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Quantitative assessment on the cloning efficiencies of lentiviral transfer vectors with a unique clone site

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Lentiviral vectors (LVs) are powerful tools for transgene expression *in vivo* and *in vitro*. However, the construction of LVs is of low efficiency, due to the large sizes and lack of proper clone sites. Therefore, it is critical to develop efficient strategies for cloning LVs. Here, we reported a combinatorial strategy to efficiently construct LVs using EGFP, hPlk2 wild type (WT) and mutant genes as inserts. Firstly, site-directed mutagenesis (SDM) was performed to create BamH I site for the inserts; secondly, pWPI LV was dephosphorylated after BamH I digestion; finally, the amounts and ratios of the insert and vector DNA were optimized to increase monomeric ligation. Our results showed that the total percentage of positive clones was approximately 48%±7.6%. Using this method, almost all the vectors could be constructed through two or three minipreps. Therefore, our study provided an efficient method for constructing large-size vectors.

Lentiviral vectors (LVs) are powerful tools for gene transduction *in vitro* and *in vivo*, because of the following advantages. Firstly, LVs can transduce not only mitotically active cells, but also slowly dividing cells, and even non-dividing terminally differentiated cells[1–4]. Secondly, transgenes delivered by LVs are more resistant to transcriptional silencing, whereas, it is a common phenomenon associated with retroviral vector transduced cells after prolonged *in vitro* or *in vivo* transplantation[2–4]. In addition, LVs can accommodate the use of various ubiquitous or tissue-specific transcriptional promoters[3,5]. Furthermore, the self-inactivating safety modification of LVs, which permanently disables the viral promoter within the viral long-terminal repeat after integration, enables transgene expression in the targeted cells to be controlled solely by internal promoters[3,6]. Finally, self-inactivating modification of LVs does not reduce viral titers significantly[6]. These advantages make LVs a powerful tool for stable gene transfer and expression[7–9].

However, it can be highly challenging to clone genes of interest into the third-generation bicistronic lentiviral transfer vectors (LVs), due to their large sizes and limited clone sites, therefore, it is important to develop efficient strategies for LV cloning. Classically, two different matched sites are used to directly clone the inserts into vectors by ligation and transformation[10]. Because of the limited clone sites, this strategy is not always applicable for LV cloning. To create matched clone sites, the incorporation of restriction sites into the primers used for PCR is the most common strategy, but this method decreases ligation efficiencies due to the inability of some restriction endonucleases to cleave sites efficiently near the termini of DNA molecules[10–13]. Another strategy is to create blunt ends for the vectors and/or inserts with the Klenow fragment of DNA Polymerase I or T4 DNA Polymerase, but this method can generate recessed ends due to their 3'–5' exonuclease activity[14]. As a result, the efficiencies of subsequent ligation and transformation are also significantly decreased. SDM was first established at 1978 by Hutchison et al., and it is essential in gene functional studies, genetic engineering, protein engineering, and vector modifications[15–17]. Currently, the QuikChange™ SDM System developed by Stratagene is a commonly used kit for mutagenesis using plasmid double-stranded DNA as templates. The advantage of this strategy is that the products after mutagenesis are circular, double-stranded DNA. After restriction endonuclease digestion and purification by Agarose gel electrophoresis, theoretically, 100% of the linearized DNA fragments will have correct-digested ends. Therefore, maximal ligation efficiency can be achieved.

To our knowledge, there are no reports to date on how to efficiently construct LVs with restriction-dependent cloning strategies. The aim of this study is to create a combinatorial method to efficiently construct LVs. The strategy described herein is also suitable for constructing other vectors. In order to investigate the relationship between human polo-like kinase 2 (hPlk2) expression and α-synuclein phosphorylation *in vitro*[26], we adopted a

Figure 1 | **Schematic representation of the LV cloning strategy.** There is a BamH I clone site at the 5'-end of the original vectors pcDNA3.1/V5-His-Snk/hPlk2, and pEGFP-N1, respectively. A BamH I clone site was inserted at the 3'-ends of EGFP and hPlk2 WT by SDM, respectively. Then, K111M, T239D and T239V mutants were created through SDM using hPlk2 WT gene as template. The inserts were digested with BamH I, and purified. At the same time, pWPI vector was digested by BamH I, and treated with CIP to protect the self-circularization of the vector DNA. Finally, LVs were cloned through ligation and transformation.

modified pWPI LV, which carries an EF1α-IRES-Neomycin (Neo) cassette for the expression of dual genes, to construct LVs of hPlk2 WT and mutants, K111M, T239D, T239V, and enhanced fluorescence protein gene (EGFP), respectively. The size of the pWPI vector is about 11.4 kb, and there is a unique BamH I clone site within the vector. Here, we report a combinatorial method, which includes insertion of BamH I sites at the 3'-ends of hPlk2 and EGFP by SDM, dephosphorylating the vector DNA after BamH I digestion by calf intestinal phosphatase (CIP) treatment, optimizing the amounts and ratios of the insert and vector DNA, and using Top10 competent cells for transformation. With this approach, we successfully constructed five LVs, and the percentages of positive clones containing EGFP, hPlk2 WT, K111M, T239D, and T239V inserts, were 37%±12.4%, 43%±16.6%, 52%±21.2%, 54%±9.8%, and 54%±12.8%, respectively. This study provides an important quantitative method for efficient construction of LVs.

Results

BamH I site insertion and mutagenesis of hPlk2 mutants. SDM was performed by PCR, and the parent template DNA was digested with Dpn I. Different annealing temperatures were used for pcDNA3.1 BamH I insertion and hPlk2 mutagenesis, representing approximately $T_m-5°C$, $T_m*+2°C$, and $T_m*-5°C$ (Figure 1, Table 1, 2 and 3), respectively. After transformation and identification by sequencing, we found that, when lower annealing temperatures (approximately $T_m*-5°C$) were adopted, more colonies could be obtained, and the mutagenesis efficiencies were approximately 90% for pcDNA3.1/hPlk2/BamH I insertion, 50% for K111M mutagenesis, 60% for T239D and 90% for T239V mutagenesis, respectively (Table 1, 2). Whereas, when higher annealing temperatures (approximately $T_m-5°C$) were used, no colonies were obtained (Table 1, 3). Interestingly, when the mutagenesis reactions were annealed at $T_m*+2°C$, a few colonies were obtained after transformation. The mutagenesis efficiencies were 100% (Table 1, 3). These data suggested that lower annealing temperatures were more efficient for BamH I insertion, and hPlk2 mutagenesis. However, higher annealing temperatures resulted in decreased transformation colonies (Table 1, 2 and 3). Our results were in accord with another report that the annealing temperatures should be determined empirically case by case[10]. Although the Stratagene protocol suggested that the primer pairs should be of 25∼45 bases in length with melting temperature $(T_m) \geq 78°C$, our experiments showed that correct mutagenesis was achieved with longer primers (up to 56 bases). The mutations were ranged from 2 to 6 bases (Table 1). DH5α competent cells were also used for transformations, but no positive clones were obtained after sequencing (data not shown). Therefore, for low efficiency mutagenesis,

Table 1 | Characteristics of mutagenesis primer pairs

Primer name	Length/ mutation (bases)	T_m (°C)	T_m* (°C)	Hairpin formation	Self-Dimer formation
pcDNA3.1/hPlk2 WT/ BamH I insertion forward	48/6	87.9	68.6	No	Yes
pcDNA3.1/hPlk2 WT/BamH I insertion complement	48/6	87.9	68.6	No	Yes
pEGFP-N1/BamH I insertion forward	44/6	86.7	66.7	No	Yes
pEGFP-N1/BamH I insertion complement	44/6	86.7	66.7	No	Yes
pcDNA3.1/hPlk2 K111M forward	36/2	76.6	64.1	No	Yes
pcDNA3.1/hPlk2 K111M complement	36/2	76.6	64.1	No	Yes
pcDNA3.1/hPlk2 T239D forward	51/3	80.9	66.5	No	Yes
pcDNA3.1/hPlk2 T239D complement	51/3	80.9	66.5	No	Yes
pcDNA3.1/hPlk2 T239V forward	56/2	84.2	66.8	No	Yes
pcDNA3.1/hPlk2 T239V complement	56/2	84.2	66.8	No	Yes

Note: T_m: primer-to-template annealing temperature, which considered the mismatches of the bases; T_m*: primer-pair self-annealing temperatures.

Top10 cells were recommended for transformation, because the transformation efficiency of Top10 could reach up to 1×10^9 cfu/μg supercoiled DNA, whereas, DH5α just about 10^6 cfu/μg supercoiled DNA (Invitrogen). In addition, we also performed the insertion of BamH I site at the 3'-end of EGFP using pEGFP-N1 vector as template. After annealing at 62°C, the mutation efficiency was about 83.5% (Figure 1, Table 2). These data demonstrated that the designed mutagenesis was successfully achieved.

CIP treatment improved the rates of recombinant vectors. A unique BamH I site of pWPI/Neo/BamH I LV was employed as a clone site. To protect the vector self-circularization, the 5'-phosphate groups of the vector DNA were removed with CIP treatment following BamH I digestion. This treatment could diminish the background of transformed bacterial colonies that carried empty plasmids. At the same time, the colony number would be significantly decreased after transformation[10,18,19]. Insert (EGFP, hPlk2 WT, K111M, T239D and T239V, respectively) and vector DNA were pooled together in 10μl ligation reactions with total DNA concentrations around 19~30ng/μl, and insert to vector molar ratios about 1~3.2:1 (Table 4, 5). To improve the transformation efficiencies, Top10 cells were used for transformation. Four independent ligation and transformation experiments were conducted with Top10 cells (n=4, Table 4, 5). Compared with DH5α cells, more colonies were obtained from Top10 cell transformations, whereas, a few colonies were gained from three DH5α cell transformations of each, respectively (Table 5). 41 of those colonies were selected for identification, respectively. The results showed that the total percentage of recombinant vectors was up to 93.7% (192/205). Among them, the individual percentages of inserted vectors of EGFP, hPlk2 WT, K111M, T239D, and T239V, were 97%±5.5%, 95%±10.5%, 91%±10.9%, 95%±6.4%, and 93%±5.2%, respectively (Figure 2, Table 5). The percentages of inserted vectors between them had no significant differences (*P>0.05*) (Table 5). These data demonstrated that the self-ligation of the vectors was efficiently protected by CIP treatment, and the rates of recombinant vectors were radically increased.

Efficient construction of LVs with monomeric, correct orientations. After miniprep, the insert number and orientation were primarily identified by Not I digestion. Because there was a unique Not I restriction endonuclease site inside pWPI/Neo/BamH I vector at 1145 nucleotide (nt) and the BamH I clone site at 3502 nt. There was also a Not I site at the 3'-end of EGFP (about 0.75kb). Therefore, after Not I digestion, if the vector was empty, one band was detected by Agarose gel electrophoresis; if the vector contained one copy of the insert and in correct orientation, two bands of ~3.1 kb and ~9 kb sizes were detected; and if the insert was in opposite orientation, two bands of ~2.4 kb and ~9.75 kb sizes were detected (Figure 2, A and B). In addition, within the 5'-BamH I-hPlk2-3'-BamH I fragment (about 2.1kb), there was a Not I site at 118 nt. Therefore, if the vector contained one copy of the insert in the correct orientation, two bands of ~2.5 kb and ~11 kb sizes were detected; and if the insert was in the opposite orientation, two bands of ~4.4 kb and ~9.2 kb sizes were detected (Figure 2, B, C, D, E, F, G and H). Some of the positive clones were further confirmed by sequencing. Our data revealed that the total percentage of monomeric inserted vectors of EGFP, hPlk2 WT, K111M, T239D, and T239V, was approximately 90.2% (185/205), and the individual percentages of those were 93%±5.2%, 89%±12.4%, 88%±15.6%, 91%±6.2%, and 93%±5.2%, respectively. In addition, the percentages of monomeric inserted vectors between them had no significant differences (*P>0.05*) (Table 5). Furthermore, the total percentage of positive clones of EGFP, hPlk2 WT, K111M, T239D, and T239V, in which monomeric, correct-oriented inserts were carried, was about 48%±7.6%, and the individual percentages of those were about 37%±12.4%, 43%±16.6%, 52%±21.2%, 54%±9.8%, and 54%±12.8%, respectively. Finally, the percentages of positive clones between them were not significantly different (*P>0.05*) (Table 5).

As controls, pWPI vector DNA, which was not treated with CIP, was also used for ligation with the inserts, EGFP, hPlk2WT, K111M, T239D and T239V, respectively. After transformation into Top10 cells, 10 clones of each were selected for identification. Our results revealed that, among the 50 clones identified, 5 clones were with monomeric insert, and one clone of pWPI/hPlk2T239V/Neo was

Table 2 | Mutagenesis efficiency of BamH I insertion and hPlk2 mutants

Mutagenesis	Annealing Temperature (°C)	Hosts of transformation	No. of obtained colonies	No. of sequenced colonies	Percentage of positive colonies
pcDNA3.1/BamH I	59	Top10	13 (n=1)	10	90% (9)
pEGFP-N1/BamH I	62	Top10	6 (n=1)	6	83.3% (5)
K111M	59	Top10	~200 (n=1)	10	50% (5)
T239D	63	Top10	~200 (n=1)	10	60% (6)
T239V	63	Top10	19 (n=1)	10	90% (9)

Note. pcDNA3.1/BamH I: pcDNA3.1/hPlk2WT/ BamH I insertion; pEGFP-N1/BamH I: pEGFP-N1/BamH I insertion; K111M: pcDNA3.1/hPlk2K111M/BamH I; T239D: pcDNA3.1/hPlk2T239D/BamH I; T239V: pcDNA3.1/hPlk2T239V/BamH I.

Table 3 | Mutagenesis efficiencies with different annealing temperatures

Mutagenesis	Hosts of transformation	Annealing temperature (°C) & total No. of transformation colonies		No. of identified colonies	Percentages of positive mutagenesis
pcDNA3.1/BamH I	Top10	83/0 (n=1)	70/8 (n=1)	4	100% (4/4)
K111M	Top10	72/0 (n=1)	66/1 (n=1)	1	100% (1/1)
T239D	Top10	76/0 (n=1)	68/0 (n=1)	0	0/0
T239V	Top10	79/0 (n=1)	68/1 (n=1)	1	100% (1/1)

with monomeric correct-oriented insert. The total percentage of positive clones was 2%±4.5% (n=5), and when compared with the CIP-treated experiment data, the difference between them was highly significant (2%±4.5% vs 48%±7.6%, $P<0.01$).

Transient expression of hPlk2 WT, mutants, and EGFP genes. To test the function of constructed vectors, four pWPI/hPlk2WT/Neo, two of each pWPI/K111M/Neo, pWPI/T239D/Neo, pWPI/T239V/Neo, and pWPI/EGFP/Neo positive clones were randomly chosen for transient expression in HEK293T cells. The hPlk2 WT and mutant gene sequences contained a 6xHis tag at their C-termini, therefore, their expression was tested with Western blotting for the 6xHis tag expression. EGFP transient expression was observed by fluorescence microscopy. All the vectors were efficiently expressed in HEK293T cells, 48 hours after transfection (Figure 3).

Discussion

Creation of compatible ends between vectors and inserts is the first step for cloning. It is critical that the ends of DNA fragments are correctly generated after restriction digestion, particularly for large-size, low-efficiency cloning. Regular vectors, such as pcDNA3 and pcDNA4 (Invitrogen), contain multiple clone sites. Therefore, it is convenient to choose two different clone sites for directional cloning. Generally, LVs carry limited clone sites, and a unique clone site is usually used for cloning, such as BamH I or EcoR V[5,20] etc. The incorporation of restriction endonuclease sites into the primers for PCR is a commonly used strategy. But the extra 3-4 bases at the terminal restriction sites are insufficient for stable association with and cutting by certain restriction endonucleases. Therefore the cloning efficiencies were very low, for example, as reported ≤0.05%[11,13]. Another strategy is to create blunt ends for the vectors and/or inserts with the Klenow fragment of DNA polymerase I or T4 DNA polymerase. This method is also of low efficiency due to two reasons. One is the low efficiency of blunt end ligation compared with cohesive ends. The other is the inefficiency of creation of correct blunt ends, for example, the filling-in efficiency of Klenow was reported as ≤50%[14,21,22]. In our previous LV vector cloning experiments, blunt ends were created for the inserts with Klenow and T4 DNA polymerase treatment. After dozens of ligations and transformations with Top10 cells, no positive clones were obtained (data not shown). SDM is a powerful tool to change DNA sequences at specific positions in genetic engineering, including insertion of restriction sites[16,17,23]. According to the working format of the QuikChange™ SDM System, the mutagenesis products after Dpn I digestion and transformation are circular double-stranded plasmid DNA (Stratagene). Therefore, after restriction digestion, 100% of the purified linearized DNA fragments through Agarose gel electrophoresis are theoretically with correct ends. Whereas, the correct cutting ends can not be confirmed with the method of incorporating of restriction sites into PCR primers[11,13]. Our data demonstrated that the clone efficiencies were significantly improved with this strategy. The total percentage of recombinant clones was about 93.7%, and the percentage of positive clones with monomeric, correct-oriented inserts was around 48%±7.6% (Table 5, Figure 2).

Design of the primers is crucial for successful mutagenesis. According to the guide of the QuikChange™ SDM System of Stratagene, complementary primer pairs were employed in the same PCR. One disadvantage of using complementary primer pairs is the formation of "primer dimers" in PCR reactions. Therefore the yield of successful transformants is reduced. This phenomenon is particularly severe in SDM, because the primers used include mismatched nucleotides for generating the desired mutations[23]. Another disadvantage of the Stratagene strategy is that the newly synthesized DNA is "nicked". They cannot be used as templates for subsequent amplification compared with regular PCR. This constraint also leads to lower PCR efficiency[17]. To circumvent these problems, several modifications were developed, such as using primers containing extended non-overlapping sequences at the 3' end (significantly larger than suggested in Ref. 24) and primer-primer complementary sequences at the 5' end[17], running two PCR reactions in parallel with each one of the forward and reverse primers[23] and so on. At the present study, we adopted the classical Stratagene strategy, and encountered problems in transformation using DH5α cells with its suggested annealing temperatures. After optimizing the annealing temperatures (approximately T_m*-5°C), and employing Top10 cells for transformation, the mutagenesis efficiencies were approximately 90% for pcDNA3.1/hPlk2/BamH I insertion, 83.3% for pEGFP-N1/BamH I insertion, 50% for K111M mutagenesis, 60% for T239D and 90% for T239V mutagenesis, respectively (Table 1, 2). Furthermore, compared with DH5α cells, Top10 cells were more applicable for

Table 4 | ligation reactions of pWPI (CIP-treated) with hPlk2 WT, mutants and EGFP

Experiment No.	#1	#2	#3	#4
pWPI [volume (μl), concentration (ng/μl)]	5* (31.8)	2 (98)	2 (124)	2 (107)
EGFP [volume (μl), concentration (ng/μl)]	2 (11.8)	2 (11)	2 (11)	2 (11)
hPlk2WT [volume (μl), concentration (ng/μl)]	3.5 (15.8)	2 (23)	2 (23)	2 (23)
K111M [volume (μl), concentration (ng/μl)]	3.5 (26.8)	2 (26)	2 (26)	2 (26)
T239D [volume (μl), concentration (ng/μl)]	3.5 (16.8)	2 (26)	2 (26)	2 (26)
T239V [volume (μl), concentration (ng/μl)]	3.5 (8.9)	2 (26)	2 (26)	2 (26)
ddH_2O (μl)	0	4.5	4.5	4.5
10X T4 DNA ligase buffer (μl)	1	1	1	1
T4 DNA ligase (2000U/μl) (μl)	0.5	0.5	0.5	0.5

Note: *6.5 μl for EGFP ligation.

Table 5 | Construction efficiencies of lentiviral vectors with CIP-treated vector DNA

Vector	Molar ratio & concentration of inserts/vector	Hosts of transformation	Total No. of transformed colonies	Total No. of identified colonies	Percentage of inserted vectors (Mean±SD)	Percentage of monomeric inserted vectors (Mean±SD)	Percentage of Correct-oriented inserts (Mean±SD)
EGFP	1.3~1.7 **:** 1 (n=4) (21.8~27.0ng/μl)	**Top10**/DH5α	**149±100 (n=4)**[a] /2[a] (n=3)	**41 (n=4)** /2 (n=1)	**97%±5.5%**[a] **(40) /**100% (2)	**93%±5.2%**[a] **(38) /**100% (2)	**37%±12.4%**[a] **(16) /**0 (0)
hPlk2 WT	1.1~1.8 **:** 1 (n=4) (21.4~29.4ng/μl)	**Top10**/DH5α	**123±108 (n=4)**[a] /1[a] (n=3)	**41 (n=4)** /1 (n=1)	**95%±10.5%**[a] **(38)** /100% (1)	**89%±12.4%**[a] **(36)** /100% (1)	**43%±16.6%**[a] **(17)** /100% (1)
K111M	1.2~3.2 **:** 1 (n=4) (24.8~30.0ng/μl)	**Top10**/DH5α	**123±88 (n=4)**[a] /3[a] (n=3)	**41 (n=4)** /3 (n=1)	**91%±10.9%**[a] **(37) /**100% (3)	**88%±15.6%**[a] **(36)** /100% (3)	**52%±21.2%**[a] **(21)** /66.7% (2)
T239D	1.2~2 **:** 1 (n=4) (21.8~30.0ng/μl)	**Top10**/DH5α	**126±78 (n=4)**[a] /7[a] (n=3)	**41 (n=4)** /6 (n=1)	**95%±6.4%**[a] **(39)** /100% (6)	**91%±6.2%**[a] **(37)** /100% (6)	**54%±9.8%**[a] **(22)** /83.3% (5)
T239V	1~1.5 **:** 1 (n=4) (19.0~30.0ng/μl)	**Top10**/DH5α	**98±60 (n=4)**[a] /4[a] (n=3)	**41 (n=4)** /4 (n=1)	**93%±5.2%**[a] **(38)** /100% (4)	**93%±5.2%**[a] **(38)** /100% (4)	**54%±12.8%**[a] **(23)** /100% (4)

Note: Data in boldfaces are obtained from Top10 cell transformation. Percentage of inserted vectors=No. of inserted vectors/total No. of identified colonies; Percentage of monomeric inserted vectors=No. of monomeric inserted vectors/total No. of identified colonies; Percentage of positive colonies=No. of vectors with monomeric correct-oriented insert/total No. of identified colonies. [a]Values in the same column indicates no significant difference (*P>0.05*); EGFP: pWPI/EGFP/Neo; hPLK2WT: pWPI/hPlk2WT/Neo; K111M: pWPI/hPlk2K111M/Neo; T239D: pWPI/hPlk2T239D/Neo; T239V: pWPI/hPlk2T239V/Neo.

low-efficiency transformation. This was further proved by our transformation experiments (Table 5).

Ligation and transformation are complicated procedures. Many factors can affect their efficiencies, such as the dephosphorylation of the vectors, the concentrations and ratios of the vector and insert DNA, the amount of DNA used for transformation, and so on. Removing the 5'-phosphate residues from both termini of the vector DNA can efficiently minimize the re-circularization of vector DNA (Figure 1), and therefore, decrease the background with empty transformants. In our experiment, a unique BamH I site was used as clone site (Figure 1), and the total percentage of recombinant clones after CIP treatment of the vector was up to about 93.7%. Our data were consistent with other reports[18,19]. The difference of clone efficiencies between non-CIP treated vectors and CIP-treated vectors was highly significant (2%±4.5% VS 48%±7.6%, *P<0.01*). Some reports recommend that DNA concentration of ligation is about 10 ng/μl, the ratio of insert to vector DNA can be around 1 to 1, and the transformation volumes are less than 10% of the competent cells[10,25]. Considering these recommendations, we used around 19~30 ng/μl DNA, low insert to vector molar ratios (1~3.2:1) for ligation, and 2μl (about 38–60 ng) volume of the ligation products for transformation. We found that these optimizations resulted in high efficiencies for the construction of LVs with monomeric, correct-oriented inserts (Table 5).

Compared with ligation-independent cloning strategies, such as the In-Fusion method[28], here we report an efficient ligation-dependent method to construct LVs, which circumvents the barriers for efficient cloning of large-size vectors. Firstly, creation of clone site by SDM guaranteed that 100% of the linearized DNA fragments were with correct cutting-ends. Secondly, dephosphorylation of vector DNA confirmed that most of the transformants (93.7%) were with recombinants. Thirdly, optimization of the amount and ratio of the insert and vector DNA increased the rate of monomeric, correct-oriented recombinants (48%±7.6%). Finally, Top10 cells improved the transformation efficiencies, and therefore facilitated sufficient colonies for identification. As a result, five bicistronic LVs were successfully constructed. These vectors were efficiently expressed in HEK293T cells after transfection (Figure 3). pWPI/hPlk2WT/Neo and pWPI/hPlk2K111M/Neo vectors were further used to infect neural progenitor cells for stable expression expreiments[26]. This study provided an important quantitative method for cloning large-size vectors, and furthermore, accelerated the establishment of *in vitro* gain-of-function models for gene function analyses.

Methods

Design of SDM primers. Two-step SDM strategies were performed to sequentially insert BamH I sites for plasmids pcDNA3.1/V5-His-Snk/hPlk2[27] (pcDNA3.1/hPlk2, Addgene plasmid 16015) and pEGFP-N1 (Clontech) at the 3'-end of hPlk2WT and EGFP open reading frames, respectively, and then create hPlk2 mutants: K111M, T239D, and T239V with pcDNA3.1/V5-His-Snk/hPlk2WT/BamH I as template (Figure 1). All primers were designed according to the guide of Stratagene's QuickChange™ SDM kit, synthesized and purified by Integrated DNA Technologies. For all primers, mutagenized positions were denoted in lower case and underlined.

pcDNA3.1/hPlk2 WT/ BamH I insertion forward: 5'-CATCATCACCATCACCATTGAggatccGTTTAAACCCGCTGATCAGCC-3';

pcDNA3.1/hPlk2 WT/BamH I insertion complement: 5'-GGCTGATCAGCGGGTTTAAACggatccTCAATGGTGATGGTGATGATG-3';

pcDNA3.1/hPlk2 K111M/BamH I forward: 5'-CAAAGTCTACGCCGCAAtgATTATTCCTCACAGCAG-3';

pcDNA3.1/hPlk2 K111M/BamH I complement: 5'-CTGCTGTGAGGAATAATcaTTGCGGCGTAGACTTTG-3';

pcDNA3.1/hPlk2 T239D/BamH I forward: 5'-GAACCCCTTGGAACACAGAAGGAGAgacATATGTGGTACCCCAAATTATCTC-3';

pcDNA3.1/hPlk2 T239D/BamH I complement: 5'-GAGATAATTTGGGGTACCACATATgtcTCTCCTTCTGTGTTCCAAGGGTTC-3';

pcDNA3.1/hPlk2 T239V/BamH I forward: 5'-CTAGAACCCTTGGAACACAGAAGGAGAgtGATATGTGGTACCCCAAATTATCTCTC-3';

pcDNA3.1/hPlk2 T239V/BamH I complement: 5'-GAGAGATAATTTGGGGTACCACATATCacTCTCCTTCTGTGTTCCAAGGGTTCTAG-3';

pEGFP-N1/BamH I insertion forward: 5'-GTACAAGTAAAGCGGCCGCggatccGACTCTAGATCATAATCAG-3';

pEGFP-N1/BamH I insertion complement: 5'-CTGATTATGATCTAGAGTCggatccGCGGCCGCTTTACTTGTAC-3'.

The melting temperatures (T_m, primer-to-template annealing temperature) and primer-pair self-annealing temperatures (T_m*) were calculated by Stratagene Quikchange Primer T_m Calculator (http://www.stratagene.com/QPCR/tmCalc.aspx), and Integrated DNA Technologies SciTools OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx), respectively (Table 1). Hairpin and self-dimer formation of the primers were analyzed by Integrated DNA Technologies SciTools OligoAnalyzer 3.1 as well (Table 1). All the DNA preparation kits, including Miniprep, Maxiprep, and Gel extraction kits, were purchased from QIAGEN. The DNA purities used in experiments were tested by NanoDrop-1000 Spectrophotometer (NanoDrop Technologies), and all the A260/280 values were ≥ 1.80.

Mutagenesis. The PCR reactions were carried out with GeneAmp® PCR System 2700 (AB Applied Biosystems). The 50 μl PCR reaction was carried out with 50ng templates, 125 ng of each forward and complement primers, 20μM of each dNTP (Invitrogen), 2.5 U of PfuUltra DNA polymerase, in 1 X reaction buffer (Stratagene). The thermal cycler programs for amplifications were as follows: denaturation at 94°C for 2 min; 18 cycles at 94°C for 30 sec, annealing for 30 sec, at 59°C for pcDNA3.1 BamH I insertion

Figure 2 | Agarose gel electrophoresis for identification of constructed vectors digested by Not I (three independent experiments, n=3). A. lanes 1–9, 10–18, and B, lanes 1–9: pWPI/EGFP/Neo. A, lanes #2, 4, 6, 7, 10, 11, 15, and B. lanes #4, and 5, were positive with correct orientation; A. lanes #1, 3, 5, 8, 9, 12, 13, 16, 17, 18, and B, lanes #1, 3, 6, 7, 8, and 9, were negative with opposite orientation. Lane M: GeneRuler™ 1kb DNA Ladder Plus (Fermentas). **B. lanes 10–18, and C, lanes 1–9, 10–18: pWPI/hPlk2WT/Neo.** B, lanes #10, 13, 16, 17, 18, and C, lanes #1, 5, 6, 8, 9, 10, and 17, were positive with correct orientation; B, lanes #11, 12, 14, 15, and C. lanes #2, 3, 4, 7, 12, 13, 14, 16, and 18, were negative with opposite orientation. **D. lanes 1–9, 10–18 and E, lanes 1–9: pWPI/hPlk2K111M/Neo.** D, lanes #5, 8, 11, 13, 14, 15, 16, 18, and E, lanes #1, 2, 6, 7, 8, and 9, were positive with correct orientation; D. lanes #1, 4, 7, 9, 10, 12, 17, and E. lanes #3, 4, 5, were negative with opposite orientation. **E, lanes 10–18, and F, lanes 1–9, 10–18: pWPI/hPlk2T239D/Neo.** E. lanes #10, 11, 13, 15, 16, 18, and F. lanes #1, 4, 5, 8, 9, 10, 11, 12, 18, were positive with correct orientation; E. lanes #12, 14, 17, and F, lanes #3, 6, 7, 13, 14, 15, 16, were negative with opposite orientation. **G. lanes 1–9, 10–18 and H. lanes 1–9: pWPI/hPlk2T239V/Neo.** G. lanes #1, 5, 6, 9, 11, 12, 13, 17, 18, and H, lanes #1, 2, 5, 6, were positive with correct orientation; G. lanes #2, 4, 7, 8, 14, 15, 16, and H. lanes #3, 4, 7, 8, 9, were negative with opposite orientation.

and K111M mutagenesis, 63°C for T239D and T239V mutagenesis, 62°C for pEGFP-N1 BamH I insertion, respectively (Table 2), and at 72°C for 8 min for pcDNA3.1/hPlk2 template, 5 min for pEGFP-N1 template; followed by a final extension at 72°C for 10 min. When the amplifications were finished, 1 µl (10 U) of Dpn I (Stratagene) was added into each reaction, and incubated at 37°C for 1 hour. Finally, 1 µl reaction products were used for transformation into 50 µl DH5α or Top10 competent cells (Invitrogen), respectively, according to the manufacturer's guides. All the transformants were used to spread plates. Transformation colonies were selected and their plasmids were isolated by Miniprep, and the positive mutants were identified by sequencing.

Preparation of vector and insert DNA. The bicistronic LV pWPI (Addgene plasmid 12254) was modified by creating a BamH I site at 3502 nt and replacing EGFP sequence with Neo, to form pWPI/Neo/BamH I. pWPI/Neo/BamH I DNA was digested with BamH I (NEW ENGLAND BioLabs), then divided into two aliquots, one aliquot of the vector DNA was used directly for ligation, and another aliquot was treated with CIP (NEW ENGLAND BioLabs) to remove the 5'-phosphate groups[10] (Figure 1) as follows: in a 300µl reaction, containing digested pWPI/Neo DNA (about 15 µg), 50 U CIP in 1 X NEBuffer 3, at 37°C water bath for 1 hours. Both aliquots of pWPI/Neo vector DNA (CIP-treated and not treated) were purified by 1% Agarose gel electrophoresis, and recovered by QIAGEN Gel Extraction Kit. pcDNA3.1/BamH I plasmids, carrying hPlk2 WT and mutants K111M, T239D, and T239V, respectively, and plasmid pEGFP-N1/BamH I were digested with BamH I. Then the inserts, hPlk2 WT, K111M, T239D, T239V and EGFP, were recovered by 1% Agarose gel electrophoresis and gel extraction procedure, respectively.

Ligation. To achieve the molar ratios of inserts to vector about 1~3.2:1, the ligation reactions were set up according to Table 4. In 10µl ligation systems, at first, the vector and insert were pooled together, warmed at 45°C for 5 min to melt any cohesive termini that had re-annealed during fragment preparation, then chilled on ice for 2 min, and then ligated with 0.5µl (1000 U) high-concentration T4 DNA ligase (NEW ENGLAND BioLabs) in 1 X T4 DNA ligase buffer (Figure 1). The 10µl reaction mixtures were incubated in GeneAmp® PCR System 2700 at 16°C for 16 hours followed by inactivation at 65°C for 10 min, and then set at 4°C until transformation[10].

Transformation and identification. 2µl (about 38~60 ng) volumes of the ligation products were used to transform 50µl of DH5α and Top10 competent cells according to the manufacturer's instructions (Invitrogen, Table 5). Briefly, the ligation reaction tubes were centrifuged briefly and placed on ice. One 50µl vial of One Shot® Top10 cells or DH5α cells was thawed on ice for 30 min for each ligation/transformation. 2µl of each ligation reaction were added directly into the vial of competent cells and mixed by tapping gently rather than pipetting up and down. The vials were incubated on ice for 30 min. Then, Top10 cells were incubated for exactly 30 seconds in a 42°C water bath, but for DH5α cells, 20 seconds were used without shaking. The vials were

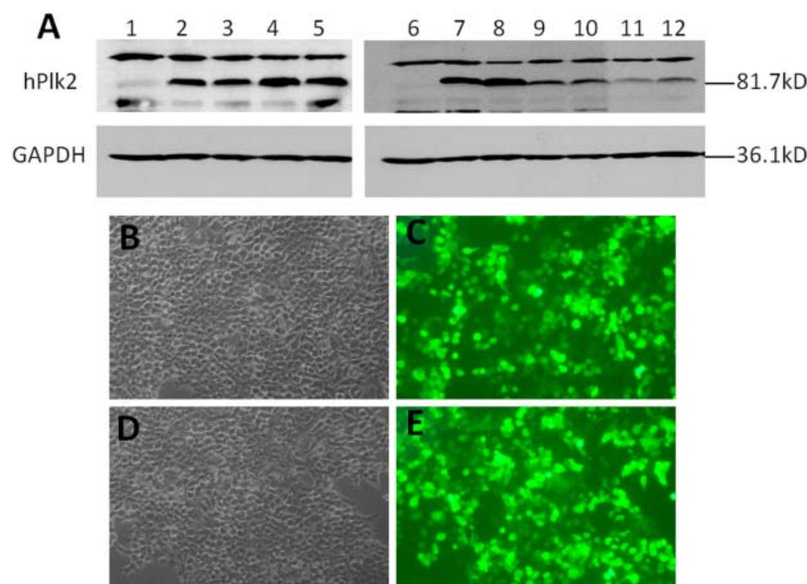

Figure 3 | Transient expression of hPlk2 WT, K111M, T239D, T239V mutants and EGFP in HEK293T cells 48 hours after transfection. (A) Cell lysates from HEK293T cells, hPlk2 WT and mutant transfected cells were run on SDS-PAGE gels and probed with anti-TetraHis antibody. lanes 1 and 6: HEK293T cells; lane 2: HEK293T/hPlk2WT #1; lane 3: HEK293T/hPlk2WT #2; lane 4: HEK293T/hPlk2WT #3; lane 5: HEK293T/hPlk2WT #4; lane 7: HEK293T/K111M #1; lane 8: HEK293T/K111M #2; lane 9: HEK293T/T239D #1; lane 10: HEK293T/T239D #2; lane 11: HEK293T/T239V #1; lane 12: HEK293T/T239V #2. B and C. HEK293T cells transfected with pWPI/EGFP/Neo #1 clone DNA, under a fluorescence microscope, the same microscopic field (at 20X original magnification) was documented by digital camera images taken with grayscale (B) and green fluorescence (C). (D) and (E) HEK293T cells transfected with pWPI/EGFP/Neo #2 clone DNA, the same microscopic field (at 20X original magnification), grayscale (D), and green fluorescence (E).

then removed from the 42°C bath and placed on ice for 2 min. 250μl of pre-warmed S.O.C medium (Invitrogen) were added to each vial, and the vials were shook at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. In order to obtain more colonies, all the transformation cells were used to spread plates. After the plates were dried, they were inverted and incubated at 37°C overnight. Positive colonies were primarily analyzed by Not I (NEW ENGLAND BioLabs) digestion. And some of them were further confirmed by DNA sequencing.

Cell culture and transfection. HEK293T cells were thawed from liquid nitrogen and cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Wisent Biocenter) for a week in 10-cm dishes. 6-well plates were coated with 0.001% poly-L-lysine (Sigma) for 15 minutes at room temperature[29]. When HEK293T cells were split, the cells were washed with Ca^{2+}, Mg^{2+}-free DPBS twice (Wisent Biocenter), then treated with 0.25% Trypsin-EDTA (Wisent Biocenter) for 1 min at room temperature, and then pipetted up and down several times to separate them into single cells. In order to obtain even distribution of the cells across the surface of the plates, the cells were swirled thoroughly before seeding[29]. When the cells were 50–70% confluent, transfection was performed with Lipofectamine® 2000 according to the manufacturer's guide (Invitrogen). Briefly, for each transfection, 4μg DNA of pWPI/hPlk2WT/Neo, pWPI/hPlk2K111M/Neo, pWPI/hPlk2T239D/Neo, pWPI/hPlk2T239V/Neo, and pWPI/EGFP/Neo, respectively, was diluted in 250μl DMEM medium (without FBS), mixed thoroughly by low-speed vortex. 10μl Lipofectamine 2000 was diluted in 250μl DMEM, mixed gently by inversion, and incubated for 5 min at room temperature. The diluted DNA and Lipofectamine 2000 were combined, mixed gently by inversion, and incubated for 20 min at room temperature to form Lipofectamine-DNA complexes. Finally, the 500μl complexes were added to each well containing HEK293T cells and 2 ml DMEM + 10% FBS medium, mixed gently by rocking the plates. The cells were incubated at 37°C in a CO_2 incubator for 6 hours or overnight. The medium was replenished with fresh DMEM + 10% FBS medium. 48 hours after transfection, the cells were collected for Western blotting to test the transgene expression.

Western blotting. Cell lysates were prepared in cell lysis buffer (50 mM Tris, pH8.3, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, and 1 X Protease inhibitor cocktail (Sigma)). Briefly, cell culture medium was removed from 6-well plates, and washed with ice-cold DPBS twice. Then 300μl lysis buffer was added into each well, incubated on ice for 10 min. 75μl 5 X Laemmli buffer (312.5 mM Tris-HCl, pH6.8, 10% SDS, 250 mM DTT, 50% Glycerol, and 0.05% Bromophenol blue) was added into each tube, mixed and heated at 95°C for 10 min. Finally, the samples were stored at −80°C until running SDS-PAGE.

10%, 1.0 mm SDS-PAGE gels were run to separate the sample proteins. 10μl of BenchMark™ prestained protein ladder (Invitrogen), and 20μl of each sample were loaded. HEK293T cell lysate was used as negative controls for the transgene expression. The gels were run in 1XTris-glycine SDS buffer (Bioshop). The proteins were transferred onto nitrocellulose and processed for Western blotting, and protein bands were visualized with ECL-plus Western blotting detection system (GE Healthcare).

Antibodies used in this study included: mouse anti-tetraHIS primary antibody (1:2000 dilution) (Qiagen), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (1:2000 dilution) (Sigma). The goat anti-mouse secondary antibody was Immunopure® goat anti-mouse IgG, H+L, Peroxidase conjugated, purchased from Thermo Scientific. Goat anti-mouse secondary antibody was 1:2500 diluted for probing tetraHIS expression, and 1:8000 diluted for GAPDH expression. All the antibodies were diluted in Tris buffered saline (TBS, Wisent Biocenter) with 0.05% Tween-20 and 5% milk.

Fluorescence microscopy. A Axiovert 100TV (Zeiss) fluorescence microscope equipped with a AttoArc™ HBO 100W (Carl Zeiss) lamp power supply was used to observe the transient expression of EGFP in HEK293T cells, 48 hours after transfection. Microscopic images were photographed using a digital camera (Kodak DC290 ZOOM).

Data Statistics. Data were analyzed by Mean±SD and Student's t-Test.

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Author contributions

G.Z. and A.T. conceived and designed the experiments. G.Z. performed the experiments and carried out data analysis. G.Z. wrote the paper. A.T. edited the paper.

Additional information

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