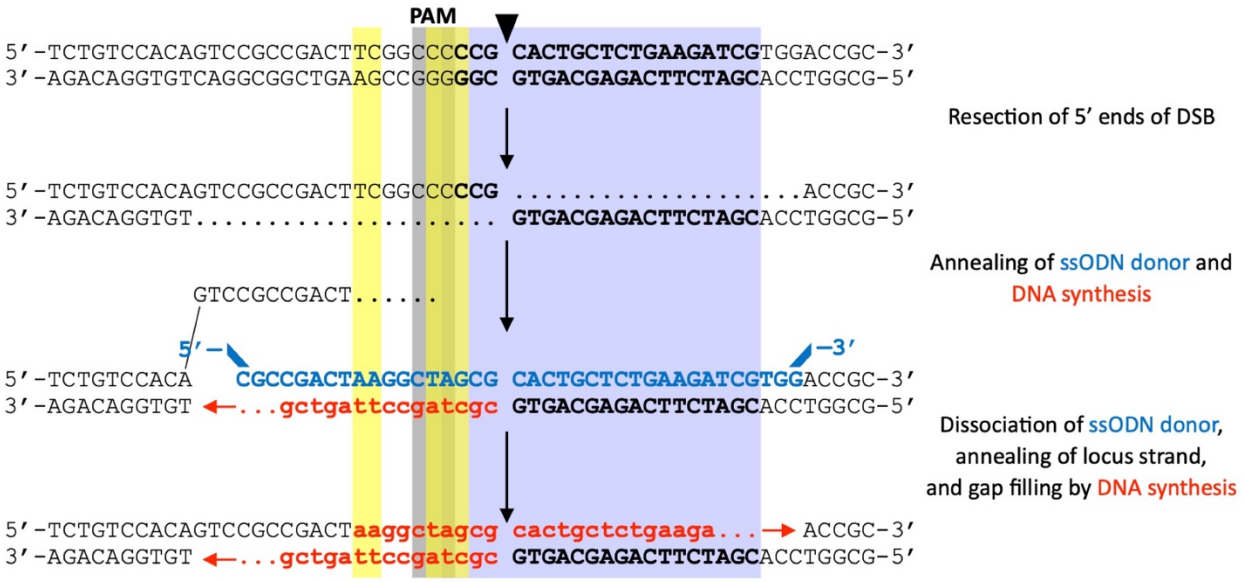
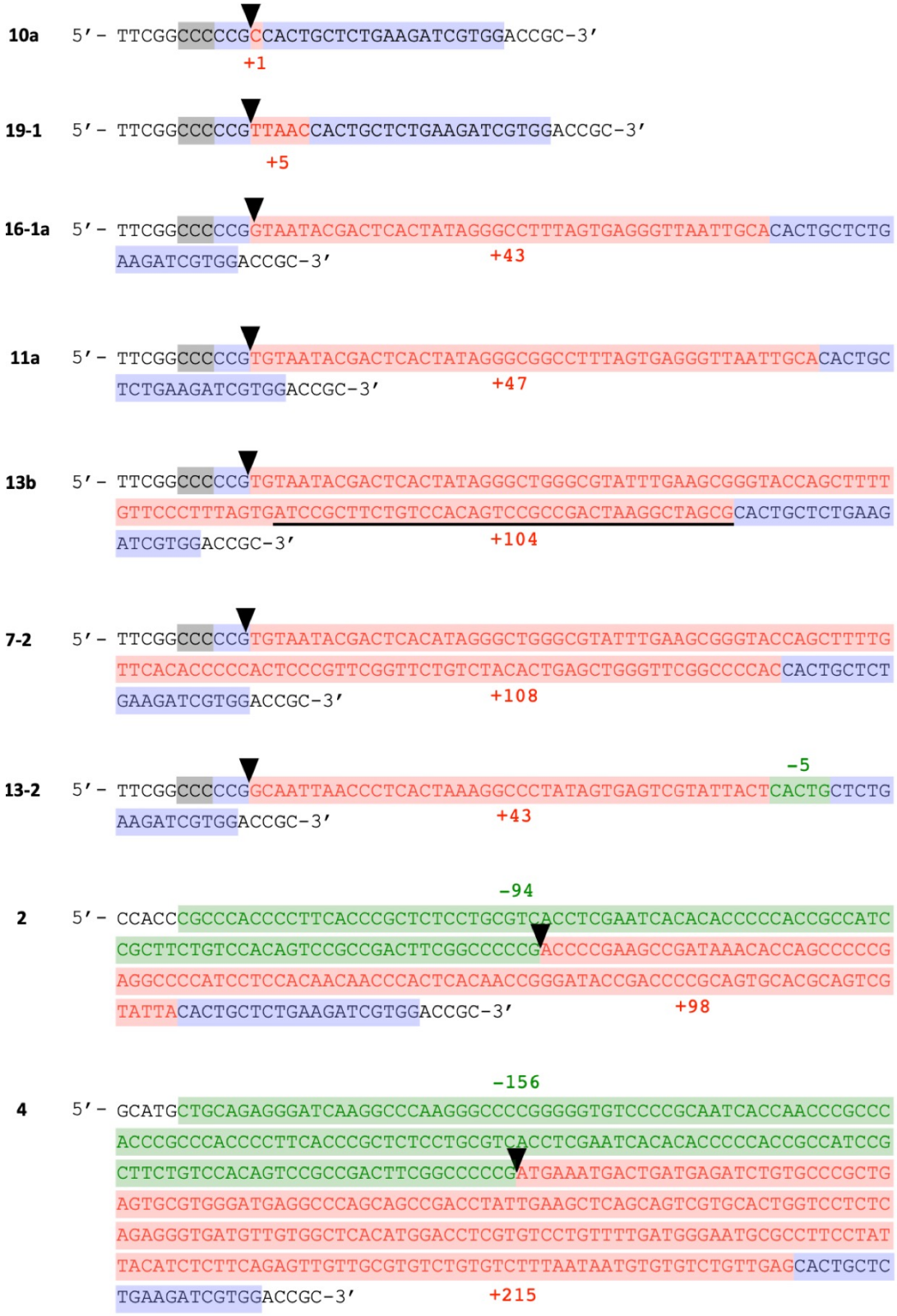
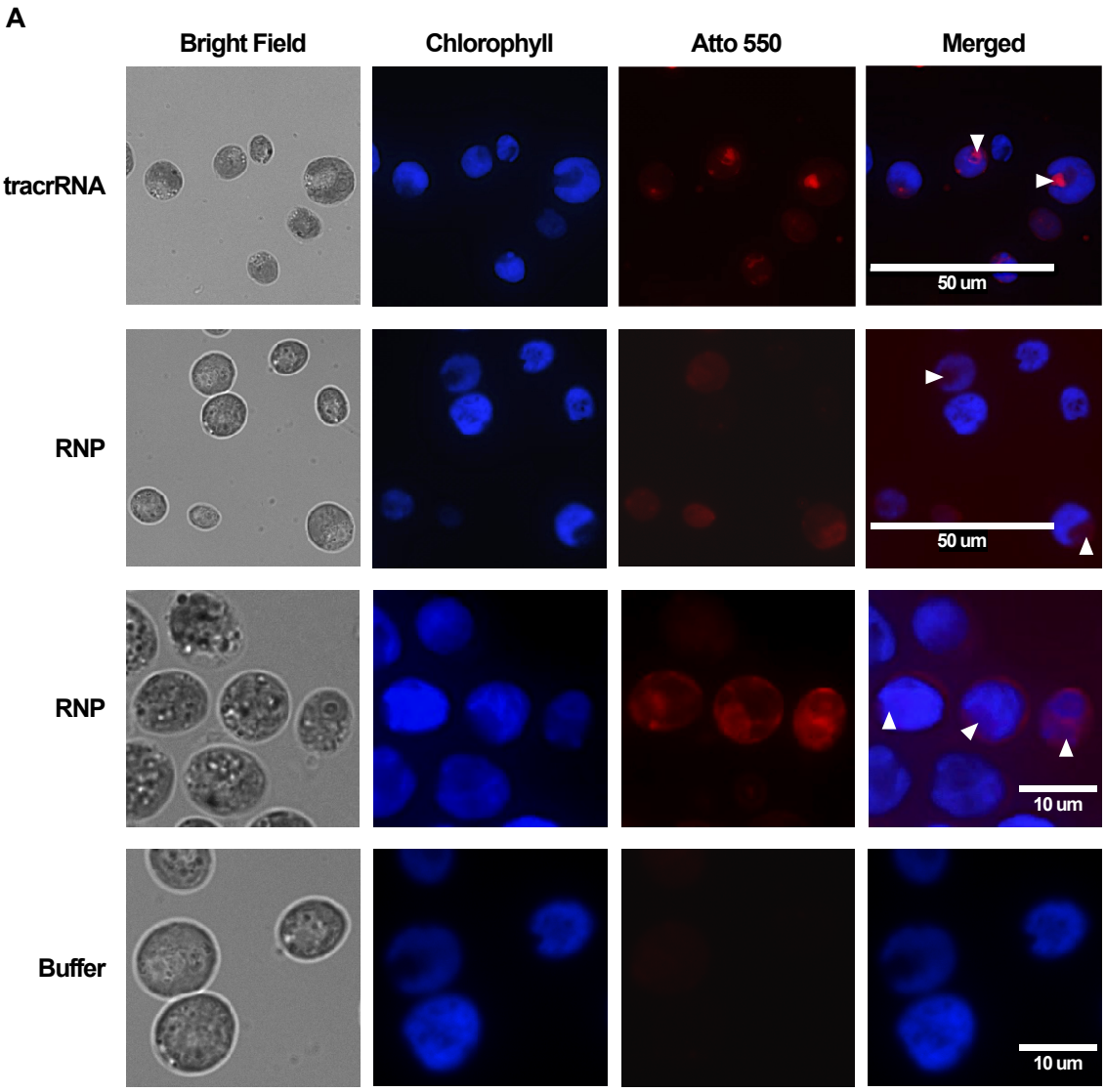


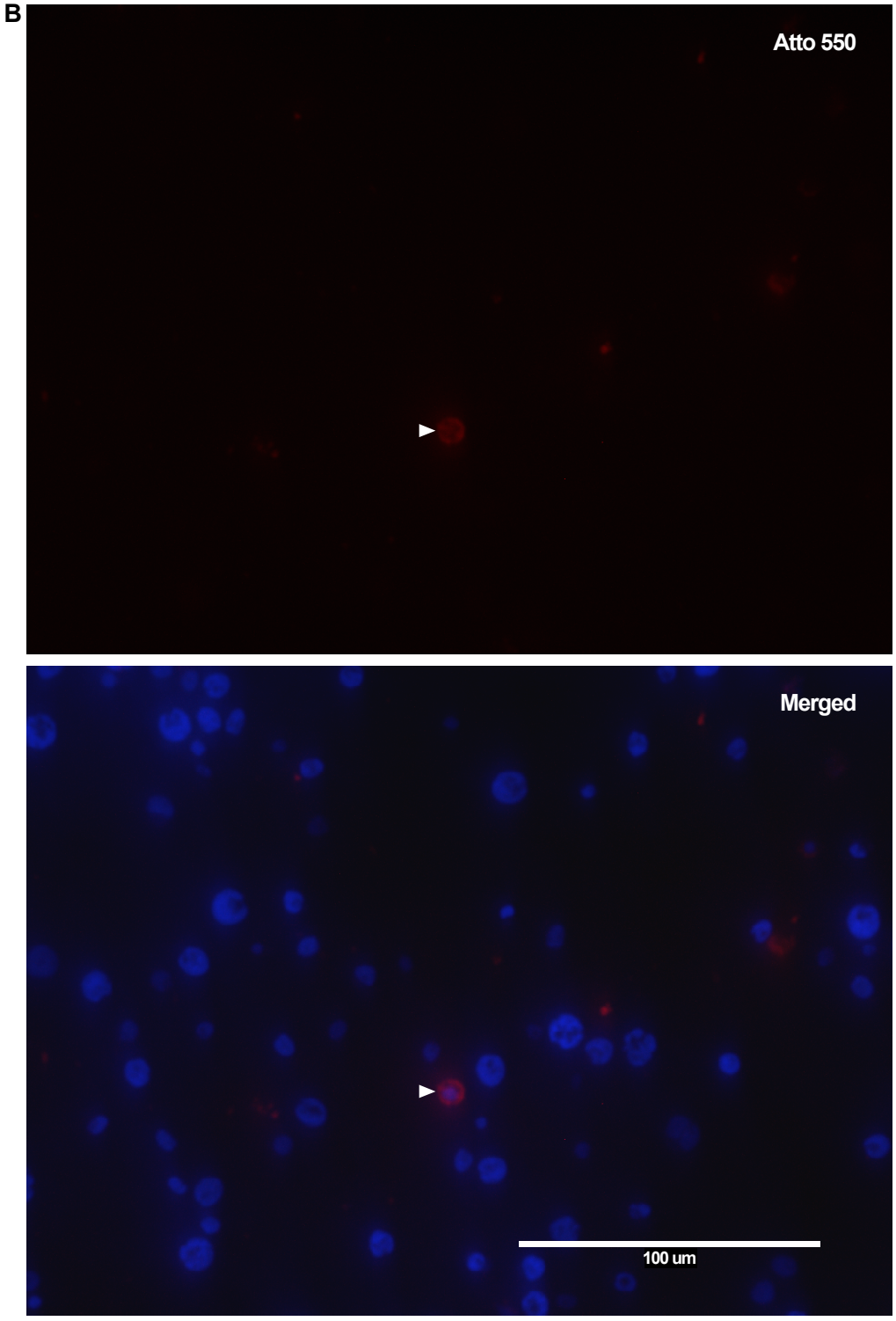
Supplemental Figure S1. Single-strand template repair (SSTR) model for homology directed repair of the *FTSY* gene, using as complementary template the transfected ssODN (adapted from Paix et al., 2017). The *FTSY* target sequence is shaded in purple, the PAM sequence is shaded in gray and the sites of base substitutions are shaded in yellow. A black arrowhead indicates the Cas9 cleavage site. After CRISPR/Cas9 (*FTSY*) RNP-mediated DNA double strand cleavage, the 5' ending strands are expected to be resected creating 3' end overhangs on each side of the DSB (Gallagher and Haber, 2017; Scully et al., 2020). The ssODN donor (depicted in dark blue) anneals to the complementary 3' overhang, which is then extended by DNA synthesis (sequence depicted in red lower case). The newly synthesized strand eventually dissociates from the ssODN donor and anneals with the complementary strand at the locus, which often may be partly eroded at its 3' end (Dorsett et al., 2014; Harmsen et al., 2018). Further DNA synthesis and ligation (not shown) completes the DSB repair. In this example, designed edits to the wild-type sequence are incorporated into both DNA strands as newly synthesized DNA (i.e., by gap filling). In mammalian cells, this appears to be the preferred mechanism of HDR using ssODN donors and generates short unidirectional conversion tracts proximal to the DSB (Kan et al., 2017; Harmsen et al., 2018). However, if the complementary strand at the locus is not eroded at its 3' end, annealing to the newly synthesized strand would generate heteroduplex DNA, which would likely be resolved by DNA mismatch repair mechanisms (Harmsen et al., 2018; Gallagher et al., 2020).



Supplemental Figure S2. DNA sequences of *FTSY* disrupted mutants obtained by co-transfection of CRISPR/Cas9 (*FTSY*) RNP, a dsDNA PCR product encoding the *aphVIII*

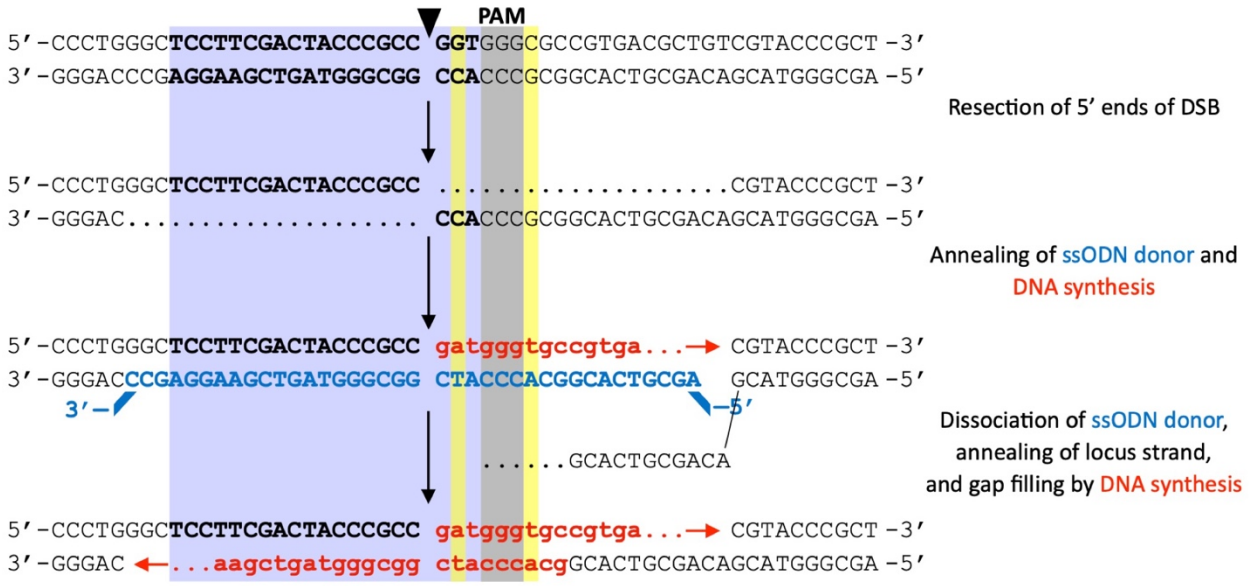
transgene and the *FTSY* ssODN donor. The *FTSY* target sequence and the reverse complement of the PAM sequence are shaded in purple and gray, respectively. A black arrowhead indicates the Cas9 cleavage site. Inserted sequences are depicted in red and deleted sequences are depicted in green. Colony 13b showed insertion of part of the ssODN donor sequence (underlined) at the cleavage site.


A
Bright Field
Chlorophyll
Atto 550
Merged
tracrRNA
RNP
RNP
Buffer
50 um
50 um
10 um
10 um

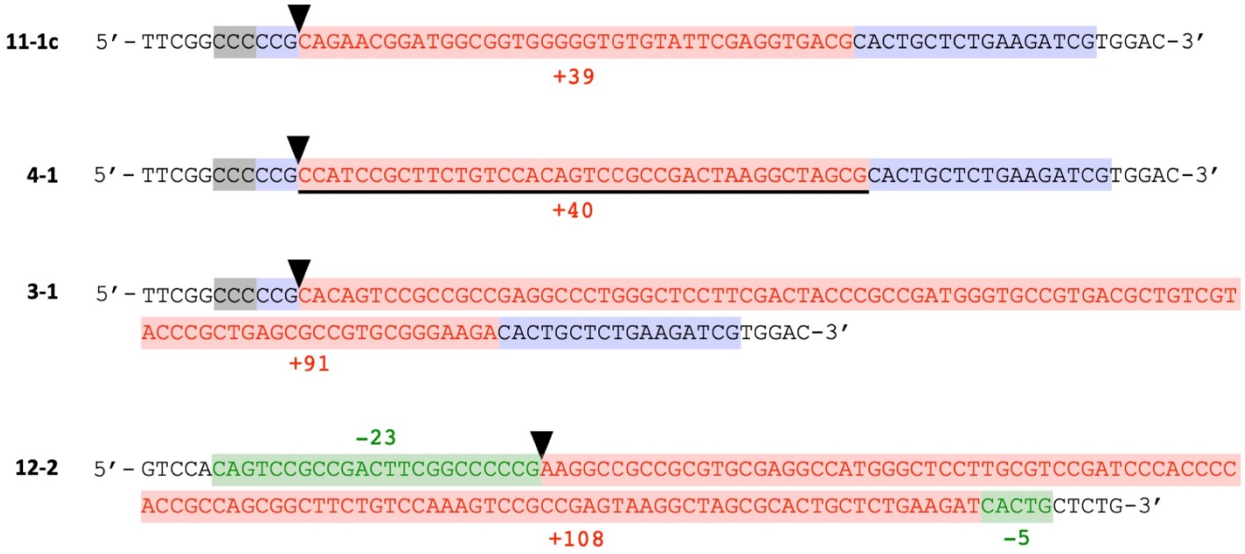


Supplemental Figure S3. Fluorescence microscopy analysis of the cellular uptake of the CRISPR/Cas9 (*FTSY*) RNP after electroporation. Images were collected 4 hours after electroporation. A, The panels correspond to bright field images of the cells (Bright Field), chlorophyll fluorescence (Chlorophyll), fluorescence of the trans-acting CRISPR RNA conjugated

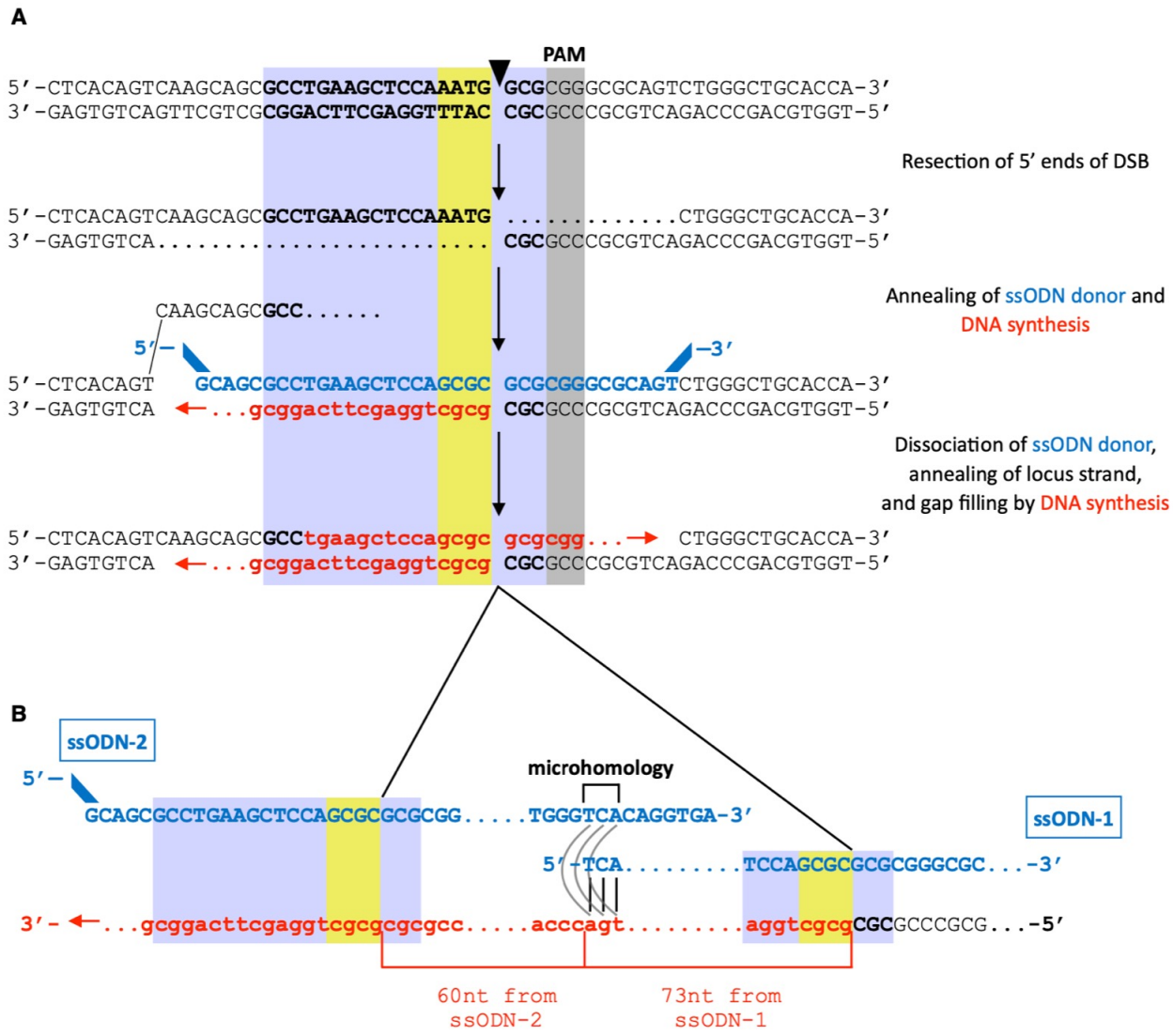
to the ATTO 550 fluorophore (Atto 550), and the merged images (Merged). Cells were electroporated with buffer alone (Buffer), as a negative control, with the tracrRNA-ATTO 550 alone (tracrRNA) or with *in vitro* assembled CRISPR/Cas9 (*FTSY*) RNP containing the tracrRNA-ATTO 550 (RNP). Pseudo-colored selected images are shown and the location of the nucleus is indicated with arrowheads. B, Representative images indicating that very few cells appear to take up the CRISPR/Cas9 (*FTSY*) RNP in the walled g1 Chlamydomonas strain.



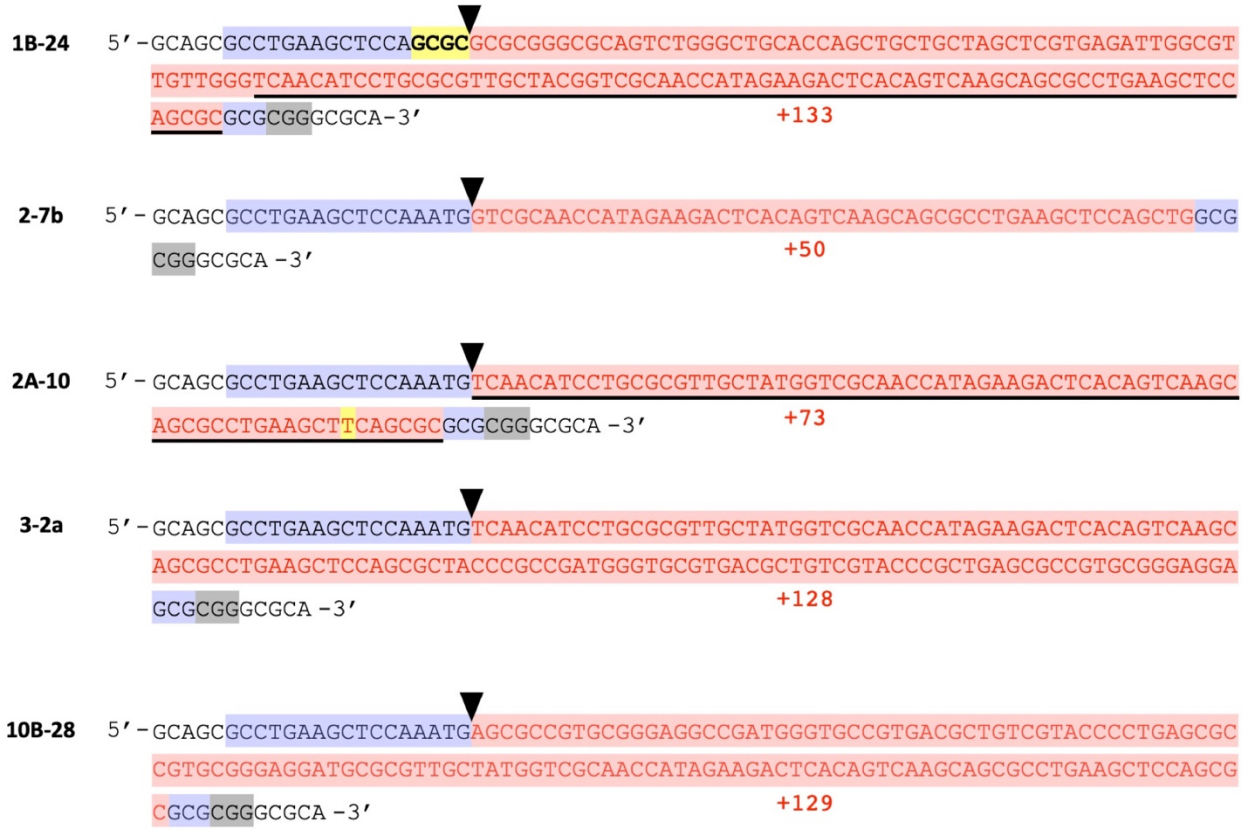
Supplemental Figure S4. Single-strand template repair (SSTR) model for homology directed repair of the *PPX1* gene, using as complementary template the transfected ssODN (adapted from Paix et al., 2017). The *PPX1* target sequence is shaded in purple, the PAM sequence is shaded in gray and the sites of base substitutions are shaded in yellow. A black arrowhead indicates the Cas9 cleavage site. See the legend to Supplemental Fig. 1 for an explanation.



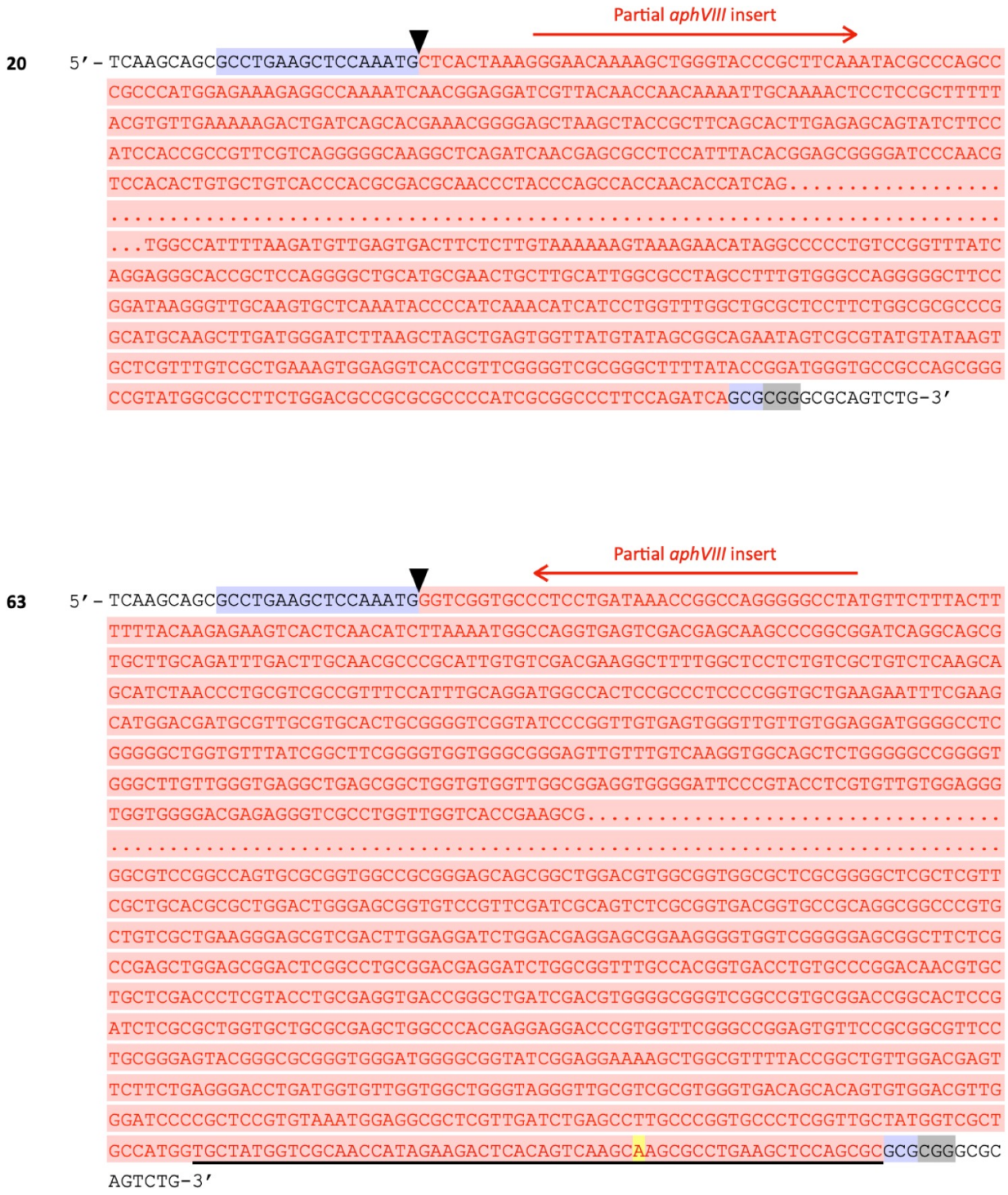
Supplemental Figure S5. DNA sequences of *FTSY* insertional mutants obtained by co-targeting the *PPX1* and *FTSY* genes for CRISPR/Cas9 editing. Color schemes and symbols are as described under Supplemental Fig. 2. Colony 4-1 showed insertion of part of the ssODN donor sequence (underlined) at the cleavage site.



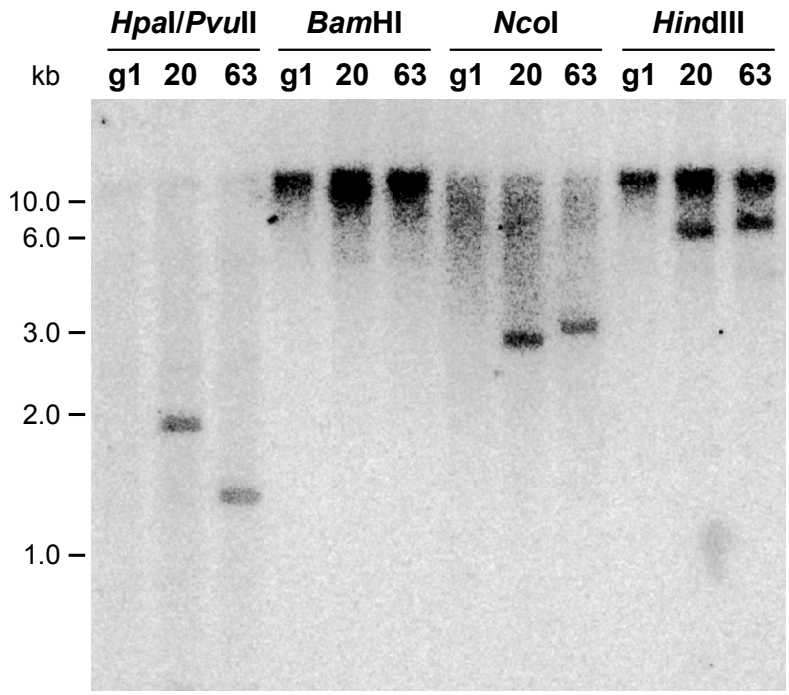
Supplemental Figure S6. Single-strand template repair (SSTR) model for homology directed repair of the *WDTC1* gene, using as complementary template the transfected ssODN (adapted from Paix et al*.,* 2017). A, SSTR model for HDR of the *WDTC1* gene. The *WDTC1* target sequence is shaded in purple, the PAM sequence is shaded in gray and the sites of base substitutions are shaded in yellow. A black arrowhead indicates the Cas9 cleavage site. See the legend to Supplemental Fig. 1 for an explanation. B, Template switching mechanism (Paix et al., 2017; Boel et al., 2018) that may explain the insertion of tandem ssODN donor sequences at the *WDTC1* target site in colony 1B-24 (Supplemental Fig. 7). Repair starts according to the SSTR model, using one molecule of ssODN donor (ssODN-1) as template for DNA synthesis. However, the newly synthesized strand anneals, through a region of microhomology (indicated in black/gray lines), to a second molecule of ssODN donor (ssODN-2) and is extended further by DNA synthesis. This extended newly synthesized strand eventually anneals with the complementary strand at the locus for completion of the DSB repair. As a result, 73 base pairs copied from ssODN-1, 60 base pairs copied from ssODN-2 and the intended four base pair edits are incorporated into the genome sequence.



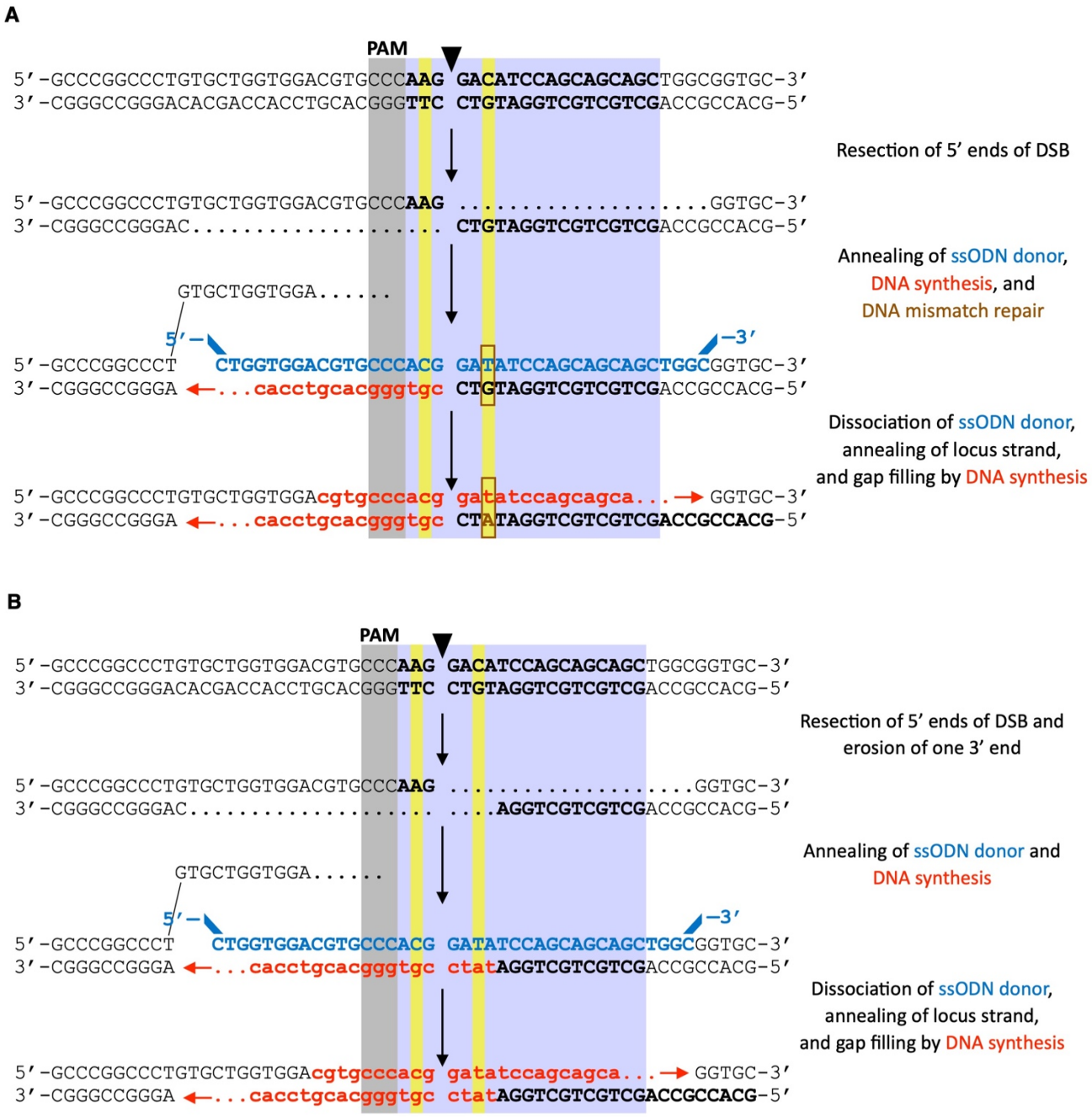
Supplemental Figure S7. DNA sequences of *WDTC1* insertional mutants obtained by co-targeting the *PPX1* and *WDTC1* genes for CRISPR/Cas9 editing. The *WDTC1* target sequence and the PAM sequence are shaded in purple and gray, respectively. A black arrowhead indicates the Cas9 cleavage site. Inserted sequences are depicted in red and base substitutions are shaded in yellow. Colony 2A-10 showed insertion of part of the ssODN donor sequence (underlined) at the cleavage site. Colony 1B-24 showed insertion of tandem ssODN donor sequences (only the first copy, designated as ssODN-1, is underlined), likely generated by template switching during DNA synthesis (Supplemental Fig. 6B).



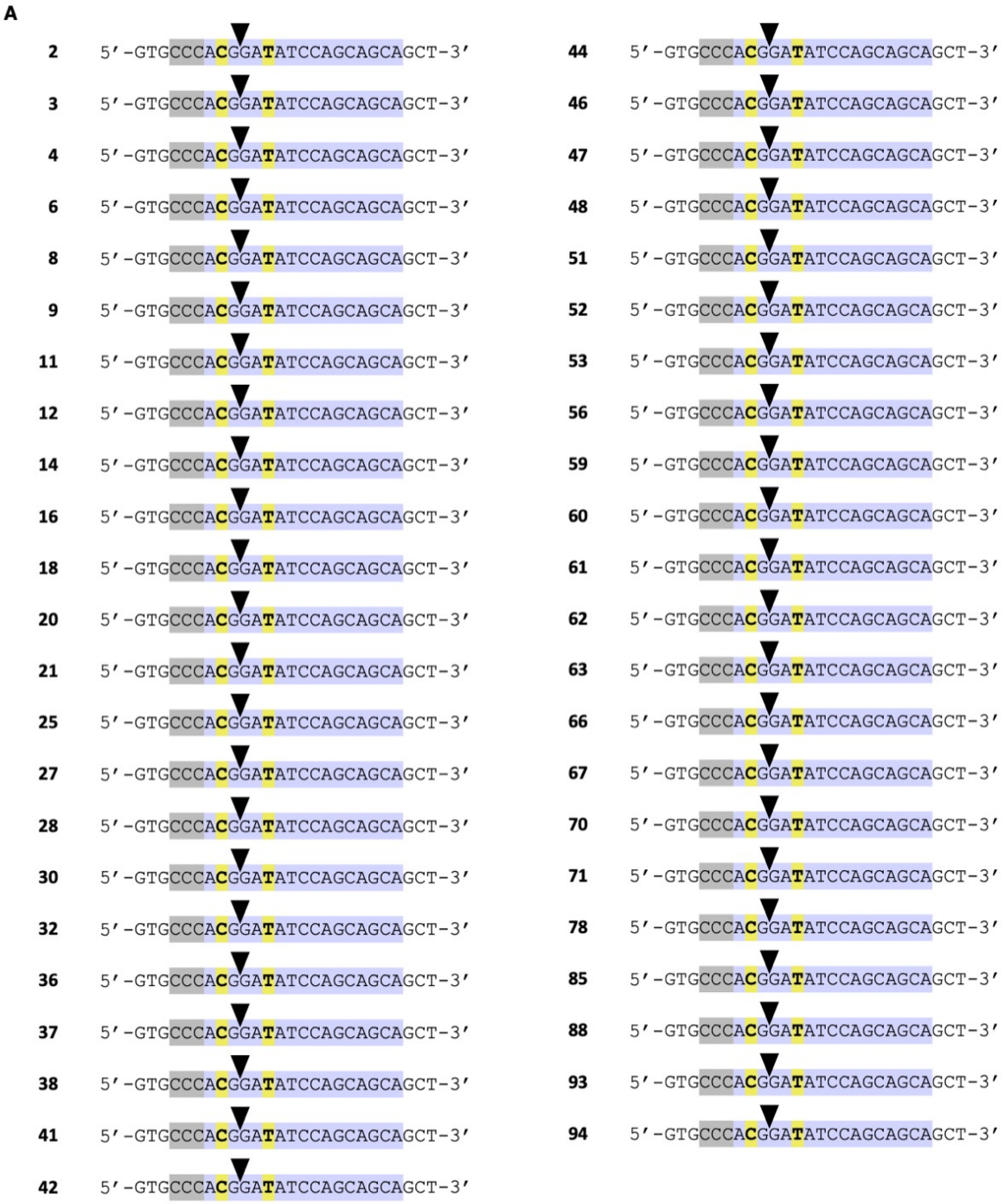
Supplemental Figure S8. DNA sequences of *WDTC1* insertional mutants obtained by co-transfection of CRISPR/Cas9 (*WDTC1*) RNP, a dsDNA PCR product encoding the *aphVIII* transgene and the *WDTC1* ssODN donor. Color schemes and symbols are as described under Supplemental Fig. 7. Base substitutions are shaded in yellow. Colony 63 showed insertion of part of the ssODN donor sequence (underlined) at the cleavage site. Both mutants displayed insertion of part of the *aphVIII* transgene, albeit in opposite orientations (as indicated by the red arrows).

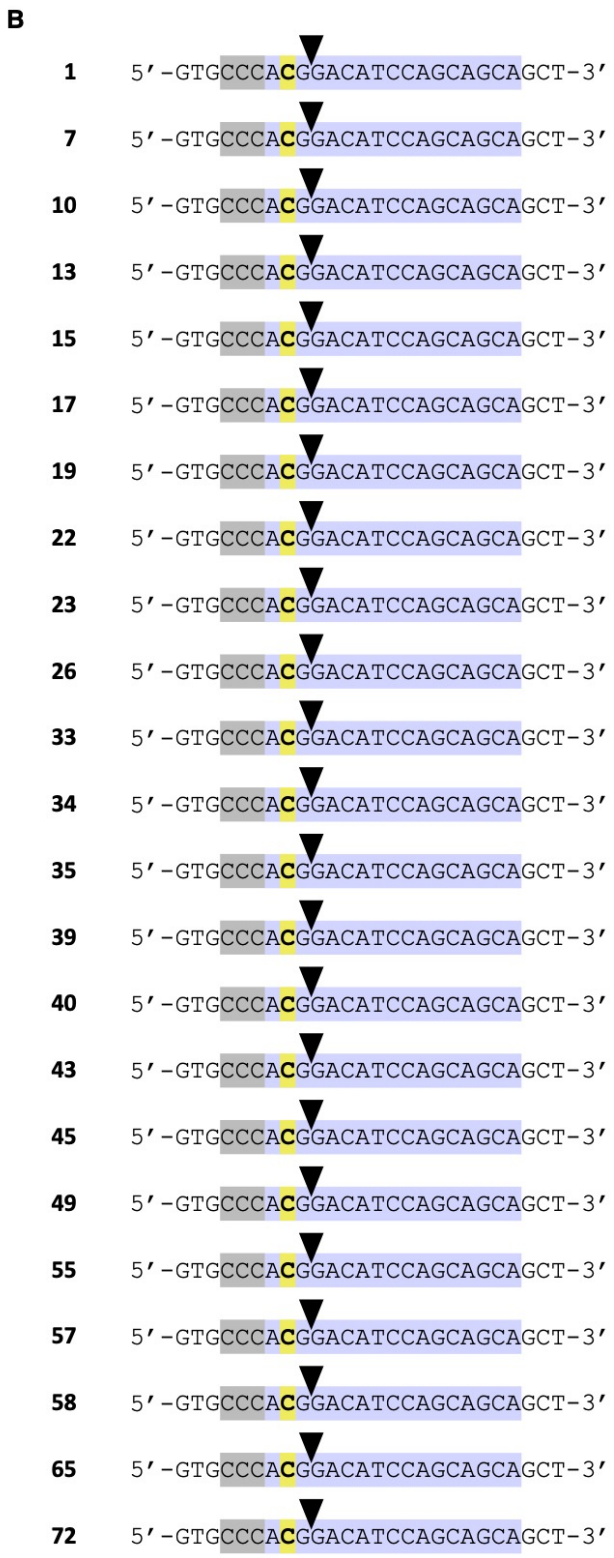


Supplemental Figure S9. Southern blot analysis of CRISPR/Cas9 induced insertional mutants of the *WDTC1* gene. Genomic DNA of the parental strain (g1) and two insertional mutants (20 and 63) was digested with the indicated restriction enzymes, separated by agarose gel electrophoresis and hybridized with a probe corresponding to the *aphVIII* coding sequence, to examine integration of the transgene conferring paromomycin resistance. The blot was washed at relatively low stringency to detect even short segments integrated into the genome and this resulted in some cross-hybridization to sequences in the untransformed wild type strain. Nonetheless, no obvious additional fragments, beyond those predicted from integration at the Cas9 cleavage site, were observed.



Supplemental Figure S10. Single-strand template repair (SSTR) model for homology directed repair of the *ALS1* gene, using as complementary template the transfected ssODN (adapted from Paix et al., 2017). The *ALS1* target sequence is shaded in purple, the PAM sequence is shaded in gray and the sites of base substitutions are shaded in yellow. A black arrowhead indicates the Cas9 cleavage site. See the legend to Supplemental Fig. 1 for an explanation. A, Schematic of the introduction of the C->T edit (on the right side of the Cas9 cleavage site) by DNA mismatch repair, instructed by the 3' half of the ssODN donor. B, Schematic of the introduction of the C->T edit (on the right side of the Cas9 cleavage site) by DNA synthesis (i.e., by gap filling), instructed by the 3' half of the ssODN donor after erosion of the 3' end of the complementary locus strand.


A
2 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
3 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
4 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
6 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
8 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
9 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
11 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
12 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
14 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
16 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
18 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
20 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
21 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
25 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
27 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
28 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
30 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
32 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
36 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
37 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
38 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
41 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
42 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
44 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
46 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
47 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
48 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
51 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
52 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
53 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
56 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
59 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
60 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
61 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
62 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
63 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
66 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
67 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
70 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
71 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
78 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
85 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
88 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
93 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′
94 5′-GTGCCCACGGATATCCAGCAGCAGCT-3′



Supplemental Figure S11. DNA sequence of the *ALS1* target site in sulfometuron methyl resistant colonies. The *ALS1* target sequence and the reverse complement of the PAM sequence are shaded in purple and gray, respectively. A black arrowhead indicates the Cas9 cleavage site.

Base substitutions are shaded in yellow. A, Forty-five colonies containing both intended nucleotide changes (i.e., A->C and C->T) (Fig. 6B; Supplemental Table 6). B, Twenty-three colonies containing only the A->C change (Fig. 6B; Supplemental Table 6).

Supplemental Table S1. Efficiency of *FTSY* targeted gene disruption by electroporation with CRISPR/Cas9 (*FTSY*) RNP and a PCR product encoding the *aphVIII* transgene

Experiment	*aphVIII* Marker	ssODN Donor	Heat Shock	Frequency[a]	Percentage[b]	Number of Electroporations
1	+	no	no	5.3×10^{-8}	0.15 (5/3350)	12
	+	+	no	6.3×10^{-8}	0.19 (6[c]/3137)	12
2[d]	+	+	no	4.2×10^{-8}	0.24 (2/819)	6
	+	+	+	2.1×10^{-7}	2.23 (10[c]/449)	6
3[d]	+	+	no	2.1×10^{-8}	0.19 (1/536)	6
	+	+	+	1.1×10^{-7}	1.57 (5[c]/318)	6

[a]Frequency of pale green colonies (i.e., with a presumably disrupted *FTSY* gene) relative to the total number of electroporated cells.
[b]Percentage of pale green colonies (i.e., with a presumably disrupted *FTSY* gene) relative to the number of paromomycin resistant colonies. In between parentheses, number of pale green colonies over number of paromomycin resistant colonies.
[c]In these colonies, disruption of the *FTSY* gene was verified by PCR analyses and sequencing.
[d]Replicate experiments.

Supplemental Table S2. Single-stranded oligodeoxynucleotides (ssODNs) electroporated into cells as templates for HDR

ssODN Donor DNA	Sequence[a]
FTSY	T*G*C*GTCACCTCGAATCACACACCCCCACCGCCATCCGCTTCTGTCCACAGT CCGCCGACT**aa**GGC**tag**CGCACTGCTCTGAAGATCGTGGACCGCAT*C*C*G
PPX1	T*C*C*TCCCGCACGGCGCTCAGCGGGTACGACAGCGTCACGGC**a**CCCA**t**CGGC GGGTAGTCGAAGGAGCCCAGGGCCTCGGCGGCGG*C*G*G
WDTC1	T*C*A*ACATCCTGCGCGTTGCTATGGTCGCAACCATAGAAGACTCACAGTCAA GCAGCGCCTGAAGCTCCA**gcgc**GCGCGGGCGCAGTCTGGGCTGCACCAGCTGCT GCTAGCTCGTGAGATTGGCGTTGTTGGGTCACAGG*T*G*A
ALS1	C*C*G*CACCGGCCGGCCCGGCCCTGTGCTGGTGGACGTGCCCA**c**GGA**t**ATCCA GCAGCAGCTGGCGGTGCCGGACTGGGAGG*C*G*C

[a]Modifications to the wild-type sequences are shown in red. The asterisks indicate phosphorothioate bonds, expected to inhibit degradation by cellular exonucleases.

Supplemental Table S3. Efficiency of *PPX1* gene editing by electroporation with CRISPR/Cas9 (*PPX1*) RNP and ssODN donor DNA

Experiment	CRISPR/ Cas9 RNP	ssODN Donor	Heat Shock	Frequency[a]	Number of OxyfluorfenR[b]	Number of Electroporations
1	no	no	no	$<2.1 \times 10^{-8}$	0	6
	no	+	no	$<2.1 \times 10^{-8}$	0	6
	+	+	no	3.2×10^{-8}	3	12
2	no	+	no	$<2.1 \times 10^{-8}$	0	6
	+	+	no	4.2×10^{-8}	2	6
	+	+	+	1.3×10^{-7}	6	6
3[c]	no	+	+	$<2.1 \times 10^{-8}$	0	6
	+	+	+	6.3×10^{-7}	30	6
4[c]	no	+	+	$<2.1 \times 10^{-8}$	0	6
	+	+	+	1.6×10^{-6}	75	6

[a]Frequency of oxyfluorfen resistant colonies (i.e., with a presumably edited *PPX1* gene) relative to the total number of electroporated cells.
[b]Number of oxyfluorfen resistant colonies (i.e., with a presumably edited *PPX1* gene).
[c]Replicate experiments carried out with an optimized protocol (see Materials and Methods).

Supplemental Table S4. Efficiency of *FTSY* gene editing by co-electroporation with CRISPR/Cas9 (*FTSY*) RNP, CRISPR/Cas9 (*PPX1*) RNP and the corresponding ssODN donor DNAs

Experiment	Number of OxyfluorfenR[a]	*FTSY* Edited[b]			Number of Electroporations
		HDR[c]	Mixed[c]	NHEJ[c]	
1	50	1 (2.0%)	1 (2.0%)	0 (0.0%)	10
2	125	1 (0.8%)	0 (0.0%)	3 (2.4%)	12
3	48	2 (4.2%)	0 (0.0%)	1 (2.1%)	10

[a]Number of oxyfluorfen resistant colonies (i.e., with a presumably edited *PPX1* gene).
[b]Number of *FTSY* edited colonies characterized by PCR analysis and sequencing. In between parentheses, percentage of *FTSY* edited colonies relative to the number of oxyfluorfen resistant colonies.
[c]Repair of DSBs induced by CRISPR/Cas9 (*FTSY*) RNP carried out by homology directed repair (HDR), non-homologous end joining (NHEJ) or a combination of these pathways (Mixed).

Supplemental Table S5. Efficiency of *WDTC1* gene editing by co-electroporation with CRISPR/Cas9 (*WDTC1*) RNP, CRISPR/Cas9 (*PPX1*) RNP and the corresponding ssODN donor DNAs

Experiment	Number of OxyfluorfenR[a]	*WDTC1* Edited[b]			Number of Electroporations
		HDR[c]	Mixed[c]	NHEJ[c]	
1	64	1 (1.6%)	0 (0.0%)	2 (3.1%)	8
2	331	3 (0.9%)	2 (0.6%)	1 (0.3%)	12

[a]Number of oxyfluorfen resistant colonies (i.e., with a presumably edited *PPX1* gene).
[b]Number of *WDTC1* edited colonies characterized by PCR analysis and sequencing. In between parentheses, percentage of *WDTC1* edited colonies relative to the number of oxyfluorfen resistant colonies.
[c]Repair of DSBs induced by CRISPR/Cas9 (*WDTC1*) RNP carried out by homology directed repair (HDR), non-homologous end joining (NHEJ) or a combination of these pathways (Mixed).

Supplemental Table S6. Mutagenesis of the Chlamydomonas acetolactate synthase (*ALS1*) gene using CRISPR/Cas9 (*ALS1*) RNP and *ALS1* ssODN donor DNA

Treatment[a]	SMM^R Colonies[b]	Sequenced Colonies[c]
None	1	None
NEB Cas9 buffer	3	3 [B1 = CCCA**T**GGACATC -> K257M B2, B3 = CCCAAGGACATC -> K257 WT]
ssODN + NEB Cas9 buffer	6	3 [AD1, AD2, AD6 = CCCAAGGACATC -> K257 WT]
CRISPR/Cas9 RNP + ssODN + NEB Cas9 buffer	483[d]	45 (59.2%) [CCCA**C**GGA**T**ATC -> K257T] 23 (30.2%) [CCCA**C**GGACATC -> K257T] 2 (2.6%) [CCCA**C**GGA**T**ATC.(34).C**TCC**G->K257T and M272P] 2 (2.6%) [CCCAA**C**GACATC -> K257N] 1 (1.3%) [CCC**C**AGGACATC -> K257Q] 1 (1.3%) [C**TGGT**GGACATC -> P256L and K257V] 1 (1.3%) [CCCA**T**GGACATC -> K257M] 1 (1.3%) [CCCAAGGACATC -> K257 WT]

[a]CC-124 cells were electroporated in TAP medium containing 60 mM sucrose plus the indicated components.
[b]Total number of sulfometuron methyl resistant (SMM^R) colonies obtained in each electroporation.
[c]Number of sequenced colonies and corresponding sequence(s) at the *ALS1* target site. Changes to the wild-type sequence are indicated in red and a new *Eco*RV restriction enzyme site is underlined.
[d]The mutation frequency is ~1.9 x 10^{-5}, expressed as number of SMM^R colonies relative to the total number of electroporated cells. Based on 76 sequenced colonies, 70 (92%) SMM^R colonies are consistent with editing by HDR (see text for details).

Supplemental Table S7. Efficiency of *FTSY* gene editing, in cells pre-treated with autolysin, by co-electroporation with CRISPR/Cas9 (*FTSY*) RNP, CRISPR/Cas9 (*PPX1*) RNP and the corresponding ssODN donor DNAs

Experiment[a]	Number of OxyfluorfenR[b]	Number of Pale Green[c]	Number Examined by PCR[d]	*FTSY* Edited[e]		Number of Electroporations
				HDR[f]	NHEJ[f]	
1 (Autolysin)	1013	61 (6.0%)	52 (5.1%)	34 (3.4%)	18 (1.8%)	7
1 (Control)	124	5 (4.0%)	4 (3.2%)	4 (3.2%)	0 (0.0%)	4
2 (Autolysin)	366	23 (6.3%)	ND[g]	ND[g]	ND[g]	8
2 (Control)	153	8 (5.2%)	ND[g]	ND[g]	ND[g]	4

[a]Two independent experiments were performed, comparing in each case cells pre-treated with autolysin with untreated walled cells (Control).
[b]Number of oxyfluorfen resistant colonies (i.e., with a presumably edited *PPX1* gene).
[c]Number of pale green colonies (i.e., with a presumably edited *FTSY* gene). In between parentheses, percentage of pale green colonies relative to the number of oxyfluorfen resistant colonies.
[d]Number of pale green colonies examined by PCR analyses. In between parentheses, percentage of pale green colonies examined by PCR relative to the number of oxyfluorfen resistant colonies.
[e]Number of *FTSY* edited colonies verified by PCR analyses of target fragment size and presence of a newly created *Nhe*I restriction enzyme site. In between parentheses, percentage of *FTSY* edited colonies relative to the number of oxyfluorfen resistant colonies.
[f]Repair of DSBs induced by CRISPR/Cas9 (*FTSY*) RNP carried out by homology directed repair (HDR), characterized by positive *Nhe*I digestion of a PCR product of expected size, or non-homologous end joining (NHEJ), characterized by PCR products of abnormal size (most commonly longer than expected, suggesting sequence insertions).
[g]ND, not done.

Supplemental Table S8. CRISPR RNAs (crRNAs) used in the study

Target Gene	crRNA Sequence[a]
FTSY	CGAUCUUCAGAGCAGUGCGG[b]
PPX1	UCCUUCGACUACCCGCCGGU
WDTC1	GCCUGAAGCUCCAAAUGGCG
ALS1	GCUGCUGCUGGAUGUCCUU

[a]The displayed sequences correspond to the target-specific protospacer regions but each crRNA contained an additional 16-nt common sequence (GUUUUAGAGCUAUGCU) at its 3' end for annealing to the tracrRNA (IDT system).
[b]This crRNA was described by Baek et al. (2016).

Supplemental Table S9. PCR primers used in the study

Use	Forward primer		Reverse primer	
	Name	**Sequence**	**Name**	**Sequence**
PCR of *FTSY* target region[a]	F1A	GGTCGTTGCACACAAGAACG	R1A	ATTGGGCGGGTTTTCGTTTC
	F1	CTTTCTTGGCATGACCTGCAT	R1	CTAACACACCCACACCCACCT
PCR of *PPX1* target region[a]	F2A	CTCCTCCCCCACCTAGACTGT	R2	CTCCCCTCCGCAACACAC
	F2	TGTCTACCGTCTACCAGTTTCTTGG	R2	CTCCCCTCCGCAACACAC
PCR of *WDTC1* target region[a]	F3A	TTGGTGCAATCTGATACTGCTG	R3	CAACAAGAGGTGAGGCAAAAAG
	F3	TAACTGCTGGCTTTCAACATCC	R3	CAACAAGAGGTGAGGCAAAAAG
PCR of *ALS1* target region[b]	F5	CAGTGTGATCAAGGAGGCCTTTTAC	R5	CAGTGTTCACGTACTGATGCTGCAC
RT-PCR of *WDTC1* transcript	2F	CAGTGTCGCGCACACCTATAA	3R	AGGAACTGCACGCCAAAGAT
RT-PCR of *WDTC1* transcript	13F	ACATCAAAGAGGTGGGCTTCA	14R	ACCGTCTCAATACCGCTGGTG
RT-PCR of *ACTIN* transcript	F4	GACATCCGCAAGGACCTCTAC	R4	GATCCACATTTGCTGGAAGGT

[a]Nested polymerase chain reactions, with two sets of primers, were used to amplify the target regions of these genes.
[b]This PCR product was sequenced with primer AEP138 (TGATGCTGCACCGCGTGGCGTTTC).