

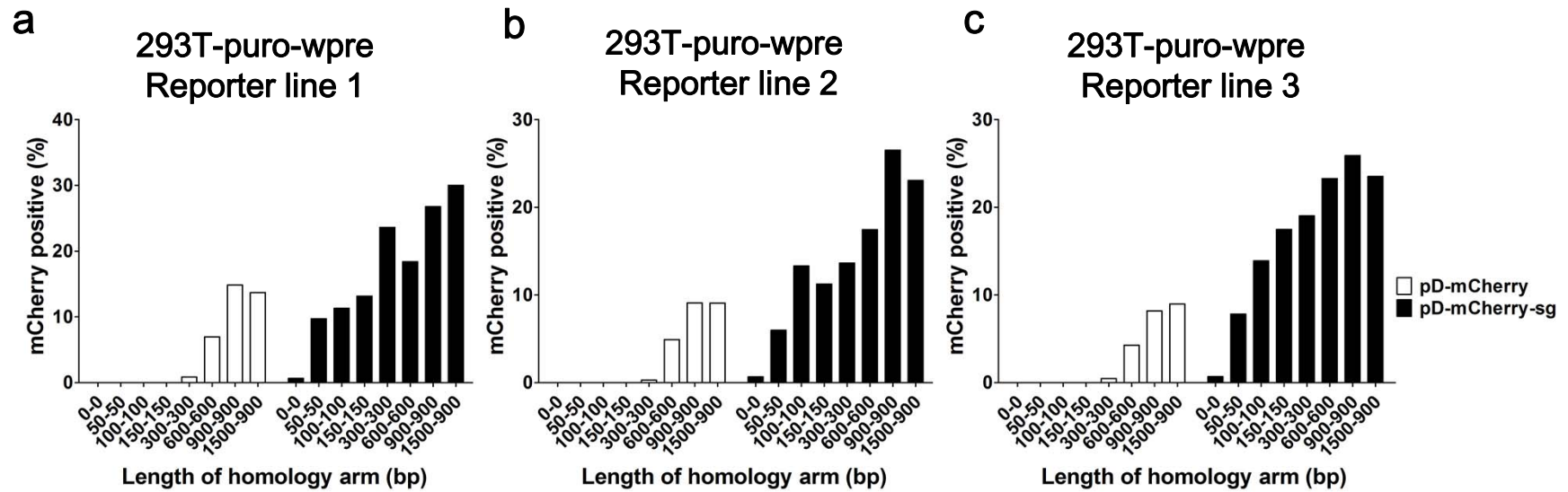
Supplementary Table 1: Small molecules that are reported to increase HDR efficiency.

| Type | Small molecule | Concentration | Time | Cell line | Fold change | Reference | Function |
|-----------------|----------------|---------------|-------------------------------------|-----------------------------|-------------------------------|---------------------------------|--|
| NHEJ inhibitors | SCR7 | 0.01~1μM | 24-48h | A549 | 1.8-3.1 | Byrne SM, <i>et al</i> , 2015 | SCR7 targets the DNA binding domain of DNA ligase IV, reducing its affinity for DSBs and inhibiting its function |
| | | 0.01~1μM | 24-48h | MeJuSo (melanoma) | 10.9-19.1 | | |
| | | 1μM | 24-48h | DC2.4 (dendritic cell line) | 9.3-14.1 | | |
| | | 1mM | Inject together with Cas9 and sgRNA | Mouse zygote | 1.0-4.0 | | |
| | | 1μM | 12-72h | HEK293 | 5 | Maruyama T, <i>et al</i> , 2015 | |
| | | 1μM | 0-72h | HEK293A | 1.2 | Pinder J, <i>et al</i> , 2015 | |
| | | 20-80μM | 0-20h | Rabbit embryo | NS | Song J, <i>et al</i> , 2016 | |
| | 1μM | 16h-64h | HEK293 | 2 | Robert F, <i>et al</i> , 2015 | | |
| | NU7441 | 2μM | 16h-64h | HEK293 | | 2 | Pharmacological inhibitors of DNA-PKcs |
| HR activator | RS-1 | 10μM | 0-72h | HEK293A | 3-6 | Pinder J, <i>et al</i> , 2015 | Stabilize association of RAD51 with DNA |
| | | 10μM | 0-72h | U2OS | 1.5-3 | | |
| | | 7.5μM, 15μM | 0-20h | Rabbit embryo | 2-5 | Song J, <i>et al</i> , 2016 | |

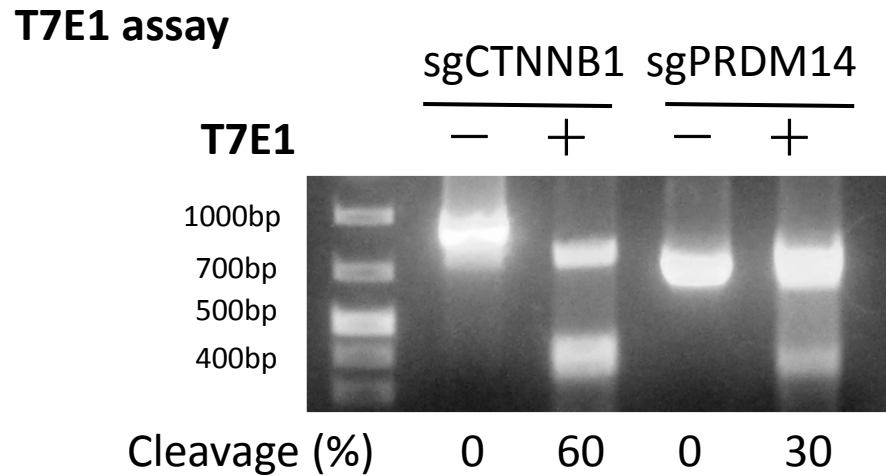
Table 1 (Continued)

| Type | Small molecule | Concentration | Time | Cell line | Fold change | Reference | Function | | | | | | | |
|-----------------------|-------------------|---------------|------------|------------|----------------------------|----------------------------|--|---|---|---|----------------------------|---|----------------------------|---|
| Cell cycle inhibitors | Lovastatin (Lov) | 40μM | 0-17h | HEK293T | NS | Lin S, <i>et al</i> , 2014 | Blocks at early G1 and partially at G2/M phase | | | | | | | |
| | | | | Fibroblast | 0.8% | | | | | | | | | |
| | Mimosine (Mim) | 200μM | 0-17h | HEK293T | NS | | Lin S, <i>et al</i> , 2014 | Arrest cells at the G1-S border prior to onset of DNA replication | | | | | | |
| | | | | Fibroblast | 0.7% | | | | | | | | | |
| | Aphidicolin (Aph) | 2μg/ml | 0-17h, | HEK293T | 1.1-1.5 | | | | Lin S, <i>et al</i> , 2014 | Arrest cells at the G1-S border prior to onset of DNA replication | | | | |
| | | | 25h-42h | Fibroblast | 0.6%-1.3% | | | | | | | | | |
| | Thymidine (Thy) | 5mM | 0-17h, | HEK293T | NS | | | | | | Lin S, <i>et al</i> , 2014 | Arrest cells at the G1-S border prior to onset of DNA replication | | |
| | | | 25h-42h | Fibroblast | 0.6% | | | | | | | | | |
| | Hydroxyurea (HU) | 2mM | 0-17h | HEK293T | NS | | | | | | | | Lin S, <i>et al</i> , 2014 | Arrest cells at the G1-S border prior to onset of DNA replication |
| | | | | Fibroblast | 0.6% | | | | | | | | | |
| Nocodazole (Noc) | 200ng/ml | 0-17h | HEK293T | 1.5-3 | Lin S, <i>et al</i> , 2014 | Arrest at G2/M phase | | | | | | | | |
| | | 0-17h | Fibroblast | NS | | | | | | | | | | |
| | | 0-16h, Aph 3h | Human ESC | 2% | | | | | | | | | | |
| Others | L755507 | 5μM | 0-72h | HEK293A | | | NS | Pinder J, <i>et al</i> , 2015 | | | | | | |
| | L755507 | 5μM | 0-24h | Mouse ESC | | | 3 | Yu C, <i>et al</i> , 2015 | | | | | | |
| | Brefeldin A | 0.1μM | 0-24h | Mouse ESC | | | 2 | | An NHEJ inhibitor of intracellular protein transport from the ER to the Golgi apparatus | | | | | |

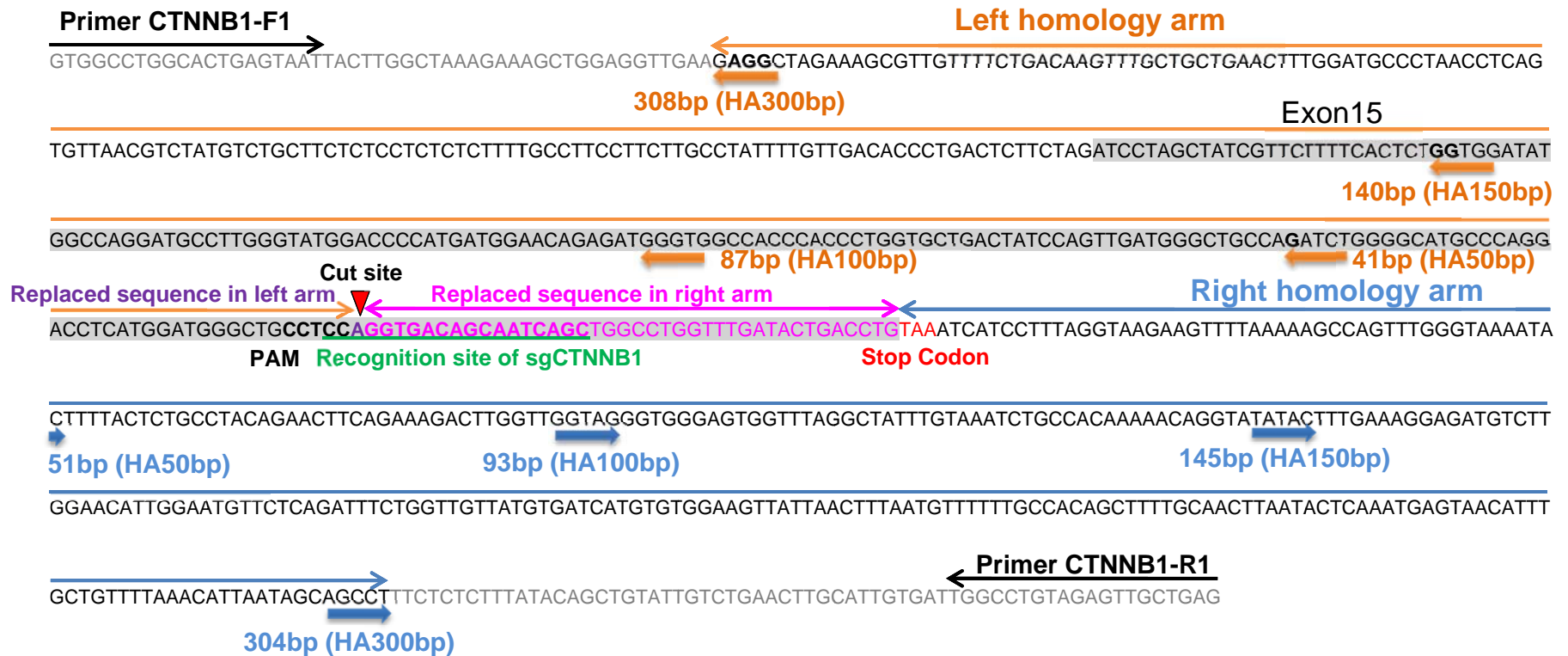
NS: not significant.



Supplementary Figure 1: The HDR efficiency of different homology arm length in three 293T reporter lines. Single 293T-puro-wpre cell clones 1# (a), 2# (b) and 3# (c) were isolated by sorting. The HDR-mediated knockin efficiencies were determined by FACS at day 7 after co-transfection with Cas9/sgRNA1 and the donor plasmids with various homology arm length. The data presented are average of three technical replicates.

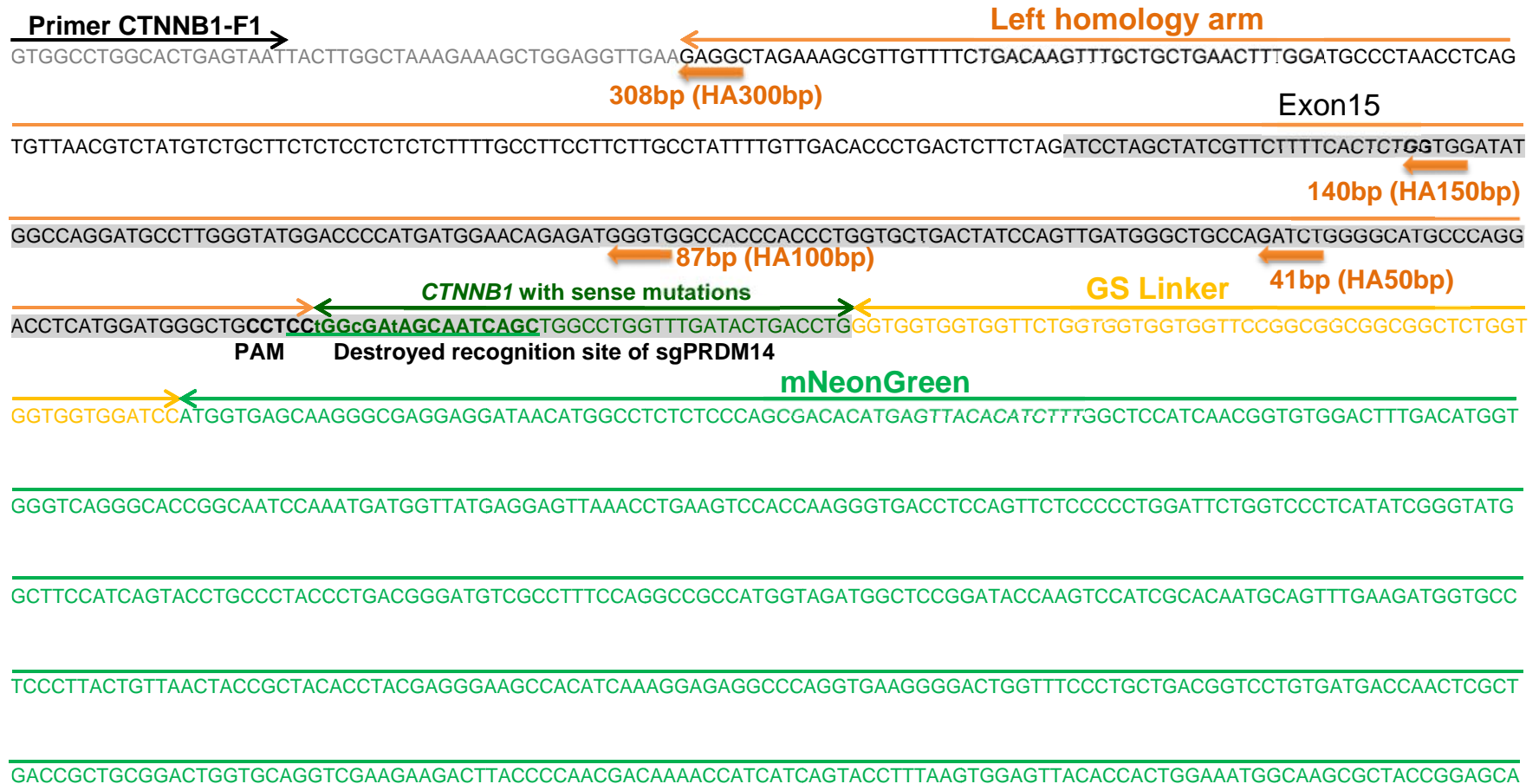


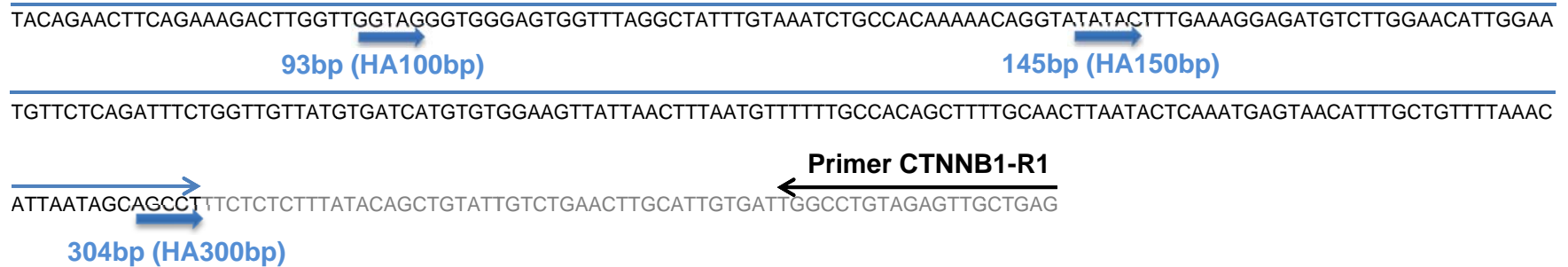
Supplementary Figure 2: The cleavage efficiency of sgCTNNB1 and sgPRDM14 in iPSCs. To confirm the target locus cleavage activity of sgCTNNB1 and sgPRDM14, we nucleofected iPSCs of each sgRNA together with Cas9 plasmid, and carried out T7E1 assay using total genomic DNA. CTNNB1-F1 and CTNNB1-R1 primers, PRDM14-F2 and PRDM14-R2 primers were used for PCR. The cleavage frequency was calculated from the proportion of cut bands intensity to total bands intensity.



Supplementary Figure 3a: Design of gene editing at the human *CTNNB1* exon15. The exon sequence of *CTNNB1* gene is in gray shadow. The stop codon is indicated in red. The recognition site of sgCTNNB1 is in bold and underlined by a green line, The PAM is indicated in bold and the predicted cut site is indicated by a red triangle. The left homology arm is indicated with an orange line and right homology arm with a blue line. The precise length of homology arm is indicated under a thick arrow. Replaced sequences in left and right arms are separately indicated in purple and pink. Sequences in grey indicate genomic DNA outside of homology arms. The primers used for knockin pattern analysis by PCR are indicated by black arrows.

Supplementary Figure 3b: Precisely edited *CTNNB1* locus.





Supplementary Figure 3b: Precisely edited *CTNNB1* locus. The exon of *CTNNB1* gene is in gray shadow. The stop codon is marked in red. Silent mutations are introduced to destroy the recognition site of sgCTNNB1, which is in bold and underlined by a green line. The left homology arm is indicated with an orange line and the right HA with a blue line. The precise length of homology arm is indicated under a thick arrow. The GS linker sequence is in yellow and mNeonGreen-Wpre-ployA sequence in green color. Sequences in grey indicate genomic DNA outside of homology arms. The primers used for knockin pattern analysis are indicated by black arrows.

Supplementary Figure 3c: Backbone sequence cut off from pD-mNeonGreen-sg (*CTNNB1*).

Partial recognition site of sgCTNNB1 ← Donor plasmid backbone sequence

ggtgacagcaatcagcccaggtggcacttttcggggaaatgtgctgcggaacccctatttgttatttttctaaatacattcaaataatgtatccgctcatgagacaataaacctgataaatgcttcaataatattgaa
aaaggaagagatgagatattcaacatttccgtgctgcccttattccctttttcgggcattttgccttctgttttctcaccagaaacgctgggaaagtaaaagatgctgaagatcagttgggtgcacgagtg
gttaccatcgaactggatctcaacagcggtaagatccttgagagtttgcctccgaagaacgctttccaatgatgagcacttttaaagtctgctatgtggcgcggtattatcccgtattgacgcccgggcaagagc
aactcggctgcccatacactattctcagaatgacttgggtgagtactaccagtcacagaaaagcatcttaccggatggcatgacagtaagagaattatgcagtgctgccataacatgagtgataaactg
cggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactgccttgatcgttgggaaccggagctgaatgaagccatacctaaacgacga
gcgtgacaccacgatgctgtagcaatggcaacaacgcttgcgcaaactattaactggcgaactacttactctagctcccggcaacaattaatagactggatggaggcggataaaagtgcaggaccacttc
tgcgctcggccctccggctggctggttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggg
agtcaaggcaactatggatgaacgaaatagacagatcgtgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatacttttagattgatttaaaactcatttttaattaaaag
gatctaggtgaagatccttttgataatctcatgacccaaaatccctaacgtgagtttctgtccactgagcgtcagaccccgtagaaaagatcaaaggatcttctgagatcctttttctgcgctgaatctgctgct
tgcaaacaaaaaaaaaccaccgctaccagcgggtggttggttgcgggatcaagagctaccaactctttccgaaggtaactggctcagcagagcgcagatacctaaatactgtccttctagtgtagccgtagtt
ggccaccactcaagaactctgtagcaccgcctacatacctcgtctgctaatacctgttaccagtggtgctgctccagtgggcagataagtcgtgcttaccgggtggactcaagacgatagttaccggataaggc
gcagcggctcgggctgaacggggggtcgtgcacacagcccagctggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcggccacgctcccgaaggagaaagg
cggacaggtatccgtaagcggcagggcggaaacaggagagcgcacgagggagctccaggggaaacgcctggatctttatagctcgtcgggttcgccacctctgactgagcgtcgatttttgtgat
gctcgtcagggggcggagcctatggaaaaacgacgcaacgccccttttacggctcctggccttttgcctcctttgctcacatgttcttctcgtgtatcccctgattctgtggataaccgtattaccgcttt
gagtgagctgataccgctcggcagccgaacgaccgagcgcagcagtgagcaggaagcgggaagagcggccaatacgcgaaaccgctctcccgcgctgtggccgattcattaatgcagc
tggcacgacaggtttcccgactgaaagcgggacgtgagcgcgaacgcaattaatgtgagttagctcactcattagccacccaggctttacactttatgcttccgctcgtatggtgtggaattgtgagcgg
ataacaatttcacaca**gctgattgctgcacc**

Partial recognition site of sgCTNNB1

Supplementary Figure 3c: Backbone sequence cut off from pD-mNeonGreen-sg (*CTNNB1*). The sequence of donor plasmid backbone is in lower case and indicated by a red line. The residual partial sgCTNNB1 recognition sequence after linearization by CRISPR is in bold and indicated by green lines.

Supplementary Figure 3d: Knockin pattern analysis at the *CTNNB1* locus.

pD-mNeonGreen-sg-HA50-50bp (*CTNNB1*)

| | Left homology arm | Replaced sequence in left arm | Replaced sequence in right arm | Right homology arm | |
|-----------|--------------------------------|--|---|--------------------|---------------------------------------|
| Wild type | TGCCA GATCTGGGGC:::GGCTGCCTCCa | | ggtgacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TTTTTA | |
| Expected | Left homology arm | <i>CTNNB1</i> with sense mutations | GS-mNeonGreen-Wpre-ployA | Right homology arm | |
| Cl1-19 | TGCCA GATCTGGGGC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | HDR |
| Cl20-21 | TGCCA GATCTGGGGC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | |
| Clone22 | TGCCA GATCTGGGGC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 3-NHEJ 71bp ins |
| Clone23 | TGCCA GATCTGGGGC:::GGCTGCCTCC | agtgagattttaccacaaactg:::cctgggatgcccagatcctcaggggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 3-NHEJ 80bp ins |
| Clone24 | TGCCA GATCTGGGGC:::GGCTGCCTCC | aggacagcaatcagccagg:::tcacacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ mNeonGreen reverse insertion |
| Clone25 | TGCCA GATCTGGGGC:::GGCTGCCTCC | agtggtgacagcaatcagccagg:::tcacacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ Backbone forward insertion |
| Clone26 | TGCCA GATCTGGGGC:::GGCTGCCTCC | cagcaatcagccagggtggcac:::tcacacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ Backbone forward insertion |
| Cl27-28 | TGCCA GATCTGGGGC:::GGCTGCCTCC | agggtgacagcaatcagctcctg:::agtccaactggctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ Backbone reverse insertion |
| Clone29 | TGCCA GATCTGGGGC:::GGCTGCCTCC | tcagctcctg:::agtccaactggctgattgctg | ggtgacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ Backbone reverse insertion |
| Clone30 | TGCCA GATCTGGGGC:::GGCTGCCTCC | ggtgacagcaatcagctcctg:::ccccgaaaagtgccacctgctgatt | gctggcctggttgatactgacctgTAAATCATCC:::GGTAAAATAC | TTTTTA | 5,3-NHEJ Backbone reverse insertion |

pD-mNeonGreen-sg-HA100-100bp (*CTNNB1*)

| | Left homology arm | Replaced sequence in left arm | Replaced sequence in right arm | Right homology arm | |
|-----------|--------------------------------|---|---|--------------------|-------------------------------------|
| Wild type | GAGAT GGGTGGCCAC:::GGCTGCCTCCa | | ggtgacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | GGTGG | |
| Expected | Left homology arm | <i>CTNNB1</i> with sense mutations | GS-mNeonGreen-Wpre-ployA | Right homology arm | |
| Cl1-16 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | HDR |
| Clone17 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | |
| Clone18 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | GGcGAIAGCAATCAGCTGGCCTGGTTTGATACTGACCTGGTGGTGGTG:::CAGCCATGAGTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 3-NHEJ 114bp ins |
| Clone19 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | tgagggtggccac:::ggctgcctcctggcctgatagcaatcagctggcctggttgatactgacctggggtgggtg:::cagccatgagTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 3-NHEJ 493bp del; 31bp ins |
| Clone20 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | atggaagggtggccac:::ggctgcctcctggcctgatagcaatcagctggcctggttgatactgacctggggtgggtg:::cagccatgagTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5-NHEJ 41bp del; 131bp ins |
| Clone21 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | aggtgacagcaatcagccagg:::cacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5-NHEJ 91bp ins; 3-NHEJ 139bp ins |
| Cl22-23 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | agggtgacagcaatcagccagg:::cacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Clone24 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | gggtgacagcaatcagccagg:::cacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Cl25-26 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | agcaatcagccagg:::cacaggagctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Clone27 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | gacagcaatcagccagg:::cacaggagctgattgctgca | tcagctgcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Clone28 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | gacagcaatcagccagg:::cacaggagctgattgctg | gctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Clone29 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | agcaatcagccagg:::cacaggagctgattgctgca | gctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |
| Clone30 | GAGAT GGGTGGCCAC:::GGCTGCCTCC | agggtgacagcaatcagctcctg:::cacctggctgattgctgaccgggacagcaatcagctggcctggttgatactgacctgTAAATCATCC:::TGGTTGGTAG | TAAATCATCC:::TGGTTGGTAG | GGTGG | 5,3-NHEJ Backbone forward insertion |

pD-mNeonGreen-sg-HA150-150bp (*CTNNB1*)

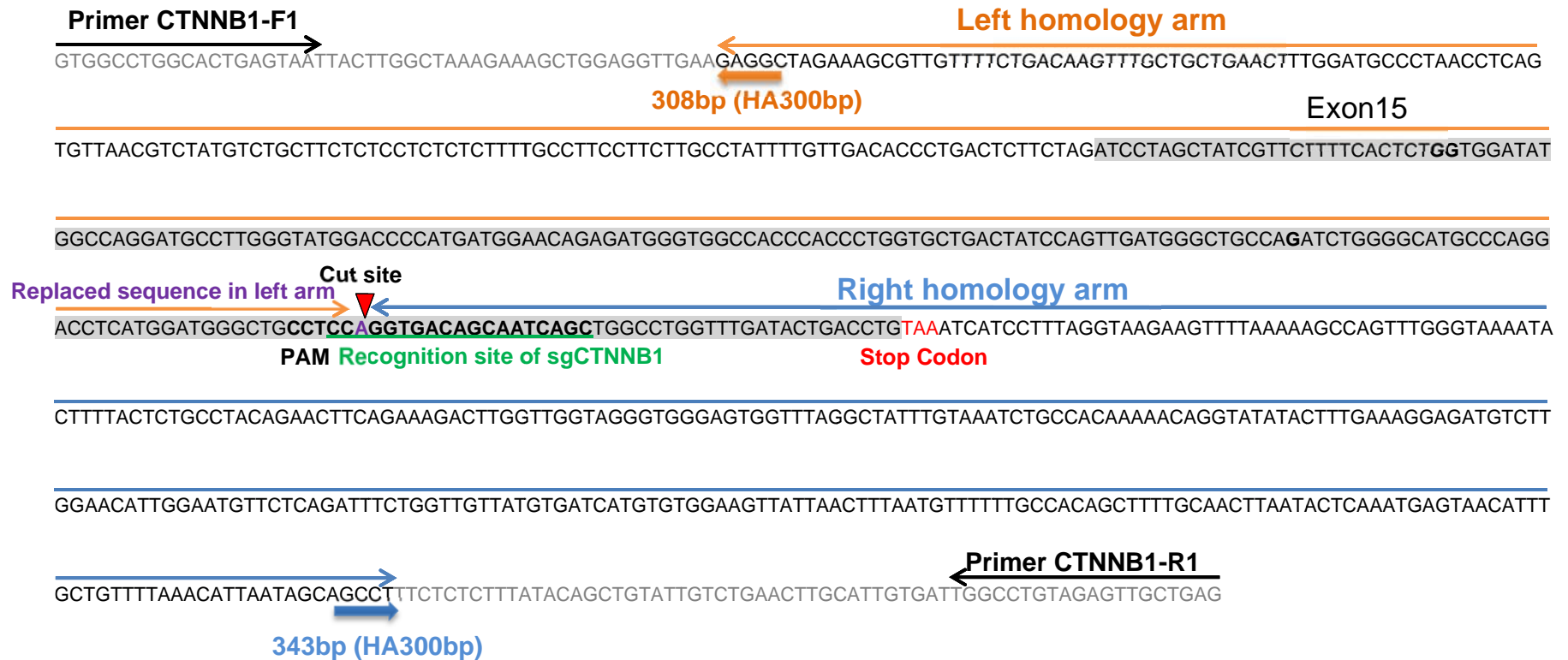
| | Left homology arm | Replaced sequence in left arm | Replaced sequence in right arm | Right homology arm | |
|-----------|---------------------------------|--|--|--|-------------------------------------|
| Wild type | ACTCTGGTGGATATG.....GGCTGCCTCCa | | | ggtgacagcaatcagctggcctggttgatactgacctgTAAATCATCC.....AGGTATATACTTTGA | |
| Expected | ACTCTGGTGGATATG.....GGCTGCCTCC | CTNNB1 with sense mutations | GS-mNeonGreen-Wpre-ployA | Right homology arm | |
| C11-24 | ACTCTGGTGGATATG.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | HDR |
| Clone25 | ACTCTGGTGGATATG.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | 3-NHEJ 163bp ins |
| Clone26 | ACTCTGGTGGATATG.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | 3-NHEJ 156bp ins |
| Clone27 | ACTCTGGTGGATATG.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | 3-NHEJ 339bp del, 38bp ins |
| Clone28 | ACTCTGGTGGATATG.....GGCTGCCTCC | agcctggaagtgatag.....ggctgcctccggcgcgatagcaatcagctggcctggttgatactgacctg | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | 5-NHEJ 148bp ins |
| Clone29 | ACTCTGGTGGATATG.....GGCTGCCTCC | agcctggaagtgatag.....ggctgcctccggcgcgatagcaatcagctggcctggttgatactgacctg | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....AGGTATATAC | TTTGA | 5-NHEJ 147bp ins; 3-NHEJ 165bp ins |
| Clone30 | ACTCTGGTGGATATG.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....AGGTATATAC | TTTGA | 5,3-NHEJ Backbone forward insertion |
| Clone31 | ACTCTGGTGGATATG.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....AGGTATATAC | TTTGA | 5,3-NHEJ Backbone forward insertion |
| C132-35 | ACTCTGGTGGATATG.....GGCTGCCTCC | agcctggaagtgatag.....ggctgcctccggcgcgatagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....AGGTATATAC | TTTGA | 5,3-NHEJ Backbone forward insertion |
| Clone36 | ACTCTGGTGGATATG.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....AGGTATATAC | TTTGA | 5,3-NHEJ Backbone forward insertion |

pD-mNeonGreen-sg-HA300-300bp (*CTNNB1*)

| | Left homology arm | Replaced sequence in left arm | Replaced sequence in right arm | Right homology arm | |
|-----------|---------------------------------|--|--|--|-------------------------------------|
| Wild type | TTGAAAGGGCTAGAA.....GGCTGCCTCCa | | | ggtgacagcaatcagctggcctggttgatactgacctgTAAATCATCC.....ATAGCAGCCTTTCTC | |
| Expected | TTGAAAGGGCTAGAA.....GGCTGCCTCC | CTNNB1 with sense mutations | GS-mNeonGreen-Wpre-ployA | Right homology arm | |
| C11-33 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....ATAGCAGCCT | TTCTC | HDR |
| Clone34 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | GGcGAIAGCAATCAGCTGGcCTGGTTTGATACTGACCTG | GGTGGTGGTG.....CAGCCATGAGTAAATCATCC.....ATAGCAGCCT | TTCTC | 3-NHEJ 339bp ins |
| Clone35 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone forward insertion |
| Clone36 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone forward insertion |
| Clone37 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone forward insertion |
| Clone38 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone forward insertion |
| Clone39 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone forward insertion |
| Clone40 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone reverse insertion |
| Clone41 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone reverse insertion |
| C142-43 | TTGAAAGGGCTAGAA.....GGCTGCCTCC | aggtgacagcaatcagccagggtg.....cacaggagctgattgctgtcaccggtgacagcaatcagctggcctggttgatactgacctg | TAAATCATCC.....ATAGCAGCCT | TTCTC | 5,3-NHEJ Backbone reverse insertion |

Supplementary Figure 3d: Knockin pattern analysis at the *CTNNB1* locus. The stop codon is indicated in red. The left homology arm is highlighted in yellow shadow and right homology arm in blue shadow. Replaced sequences at upstream and

downstream of DSB are separately indicated in purple and pink lowercase. The inserted sequence is in red lowercase. Sequences in grey indicate genomic DNA outside of homology arms.



Supplementary Figure 4a: Improved design of genome editing at human *CTNNB1* exon15 (pD-mNeonGreen-sg-HA300-300bp-RR1-0bp). The exon sequence of *CTNNB1* is in gray shadow. The stop codon is indicated in red. The recognition site of sgCTNNB1 is in bold and underlined by a green line. The PAM is indicated in bold and the expected cut site is indicated by a green triangle. The left homology arm is indicated with an orange line and the right arm with a blue line. The precise length of homology arm is indicated under a thick arrow. Replaced sequence at upstream of DSB is indicated in purple. Sequences in grey indicate genomic DNA outside of homology arms. The primers used for knockin pattern analysis are indicated by black arrows.

Supplementary Figure 4b: Precisely edited *CTNNB1* locus with pD-mNeonGreen-sg-HA300-300bp-RR1-0bp



CTGCGCGGACCACCTACACCTTTGCCAAGCCAATGGCGGCTAACTATCTGAAGAACCAGCCGATGTACGTGTTCCGTAAGACGGAGCTCAAGCACTCCAAGACCGAGCTC

AACTTCAAGGAGTGGCAAAAGGCCTTTACCGATGTGATGGGCATGGACGAGCTGTACAAGTAA  **Wpre** GTTTAAACGCGTCGACAATCAACCTCTGGATTACAAAATTTGTGAAAGA

TTGACTGGTATTCTTAACTATGTTGCTCCTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTCTCCTCCTTGATA

AATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGTGACGCAACCCCCACTGGTTGGGGCATTGCCACC

ACCTGTCAGCTCCTTTCCGGGACTTTGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCAC


TGACAATCCGTGGTGTTCGGGGAAGCTGACGTCTTTCCATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTACGTCCTTCGGCCC

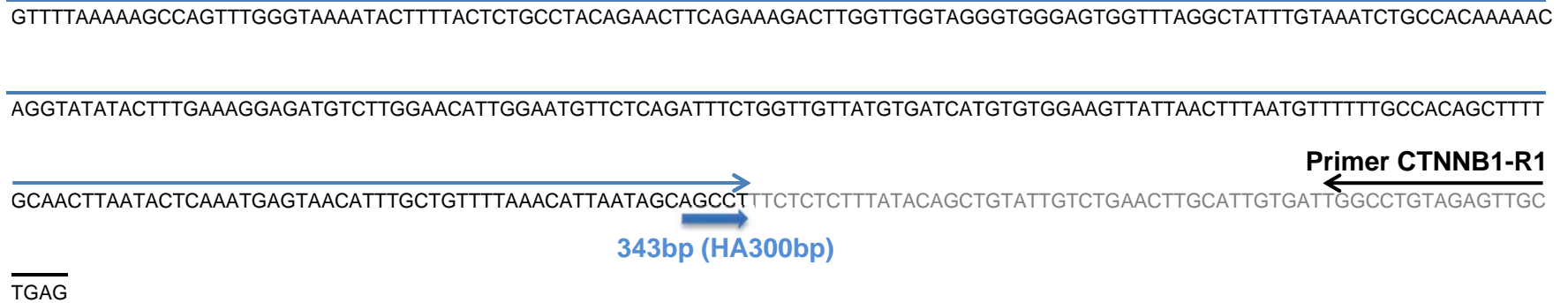
TCAATCCAGCGGACCTTCTTCCGCGGCCTGCTGCCGGCTCTGCCGCTTCCGCGTCTTCGCCTTCGCCCTCGACGAGTCGGATCTCCCTTTGGGC  **ployA** GGATCCAGACA

TGATAAGATACATTGATGAGTTTGGACAAACCACA ACTAG AATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCA

ATAACAAGTTAAACAACA AATTGCATTCA TTTATGTTTCAGGTT CAGGGGAGGTGTGGGAGGTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTAT

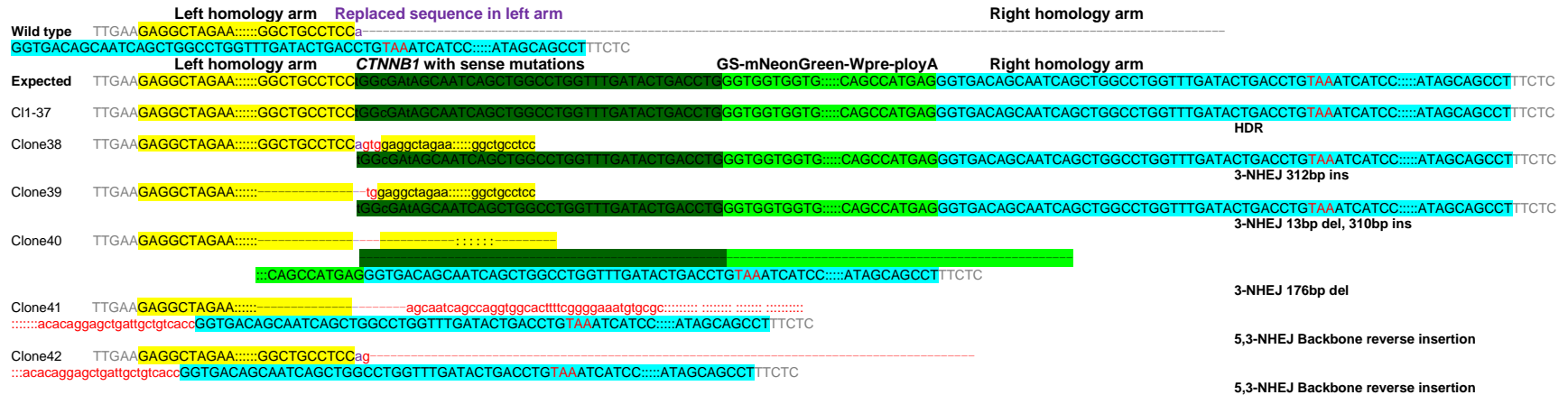
GATCCGGCTGCCTCGCGCTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGC

CCGTCAGGCGTCAGCGGTGTTGGCGGGTGTGGGGCGCAGCCATGAG  **Right homology arm** GGTGACAGCAATCAGCTGGCCTGGTTTGATACTGACCTGTAAATCATCTTTAGGTAAGAA
Stop Codon



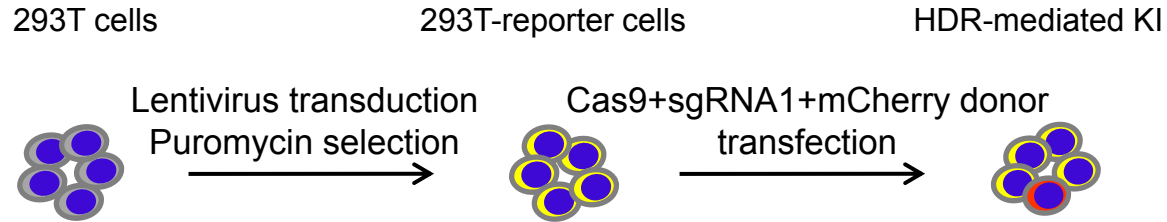
Supplementary Figure 4b: Precisely edited *CTNNB1* locus with pD-mNeonGreen-sg-HA300-300bp-RR1-0bp. The exon sequence of *CTNNB1* is in gray shadow. The stop codon is indicated in red. Silent mutations (indicated in lowercase) were introduced to destroy the recognition site of sgCTNNB1, which is in bold and underlined by a green line. The PAM is indicated in bold. The left homology arm is indicated with an orange line and right homology arm with a blue line. The precise lengths of homology arm are indicated under thick arrows. The GS linker sequence is in yellow and mNeonGreen-Wpre-*polyA* sequence in green color. Sequences in grey indicate genomic DNA outside of homology arms. The primers used for knockin pattern analysis are indicated by black arrows.

pD-mNeonGreen-sg-HA300-300bp-RR1-0bp (*CTNNB1*)

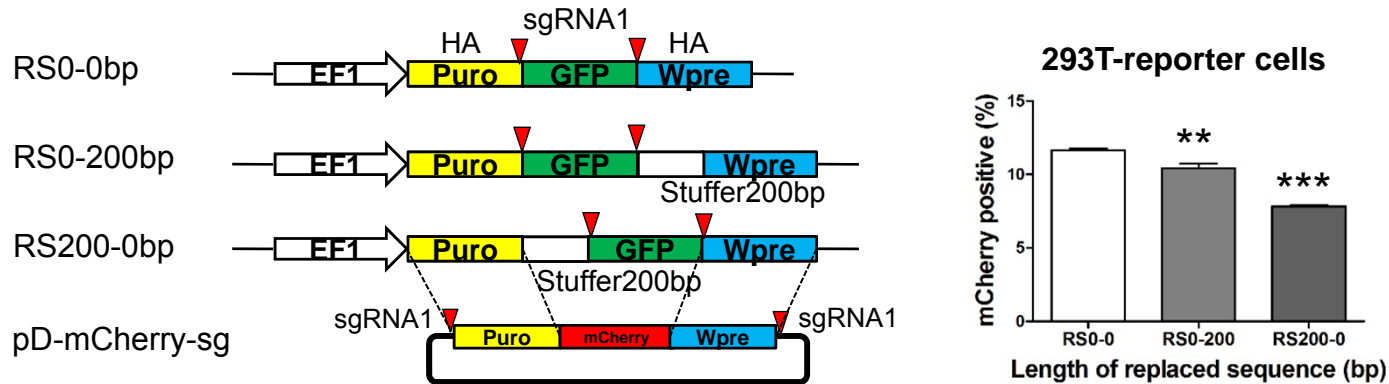


Supplementary Figure 4c: Knockin pattern analysis at the *CTNNB1* locus edited by pD-mNeonGreen-sg-HA300-300bp-RR1-0bp (*CTNNB1*). The stop codon is indicated in red. The left homology arm is indicated in yellow shadow and right homology arm in blue shadow. Replaced sequence in the left arm is indicated in purple lowercase. The inserted sequence is in red lowercase. Sequences in grey indicate genomic DNA outside of homology arms.

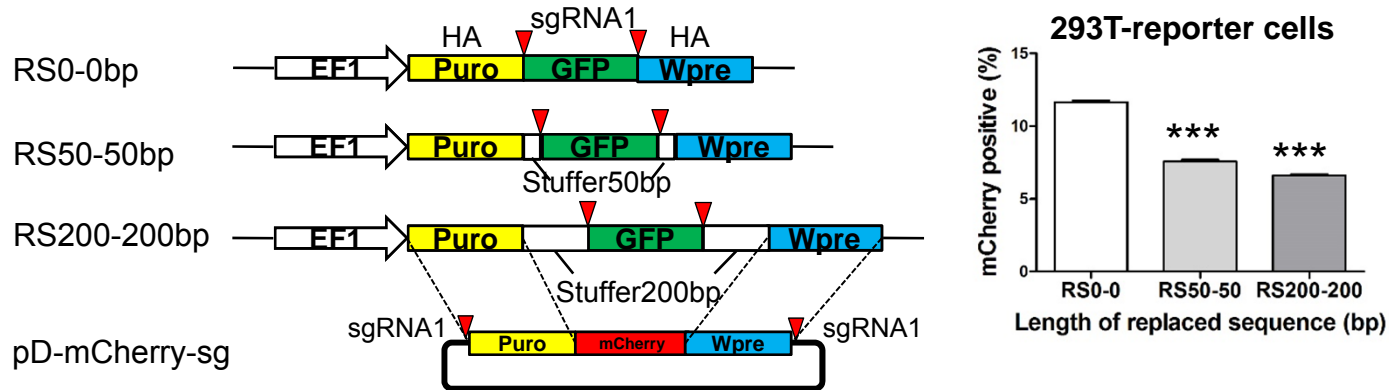
a mCherry HDR reporter system



b Reporter system with replaced sequence on one side

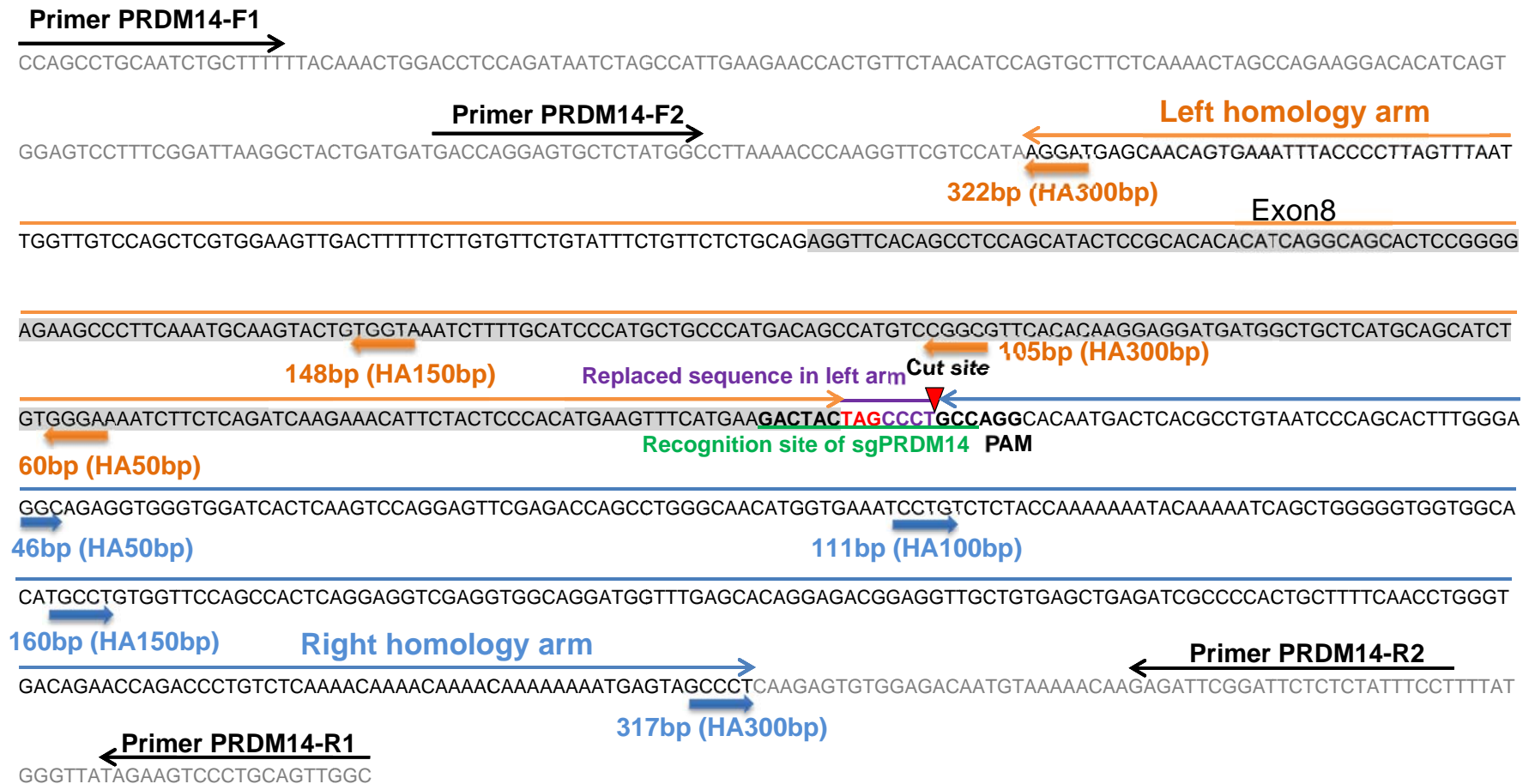


c Reporter system with replaced sequence on both sides



Supplementary Figure 5: The effects of replaced sequence length adjacent to DSB site (sgRNA1 cleavage site) on HDR efficiency. (a) Schematic outline of the HDR reporter systems. 293T cells transduced with lentiviral vectors harboring different lengths of replaced sequences in (b) and (c) were used as reporter cell lines. (b-c) The HDR-mediated knockin efficiency was determined by FACS at day 7 after co-transfection with Cas9/sgRNA1 and the donor plasmid pD-mCherry-sg-HA300-300bp. Significance was calculated using Student's paired t-test: ** $P \leq 0.01$; *** $P \leq 0.001$.

Supplementary Figure 6a: Design of genome editing at the *PRDM14* locus



Supplementary Figure 6a: Design of genome editing at the human *PRDM14* exon8. The exon8 of *PRDM14* is indicated in gray shadow. The stop codon is indicated in red. The recognition site of sgPRDM14 is in bold and underlined by a green line. The PAM is indicated in bold and the cut site is indicated by a red triangle. The left homology arm is indicated with an orange line and right homology arm with a blue line. The precise length of homology arm is indicated under a thick arrow. Replaced sequence upstream of DSB is indicated in purple. Sequences in grey indicate genomic DNA outside of homology arms. The two pairs of primers used for knockin pattern analysis by PCR are indicated by black arrows.

Supplementary Figure 6b: Precisely edited *PRDM14* locus.



TCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA

Wpre

GCGCGATCACATGGTCTCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGTTTAAACGCGTCGACAATCAACCTCTGGATTAC

AAAATTTGTGAAAGATTGACTGGTATTCTTAACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTTCATT

TCTCCTCCTTGATAAATCCTGGTTGCTGTCTCTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGTGACGCAACCCCCACTGGTT

GGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAACTCATCGCCGCTGCCTTGCCCGCTGCTGGACAGGGGGCT

CGGCTGTTGGGCACTGACAATTCCGTGGTGTGTCGGGGAAGCTGACGTCTTCCATGGCTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCCTTCTGCTA

CGTCCCTTCGGCCCTCAATCCAGCGGACCTTCTTCCCGCGGCCTGCTGCCGGCTCTGCGGCCTTCCGCGTCTTCGCCTTCGCCCTCGACGAGTCGGATCTCCCTTG

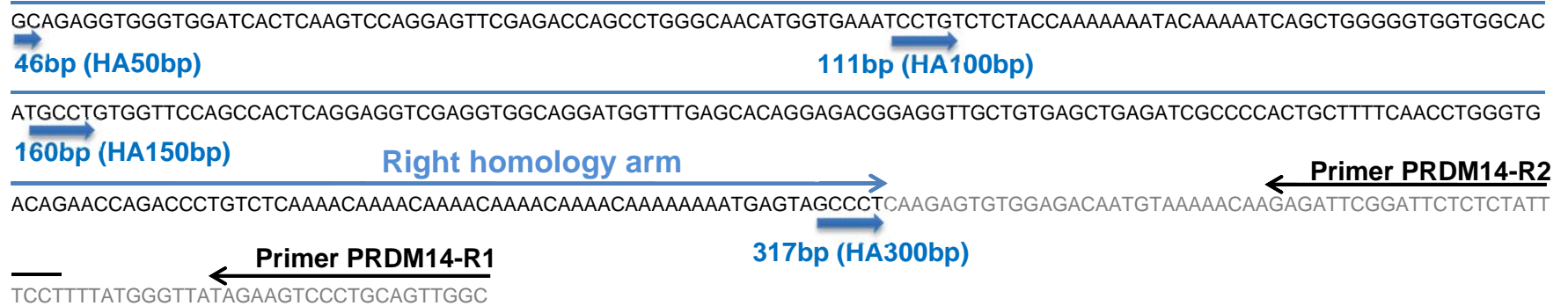
PolyA

GGCGGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACCTAGAATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAAC

CATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTTCAGGGGGAGGTGTGGGAGGTTTTTAAAGCAAGTAAAACCTCTACAAATGTG

GTATGGCTGATTATGATCCGGCTGCCTCGCGGTTTTCCGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCG

GGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTCCGGGCGCAGCCATGAGGCCAGGCACAATGACTCACGCCTGTAATCCCAGCACTTTGGGAG
PAM



Supplementary Figure 6b: Precisely edited *PRDM14* locus. The exon8 of *PRDM14* is indicated in gray shadow. The stop codon is indicated in red. The destroyed recognition site of sgPRDM14 is in bold and underlined by a green line, and PAM is indicated in bold. The left homology arm is indicated with an orange line and right homology arm with a blue line. The precise lengths of homology arm are indicated with thick arrows. The ribosome-skipping sequence 2A is in yellow and GFP-Wpre-ployA sequence in green color. Sequences in grey indicate genomic DNA outside of homology arms. The two pairs of primers used for knockin pattern analysis by PCR are indicated by black arrows.

Supplementary Figure 6c: Backbone sequence cut off form pD-GFP-sg (*PRDM14*)

Partial recognition
site of sgPRDM14

Donor plasmid backbone sequence

agggctagtagcttc cagggtggcacttttcggggaaatgtgcgcggaaccctattgtttattttctaaatacattcaaataatgatccgctcatgagacaataaccctgataaatgctcaataatattgaaa
aaggaagagatagattcaacatttccgtgctgccttattccctttttgcgccattttgccttctgttttctcaccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtggtg
ttacatcgaactggatctcaacagcggtaagatccttgagagtttgcggcgaagaacggtttccaatgatgagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgcccgggcaagagca
actcggctgcccatatactattctcagaatgacttgggtgagtagtaccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataacctgagtgataacactgc
ggccaacttactctgacaacgatcggaggaccgaaggagctaaccgctttttgcacaacatgggggatcatgtaactgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgag
cgtgacaccacgatgcttagcaatggcaacaacggtgcgcaactattaactggcgaactacttacttagctcccggcaacaattaatagactggatggaggcggataaaagtgcaggaccactct
gcgctcggcccttccggctggctggttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggaagccctcccgtatcgtagtatctacacgacgggga
gtcaggcaactatggatgaacgaaatagacagatcgctgagataggtcctcactgattaagcattgtaactgtcagaccaagtttactcatatatactttagattgatttaaaactcatttttaatttaaagg
atctaggtgaagatccttttgataatctcatgacaaaaatccctaacgtgagtttcttccactgagcgtcagaccccgtagaaaagatcaaaggatcttctgagatcctttttctgcgcgtaactctgctgctt
gcaaacaaaaaaaccaccgctaccagcgggtggtttgttgcgggatcaagagctaccaactcttttccgaaggtaactggctcagcagagcgcagataccaaatactgtccttctagtgtagccgtagtta
ggccaccactcaagaactctgtagcaccgcctacatacctcgtctgctaactctgttaccagtggtgctgctccagtgccgataagtcgtgcttaccgggttgactcaagacgatagttaccggataaggc
gcagcggctcgggctgaacggggggtcgtgcacacagcccagctggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcggccacgctcccgaagggagaaagg
cggacaggtatccgtaagcggcagggcggaacaggagagcgcacgagggagctccaggggaaacgcctggatctttatagctctgctgggttccgacactctgactgagcgtcgattttgtgat
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gagtgagctgataccgctcggcagccgaacgaccgagcgcagcagtgagcaggaagcgggaagagcggccaatacgcgaaaccgctctcccgcgctggccgattcattaatgcagc
tggcacgacaggttcccgactgaaagcgggacgtgagcgaacgcaattaatgtgagttagctcactcattagaccacccaggctttacactttatgctccggctcgtatgtgtgtggaattgtgagcgg
ataacaattcacaca**gaagactactagccct**
Partial recognition
site of sgPRDM14

Supplementary Figure 6c: Backbone sequence cut off from pD-mNeonGreen-sg (*PRDM14*). The sequence of donor plasmid backbone is in lowercase and indicated by a red line. The residual partial sgPRDM14 recognition sequence after plasmid linearization by CRISPR is in bold and indicated by green lines.

Supplementary Figure 6d: Knockin pattern analysis at the *PRDM14* locus.

pD-GFP-sg-HA50-50bp (*PRDM14*)

| | Left homology arm | Replaced sequence in left arm | Right homology arm | | |
|-----------|------------------------------|--------------------------------------|-----------------------------------|---------------------------------|--|
| Wild type | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccct | GCCAGGCACAA:::TTTGGGAGGC | AGAGG | |
| Expected | Left homology arm | 2A-GFP-Wpre-ployA | Right homology arm | | |
| Cl1-38 | TCTGTGGGAAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG HDR | |
| Clone39 | TCTGTGGGAAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG HDR with 1bp mutation | |
| Clone40 | TCTGTGGGAAAATCT:::TGAAGACTAC | AGCTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG HDR with 2bp mutation | |
| Clone41 | TCTGTGGGAAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | gccaggcaciaa:::tttgg | ccaggca | |
| Clone42 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctctgcccgggaaaatct:::tgaagactac | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG 3-NHEJ: 48bp ins; 290bp del |
| Clone43 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctgcccgggaaaatct:::tgaagactac | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG 5-NHEJ: 73bp ins |
| Clone44 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctcagatttcagttl | GGGCGCAGCCATGAG | GCCAGGCACAA:::TTTGGGAGGC | AGAGG 5-NHEJ: 71bp ins |
| Clone45 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctctgcccgggaaaatct:::tgaagactac | cagtgactaattat:::gggcgagccatgag | gccaggcaciaa:::tttggaggcctggca | AGAGG 5-NHEJ: 20bp ins; 531bp del |
| Clone46 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctagaacattc:::tgaagactac | cagtgactaattat:::gggcgagccatgag | gccaggcaciaa:::tttggaggcctggcat | AGAGG 5, 3-NHEJ: left 73bp ins, right 52bp ins |
| Clone47 | TCTGTGGGAAAATCT:::TGAAGACTAC | ctagtagtctctgt | ggcacttgggaagactactagcc | AGGCACAA:::TTTGGGAGGC | AGAGG 5, 3-NHEJ: left 67bp ins, right 53bp ins |
| Clone48 | TCTGTGGGAAAATCT:::TGAAGACTAC | atlaaaatgaagttl | ggcacttgggaagactactagcc | AGGCACAA:::TTTGGGAGGC | AGAGG 5, 3-NHEJ: Backbone reverse insertion |
| Clone49 | TCTGTGGGAAAATCT:::TGAAGACTAC | tagccctaggctagtagtct | acaatagggttccgcgaca | TTTGGGAGGC | AGAGG 5, 3-NHEJ: Backbone reverse insertion |

pD-GFP-sg-HA100-100bp (*PRDM14*)

| | Left homology arm | Replaced sequence in left arm | Right homology arm | | |
|-----------|-----------------------------|-----------------------------------|-------------------------|-------------------------|---|
| Wild type | ATGTCGGCGTTTAC:::TGAAGACTAC | tagccct | GCCAGGCACAA:::TGAATCCTG | TCTCT | |
| Expected | Left homology arm | 2A-GFP-Wpre-ployA | Right homology arm | | |
| Cl1-37 | ATGTCGGCGTTTAC:::TGAAGACTAC | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TGAATCCTG | TCTCT HDR | |
| Clone38 | ATGTCGGCGTTTAC:::TGAAGACTAC | CAGTGTACTAATTAT:::GGGCGCAGCCATGAG | GCCAGGCACAA:::TGAATCCTG | TCTCT | |
| Clone39 | ATGTCGGCGTTTAC:::TGAAGACTAC | tagccct | GGGCGCAGCCATGAG | GCCAGGCACAA:::TGAATCCTG | TCTCT 3-NHEJ: 252bp del |
| Clone40 | ATGTCGGCGTTTAC:::TGAAGACTAC | tagcc | GGGCGCAGCCATGAG | GCCAGGCACAA:::TGAATCCTG | TCTCT 5-NHEJ: 7bp ins, 656bp del |
| Clone41 | ATGTCGGCGTTTAC:::TGAAGACTAC | caca | GGGCGCAGCCATGAG | GCCAGGCACAA:::TGAATCCTG | TCTCT 5-NHEJ: 5bp ins, 553bp del |
| Clone42 | ATGTCGGCGTTTAC:::TGAAGACTAC | tagccctgctccagggtggcac | gggaagactactagccct | GCCAGGCACAA:::TGAATCCTG | TCTCT 5, 3-NHEJ: Backbone forward insertion |
| Cl43-44 | ATGTCGGCGTTTAC:::TGAAGACTAC | gtcagacccctag | caggaaagactactagccct | ACA:::TGAATCCTG | TCTCT 5, 3-NHEJ: Backbone forward insertion |

pD-GFP-sg-HA150-150bp (*PRDM14*)

| | | | | | |
|-----------|-------------------|-------------------------------|--------------------------------|--------------------|--|
| | Left homology arm | Replaced sequence in left arm | | Right homology arm | |
| Wild type | TACTG | TGGTAAATCT:::TGAAGACTAC | tagccct | | GCCAGGCACA:::GCACATGCCTGTGGT |
| | Left homology arm | 2A-GFP-Wpre-ployA | | Right homology arm | |
| Expected | TACTG | TGGTAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT | | GGGCGCAGCCATGAGGCCAGGCACA:::GCACATGCCTGTGGT |
| Cl1-34 | TACTG | TGGTAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT | | GGGCGCAGCCATGAGGCCAGGCACA:::GCACATGCCTGTGGT |
| Cl35-36 | TACTG | TGGTAAATCT:::TGAAGACTAC | CAGTGTACTAATTAT | | GGGCGCAGCCATGAGGCCAGGCACA:::GCACATGCCTGTGGT |
| Clone37 | TACTG | TGGTAAATCT:::TGAAGACTAC | t-gcc--tggtaaatct:::tgaagactac | CAGTGTACTAATTAT | GGGCGCAGCCATGAGGCCAGGCACA:::GCACATGCCTGTGGT |
| Clone38 | TACTG | TGGTAAATCT:::TGAAGACTAC | | | GGGCGCAGCCATGAGGCCAGGCACA:::GCACATGCCTGTGGT |
| Clone39 | TACTG | TGGTAAATCT:::TGAAGACTAC | tagccctaggctagtagtcttcagggtg | | attcacacaggaagac-----AGGCACA:::GCACATGCCTGTGGT |

HDR

3-NHEJ: 479bp del
 5-NHEJ: 326bp ins
 5-NHEJ: 1191bp del
 5,3-NHEJ: Backbone forward insertion

pD-GFP-sg-HA300-300bp (*PRDM14*)

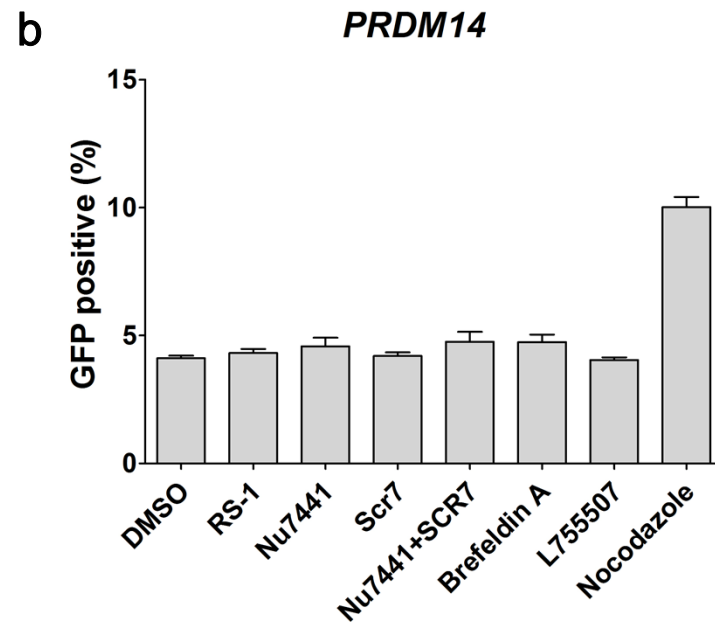
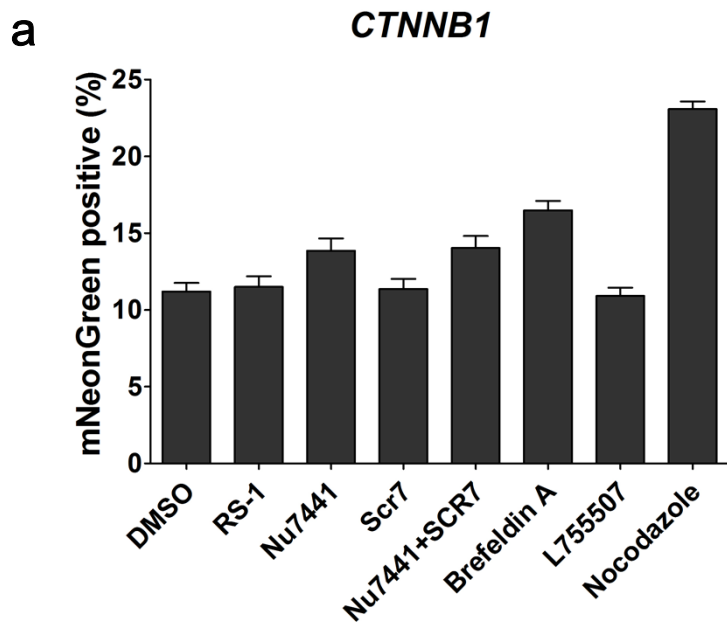
| | | | | | |
|-----------|-------------------|-------------------------------|------------------|--------------------|---|
| | Left homology arm | Replaced sequence in left arm | | Right homology arm | |
| Wild type | CCATA | AGGATGAGCA:::TGAAGACTAC | tagccct | | GCCAGGCACA:::GAGTAGCCCTCAAGA |
| | Left homology arm | 2A-GFP-Wpre-ployA | | Right homology arm | |
| Expected | CCATA | AGGATGAGCA:::TGAAGACTAC | CAGTGTACTAATTATG | | GGGCGCAGCCATGAGGCCAGGCACA:::GAGTAGCCCTCAAGA |
| Cl1-45 | CCATA | AGGATGAGCA:::TGAAGACTAC | CAGTGTACTAATTATG | | GGGCGCAGCCATGAGGCCAGGCACA:::GAGTAGCCCTCAAGA |

HDR

Supplementary Figure 6d: Knockin pattern analysis at the *PRDM14* locus. The left homology arm is indicated in yellow shadow and right HA in blue shadow. The inserted sequence is in red lowercase. Replaced sequence in left arm is indicated in purple. Sequences in grey indicate genomic DNA outside of homology arms.

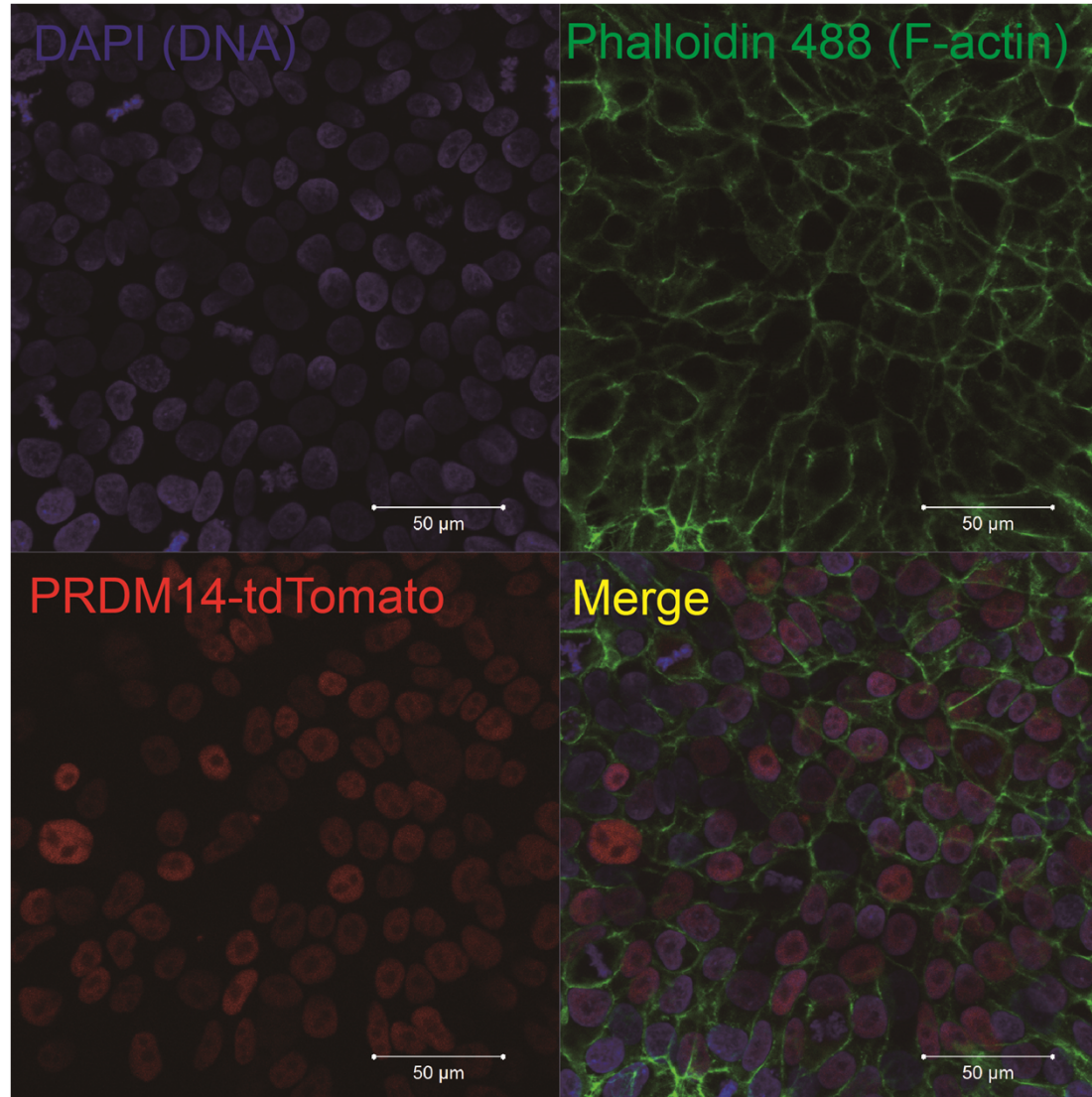
| | | |
|-----|--|-----|
| WT | CTCCCACATGAAGTTTCATGAAGACTACTAGCCCT ⁺ GCCAGGCACAATGACTCACGCCTGT | 0 |
| 2# | CTCCCACATGAAGTTTCATGAAGACTACTAGCCCT ⁺ GCCAGGCACAATGACTCACGCCTGT | +1 |
| 3# | CTCCCACATGAAGTTTCATGAAGACTACTAGCCCT ⁺ GCCAGGCACAATGACTCACGCCTGT | +1 |
| 6# | CTCCCACATGAAGTTTCATGAAGACTAC-----CTGCCAGGCACAATGACTCACGCCTGT | -5 |
| 9# | CTCCCACATGAAGTTTCATGAAGACTACTAGCC-----AGGCACAATGACTCACGCCTGT | -5 |
| 10# | CTCCCACATGAAGTTTCATGAAGACTACTAGCC;T ⁺ GCCAGGCACAATGACTCACGCCTGT | -1 |
| 12# | CTCCCACATGAAGTTTCATGAAG-----T ⁺ GCCAGGCACAATGACTCACGCCTGT | -11 |

Supplementary Figure 7: Presence of indels at the non-target allele of all iPSC clones with heterozygous HDR knockin at the *PRDM14* locus. The non-targeted allele was amplified by PCR, followed by Sanger sequencing to identify indels.

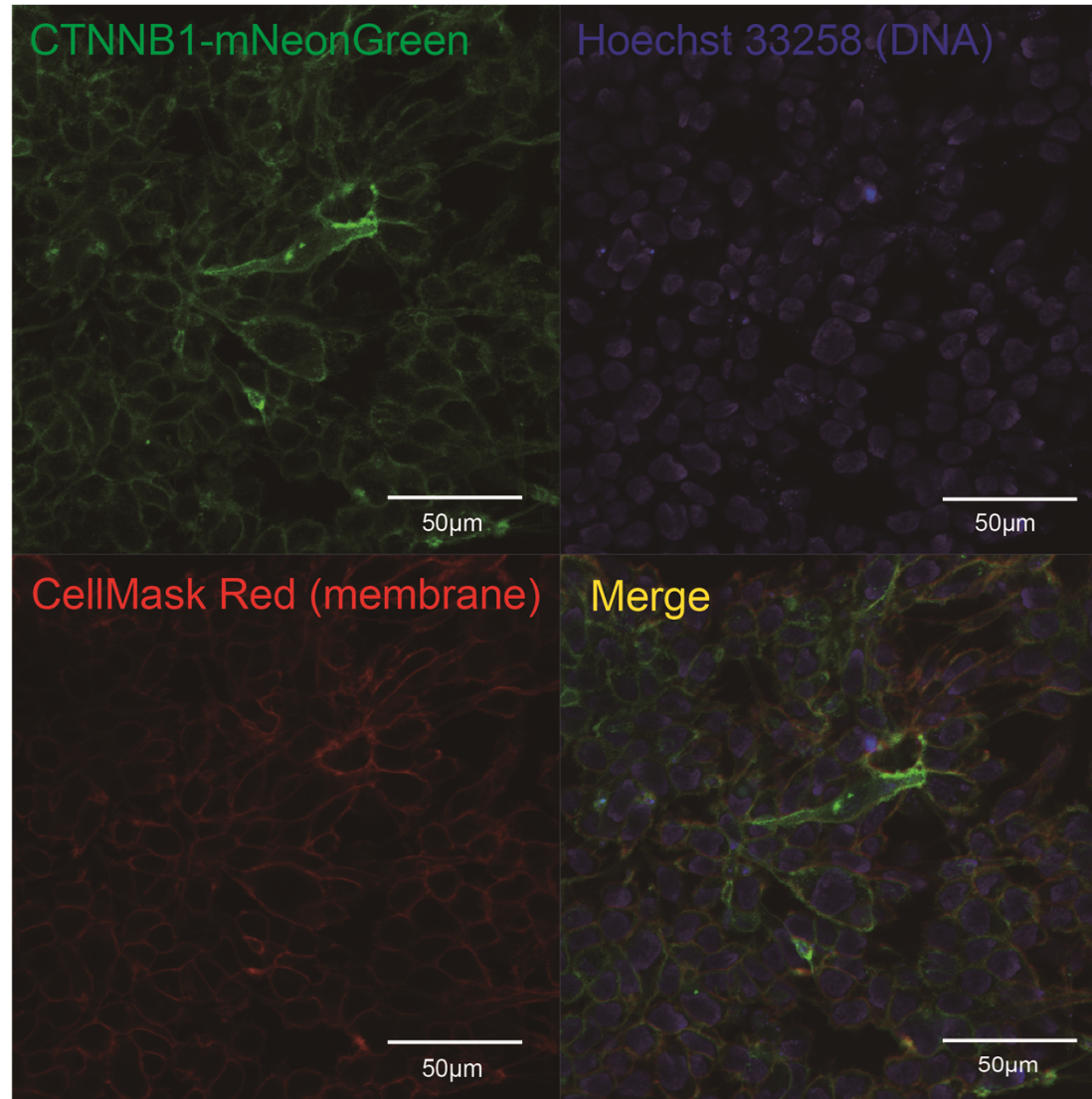


Supplementary Figure 8: The effects of small molecules on HDR in the H1 ES cell line. (a-c) The effects of small molecules on HDR efficiency at the *CTNNB1* locus (a) or *PRDM14* locus (b). The human H1 ES cells were treated with RS-1 (10 μ M), Nu7441 (2 μ M), SCR7 (1 μ M), Brefeldin A (0.1 μ M), L755507 (5 μ M) or Nocodazole (100ng/ml) at 0-24h after nucleofection and the HDR efficiency was determined by FACS on Day 3.

Supplementary Fig.9a

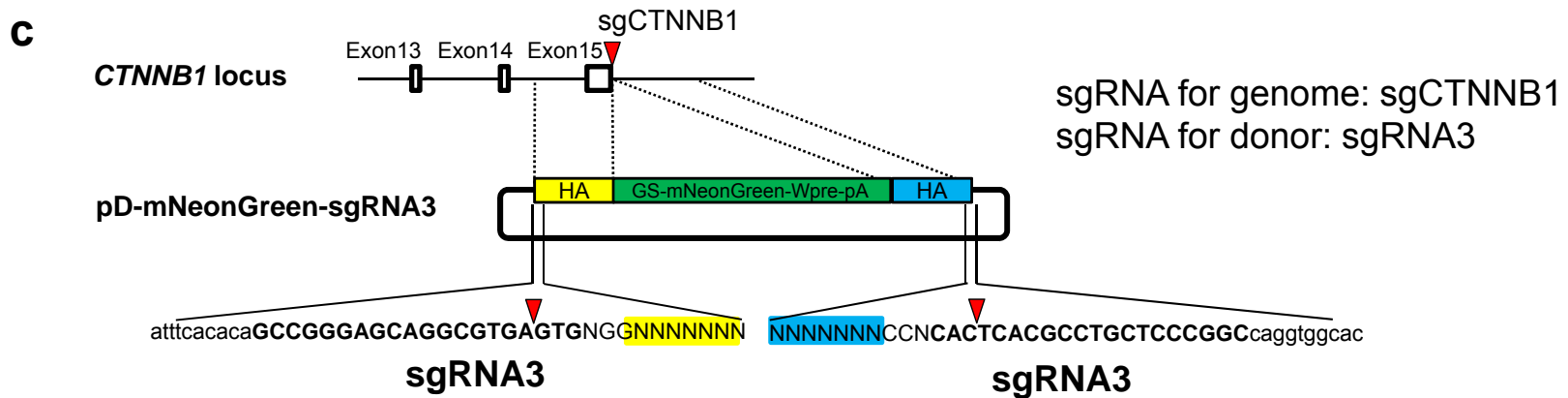
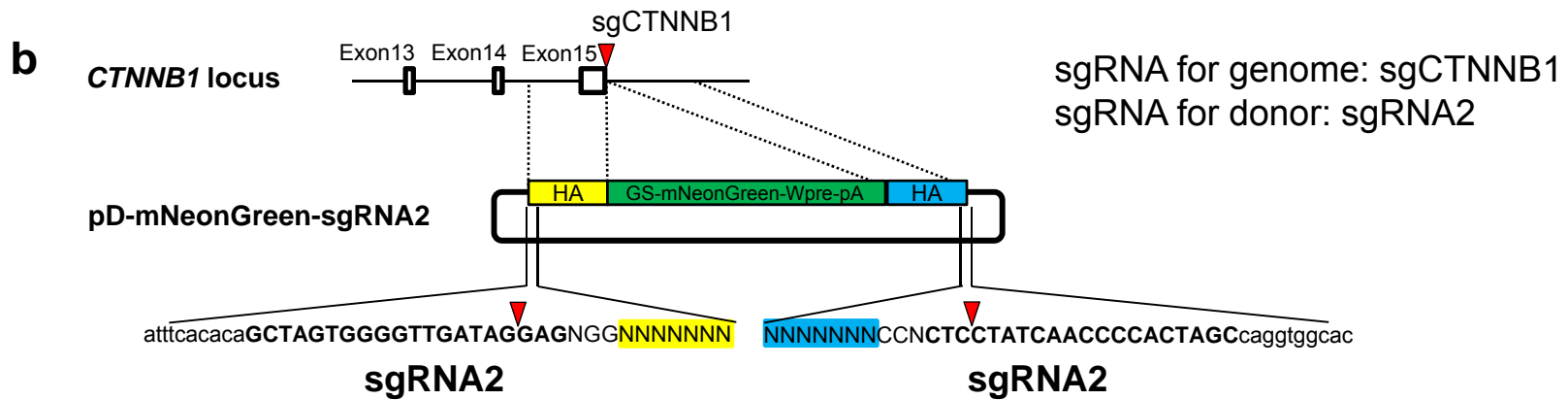
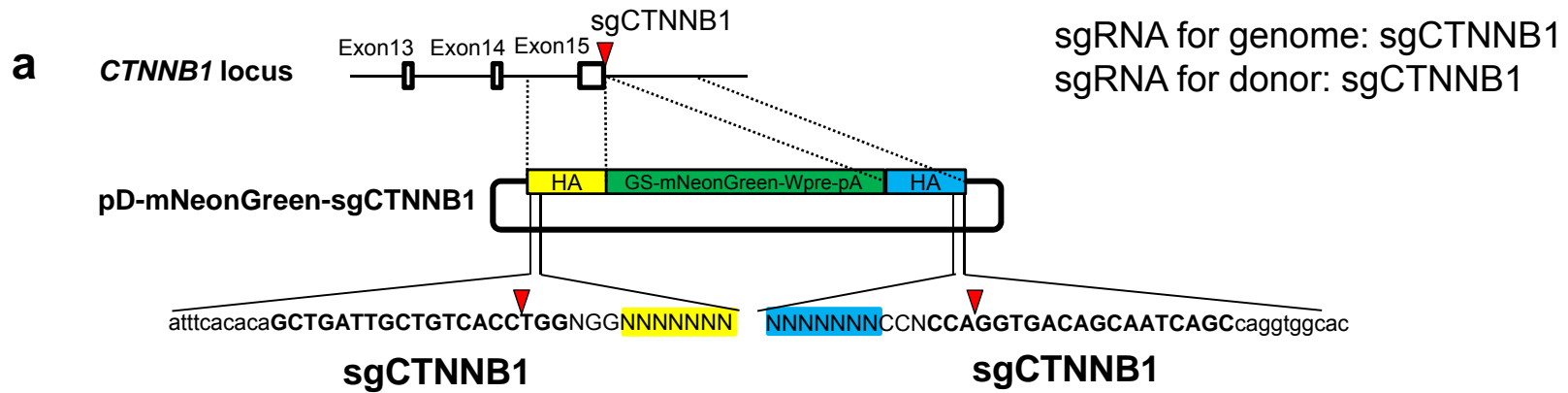


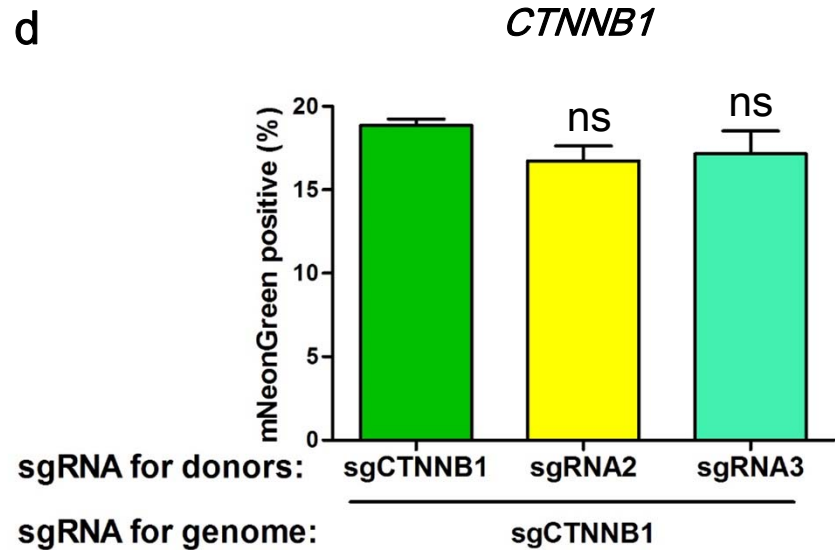
Supplementary Fig.9b



Supplementary Figure 9: Illustration of cellular location of genes of interest by fusion with a fluorescent protein after HDR targeting in iPSCs.

(a) Localization of PRDM14 at the nucleus as indicated by a tdTomato reporter. A new donor plasmid was created to generate a fusion gene of PRDM14 and tdTomato, which are separated by a GS linker. The cells were stained with DAPI and Phalloidin 488 to show the nucleus and plasma membrane. (b) Localization of CTNNB1 at plasma membrane as indicated by a mNeonGreen reporter. The cells were stained with Hoechst 33258 and CellMask Red to show the nucleus and membrane. (scale bar = 50 μm).





Supplementary Figure 10: The effects of using one vs. two sgRNAs in the double cut donor system on HDR efficiency. (a-c) Schematic outline of genome editing at the *CTNNB1* locus using double cut HDR donors, which will be linearized by Cas9 and sgCTNNB1 (a), sgRNA2 (b), or sgRNA3 (c), respectively. The donors contain a GS-mNeonGreen-Wpre-polyA sequence sandwiched by left homology arm (HA, marked in yellow shadow) and right homology arm (HA, marked in blue shadow). (d) The HDR efficiency was determined by FACS at day3 after co-transfection with Cas9/sgRNA and one of the three pD-mNeonGreen-sg donors (a-c). Statistical significance was calculated using the Student's paired t-test: ns, not significant.