Effective downregulation of *BCR-ABL* tumorigenicity by RNA targeted CRISPR-*Cas13a*

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Running title: CRISPR effectively downregulates BCR-ABL in CML cell line

Keywords: CRISPR, *Cas13a*, CML, K562 cell line

Abstract

CML is a clonal myeloproliferative disorder of pluripotent stem cells driven by a reciprocal translocation between chromosome 9 and 22, forming a *BCR-ABL* fusion gene. Tyrosine kinase inhibitor drugs like imatinib are mainstay of treatment and cases resistant to these drugs have a poor prognosis in absence of a compatible stem-cell donor. However, with rapid advancements in the gene editing technologies, most studies are now focusing on developing a translational model targeting single gene disorders with prospective permanent cure. The present study explores the potential application of the RNA targeting CRISPR *Cas13a* system for effective knockdown of *BCR-ABL* fusion transcript in K562 cell line. The CRISPR system was successfully able to lower the expression of downstream genes (*pCRKL* and *pCRK*) dependent on the activated *BCR-ABL* kinase signal by up-to 4.3 folds. The time dependent kinetics also highlighted the significant *in-vitro* suppressive activity to last up to 8 weeks (p-value: 0.025). As per the cDNA sequencing data from Oxford MinION next generation sequencer, the CRISPR activity was 57.45% resulting in 42.55% wild type, 19.15% stop-gain, 10.64% frameshift and 27.66% base substitution transcripts. These preliminary results highlight an excellent potential application of RNA targeting CRISPRs in Hematological neoplasms like CML and should pave way for further research in this direction.

1. Introduction

Chronic myeloid leukemia (CML) is caused by a reciprocal translocation between chromosome 22 and 9, resulting in a chimeric *BCR-ABL* protein having kinase activity [1]. The translocation is characteristically between band q34 of chromosome 9 bearing Abelson (*ABL*) proto-oncogene and band q11 of chromosome 22 bearing the breakpoint cluster region (*BCR*) gene [2]. The *BCR-ABL* fusion gene protein can vary from 190 kDa to 230 kDa depending upon the breakpoint site on *BCR* gene [3]. Most commonly found *BCR-ABL* fusions carry their breakpoints in the 2.9 kb region between exons 13 and 15 (b2 and b4) of *BCR* region that fuses with the large intronic region of *ABL* between exons 1b and 2b. This forms an 8.5 kb *BCR-ABL* transcript containing *BCR* exon b2/b3 and *ABL* exon 2 (a2). Both of these combinations (b1a2 and b3a2) code for the same 210 kDa protein [3]. However, irrespective of the breakpoint, the *BCR-ABL* chimeric kinase activates downstream proteins like *pCRKL*, *pCRK*, p*STAT5* etc that further modulate expression of target genes to give proliferative advantage to the Philadelphia chromosome (Ph) carrying pluripotent stem cells [4].

With recent advancements in gene editing research, much focus has drifted towards targeting single gene disorders for possible therapeutic cure. In this regard, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), has emerged as a highly versatile, flexible and cost-effective tool for gene editing experiments. This tool has circumvented the shortcomings of the previous tools like transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) with improved overall turnaround time [5-8]. In Hematological neoplasms, the chimeric fusion kinase *BCR-ABL* of CML, seems an exciting target for CRISPR as it is the driving force behind the neoplastic pathogenesis. Working on the same idea, in 2017, García-Tuñón *et al*, demonstrated the use of CRISPR *Cas9* for targeting *BCR-ABL* chimeric trans-gene [9] in a synthetically engineered Boff-p210 cell line bearing the transgene coding for the p210 variant of the *BCR-ABL* fusion gene. This extensive work showcased for the first time the application of CRISPR in down regulating

the *BCR-ABL* chimeric gene. However, in actual cases of CML, the breakpoints might differ at the DNA level due to different intronic breakpoint regions in ABL gene [10]. Hence, using DNA targeting CRISPR will require a per case targeted sequencing and custom CRISPR sgRNA/ sgRNAs to effectively knockdown the *BCR-ABL* chimeric gene. This would require a dedicated research team which will eventually incur substantial costs and increase the turnaround time per case. Also, the custom target effectiveness might vary over the range of patients and hence a definitive prognosis might never be established. All these issues can be circumvented by employing techniques that can target the *BCR-ABL* transcript rather than the DNA as the former will remain immune to the intronic changes at the DNA level. This was only possible after the recent development and assessment of RNA targeting CRISPRs [11]. These CRISPR systems, unlike DNA targeting *Cas9*, are additionally more flexible and exhibit less off-target and more on-target activities. In current experimental study, we've explored the use of CRISPR *Cas13a*, a form of RNA targeting CRISPR, in knocking down the *BCR-ABL* transcript and quantified the downstream gene products (*pCrKL* and *pCrK*) using flow-cytometry and western blot techniques. The overall activity of CRISPR has been enumerated by deep sequencing of cDNA by Oxford Nanopore Technologies MinION system. The developed CRISPR system was found to be effectively down regulating the levels of downstream genes of the CML pathogenesis pathway. Moreover, we have tried highlighting the time dependent kinetics of downstream effects of the editing. To the best of our knowledge, this is the first such *in vitro* experimental study successfully employing RNA targeting CRISPR-*Cas13a* in knocking down the *BCR-ABL* transcript and is likely to pave way for further research in the field.

2. Materials and Methods

2.1 Construction CRISPR plasmids

The CRISPR RNA (crRNA) was designed using CRISPR-RT server [12]. Only the crRNA designs overlapping *BCR* and *ABL* breakpoint region without any predicted off targets were considered. The top and bottom crRNA oligos with *BbsI* overhangs were procured from

Sigma-Aldrich (St. Louis, Missouri, US). The two oligos were phosphorylated using T4 polynucleotide kinase and ligated using T7 polymerase to form a double stranded crRNA as per the protocol described by Cong *et al*, 2014 [13]. The plasmids *pC016 - LwCas13a guide expression backbone with U6 promoter* (Addgene #91906) and *pC034 - LwCas13a-msfGFP-2A-Blast* (Addgene #91924) were obtained as gifts from Dr Feng Zhang. The plasmid pC034 carries blasticidin resistance and green fluorescent protein (GFP) expression markers. The plasmid pC016, was digested using *BbsI-HF* (New England Biolabs, MA, USA, Cat#R3539L) as per the manufacturer's protocol with slight modification of over-digesting the plasmid in 10U of the enzyme thrice in continuity with 16hrs of incubation each time without deactivating the enzyme in between ensuring a successful second cut after the plasmid is linearized by the first cut. The pC016 plasmid with insert (pC016I) was then transformed into competent *Escherichia coli DH5* cells using heat shock method as described by Sambrook and Russell [14]. Plasmid was extracted from the positive clone using QIAprep Spin Miniprep kit as per the manufacturer's protocol.

2.2 Transfection of K562 cells with CRISPR plasmids

Lipofectamine LTX (cat#15338100, Thermo-Fisher Scientific, MA, US) was used for the co-transfection of the two plasmids, pC016I and pC034 using the manufacturer's protocol in a six well plate containing approximately 50,00,00 cells from overnight culture of K562 cells in RPMI-1640 medium with 1% Penicillin-Streptomycin at a confluency of 75-80%. The cells were incubated overnight at 37°C with 5% CO_2. After the overnight incubation, old media was replaced with fresh and additionally 6 µg/mL of blasticidin antibiotic was added as the positive selection marker for the plasmid in both test and negative control cells [15]. Cultures were observed till all negative control cells were killed and GFP expression of the transfected cells became close to hundred percent. Cells were harvested at passage number eight, seventeen and thirty-six for downstream analysis and time dependent kinetics study.

2.3 Downstream gene regulation analysis

Total RNA was extracted using RNeasy kit from QIAGEN (cat#74104) as per the manufacturer's protocol. A fraction of RNA was reverse transcribed to cDNA using RevertAid first strand cDNA synthesis kit by Thermo Fisher scientific (cat#K1622) using the manufacturer's protocol. The *BCR-ABL* cDNA region was amplified using specific primers, forward 5'-ACAGAATTCCGCTGACCATCAATAAG-3', reverse 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3' [16]. The amplified region was sequenced using ABI 3500 automated Sanger sequencing platform (cat#4440462). EditR [17] and PolyPeakParser [18] were used to enumerate base changes in the 28 nucleotide long crRNA region with *BCR-ABL* cDNA of K562 cells as control. MuPro [19] and I-Mutant [20] were used to assess the effects of these base changes. For the flow cytometric analysis of *pCRKL* levels, the *pCRKL (Y207)* specific Alexa Fluor® 647 tagged antibody (cat#560790, Becton, Dickinson and Company, NJ, US) was used as per the manufacturer's protocol in a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., IN, US). The fold change was calculated by comparing the mean fluorescence intensity (MFI) using median population and at least 10,000 events per test between K562 cells as control and K562 cells bearing CRISPR plasmids (coded as K562-1634). The calculations were done in Kaluza Analysis software by Beckman Coulter.

Whole cell protein extraction for western blot was done using Radioimmunoprecipitation assay (RIPA) buffer (cat#89900, Thermo Fisher Scientific, MA, US) using the manufacturer's protocol. Bicinchoninic acid (BCA) protein assay was done for extracted protein quantification using Pierce™ BCA Protein assay kit according to the manufacturer's protocol (cat#23225, Thermo Fisher Scientific, MA, US). For western blotting, primary rabbit raised antibodies against *pCRKL Y207* (37 kDa) and *pCRK Y221* (34 kDa) (another downstream target protein) were procured from Abcam plc, Cambridge, UK with catalogue numbers ab52908 and ab76227 respectively and housekeeping mouse raised *β-tubulin* (51 kDa) primary antibody was procured from Cell Signaling Technologies, MA, US with catalogue number 2146S. The anti-rabbit and anti-mouse secondary horseradish peroxidase

linked antibodies were procured from Cell Signaling Technologies with catalogue numbers 7074P2 and 7076P2 respectively. Image J software was used for analysis of the western blot imaging data [21].

2.4 Enumeration of CRISPR activity by NGS

Total RNA from K562-1634 and K562 cells were subjected to cDNA sequencing using Oxford Nanopore Technologies MinION instrument with SQK-PCS108 library kit along with barcodes from SQK-PBK004 kit on a FLO-MIN106 R9.4 flow cell according to the manufacturer's protocol. QCAT tool was used for demultiplexing the barcoded reads (https://github.com/nanoporetech/qcat), NanoFilt was used to filter the data with minimum quality cut off of 5 [22]. NCBI local BLAST+ was used to extract raw reads carrying crRNA sequence [23]. The reads carrying the crRNA sequence were aligned with *BCR-ABL* cDNA and crRNA sequences using CLC Genomics Workbench v.12 (QIAGEN Aarhus, Silkeborgvej, Denmark). The nucleotide and protein alignment of control K562 and K562-1634 cells were compared in the same software.

The study was duly approved by the Institute's Ethics Committee and the Departmental Review Board.

2.5 Statistical analysis

SPSS Statistics software version 25 (IBM Corp, NY, US) was used to perform statistical tests. The flow cytometry and western blot data was normalized using maximum value before performing any statistical tests. Student's t test was used to check for significant change in expression over the period of time. Pearson's correlation was used to find significant relation between flow and western blot data.

3. Results

3.1 crRNA Designing

CRISPR-RT suggested a total of 175 target regions, out of which, 14 were overlapping the *BCR-ABL* chimeric region. Among these 14 candidate crRNAs, the one 5'-ATTTAAGCAGAGTTCAAAAGCCCTTCAG-3', which spanned significantly in both *BCR* and *ABL1* regions (17 and 12 bases in *BCR* and *ABL1* respectively) with the lowest GC content of 39% and no predicted off targets was chosen.

3.2 Transfection and downstream analysis

Pure colonies of K562-1634 transfected cells were selected by day 12 (figure 1). Levels of *pCRKL* in control K562 and K562-1634 cells as assessed by flow cytometry at passages eight, seventeen and thirty-six is given in table I. Flow cytometric analysis was done in duplicate for each run and the average was used for calculations. At least ten thousand events per reaction were recorded with maximum events recorded reaching around 85,000 in a single reaction. *pCRKL* level was observed to be 4.30, 4.02 and 1.80 times lower than the control K562 cell levels at passages eight, seventeen and thirty-six respectively (p-value 0.025) (figure 2A). Western blotting data of *pCRKL* and *pCRK* (table II and figure 2 B) was found to be synchronous with the flow cytometry data after normalization with a significant p-value of 0.01 (figure 2 C).

3.3 Sanger and NGS sequencing of transfected and control cell lines

EditR suggested changes in four bases and Poly Peak Parser suggested three different changes (table III and figure 3). On assessment of all combinations of the nucleotide changes, a total of four amino acid changes were found. According to MuPro and I-Mutant, one of these changes, FK(Q/G)SSKALQ, might be causing a very large decrease in stability, while two changes, FK(Q/K)SSKALQ and FKQ(S/N)SKALQ, were suggested to cause a large decrease in stability, and one FK(Q/R)SSKALQ, was predicted to be causing only a minor decrease in stability. The next generation sequencing using Oxford MinION generated a total of 5,365,731 reads with an average read length of 402.8 bp, median read

length of 253 bp and maximum read length of 232,778 bp. On alignment of the deep sequencing data with *BCR-ABL* cDNA, we found 61.70% of total 47 aligned reads with altered bases in the 28-nucleotide long crRNA region (supplementary figure S1). On translation of these changes, we found sixteen candidate amino acid changes. Among these, 42.55% were of wild type, 27.66% were base substitutions, 19.15% were stop gain and rest 10.64% were frameshift variations. Three out of four amino acid changes suggested by EditR were found in NGS data too. One of these three has a very high effect on stability of the protein as predicted by iMutant and MuPro software. The overall efficiency of CRISPR after accounting for synonymous variants was calculated to be 57.45%

4. Discussion

Tyrosine kinase inhibitor (TKI) drugs, though being the mainstay of treatment in CML, have been shown in studies to have adverse effects on growth of children in addition to neoplastic clones developing resistance to their action during long term treatment [24]. In a retrospective study by Millot *et al.,* in 2014 which included 81 children under 18 years of age with CML, it was found that imatinib adversely affected growth in the subjected children [25,26]. Additionally, there are several cases where resistance against these drugs are forged, these cases then either require a complex treatment regime including trials with other TKI drugs or ultimately a stem cell transplantation (SCT) [27]. SCT has its own set of complications and issues. First, it is hard to find a compatible donor, if found, there are chances of development of graft-versus-host diseases (GVHD) [28]. Even if everything is in place, treatment costs become unaffordable for most people, especially from developing countries like India. Gene editing based corrective techniques might prove to be beneficial in such conditions in long term considering their current efficiency and flexibility. There are four major genome editing tools, namely, Meganucleases (MNs), ZFNs, TALENs and the most recent, CRISPR. CRISPR finds its edge in being almost 10 times cheaper than its closest competitor, TALENs, has widest applications, is freely available and simplest to design and apply [29]. Earlier, in a study by García-Tuñón *et al*, it was observed that CRISPR

Cas9 was able to effectively control the oncogenic capabilities of *BCR-ABL* chimeric gene in custom Boff-p210 cell line carrying *BCR-ABL* fusion transgene [9]. In a recent study, Liu *et al* demonstrated effective down regulation of *BCR-ABL* gene using CRISPR RNA guided *FokI* Nucleases in both cell lines and murine xenograft model [30]. However, García-Tuñón *et al*, in their other research article on CRISPR, published recently, highlighted the limitation of CRISPR/Cas9 in effectively knocking out target DNA without a null variation inducing homology directed repair template in the construct. They went on to demonstrate that effective knockdown could be achieved if sgRNAs target the splice donor site of the gene [31]. Moreover, it has been shown that in CML, using a DNA targeting CRISPR would mean designing custom guide RNAs per patient as the breakpoints might differ per case at the DNA level [10]. Considering above limitations and problems in targeting *BCR-ABL* gene at DNA level by CRISPR/Cas9, we hypothesized utility of targeting the p210 transcript of CML with a RNA CRISPR since the p210 transcript wouldn't be likely affected by the differences in intronic breakpoints. Working on this hypothesis, we designed this study to explore the potential of RNA targeting CRISPR *Cas13a* in knocking down the tumorigenicity of the *BCR-ABL* chimeric gene. CRISPR *Cas13a* is a relatively newer form of CRISPR which targets RNA rather than the DNA and has been shown to have an efficiency of around 50% [11]. In the current study too, we found the efficiency to be around 57.45%. This RNA targeting CRISPR system was able to lower the expression of downstream genes of CML pathogenesis as per the KEGG [32] pathway ID hsa05220.

pCRKL levels as calculated through passage numbers 8, 17 and 36 were 23.26, 24.85 and 55.43% respectively by flow cytometric analysis and 15.75, 17.52 and 54.00% respectively by western blotting experiment. The same for *pCRK* was found to be 37.84, 38.23 and 77.80% through passage numbers 8, 17 and 36 respectively as per the western blotting data. All the cells were killed in presence of blasticidin antibiotic by passage number 42. This tells us that the cells started to lose plasmid around passage number 36 and by 42 they had all lost it. This suggests that the RNA targeting CRISPR *Cas13a* in vitro is likely to persist

till two months with no significant decrease in effectiveness. Post that time, the effectiveness starts deteriorating which gradually comes down to the cells losing the plasmid. On the analysis of the *BCR-ABL* transcript changes by both Sanger sequencing and NGS, we could assert an overall 57.45% shift from the wild type *BCR-ABL* transcript. There were three potential alternate transcripts that were confirmed by both sequencing methods. These included one very high, one large and one minor pathogenic variant as predicted by MuPro and iMutant. We could say that NGS yielded more information than Sanger sequencing as the later has a higher minimum detection threshold of around 20% [33]. This assumes importance since certain transcript changes could be at a lower level than the minimum required threshold of Sanger sequencing. Overall, our study results show that CRISPR-*Cas13a* efficiency (based on NGS) and down regulation of downstream genes (*pCRKL* & *pCRK*) go hand in hand *in vitro*. The study is limited by further analysis on primary culture of multiple CML patients. Utilizing newer, more efficient RNA targeting CRISPRs like CRISPR *Cas13b* and *Cas13c* with efficiencies going up to 90-95*%* [34] can potentially revolutionize the effects further with higher fold reduction in downstream target protein expression, ultimately reducing the tumorigenic capabilities of *BCR-ABL* chimeric gene. It is interesting to mention here that imatinib in a dose dependent manner of 10 ≥μM reduces *pCRKL* levels to 16.6% which translates to a 6.05-fold reduction [35], compared to 4.3-fold reduction achieved by the described CRISPR *Cas 13a* RNA machinery. However, as detailed above, with rapid advancements in the CRISPR technology, this gap might be filled or even surpassed in near future.

Conflicts of Interest: The authors declare no conflict of interests.

Author Contributions: AS and PB designed the research study. AS performed the experimental research and wrote the article. PB guided the research and edited the manuscript with significant inputs.

Acknowledgements: AS would like to thank Dr Meenu Singh for her valuable insights during this work.

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Table Title & or Legends

Table I (Title): Fold change of *pCRKL* assessed by flow cytometry.

Table I (Footnote): K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after 'K562-1634-' denote passage numbers.

Table II (Title): Western blot analysis data for *pCRKL* and *pCRK*

Table II (Footnote): K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after 'K562-1634-' denote passage numbers.

Table III (Title): List of predicted variants by EditR and PolyPeakParser. Bases in red color highlights are the predicted changes

Table III (Footnote): Variant names with "PPP" were predicted by PolyPeakParser

Figure Title & or legends

Figure 1: Blasticidin selection of K562-1634 cells over different passages in contrast with wild type K562 cells**.** A1-A3 and B1-B3 Inverted microscopy images taken at 10x magnification on days 08, 10 and 12 for control K562 and 1634 plasmid bearing K562 cells respectively. C: Final resulting cell population of 1634 plasmid bearing cells at 10x magnification showing widespread GFP expression.

Figure 2: Flow cytometry and western blot data for control vs K562-1634 cells. A: Flow cytometry data for comparative *pCRKL* expression levels. P08, P17 & P36 denote passage numbers 8, 17 and 36 for K562 cells bearing CRISPR plasmids (K562-1634 cells). B: Comparative western blot data for *pCRK* and *pCRKL* expression levels. K- K562 cells without plasmids (control). P08, P17 & P36 denote passage numbers 8, 17 and 36 for K562

cells bearing CRISPR plasmids (K562-1634 cells). C: Comparative trends between western blot and flow cytometry data after normalization.

Figure 3: EditR predictions. Highlighted region is the area where EditR calculates a significant edit. Top picture is of the chromatogram. Middle one shows the noise bars generated by EditR and bottom picture illustrates the probable base edits as per the software.

Supplementary Data

Supplementary Figure S1: cDNA Reads aligned to reference *BCR-ABL* region following analysis of Oxford MinION sequencing data. A: Control K562 reads. B: Reads from K562 cells bearing the CRISPR plasmids.

Table I: Fold change of *pCRKL* assessed by flow cytometry.

Passage No.	**Control mean MFI**	**K562-1634 mean MFI**	**Mean fold Change**
08	35524.43	8263.72	4.30
17	38175.67	9489.39	4.02
36	24464.225	13560.96	1.80

*K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after '1634-' denote passage numbers.

Table II: Western blot analysis data for *pCRKL* and *pCRK*

Sample	***β-tubulin***	***pCRKL***	***pCRKL* ratio**	***pCRK***	***pCRK* ratio**
K562-1634-17	1.19	0.19	0.16	0.49	0.42
K562-1634-36	1.14	0.63	0.56	0.85	0.75
K562-1634-08	1.05	0.19	0.18	0.39	0.38
K562	1	1	1	1	1

*K562-1634 denotes the K562 cells bearing the CRISPR plasmids. Numbers after 'K562-1634-' denote passage numbers.

Table III: List of predicted variants by EditR and PolyPeakParser. Bases in red colour highlights are the predicted changes

S.No.	Type	Nucelotide	Amino Acid	Destability
0.	Wild	atttaagcagagttcaaaagcccttcag	FKQSSKALQ	-
1.	Var 1	atttaaAcagagttcaaaagcccttcag	FKQSSKALQ	Unchanged
2.	Var 2	atttaagAagagttcaaaagcccttcag	FKKSSKALQ	Large
3.	Var 3	atttaagcGgagttcaaaagcccttcag	FKRSSKALQ	Minor
4.	Var 4	atttaagcagaAttcaaaagcccttcag	FKQNSKALQ	Large
5.	Var 1-4	atttaaAAGAagttcaaaagcccttcag	FKRSSKALQ	Minor
6.	Var 1,2	atttaaAAagagttcaaaagcccttcag	FKKSSKALQ	Large
7.	Var 1,3	atttaaAcGgagttcaaaagcccttcag	FKRSSKALQ	Minor
8.	Var 1,4	atttaaAcaAagttcaaaagcccttcag	FKQSSKALQ	Unchanged
9.	Var 1-3	atttaaAAGgagttcaaaagcccttcag	FKRSSKALQ	Minor
10.	Var 2,3	atttaagAGgagttcaaaagcccttcag	FKRSSKALQ	Minor
11.	Var 2,4	atttaagAaAagttcaaaagcccttcag	FKKSSKALQ	Large
12.	Var 2-4	atttaagAGAagttcaaaagcccttcag	FKRSSKALQ	Minor
13.	Var 3,4	atttaagcGAagttcaaaagcccttcag	FKRSSKALQ	Minor
14.	Var PPP-C	atttaaAcgaagttcaaaagcccttcag	FKRSSKALQ	Minor
15.	Var PPP-A	atttaaAAgaagttcaaaagcccttcag	FKRSSKALQ	Minor
16.	Var PPP-G	atttaaAGGAagttcaaaagcccttcag	FKGSSKALQ	Very High

* Variant names with “PPP” were predicted by PolyPeakParser

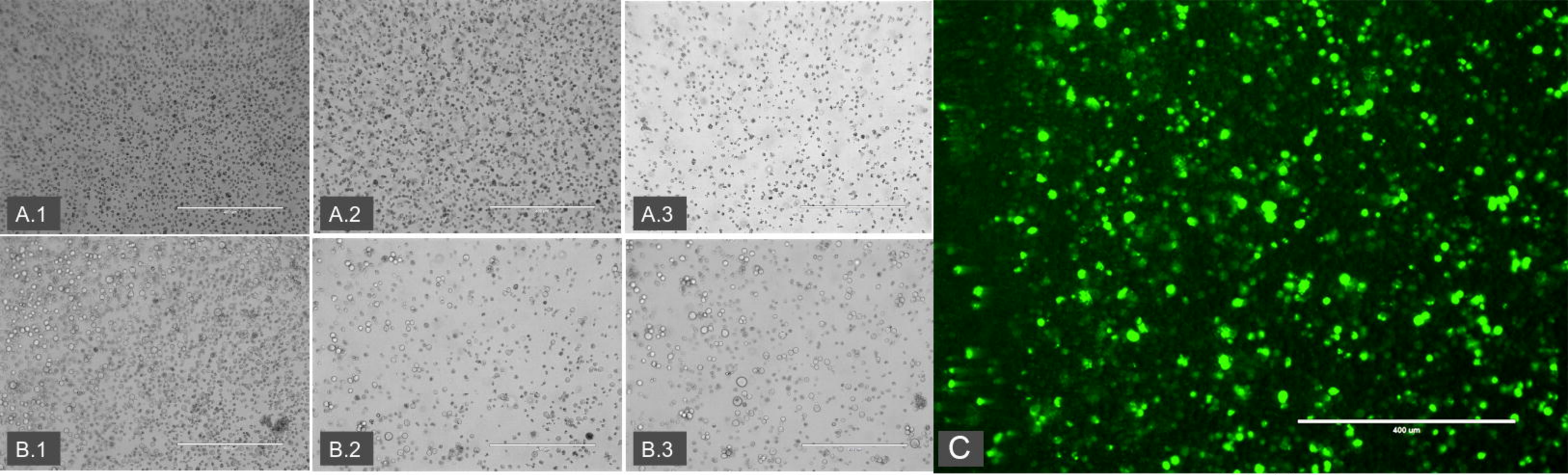


Figure 1

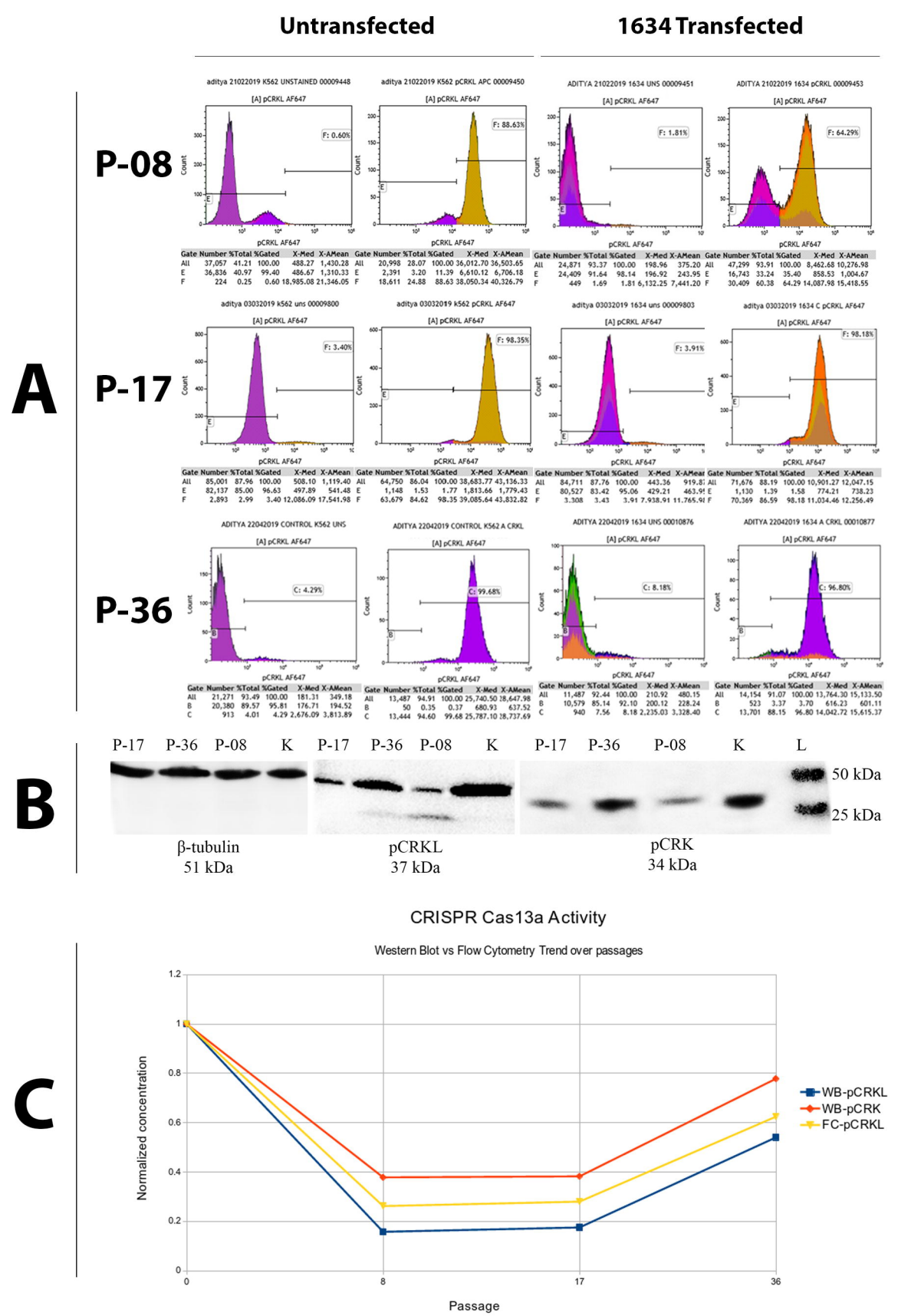
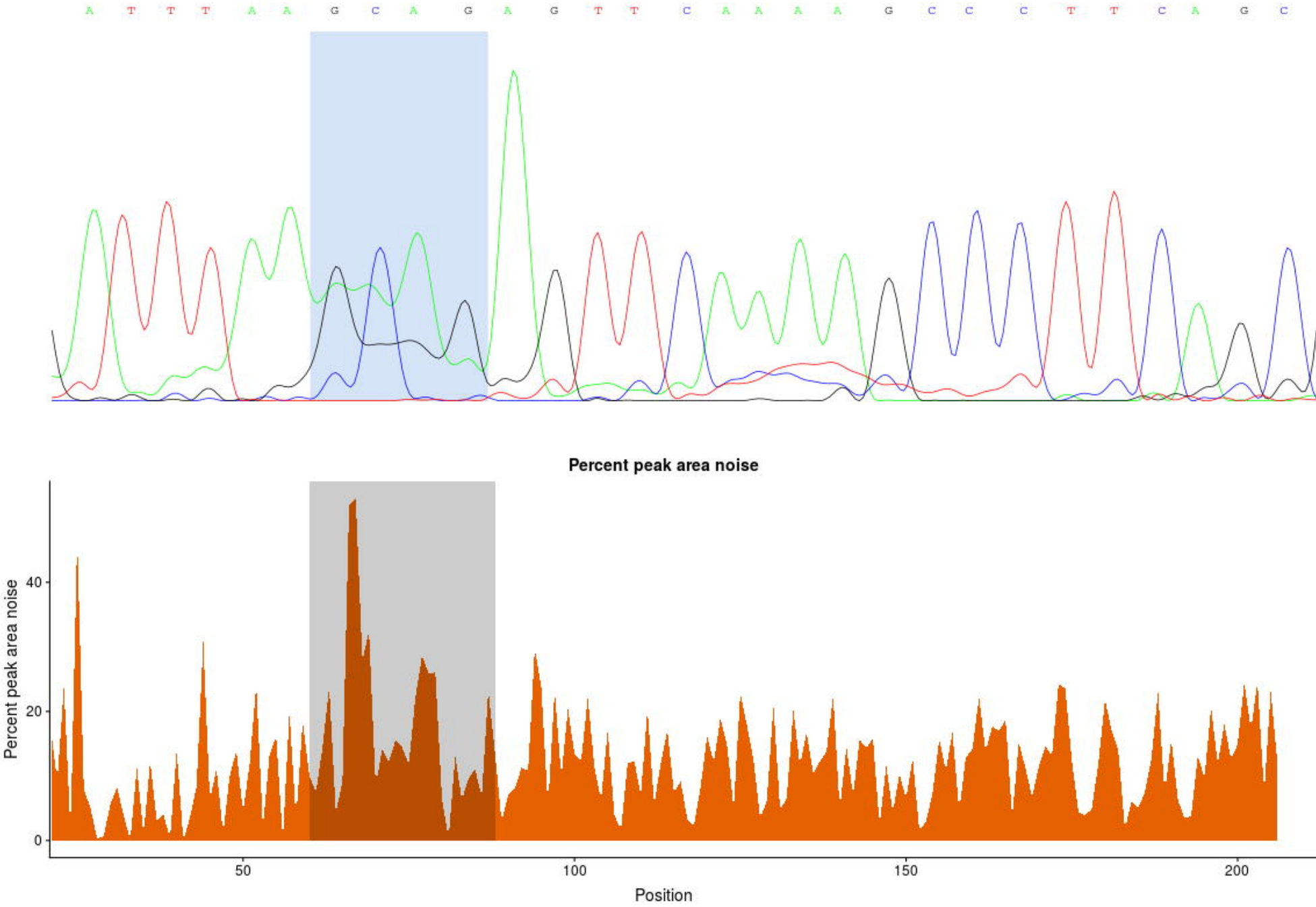

Untransfected
1634 Transfected
A
P-08
P-17
P-36
B
P-17 P-36 P-08 K P-17 P-36 P-08 K P-17 P-36 P-08 K L
50 kDa
25 kDa
β-tubulin
51 kDa
pCRKL
37 kDa
pCRK
34 kDa
C
CRISPR Cas13a Activity
Western Blot vs Flow Cytometry Trend over passages
Normalized concentration
Passage
WB-pCRKL
WB-pCRK
FC-pCRKL

Figure 2



	A	T	T	T	A	A	G	C	A	G	A	G	T	T	C	A	A	A	A	G	C	C	C	T	T	C	A	G	C
T	9	93	86	76	1	0	0	0	1	1	2	14	88	84	4	12	0	16	19	10	6	0	13	94	90	3	4	5	1
G	1	3	1	6	1	7	48	17	26	68	6	86	1	0	0	0	1	0	7	74	0	0	0	0	0	4	0	77	12
C	0	0	3	1	2	2	10	47	2	4	0	0	2	10	86	0	21	12	0	16	93	100	87	3	9	89	3	17	86
A	90	4	11	17	96	91	42	36	72	28	92	0	9	5	10	88	78	71	74	0	0	0	0	3	0	4	93	1	0

Figure 3