the absence of long pyrimidine tracts characteristic of consensus acceptor sites.[54] Notably, the sequences of both the minor and major 3′ splice acceptor sites of AAV lack extended pyrimidine tracts, and therefore may represent potential targets of splicing activation by L4-33K. Interestingly, Mouw and Pintel[49] reported a shift in the abundance of spliced AAV transcripts in HEK293 cells co-infected with AAV and adenovirus when the cells were pre-infected for 12 h with adenovirus. This observation is consistent with the involvement of a late gene product in AAV splicing. As shown here, supplementation of L4-33K in the context of an Ad5ts149 infection at 39°C was able to increase Cap protein expression and AAV production while the splicing of *rep/cap* transcripts was enhanced from 26% to 38% (Figures 7A–7D). This relatively small increase in splicing may be attributed to a relatively low level of expression of L4-33K following transient transfection of the PCL with the EF1α-33K plasmid. RT-dPCR analysis showed that expression from the supplemented L4 gene was more than one log lower than that observed following infection with wtAd5 (Figure S6). This low level of expression of L4-33K might also explain why the rAAV productivity was increased only 2-fold with supplementation and was not fully rescued. These results demonstrate a novel role for L4-33K in AAV vector production.

Interestingly, the L4-22K gene product did not seem to have an impact on splicing of AAV transcripts, but supplementation of L4-22K in cells infected with Ad5ts149 at 39°C increased AAV production along with *rep/cap* gene transcription (Figures 7A and 7B). This raises the possibility that L4-22K may regulate the level of *rep/cap* transcripts either by altering promoter activity or via an effect on mRNA stability. During the adenoviral life cycle, L4-22K has been proposed to both activate transcription of the MLP[29] and facilitate post-transcriptional processing of viral mRNAs.[26] L4-22K has also been reported to suppress early gene expression during the late phase of infection; L4-22K mutant viruses show increased E1A expression.[28] Therefore, reduced expression of the L4-22K gene product may at least partially explain the increase in E1A protein observed with Ad5ts149 infection at 39°C (Figure 4C).

Involvement of L4-22K and L4-33K in AAV production was recently demonstrated by using a novel helper virus (TESSA-E1 [Engineered Tetracycline-Enabled Self-Silencing Adenovirus with E1 gene]) that is replication competent but contains a self-repressing MLP.[24] Repression of the MLP interferes with late gene expression and consequently with adenoviral packaging. However, when TESSA-E1 was used as a helper in the HeLaRC32 AAV packaging cell line, amplification of the integrated AAV *rep/cap* genes was reduced. In the study performed by Su et al.,[24] knockdown and *trans* supplementation experiments confirmed that the L4-22K and L4-33K genes play a role in *rep/cap* gene amplification. The authors hypothesized that reduced amplification was due to sequestration of the L4-22K/33K gene products bound to replicating TESSA-E1 genomes and subsequently demonstrated that the amount of L4-22K/33K produced without MLP activation was sufficient to support *rep/cap* amplification when adenoviral replication was inhibited. In the present study, expression of the L4-100K/22K/33K genes was reduced in cells infected with Ad5ts149 at the restrictive temperature (Figures 5D–5F), but amplification of the *rep* gene was unaffected (Figure 2F; Table S3). Since replication of Ad5ts149 is reduced at the restrictive temperature, this observation is consistent with the hypothesis that replicating adenoviral genomes sequester the low levels of L4-22K/33K expressed from the L4P promoter.[24] A separate study reported that a 100K-deleted adenovirus was unable to support *rep/cap* amplification in a stable AAV2 PCL, whereas amplification was unaffected when a replication-defective ΔpTP mutant was used as a helper.[55] In agreement with Krüger-Haag et al.,[55] we found that knockdown of L4-100K in the context of a wtAd5 infection resulted in a 10-fold decrease in *rep* DNA copies, thereby reducing Rep and Cap protein expression. In addition, a 20-fold reduction in *rep* DNA copies was observed with knockdown of L4-22K/33K (Figure 6C). These results confirm previous reports and demonstrate that the L4 gene products play a role in the amplification of *rep/cap* gene sequences in AAV PCLs.

In the HEK293 transient transfection platform, Adsero et al. showed that the L4-22K gene product was essential for rAAV production, while co-expression of L4-33K contributed to a boost in productivity.[25] In those studies, deletions were introduced in the adenovirus helper plasmid encoding the E2A, E4, and VA RNAs. Deletions encompassing the E2A promoter that overlaps the L4 gene region resulted in reduced AAV vector titer. Rescue of L4-22K or L4-33K expression either via insertion into the non-productive adenovirus helper plasmid or in *trans* via transfection of a separate plasmid recovered productivity. In contrast, in the studies described here, a plasmid expressing either the L4-22K, L4-33K, or additional L4 genes was co-transfected into HEK293 cells in conjunction with an intact and functional adenovirus helper plasmid. Increased expression of L4-33K but not L4-22K or other L4 genes via this supplementation strategy resulted in a 2- to 3-fold increase in AAV productivity for multiple AAV vectors (Figure 8A). RT-dPCR analysis confirmed that in this platform as well, L4-33K appears to enhance splicing of AAV transcripts (Figure 8B). Therefore, the L4-33K gene product is an important factor contributing to AAV production across different vector

production platforms. A reduction of AAV splicing and production resulting from 100K supplementation in this experiment differs from the observation in the PCL platform, indicating potentially unique mechanisms of regulation of AAV transcription splicing and production by the L4 genes in the transient transfection platform. Further investigation would be required to delineate the specific differences.

Overall, our results advance the understanding of adenoviral helper function in the AAV production process, highlighting L4-22K and L4-33K as adenoviral helper genes necessary for robust vector production in the AAV producer cell system and revealing a novel role for L4-33K in the splicing of AAV transcripts.

MATERIALS AND METHODS

Cells and cell culture

The AAV PCL was generated and cultured as previously described.[56] Parental host cells were cultured in EX-CELL media (Sigma-Aldrich, St. Louis, MO, catalog no. 14591C) supplemented with 6 mM glutamine (Gibco, Billings, MT, catalog no. 25030081) in a suspension shake flask at 100 rpm, 37°C with 5% CO_2, and 80% humidity. A plasmid containing the AAV *rep/cap* genes, the vector genome, and a puromycin resistance gene was stably transfected into parental host cells. Clonal PCLs were screened for AAV productivity and scaled up in the culture medium. Producer cell cultures were maintained by passaging at 3- to 4-day intervals and seeded for intended experiments generally from a 3-day culture.

HEK293 cells were cultured following the manufacturer's protocol. Cells were cultured in LVMax production medium (Thermo Fisher, Waltham, MA, catalog no. A3583402) in a suspension shake flask at 37°C with 8% CO_2 and 80% humidity at 120 rpm. Cultures were passaged every 3 to 4 days and seeded at the target density for transient transfection generally from a 3-day culture.

Viruses and virus infection

WT human Adenovirus type 5 (wtAd5, ATCC-VR5) and Ad5ts149, an Ad5 mutant harboring temperature-sensitive mutation within E2B,[57,58] were used as helper viruses to infect producer cells. wtAd5 was produced and titrated according to standard procedures as reported previously.[56,59] Ad5ts149 was grown at the permissive temperature (33°C) and purified as previously described.[60] For wtAd5 infection, producer cells were seeded at the target density in proprietary production media and infected with wtAd5 at the indicated MOI.[61] For Ad5ts149 infection, three MOIs (4, 15, and 100) were used to infect producer cells at either the semi-permissive 37°C or non-permissive 39°C temperature. Infections were performed in suspension spin tubes or shake flasks and cultured at 37°C, 10% CO_2, and 80% humidity.

Plasmid, DsiRNA, and transfection

For adenoviral L4 gene overexpression, constructs of each of the human Adenovirus type 5 (GenBank: AC_000008) L4 genes, including L4-100K (base pair [bp] 24,061–26,484), L4-22K (bp 26,195–26,785), L4-33K (bp 26,195–26,510/26,713–27,086), L4-pVIII (bp 27,174–27,857), expressed from the EF1α promoter was obtained from GenScript (Piscataway, NJ).

For L4 gene knockdown, DsiRNAs targeting different L4 regions, including 100K and 22K/33K as described in Su et al.[24] were ordered from IDT (San Jose, CA) along with negative control DsiRNA (IDT, catalog no. 51011403).

For PCL transfection, Lonza 4D nucleofector and SE Cell Line 4D-Nucleofector X kit L (Lonza, Basel, Switzerland, catalog no. V4XC1024) were used for DsiRNA and plasmid DNA transfection. Transfections were performed following the manufacturer's protocols. For HEK293 transfection, Polyplus FectoVIR-AAV (Illkirch, France) was used to transfect serum-free suspension HEK293 with 0.5 μg plasmid DNA per 1E+6 viable cells. The transfection complex was generated using a plasmid DNA to FectoVIR ratio of 1 at 5% complexing volume. The transfection complex was then added to the HEK293 cells within 25 min of complexing incubation, and cells were subsequently returned to the shaker incubator for continued culturing.

AAV production and titration

AAV production from producer cells was initiated by infecting producer cells with a helper virus, as described above. AAV replication and production was analyzed two to three days post-infection in whole culture cell lysates as described below.

AAV production in the HEK293 transient transfection system was assessed by transfecting HEK293 cells with three plasmid DNAs, including pAdhelper (including E2A, E4, L4, and VA RNAs), pAAV-Rep/Cap/Transgene, and pEF1α-L4 constructs or pUC57 stuffer control. The plasmids were mixed at a 1:1:1 ratio and transfected, as described above. Transfected cultures were collected at 3 days post-transfection for analysis of rAAV replication and production, as described below.

AAV replication and production were analyzed, as previously described.[56] Briefly, whole production cultures were collected and lysed with Tween 20 (0.5%) followed by treatment with benzonase (EMD, McCook, IL, catalog no. 1.01697.0001, 10 U/mL) for AAV production titer analysis. Benzonase-treated cell lysates were further digested with DNase (Promega, Madison, WI, catalog no. M610A; 60 U/mL final concentration) for 10 min at 37°C. The DNase reaction was terminated via the addition of Proteinase K (Invitrogen, Waltham, MA, catalog no. 100005393; 0.5 mg/mL final concentration) and incubation at 65°C for 10 min, followed by heat inactivation at 95°C for 20 min. Heat-inactivated post-Proteinase K samples were then diluted and analyzed with QuantStudio 7flex using a bovine growth hormone polyadenylation (BGH polyA) primer/probe set listed in Table S2 following standard qPCR procedures. AAV genome copy numbers were calculated via comparison to a standard curve generated with a plasmid (pAF196) that contained all PCR target sequences (e.g., *rep*, *cap*, BGH polyA, E2A). For analysis of AAV replication as represented by total vector genome copies, the same procedure was followed, with the exclusion of benzonase and DNase treatment.

Analysis of adenovirus production and replication

Adenovirus production was quantified as DNase-resistant genome copies per milliliter, employing a methodology akin to the AAV titer analysis described above, but utilizing the E2A primer/probe set from Table S2 in accordance with standard qPCR protocols using QuantStudio 7 Flex. For assessing adenoviral replication, total genome copies per milliliter were measured using the same protocol, but with the exclusion of benzonase and DNase treatment.

rep/cap gene amplification analysis

rep/cap genes stably integrated in PCLs undergo amplification after wtAd5 infection. The amplified copies exist as extrachromosomal DNA (ecDNA).[36,62] To assess *rep/cap* amplification, ecDNA was extracted from infected producer cell pellets using Purelink Hipure DNA extraction mini-kit (Life Technologies, Waltham, MA, catalog no. K210003). To quantify *rep/cap* amplification, duplex dPCR using *rep/cap* specific primer/probes (Table S2) were performed using ecDNA as the template. The dPCR was carried out with QIAcuity Probe PCR Kit (Qiagen, Germantown, MD, catalog no. 250102) according to the manufacturer's protocol on a QIAcuity 8. *rep/cap* gene copies were normalized to the cell number for comparison as described previously.[62]

AAV transcription analysis via northern blot

Total RNA was isolated from wtAd5-infected producer cells using the RNeasy Plus kit (Qiagen, catalog no. 74134) according to the manufacturer's instructions. Total RNA (1–2 μg) from each sample was run on a 1% agarose-LE gel and transferred to a nylon membrane (Roche, Basel, Switzerland, catalog no. 11209299001) using the NorthernMax-Gly kit (Invitrogen, catalog no. AM1946) according to the manufacturer's protocol. A digoxigenin (DIG)-labeled probe targeting the *cap* sequence was prepared using the PCR DIG probe Synthesis kit (Roche, catalog no. 11636090910) according to the manufacturer's instructions. The forward primer sequence 5′-TGCAGGCGGGTGACAAT-3′ and the reverse primer sequence 5′-GGTTGTCGTTGCTGGCCCC-3′ were used for probe preparation. The blot was UV crosslinked and pre-hybridized in EasyHyb solution (Roche, catalog no. 11603558001) for 30 min before hybridization with denatured probe at 55°C overnight. The blot was then washed and developed using the DIG Wash and Block buffer set (Roche, catalog no. 11585762001) and the DIG Luminescent Detection kit (Roche, catalog no. 11363514910), according to the manufacturer's protocol. Blots were visualized and imaged with the Bio-Rad Chemidoc MP imaging system (Bio-Rad, Hercules, CA). The blot image was further quantified using Bio-Rad Image Lab software version 6.1.0.

Transcription and AAV splicing analysis via RT-dPCR

For the quantification of *rep/cap* transcripts, total RNA isolated from infected producer cells or transfected HEK293 cells was reverse transcribed to cDNA using SuperScript IV VILO Master Mix with ezDNase Enzyme (Invitrogen, catalog no. 11766050), followed by quantification via dPCR using QIAcuity Probe PCR Kit (Qiagen, catalog no. 250102) on a QIAcuity 8. Serial dilutions of cDNA reaction were used as the template for dPCR using primers/probes targeting total *rep*, *rep/cap* transcripts, or spliced *rep/cap* transcripts (Figure S2; Table S2). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal loading control. Relative *rep* or *rep+cap* total mRNA was calculated from GAPDH-normalized dPCR values compared against the wtAd5 infection condition at an MOI of 5, the non-targeting DsiRNA negative control, or the pUC57 control. AAV *rep/cap* splicing efficiency was assessed by dividing dPCR values obtained using primers/probe specifically targeting spliced transcripts (splicing set) by values using primers/probe targeting total *rep/cap* transcripts (*rep/cap* set). Adenoviral mRNA was analyzed with QIAcuity EG PCR kit (Qiagen, catalog no. 250112) with target-specific primers (Table S2) and then normalized to the GAPDH internal control.

Western blot analysis

Cell pellets were lysed in radioimmunoprecipitation assay lysis and extraction buffer (Life Technologies, catalog no. 89900) supplemented with benzonase (EMD, catalog no. 1.01697.0001, 10 U/mL), $MgCl_2$ (2 mM), and Halt protease inhibitor cocktail (Thermo Fisher, catalog no. 78425). Total cell lysates were subjected to electrophoresis in a 4%–12% NuPAGE Bis-Tris gel in NuPAGE 3-(*N*-morpholino)propanesulfonic acid running buffer (Invitrogen, catalog no. NP0001) and then transferred to a nitrocellulose membrane (Invitrogen, catalog no. IB23001) using the iBlot2 dry blotting system. The primary antibodies used were anti-AAV2 Rep (Fitzgerald, Gardner, MA, catalog no. 10RA140a, 1:200), anti-AAV2 Cap (American Research Products, Atlantis, FL, clone B1, catalog no. 690058, 1:1,000), anti-β-tubulin Dylight 680 (Invitrogen, catalog no. NA516308D680, 1:2,000), anti-Ad DBP (CUSABIO, Houston, TX, catalog no. CSB-PA365892ZA01HIL, 1:2,000), anti-Ad E1A (Santa Cruz, Dallas, TX, catalog no. sc-25, 1:2,000), and anti-Ad5 capsid (Abcam, Waltham, MA, catalog no. AB6982, 1:10,000). Goat anti-mouse H + L horseradish peroxidase (HRP) (Bio-Rad, catalog no. 1706516, 1:3,000) secondary antibody was used for anti-Rep and anti-Cap. Goat anti-rabbit immunoglobulin G (IgG) (H + L) secondary antibody HRP (Invitrogen, catalog no. 32460, 1:2,000) was used for anti-Ad DBP and anti-Ad E1A. Goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, DyLight 800 (Invitrogen, catalog no. SA510036) was used for anti-Ad5 capsid. Enhanced chemiluminescence (Bio-Rad, catalog no. 1705060) was used for detection and blot was imaged with the BioRad ChemiDoc MP imaging system. The blot image was further quantified using BioRad Image Lab software version 6.1.0.

DATA AND CODE AVAILABILITY

All raw research data used to calculate the relative levels presented in the main figures and supplemental figures can be obtained from the lead contact upon reasonable request.

ACKNOWLEDGMENTS

We would like to sincerely thank Denise Woodcock, Amy Frederick, Ajay Maghodia, and Chris Renzi for generously sharing reagents and methods. We would also like to thank Edith Pfister and Jeong Lee for their review of the manuscript. All are members of the

Genomic Medicine Unit CMC, Global CMC Development Sanofi R&D, 225 2nd Ave Waltham, MA 02451. This research was funded by Sanofi.

AUTHOR CONTRIBUTIONS

Y.N.: writing – original draft, project conceptualization, project administration, and supervision. H.P.: writing – original draft, project conceptualization, investigation, methodology, visualization, and formal analysis. Q.L.: investigation, methodology, and visualization. H.N.: investigation and validation. B.F.: supervision and funding acquisition. K.V.: writing – original draft, project conceptualization, project administration, and supervision. All authors: writing – review & editing.

DECLARATION OF INTERESTS

All authors are employed by Sanofi US and may hold shares and/or stock options in the company. A provisional patent covering the findings from this study has been submitted.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101370.

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