

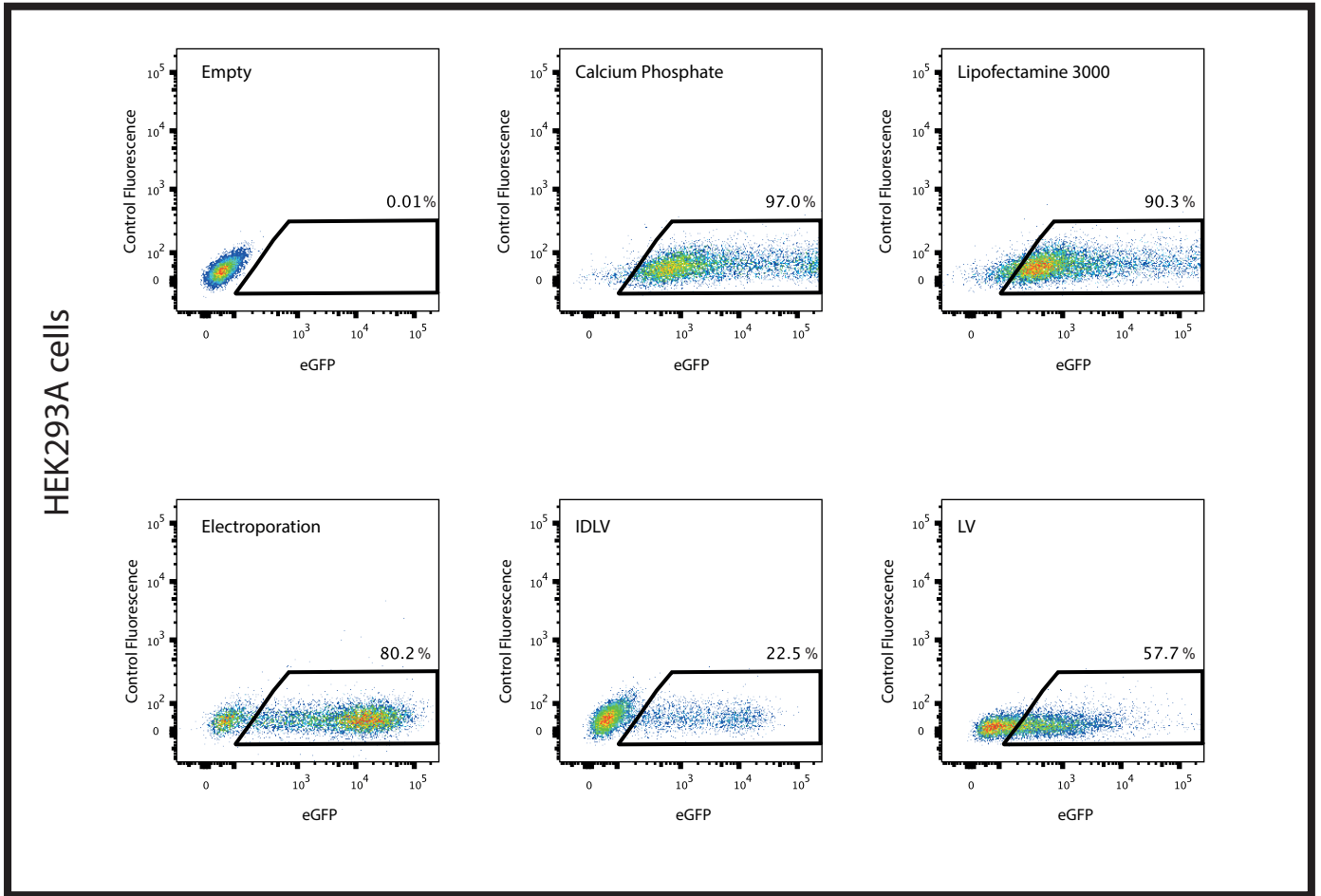
Stem Cell Reports, Volume 8

Supplemental Information

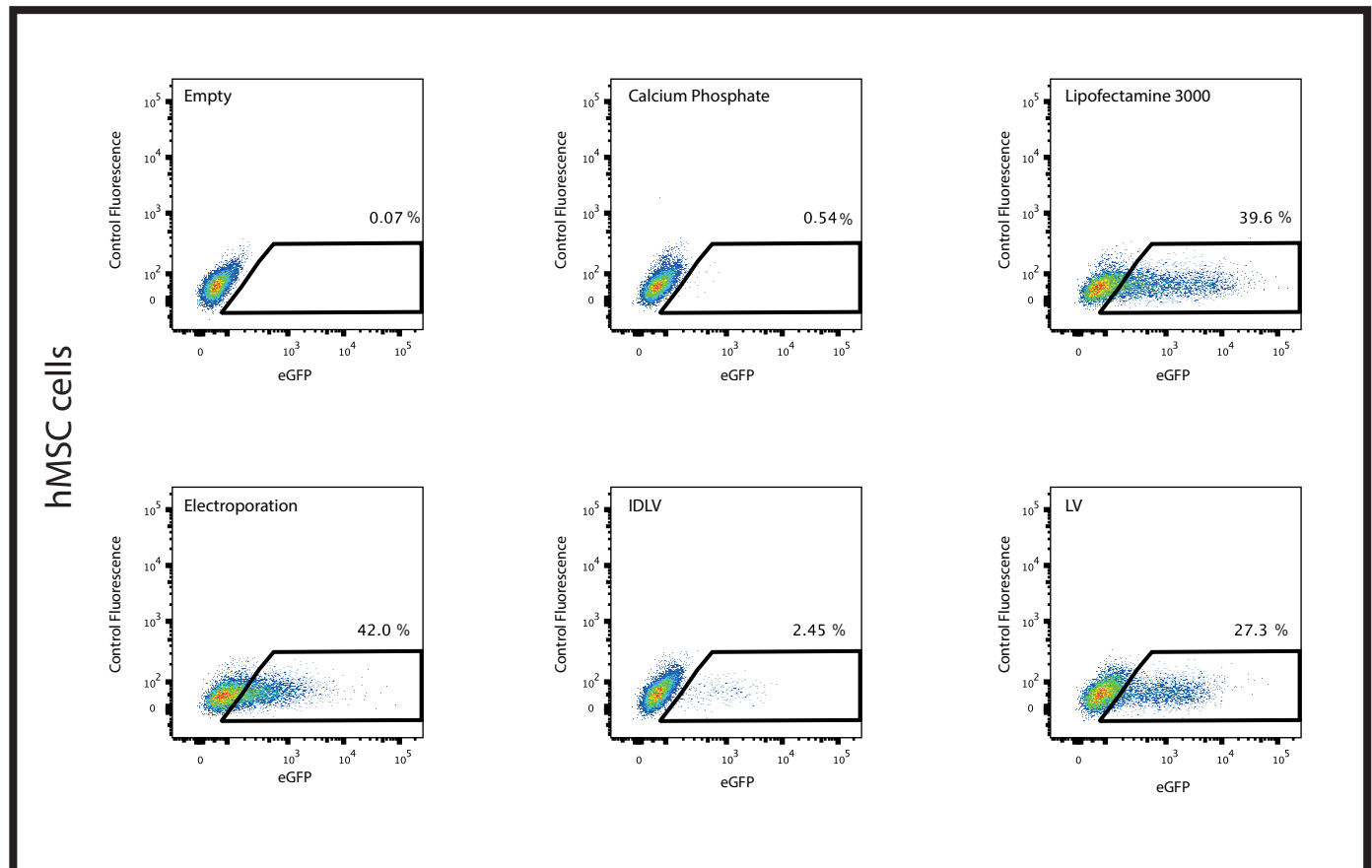
**Efficient Recreation of t(11;22) *EWSR1-FLI1*⁺ in Human Stem Cells
Using CRISPR/Cas9**

Raul Torres-Ruiz, Marta Martinez-Lage, Maria C. Martin, Aida Garcia, Clara Bueno, Julio Castaño, Juan C. Ramirez, Pablo Menendez, Juan C. Cigudosa, and Sandra Rodriguez-Perales

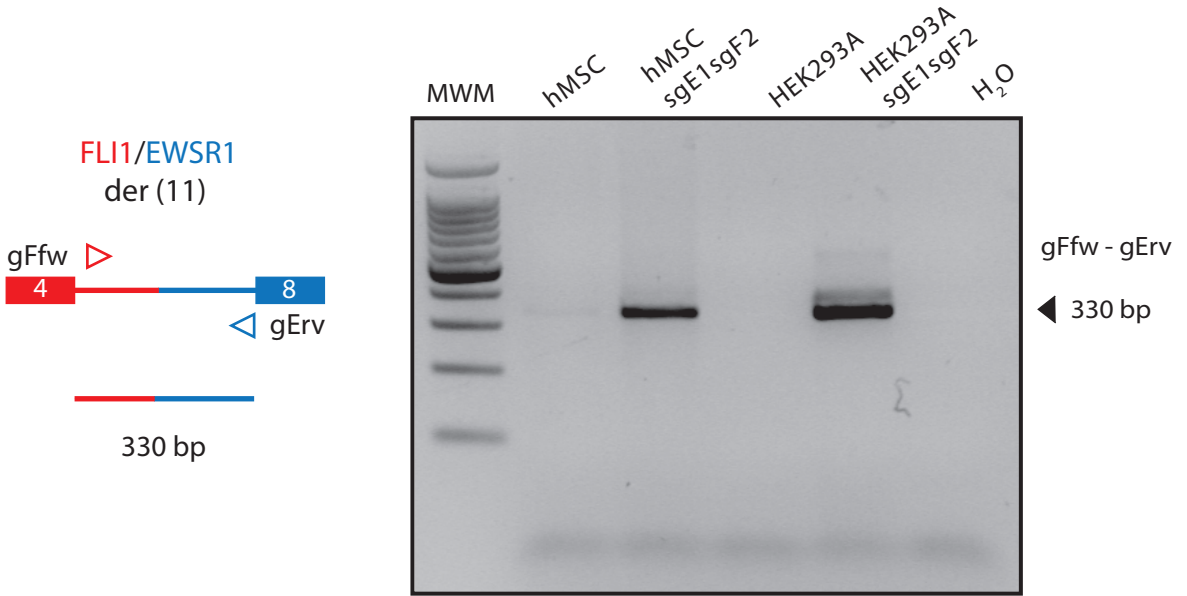
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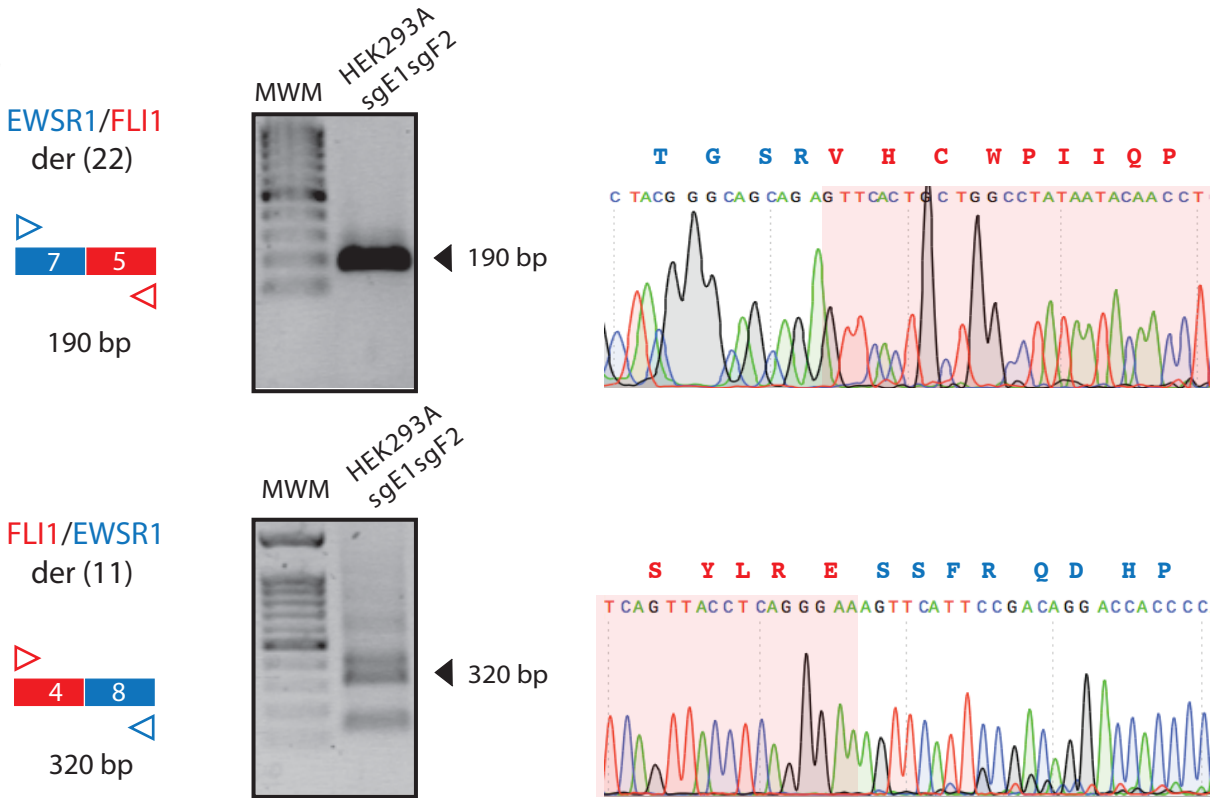
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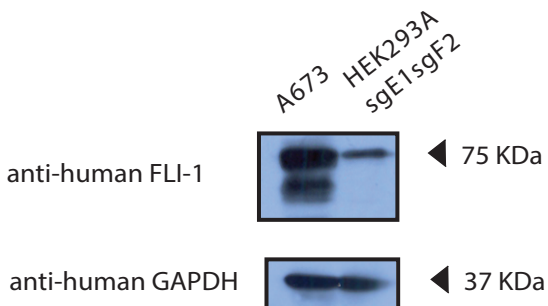
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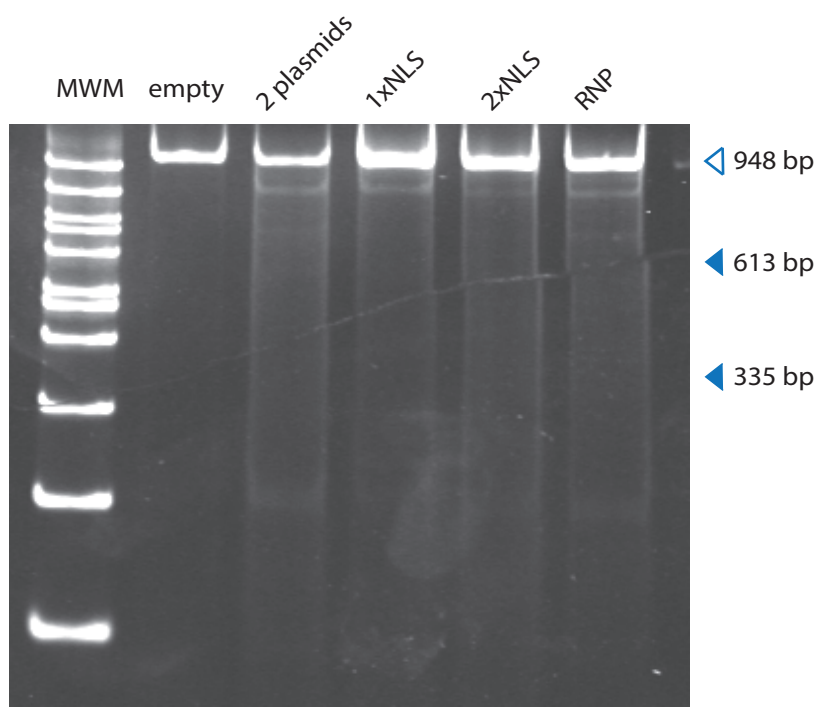
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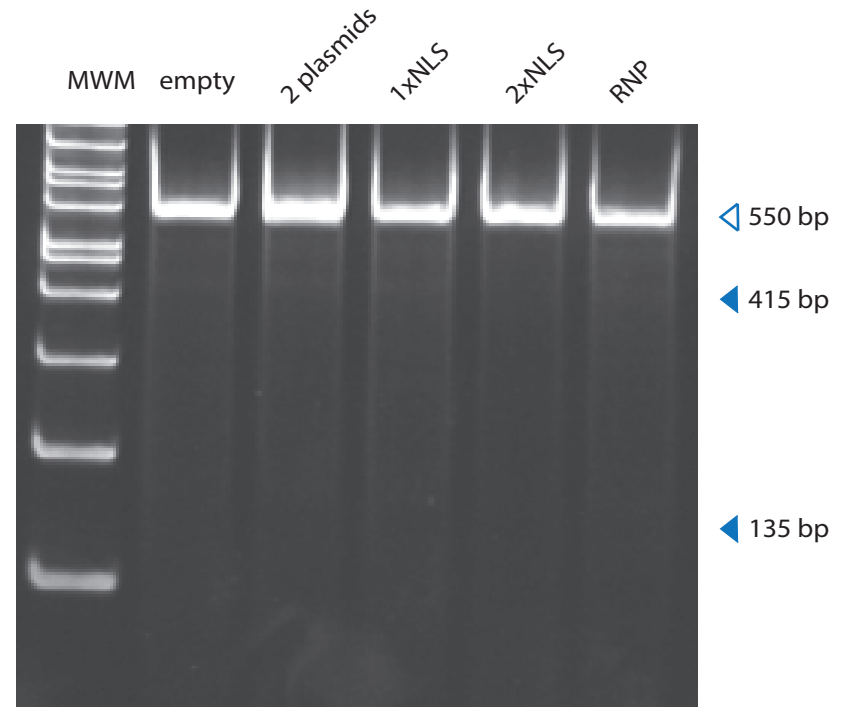
C



A

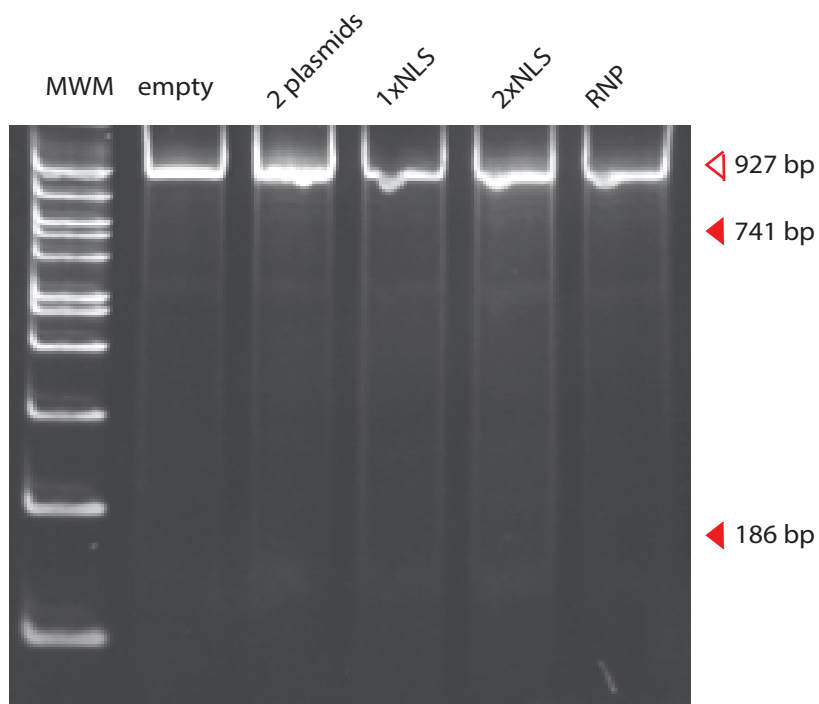


EWSR1 Off-target 1
(chromosome 14: -83517253)
AAaTTGTTTTTAGTtTGCCT aaG

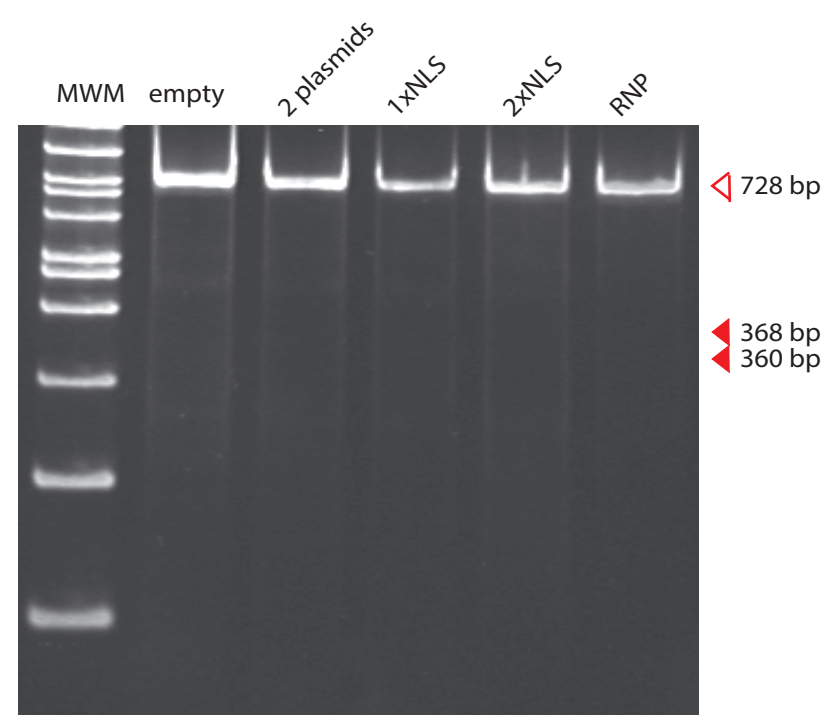


EWSR1 Off-target 2
(chromosome 14: 71328829)
AATaGgaTTTAGTATGCCT aaG

B

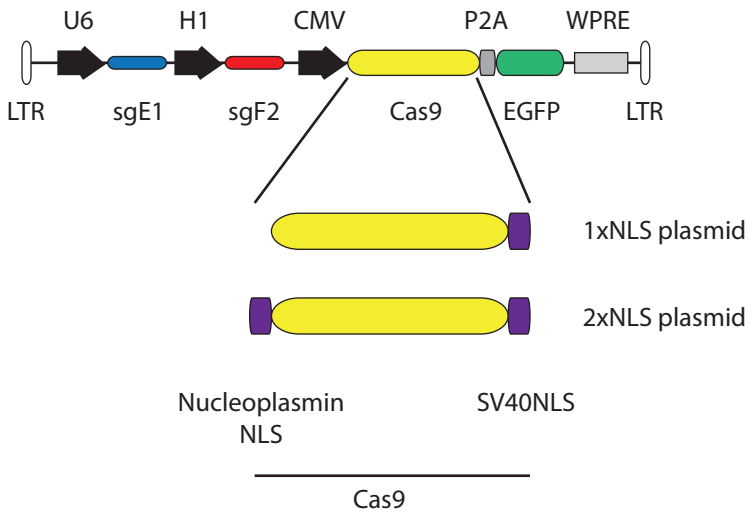


FLI1 Off-target 1
(chromosome 6: +155480)
CcaGCCTGGGCGATAGACCa caG

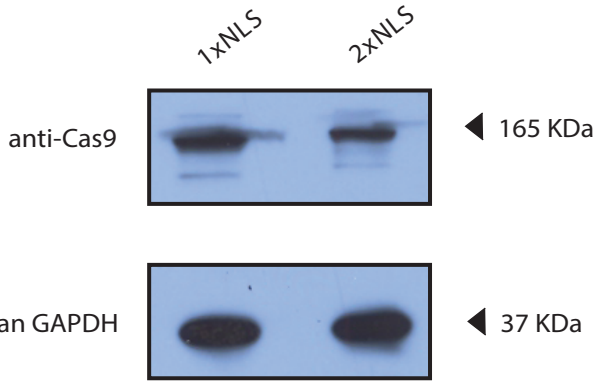


FLI1 Off-target 2
(chromosome 18: -63518093)
CcaGCCTGGGCGATAGACTg AGG

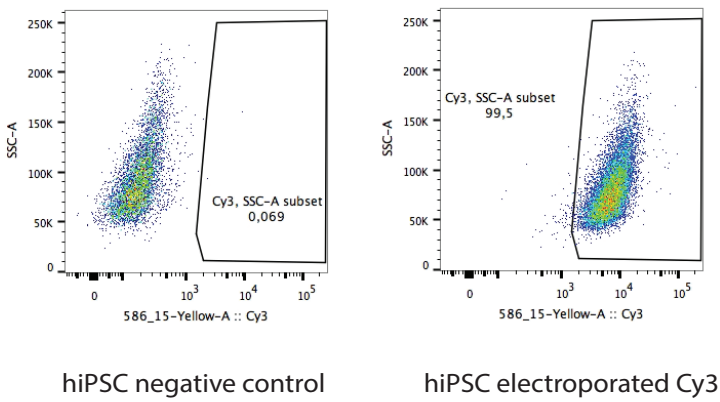
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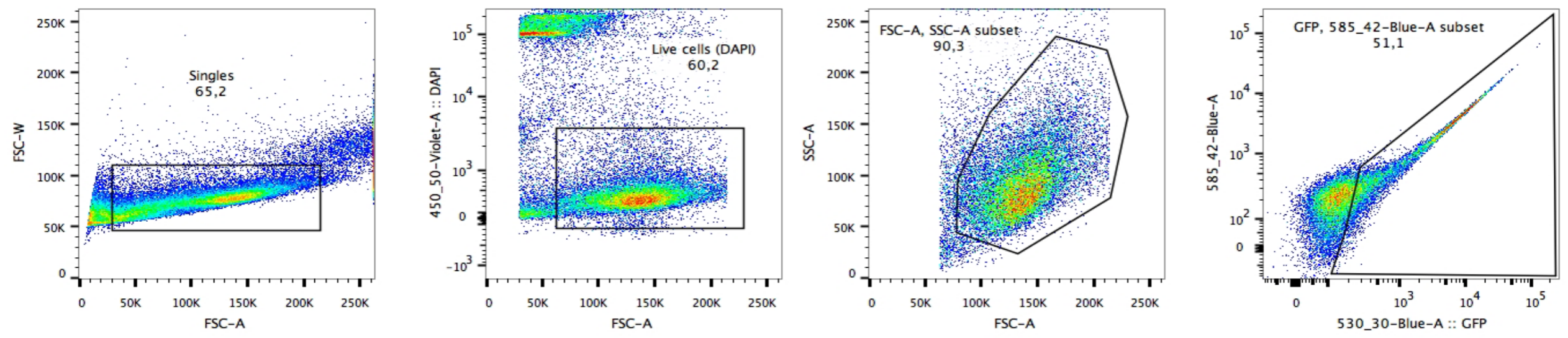
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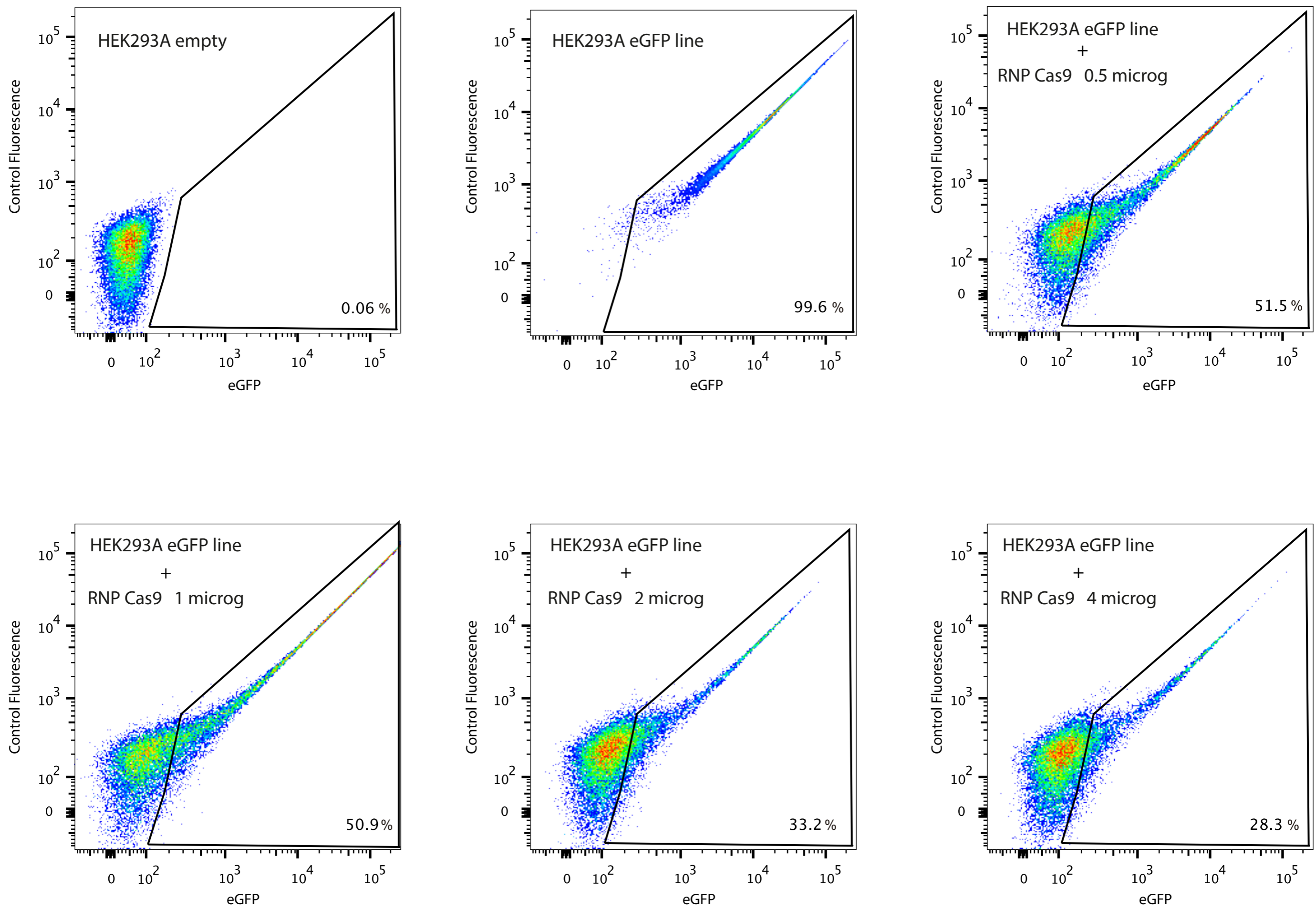
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A



B



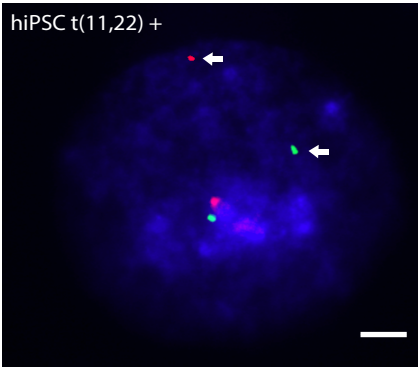
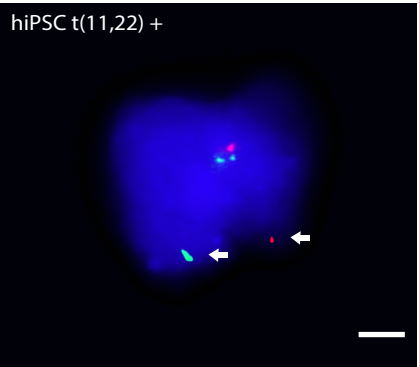
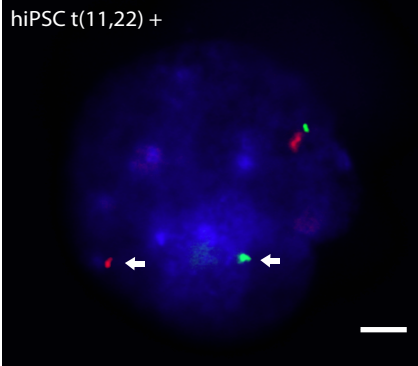
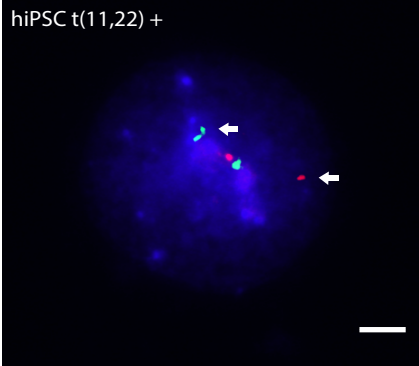


Figure S1. Flow cytometry analysis of eGFP expression achieved with different delivery systems in HEK293A cells and hMSCs. Representative eGFP flow cytometry profiles showing the transfection efficiency achieved with five distinct delivery approaches in HEK293A cells (A) and hMSCs (B). Non-transfected cells were used as negative controls. IDLV: Integrative Defective Lentivirus, LV: Lentivirus.

Figure S2. (A) Agarose-gel electrophoresis of translocation-specific PCR products from pooled HEK293 and hMSCs samples corresponding to the *FLI1-EWSR1* fusion gene mapped at der(11) chromosome. (B) Representative agarose-gel electrophoresis of EWSR1-FLI1 and FLI1-EWSR1 RT-PCR products confirming the expression of both derivative fusion genes *EWSR1-FLI1* and *FLI1-EWSR1*. Sanger sequencing chromatograms showing the breakpoint region in the fusion genes mapped at der(11) and der(22) in HEK293 cells. (C) Representative Western blot analysis of EWSR1-FLI1 chimeric protein in A673 Ewing sarcoma cell line and HEK293 cells. GAPDH was used as a loading control.

Figure S3. Off-target effects in HEK293A cells produced with different CRISPR approaches. Representative T7 endonuclease I cleavage assay in HEK293A cells after electroporation with the 2-plasmid, 1xNLS, 2xNLS, or RNP approaches. Non-electroporated cells are used as controls. Full-length PCR products are indicated by an open triangle; predicted positions of digestion products are indicated by filled triangles.

Figure S4. (A) Schematic representation of 1xNLS and 2xNLS versions of pLV-U6#1H1#2-C9G. (B) Representative Western blot analysis of Cas9 protein in HEK293 cells transfected with either 1xNLS or 2xNLS versions of pLV-U6#1H1#2-C9G. GAPDH was used as a loading control. (C) Representative flow cytometry analysis of Cy3 signals in hiPSCs transfected with a synthetic Cy3 labelled oligonucleotide.

Figure S5. (A) Representative gating strategy to remove clumps or doublets, dead cells and debris or other events of non-interest from the data set while preserving cells based on size and or complexity. This gating strategy was used in Figures S1 and S5. (B) Flow cytometry analysis of eGFP expression in HEK293A cells after nucleofection with increasing amounts of RNPs.

Figure S6. Representative FISH images showing t(11;22)+ hiPSC after 8 weeks in culture. Arrows indicate separated signals corresponding to the der(22) and der(11) breakpoint regions. Interphase nuclei are counterstained with DAPI. Scale bars, 10 μ m.

Sorting

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
|------------|-------------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------------|------------|--------|----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| 293 cells | Non Sorted | 2 | 306 | 0.65 | 2 | 311 | 0.64 | 3 | 294 | 1.02 | 7 | 911 | 0.77 | 0.22 | Non sorted | 0.3488 | ns |
| | Sorted Low | 1 | 299 | 0.33 | 3 | 315 | 0.95 | 0 | 268 | 0.00 | 4 | 882 | 0.45 | 0.48 | Low | | |
| | Sorted High | 5 | 273 | 1.83 | 5 | 303 | 1.65 | 4 | 233 | 1.72 | 14 | 809 | 1.73 | 0.09 | Low | 0.0387 | * |
| | | | | | | | | | | | | | | | High | 0.0080 | ** |
| | | | | | | | | | | | | | | Non sorted | | | |
| | | | | | | | | | | | | | | | High | | |
| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| hMSC cells | Non Sorted | 1 | 312 | 0.32 | 0 | 325 | 0.00 | 1 | 294 | 0.34 | 2 | 931 | 0.22 | 0.19 | 0.0145 | | * |
| | Sorted | 2 | 283 | 0.71 | 3 | 315 | 0.95 | 2 | 247 | 0.81 | 7 | 845 | 0.83 | 0.12 | | | |

1xNLS vs 2xNLS

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
|------------|-------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|---------|--------|----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| 293 cells | 1xNLS | 3 | 515 | 0.58 | 1 | 516 | 0.19 | 4 | 557 | 0.72 | 8 | 1588 | 0.50 | 0.27 | 1xNLS | 0.0899 | ns |
| | 2xNLS | 9 | 480 | 1.88 | 5 | 532 | 0.94 | 18 | 737 | 2.44 | 32 | 1749 | 1.83 | 0.76 | | | |
| | | | | | | | | | | | | | | | | | |
| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| hMSC cells | 1xNLS | 1 | 545 | 0.18 | 0 | 527 | 0.00 | 0 | 501 | 0.00 | 1 | 1573 | 0.06 | 0.11 | 1xNLS | 0.0145 | * |
| | 2xNLS | 2 | 495 | 0.40 | 2 | 543 | 0.37 | 3 | 500 | 0.60 | 7 | 1538 | 0.46 | 0.13 | | | |
| | | | | | | | | | | | | | | | | | |

ssODNs

| | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | | |
|-----------|----------|---------------|-----|----------|---------------|-----|----------|---------------|-----|----------|---------------|---------|------|---------|-------|--------|----|
| | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | | |
| 293 cells | 60 | 1 | 304 | 0.33 | 1 | 300 | 0.33 | 1 | 297 | 0.34 | 3 | 901 | 0.33 | 0.01 | 60 | 0.1812 | ns |
| | 90 | 1 | 347 | 0.29 | 1 | 260 | 0.38 | 1 | 308 | 0.32 | 3 | 915 | 0.33 | 0.05 | empty | | |
| | 120 | 2 | 301 | 0.66 | 1 | 300 | 0.33 | 2 | 310 | 0.65 | 5 | 911 | 0.55 | 0.19 | 90 | 0.1757 | ns |
| | 150 | 4 | 310 | 1.29 | 3 | 300 | 1.00 | 3 | 290 | 1.04 | 10 | 900 | 1.11 | 0.16 | empty | | |
| | 180 | 2 | 346 | 0.58 | 2 | 305 | 0.66 | 1 | 280 | 0.36 | 5 | 931 | 0.54 | 0.15 | 120 | 0.0469 | * |
| no ssODN | | 1 | 302 | 0.33 | 0 | 300 | 0.00 | 0 | 315 | 0.00 | 1 | 917 | 0.11 | 0.19 | empty | | |
| | | | | | | | | | | | | | | | 150 | 0.0025 | ** |
| | | | | | | | | | | | | | | | empty | | |
| | | | | | | | | | | | | | | | 180 | 0.0440 | * |
| | | | | | | | | | | | | | | | empty | | |

| | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | | |
|-----------|-------------|---------------|-----|----------|---------------|-----|----------|---------------|-----|----------|---------------|---------|------|---------|-------------|--------|-----|
| | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | | |
| 293 cells | e1f2 | 5 | 323 | 1.55 | 4 | 276 | 1.45 | 5 | 290 | 1.72 | 14 | 889 | 1.57 | 0.14 | e1f2 | 0.0006 | *** |
| | f1e2 | 2 | 194 | 1.03 | 2 | 220 | 0.91 | 4 | 234 | 1.71 | 8 | 648 | 1.24 | 0.43 | empty | | |
| | e1f2 + f1e2 | 4 | 198 | 2.02 | 5 | 263 | 1.90 | 5 | 231 | 2.16 | 14 | 692 | 2.02 | 0.13 | f1e2 | 0.0822 | ns |
| no ssODN | | 1 | 194 | 0.52 | 1 | 212 | 0.47 | 1 | 305 | 0.33 | 3 | 711 | 0.42 | 0.10 | empty | | |
| | | | | | | | | | | | | | | | e1f2 + f1e2 | 0.0058 | ** |
| | | | | | | | | | | | | | | | empty | | |

| | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | | |
|------------|-------------|---------------|-----|----------|---------------|-----|----------|---------------|-----|----------|---------------|---------|------|---------|-------------|--------|----|
| | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | | |
| hMSC cells | e1f2 | NA | NA | | NA | NA | | NA | NA | | NA | NA | | | NA | NA | |
| | f1e2 | NA | NA | | NA | NA | | NA | NA | | NA | NA | | | NA | NA | |
| | e1f2 + f1e2 | 7 | 306 | 2.29 | 8 | 250 | 3.20 | 10 | 382 | 2.62 | 25 | 938 | 2.67 | 0.46 | e1f2 + f1e2 | 0.0027 | ** |
| no ssODN | | 1 | 500 | 0.20 | 0 | 300 | 0.00 | 2 | 315 | 0.64 | 3 | 1115 | 0.27 | 0.32 | empty | | |

NA: Not Applicable

DNA-end processing factors

293 cells

| Factors | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
|---------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|---------|--------|----|
| | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| empty | 8 | 284 | 2.82 | 6 | 249 | 2.41 | 7 | 261 | 2.68 | 21 | 794 | 2.64 | 0.21 | AICDA | 0.0022 | ** |
| AICDA | 5 | 352 | 1.42 | 4 | 294 | 1.36 | 5 | 308 | 1.62 | 14 | 954 | 1.47 | 0.14 | empty | | |
| ARTEMIS | 10 | 307 | 3.26 | 9 | 273 | 3.30 | 9 | 281 | 3.20 | 28 | 861 | 3.25 | 0.05 | ARTEMIS | 0.0306 | * |
| PARP1 | 9 | 304 | 2.96 | 3 | 297 | 1.01 | 6 | 285 | 2.10 | 18 | 886 | 2.03 | 0.98 | empty | | |
| TREX2 | 9 | 273 | 3.30 | 11 | 287 | 3.83 | 8 | 268 | 2.99 | 28 | 828 | 3.38 | 0.43 | PARP1 | 0.3928 | ns |
| DCLRE1 | 8 | 280 | 2.86 | 7 | 257 | 2.72 | 6 | 241 | 2.49 | 21 | 778 | 2.70 | 0.19 | empty | | |
| FEN1 | 7 | 292 | 2.40 | 6 | 237 | 2.53 | 4 | 189 | 2.12 | 17 | 718 | 2.37 | 0.21 | TREX2 | 0.0786 | ns |
| MRE11 | 7 | 225 | 3.11 | 11 | 308 | 3.57 | 8 | 271 | 2.95 | 26 | 804 | 3.23 | 0.32 | empty | | |
| | | | | | | | | | | | | | | DCLRE1 | 0.7542 | ns |
| | | | | | | | | | | | | | | empty | | |
| | | | | | | | | | | | | | | FEN1 | 0.1682 | ns |
| | | | | | | | | | | | | | | empty | | |
| | | | | | | | | | | | | | | MRE11 | 0.0701 | ns |
| | | | | | | | | | | | | | | empty | | |

AICDA & ARTEMIS has a significant p-value but with a decreasing translocation efficiency

hMSC cells

| Factors | assay #1 | | | assay #2 | | | assay #3 | | | total | | average | SD | p-value | | |
|---------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|---------|--------|----|
| | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | | | | | |
| empty | 1 | 315 | 0.32 | 1 | 301 | 0.33 | 1 | 289 | 0.35 | 3 | 905 | 0.33 | 0.01 | ARTEMIS | 0.1248 | ns |
| AICDA | na | na | na | na | na | na | na | na | na | na | na | na | na | empty | | |
| ARTEMIS | 0 | 321 | 0.00 | 1 | 356 | 0.28 | 0 | 294 | 0.00 | 1 | 971 | 0.10 | 0.16 | PARP1 | 0.0206 | * |
| PARP1 | 2 | 321 | 0.62 | 3 | 362 | 0.83 | 2 | 278 | 0.72 | 7 | 961 | 0.73 | 0.10 | empty | | |
| TREX2 | 2 | 301 | 0.66 | 3 | 387 | 0.77 | 2 | 291 | 0.69 | 7 | 979 | 0.72 | 0.06 | TREX2 | 0.0056 | ** |
| DCLRE1 | na | na | na | na | na | na | na | na | na | na | na | na | na | empty | | |
| FEN1 | na | na | na | na | na | na | na | na | na | na | na | na | na | | | |
| MRE11 | na | na | na | na | na | na | na | na | na | na | na | na | na | | | |

RNP Cas9

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | | | p-value | | |
|-----------|------------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|------------|--------|----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | average | SD | | | |
| 293 cells | 2 plasmids | 0 | 213 | 0.00 | 1 | 200 | 0.50 | 2 | 353 | 0.57 | 3 | 766 | 0.39 | 0.31 | 2NLS | 0.0253 | * |
| | 2NLS | 4 | 301 | 1.33 | 2 | 204 | 0.98 | 4 | 328 | 1.22 | 10 | 833 | 1.20 | 0.18 | 2 plasmids | | |
| | RNP | 6 | 203 | 2.96 | 9 | 258 | 3.49 | 11 | 250 | 4.40 | 26 | 711 | 3.66 | 0.73 | RNP | 0.0081 | ** |
| | | | | | | | | | | | | | | | 2 plasmids | | |

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | | | p-value | | |
|------------|--------------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|--------------|--------|-----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | average | SD | | | |
| hMSC cells | 2NLS plasmid | 0 | 335 | 0.00 | 0 | 427 | 0.00 | 1 | 468 | 0.21 | 1 | 1230 | 0.08 | 0.12 | RNP | 0.0007 | *** |
| | RNP | 3 | 327 | 0.92 | 4 | 418 | 0.96 | 4 | 350 | 1.14 | 11 | 1095 | 1.00 | 0.12 | Cas9 plasmid | | |

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | | | p-value | | |
|------------|-------------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|-------------|--------|-----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | average | SD | | | |
| hMSC cells | RNP | 4 | 427 | 0.94 | 3 | 400 | 0.75 | 3 | 368 | 0.82 | 10 | 1195 | 0.84 | 0.09 | RNP | 0.9700 | ns |
| | RNP + TREX2 | 4 | 413 | 0.97 | 3 | 408 | 0.74 | 3 | 381 | 0.79 | 10 | 1202 | 0.83 | 0.12 | RNP + TREX2 | | |
| | RNP + PARP1 | 3 | 397 | 0.76 | 2 | 400 | 0.50 | 4 | 376 | 1.06 | 9 | 1173 | 0.77 | 0.28 | RNP | 0.7517 | ns |
| | RNP + ssODN | 6 | 385 | 1.56 | 7 | 403 | 1.74 | 6 | 363 | 1.65 | 19 | 1151 | 1.65 | 0.09 | RNP + PARP1 | | |
| | | | | | | | | | | | | | | | RNP + TREX2 | 0.0004 | *** |

| | | assay #1 | | | assay #2 | | | assay #3 | | | total | | | | p-value | |
|-------|--------------|----------|---------------|------|----------|---------------|------|----------|---------------|------|----------|---------------|---------|------|---------|----|
| | | positive | total counted | % | positive | total counted | % | positive | total counted | % | positive | total counted | average | SD | | |
| hiPSC | RNP + ssODN | 3 | 261 | 1.15 | 6 | 417 | 1.44 | 6 | 398 | 1.51 | 15 | 1076 | 1.39 | 0.19 | | |
| | 2NLS plasmid | 1 | 353 | 0.28 | 2 | 461 | 0.44 | 2 | 375 | 0.53 | 5 | 1189 | 0.42 | 0.13 | 0.0033 | ** |

TORRES-RUIZ et al. Sup. Table. 2

| Name | Application | Sequence |
|--------------------|-------------------------|--|
| OTf2 Fw | <i>FLI1</i> off target | GGAGTGCATGGGAGTTGAGT |
| OTf2 Rv | <i>FLI1</i> off target | GCAGAGGAAGTGAGAAGCGT |
| OTf4 Fw | <i>FLI1</i> off target | CATGTAGCGAGGGTAGCAGG |
| OTf4 Rv | <i>FLI1</i> off target | CTAACTGTGTGGCTGGGGAC |
| OTe1 Fw | <i>EWSR1</i> off target | TGCTTTCCAGCCCTAGAGGA |
| OTe1 Rv | <i>EWSR1</i> off target | TGCTCACGTTTGCCATGAGA |
| OTe7 Fw | <i>EWSR1</i> off target | TGCATAAGGGACAGAGCAACA |
| OTe7 Rv | <i>EWSR1</i> off target | TTTGAGTGACCCCGAGTTT |
| <i>EWSR1</i> Rv | <i>EWSR1</i> on-target | GCTGCCTCCCCACTTTACAT |
| <i>EWSR1</i> Fw1 | <i>EWSR1</i> on-target | AGGCTGGTCTCGAACTCCTG |
| <i>FLI1</i> Rv1 | <i>FLI1</i> on-target | TGGATGCCTTTGAACCAAT |
| <i>FLI1</i> Fw | <i>FLI1</i> on-target | ATGCAAGGAGGACTGTGAT |
| <i>EWSR1</i> Fw2 | Tranlocation detection | AGGACACATCTTTAGGGCA |
| <i>FLI1</i> Rv2 | Tranlocation detection | GCGTGGTGATAGGTTGGCT |
| ssODN_elf2 60 | ODN | agtgtttttccaattgttttttagtatg ccgaggctctgtcttcagagga aaaaaggt |
| ssODN_elf2 90 | ODN | cccagtgaaagatttcagtgctttttccaattgttttttagtatg ccgaggctctgtcttcagagga aaaaaggtgcccccttggac |
| ssODN_elf2 120 | ODN | taacatcttggatgccccagtgaaagatttcagtgctttttccaattgttttttagtatg ccgaggctctgtcttcagagga aaaaaggtgcccccttggacaactatctacagtac |
| ssODN_elf2 150 | ODN | gcatggcattccagctaacatcttggatgccccagtgaaagatttcagtgctttttccaattgttttttagtatg ccgaggctctgtcttcagagga aaaaaggtgcccccttggacaactatctacagtaccattttgaaagctga |
| ssODN_elf2 180 | ODN | ggacacatctttaggggcattccagctaacatcttggatgccccagtgaaagatttcagtgctttttccaattgttttttagtatg ccgaggctctgtcttcagagga aaaaaggtgcccccttggacaactatctacagtaccattttgaaagctgaaaccactgaggccca |
| sg <i>EWSR1</i> _1 | single-guide sequence | AATTTGTTTTTAGTATGCCCT TGG |
| sg <i>FLI1</i> _2 | single-guide sequence | CTGGCCTGGGCGATAGACCG AGG |

Table S1: Details of the FISH results included in this study.

Table S2: Oligonucleotide, ssODN, and sgRNA sequences used in this study.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Transfection and Electroporation

Cells were transfected with endotoxin-free DNA (Qiagen) in 6-well plates. Calcium-phosphate-based transfection was performed as previously described (Torres et al., 2014a). Briefly, HEK293A cells were seeded and grown to confluence, and then transfections were performed with 6 μ g plasmid DNA mixed with CaCl_2 . After 48-72 hours, cells were processed for subsequent analysis. For electroporation, the Neon Transfection System was used (Thermo Fisher Sci). Confluent hMSCs, hiPSCs and HEK293A cells were trypsinized, resuspended in R solution, and electroporated using the following conditions. For hMSCs, 10- μ l tips were used to electroporate 1×10^5 cells with a single 40-ms pulse of 990 V. For HEK293A cells, 4×10^5 cells were electroporated with 10- μ l tips using three 10-ms pulses of 1245 V. Finally, hiPSCs were electroporated with 10- μ l tips using 2×10^5 cells with three 5-ms pulses of 1400 V. After electroporation, cells were seeded in a 24-well plate containing pre-warmed medium. When required, cells were sorted 72 h post-transfection. For co-transfection experiments, 200 pmol of ssODN (IDT) or end processing plasmids were included.

Immunofluorescence

Immunofluorescence was carried out as previously described, with modifications (Torres and Ramirez, 2009). Briefly, after nucleofection, cells were seeded onto 8-well glass bottom μ -Slides (Ibidi). After 72 h, cells were washed twice with PBS and fixed 10 min in 4% paraformaldehyde, permeabilized with 0.5% TritonX-100/PBS, and blocked with 4% normal goat serum. For Cas9 studies, samples were incubated overnight at 4°C with anti-Cas9 antibody (1/500 dilution, 7A9-3A3, Active Motif) and then for 1 h at RT with Alexa-fluor-555 secondary antibody (dilution 1/1000, Molecular Probes). For pluripotency-associated markers, hiPSCs were incubated overnight at 4°C with anti-OCT4 (1/500 dilutions, Cell Signaling Tech) or anti-SSEA4 antibodies (1/500 dilutions, MC-813-70, StemCell Tech) and then 1h at RT with Alexa-fluor-555 and Alexa-fluor-647 secondary antibodies in the darkness. Nuclei were counterstained with DAPI, and samples air-dried and mounted in gelatin (Sigma Aldrich). Images were captured with a Leica SP5 confocal laser-scanning microscope.

Genomic DNA Extraction and PCR Analysis

Genomic DNA was extracted using standard procedures. Briefly, $5-10 \times 10^6$ cells were either trypsinized or scraped, washed in PBS, pelleted, and lysed in 100 mM NaCl, Tris (pH 8.0) 50 mM, EDTA 100 mM, and 1% SDS. After overnight digestion at 56°C with 0.5 mg/ml of proteinase K (Roche), DNA was cleaned by precipitation with saturated NaCl, and the clear supernatant was precipitated with isopropanol, washed with 70% ethanol, air-dried, and resuspended overnight at

RT in 1xTE buffer. Serial DNA dilutions were quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Standard PCR was performed under the following conditions: denaturation at 95°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62.5°C for 30 s, extension at 72°C for 60 s, and a final extension of 5 min at 72°C. Primers used are listed in **Table S1**.

Lentivirus Generation, Titration, and Transduction

Viruses were produced by transient plasmid transfection into HEK293T cells using the calcium phosphate method (Torres et al., 2011). Briefly, cells were seeded at 1.1×10^7 cells/dish in 15-cm dishes the day before transfection. Cells were transfected using calcium phosphate with 3 µg pRSV-Rev, 3.75 µg pMD.2G (VSV-G), 13 µg pMDLg/pRRE (or 13 µg pMDLg/pD64VRRE for production of non-integrative lentiviral vectors), and 35 µg of transfer plasmid (pLVX-U6-sgRNA^{#1}-H1-sgRNA^{#2}-Cas9-2A-eGFP). The medium was collected after 48 h, cleared by low-speed centrifugation, and filtered through 0.45-µm pore PVDF filters (Millipore). Viral stocks were concentrated by ultracentrifugation at 20,000 rpm for 2 h at 16°C. Pellets containing lentivirus were air-dried and resuspended overnight at 4°C in 400 µl of medium. Viral titers in HEK293T cells transduced with vectors expressing fluorescent proteins were determined by FACS analysis (transduction units/ml). In the absence of a fluorescent reporter, titer was estimated by qPCR of particles in supernatants (particles/ml). Values ranged from 10^7 to 10^8 TU/ml and were at a TU/particle ratio of 1:100. Cells were transduced at different multiplicities of infection in medium containing Polybrene (8 µg/ml final concentration). Cells were incubated at 37°C for 6 h, and the viral supernatant was then replaced with fresh medium and analysed 72 h later.