**Supplementary Figure 3.** Cassettes for knock-in of the Cre recombinase gene into targeting vectors for gene specific expression.

**a.**
Cre in pC2A

<table>
<thead>
<tr>
<th></th>
<th>BglII</th>
<th>NotI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATTCC</td>
<td>AGATCT</td>
<td>GGGCCGG</td>
</tr>
<tr>
<td>s a a a M P K K K R K V S N L L T V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nls</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**b.**
Rag1 genomic

<table>
<thead>
<tr>
<th></th>
<th>GATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGGCTGCCTCCTTGCCGTCTACCTGGATCTTCAGTCTGCAACCGATGAAATTCAGACAC</td>
<td></td>
</tr>
<tr>
<td>M A A S L P S T L S F S S A P D E I Q H</td>
<td></td>
</tr>
</tbody>
</table>

d.  
Rag1-Cre KI

<table>
<thead>
<tr>
<th></th>
<th>BamHI/BglII</th>
<th>NotI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGGCTGCTGATCTGGCGCGCGCATGCACCCGATGCGATGAAATTCAGACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M A Q s a a a M P K K K K R K V S N L L T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rag1 linker Cre (from pC2A-neo)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d.  
\[
\begin{align*}
    &\text{Cre} \\
    &\text{BglII} \\
    &\text{NotI} \\
    &\text{BamHI} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{BglII} \\
    &\text{SV40pA} \\
    &\text{MC1-neomycin} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{Cre} \\
    &\text{NotI} \\
    &\text{(BamHI)} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{BglII} \\
    &\text{SV40pA} \\
    &\text{MC1-neomycin} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{Cre} \\
    &\text{NotI} \\
    &\text{(BamHI)} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{BglII} \\
    &\text{SV40pA} \\
    &\text{PGK-neomycin} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{Cre} \\
    &\text{NotI} \\
    &\text{(BamHI)} \\
    &\text{BglII} \\
\end{align*}
\]

\[
\begin{align*}
    &\text{BglII} \\
    &\text{SV40pA} \\
    &\text{FRT-neo-FRT} \\
    &\text{BglII} \\
\end{align*}
\]
Three vectors are available for the knock-in of Cre (or of FLP) recombinase genes into target genes of interest (illustrated in d).

(a) In each case, the Cre cDNA sequence was cloned as a NotI flanked PCR fragment into the NotI site of pC2A vectors giving the ORF shown (the single letter code is used for protein and those residues in upper case correspond to the N-terminus of Cre, including the added nuclear localisation signal, nls).

(b) To create a Cre knock-in into the coding sequence of any gene of interest, a mutation would normally be required just 3′ of the initiation codon of the target gene for insertion of a pC2A-Cre clone in frame. In the example here, an in-frame knock-in of Cre into Rag1 was achieved by mutagenesis of Rag1 to include a BamHI site adjacent to the Rag1 initiator converting the sequence as shown.

(c) Cre was cloned into the pC2A-Cre clone (described in Supplementary Fig. 1 online) digested with NotI and the cassette cleaved with BgIII and cloned into the Rag1 mutated BamHI site. This yielded in-frame knock-in of Cre into Rag1 which produces a fusion protein comprising two amino-acids of Rag1, one amino-acid (gly) from the linker and the whole Cre sequence, including the nuclear localisation signal.

(d) Three Cre transfer vectors are available as shown. In the case of pC2A-Cre-frt-neo-frt, the frt-neo-frt portion (frt-PGK-neo-frt) was cloned as (XhoI-Sall) from pfrt-neo-frt (a gift from Dr A. McKenzie) into the filled-in BamHI site of pC2A (note: a single BgIII site in pfrt-neo-frt was removed by linearization with BgIII, repairing the cohesive ends followed by re-ligation to form circular plasmid). Cre was cloned as the NotI PCR fragment (the Cre reading frame as in part c).

NOTE: If required as an alternative to Cre, the FLP recombinase gene can be cloned into pC2A-lox-neo-lox using PCR to clone the relevant coding region of FLP, facilitating knock-in of FLP into genes of interest.