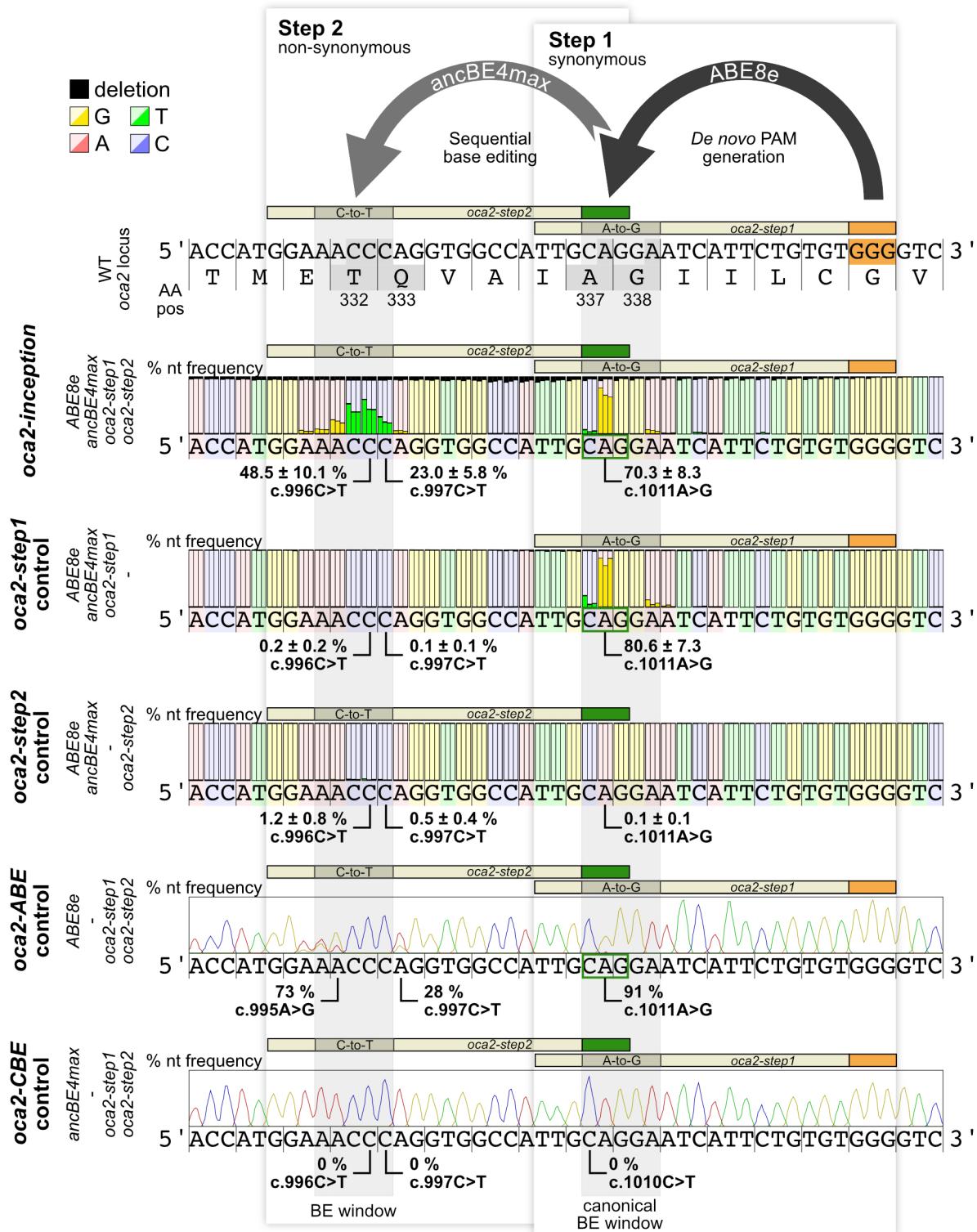


**Fig. S1. Range of possible codon outcomes after base editing in oca2 via inception**

Expected range of all potential translated codon outcomes after inception editing using the ABE8e and ancBE4max base editors at the *oca2* locus. The anticipated *de novo* PAM (green outlined box) introduced by *oca2-step1* guide RNA, T332I and Q333\* edits introduced by *oca2-step2* guide RNA are indicated by black arrows. Further possible edits are indicated by white arrows. Resulting codon range per amino acid position given.

AA pos, amino acid position; canonical PAM (orange); *de novo* PAM (green); PAM, protospacer adjacent motif; PTC, pre-termination STOP codon; WT, wild-type



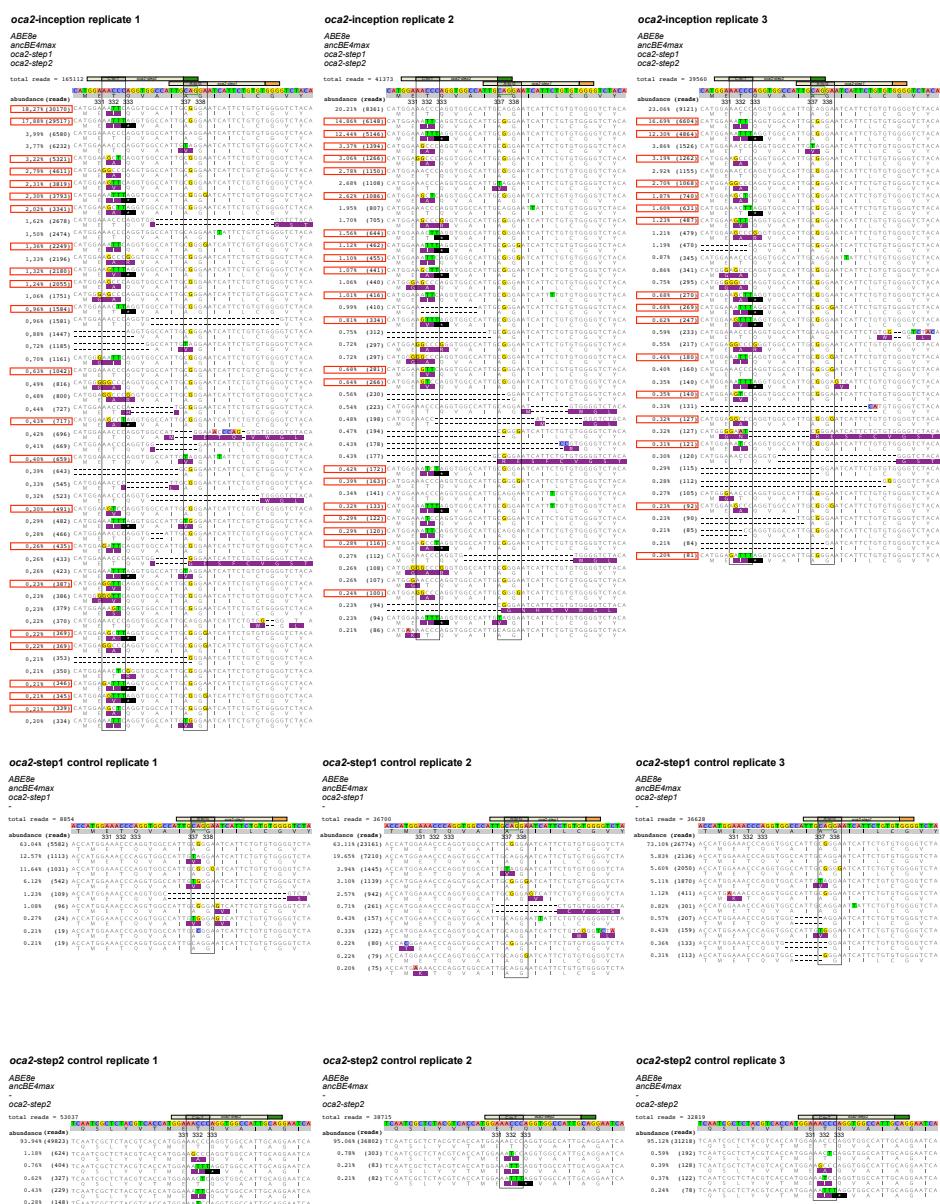
**Fig. S2. Sequencing results of oca2 inception and control editants**

Illumina amplicon sequencing analyses of oca2-editants (three replicates each) injected with the respective injection mixes: oca2-inception (3 pools of 8 editants; 246045 reads total), oca2-step1 control (3 pools of 1 to 8 editants, 82182 reads total) and oca2-step2 control (3 pools of 8 editants, 124571 reads total). Barplot representation of nucleotide frequency of the three replicates per nucleotide position.

Highlighted nucleotide changes represented as mean  $\pm$  standard deviation.

Chromatograms of oca2-*ABE* (5 editants) and oca2-*CBE* controls (8 editants) resulting from Sanger sequencing with indicated nucleotide changes derived from EditR (Kluesner et al., 2018) analysis.

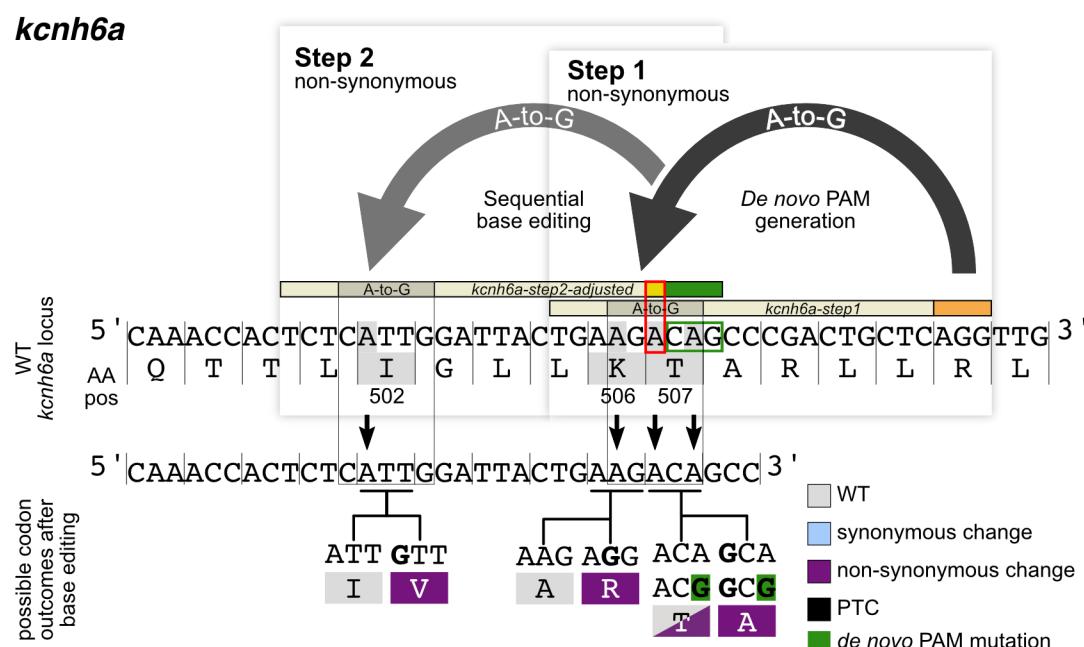
AA pos, amino acid position; canonical PAM (orange); de novo PAM (green); nt, nucleotide; PTC, pre-termination STOP codon; WT, wild-type



**Fig. S3. Allele frequency table and translation of aligned targeted Illumina amplicon sequencing reads of oca2 inception and control editants**

Canonical (step 1) and inception (step 2) guide RNA target sites indicated. Analysis based on the Allele frequency table output files derived from CRISPResso2 tool (Clement et al., 2019), cut off at 0.2 % read abundance per replicate. Alleles sorted by frequency (reads in parentheses). Alignment differences to wild-type reference indicated by color: adenine, red; guanine, yellow; thymidine, green; cytosine, blue; -, deletions. Injection mix components and replicate numbers provided.

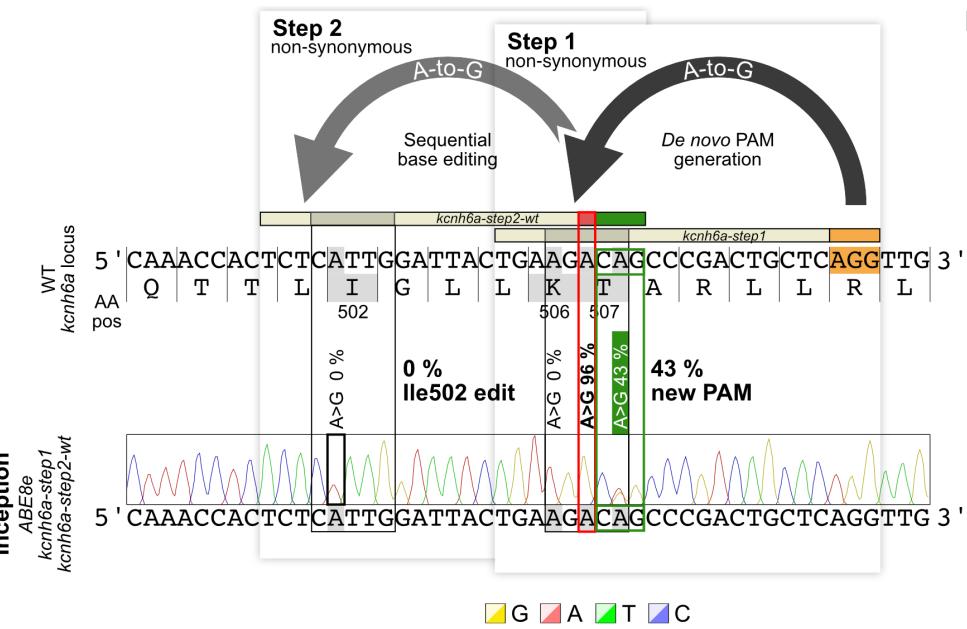
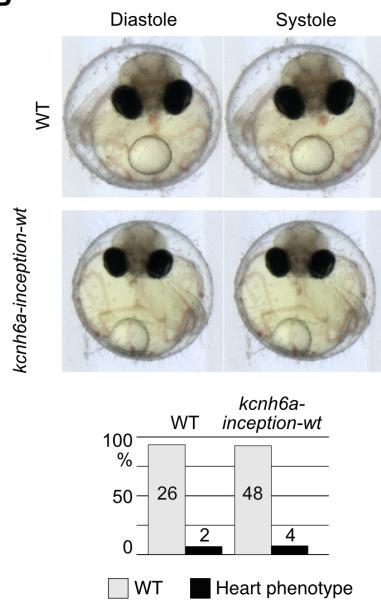
Anticipated alleles, red outlined box; base editing window, black outlined box; canonical PAM (orange); *de novo* PAM (green); non-synonymous codon changes, purple box; pre-termination STOP codon, black box



**Fig. S4. Range of possible codon outcomes after base editing in *kcnh6a* via inception**

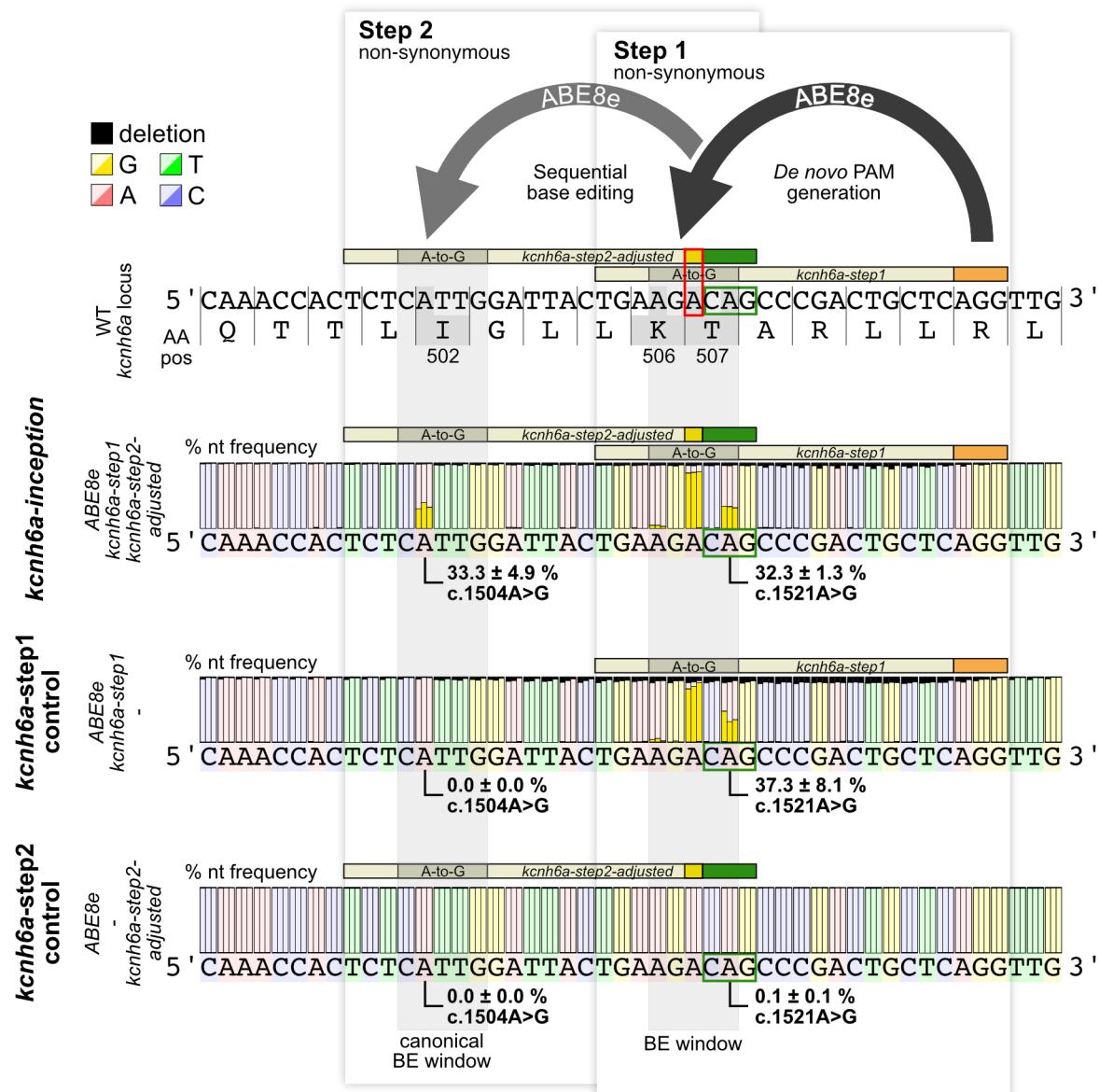
Expected range of all potential translated codon outcomes after inception editing using the ABE8e base editor at the *kcnh6a* locus. The anticipated *de novo* PAM (green outlined box) introduced by *kcnh6a-step1* guide RNA and K506R/T507A edits as well as the *kcnh6a-step2-adjusted* guide RNA induced I502V mutations are indicated by black arrows. Resulting codon range per amino acid position given.

AA pos, amino acid position; canonical PAM (orange); *de novo* PAM (green); PAM, protospacer adjacent motif; WT, wild-type

**A****B****C**

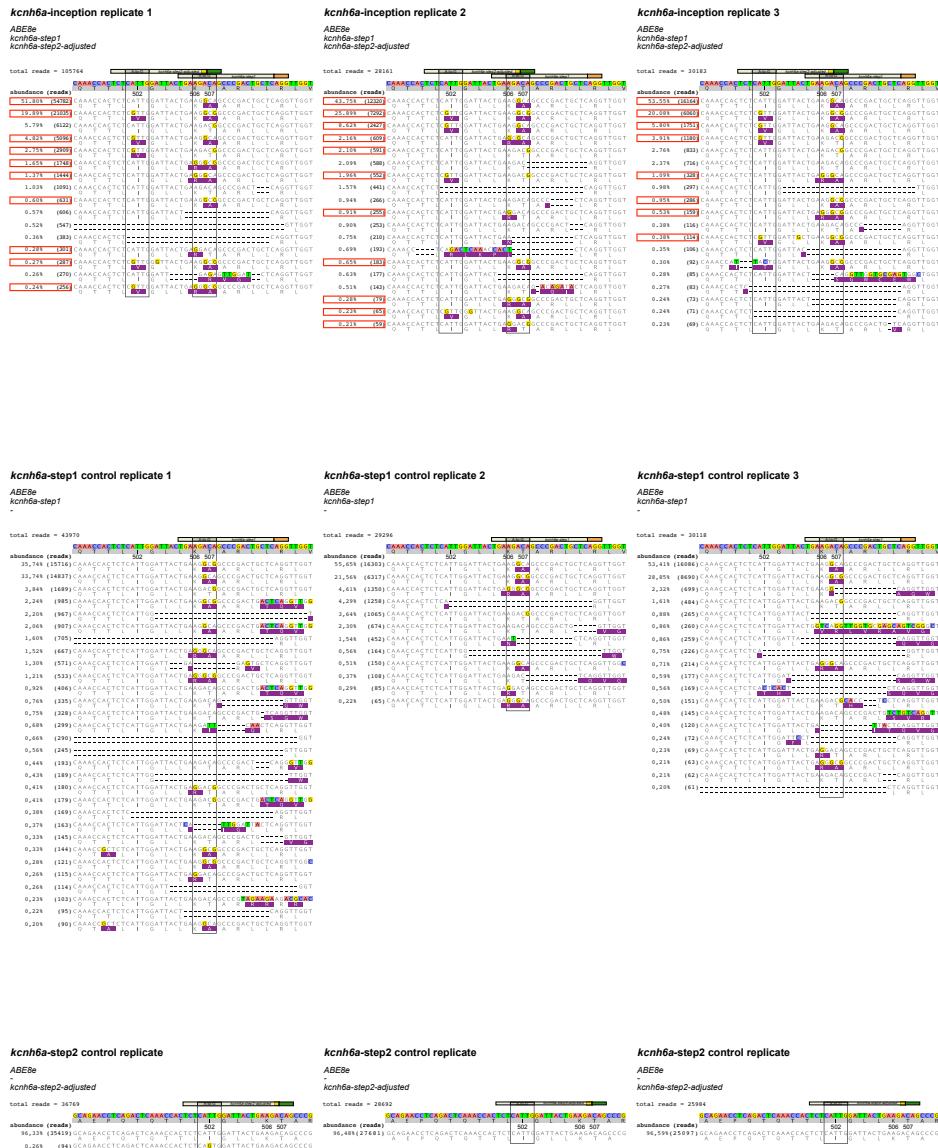
### Fig. S5. No inception editing events using wild-type *kcnh6a-step2* guide RNA

(A) Inception mix containing the *kcnh6a-step2-wt* guide RNA fails to edit p.I502 despite the efficient *de novo* PAM generation validated via Sanger sequencing and EditR (Kluesner et al., 2018). The 96 % c.1519A>G editing is in position 1 of the *kcnh6a-step2-wt* target-site preventing the second editing event. (B) At 4 days post fertilization the two-chambered embryonic heart shows Diastole/Systole periodicity. Phenotypically the inception editants were indifferent from wild-type (WT) controls. AA pos, amino acid position; canonical PAM (orange); de novo PAM (green); red box, sequence difference to edited locus in *kcnh6a-step2-wt* guide RNA



**Fig. S6. Sequencing results of *kcnh6a* inception and control editants** Illumina amplicon sequencing analyses of *kcnh6a*-editants (three replicates each) injected with the respective injection mixes: *kcnh6a*-inception (3 pools of 5-10 editants, 164108 reads total), *kcnh6a*-step1 control (3 pools of 5 editants, 103384 reads total) and *kcnh6a*-step2 control (3 pools of 5, 91445 reads total). Barplot representation of nucleotide frequency of the three replicates per nucleotide position. Highlighted nucleotide changes represented as mean ± standard deviation.

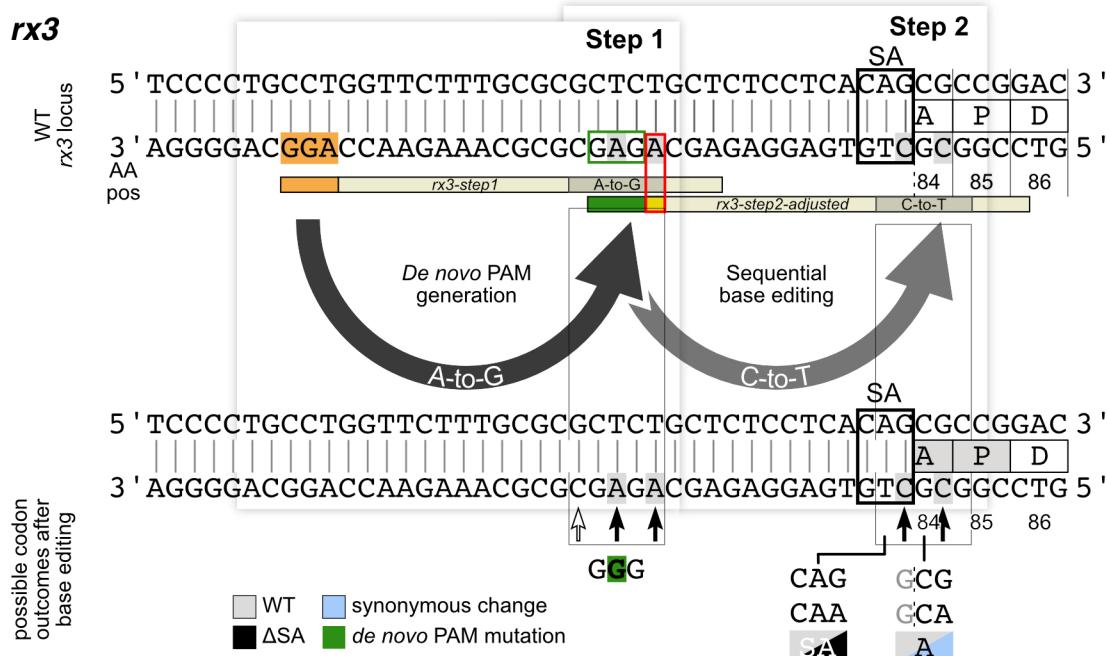
AA pos, amino acid position; canonical PAM (orange); de novo PAM (green); nt, nucleotide; PTC, pre-termination STOP codon; WT, wild-type



**Fig. S7. Allele frequency table and translation of aligned targeted Illumina amplicon sequencing reads of *kcnh6a* inception and control editants**

Canonical (step 1) and inception (step 2) guide RNA target sites indicated. Analysis based on the Allele frequency table output files derived from CRISPResso2 tool (Clement et al., 2019), cut off at 0.2 % read abundance per replicate. Alleles sorted by frequency (reads in parentheses). Alignment differences to wild-type reference indicated by color: adenine, red; guanine, yellow; thymidine, green; cytosine, blue; -, deletions. Injection mix components and replicate numbers provided.

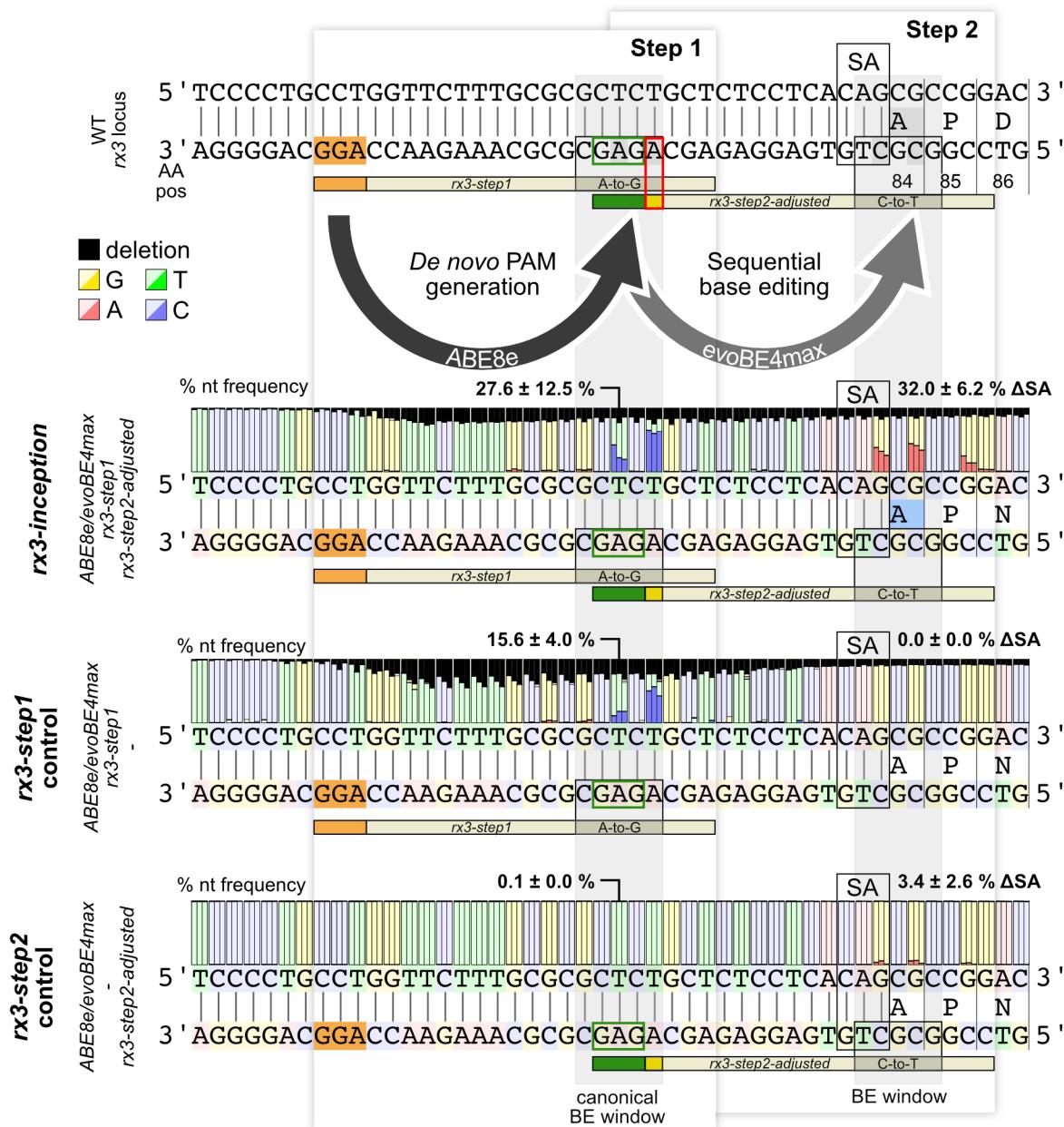
Anticipated alleles, red outlined box; base editing window, black outlined box; canonical PAM (orange); *de novo* PAM (green); non-synonymous codon changes, purple box



**Fig. S8. Range of possible codon outcomes after base editing in *rx3* via inception**

Expected range of all potential translated codon outcomes after inception editing using the ABE8e base editor at the *rx3* locus. The anticipated *de novo* PAM (green outlined box) introduced by *rx3-step1* guide RNA and splice acceptor edits ( $\Delta$ SA) as well as the *rx3-step2-adjusted* guide RNA induced A84A mutations are indicated by black arrows. Resulting codon range per amino acid position given. Further possible edits are indicated by white arrows.

AA pos, amino acid position; canonical PAM (orange); *de novo* PAM (green); PAM, protospacer adjacent motif; SA, splice acceptor; WT, wild-type



**Fig. S9. Sequencing results of rx3 inception and control editants**

Illumina amplicon sequencing analyses of rx3-editants (three replicates each) injected with the respective injection mixes: *rx3-inception* (3 pools of 5-9 editants, 136642 reads total), *rx3-step1* control (3 pools of 5 editants, 97976 reads total) and *rx3-step2* control (3 pools of 5 editants, 82283 reads total). Barplot representation of nucleotide frequency of the three replicates per nucleotide position. Highlighted nucleotide changes represented as mean  $\pm$  standard deviation. AA pos, amino acid position; canonical PAM (orange); de novo PAM (green); nt, nucleotide; PTC, pre-termination STOP codon; SA, splice acceptor site; WT, wild-type



**Fig. S10. Allele frequency table and translation of aligned targeted Illumina amplicon sequencing reads of rx3 inception and control editants**

Canonical (step 1) and inception (step 2) guide RNA target sites indicated. Analysis based on the Allele frequency table output files derived from CRISPResso2 tool (Clement et al., 2019), cut off at 0.2 % read abundance per replicate. Alleles sorted by frequency (reads in parentheses). Alignment differences to wild-type reference indicated by color: adenine, red; guanine, yellow; thymidine, green; cytosine, blue; -, deletions. Injection mix components and replicate numbers provided.

Base editing window, black outlined box; canonical PAM (orange); *de novo* PAM (green); non-synonymous codon changes, purple box; SA, splice acceptor site

**Table S1. Raw data of base editor target site abundances in the top ten studied human genes and the orthologs in mouse, zebrafish, medaka and drosophila**

[Click here to download Table S1](#)

**Table S2. Overview of injection mixes and analyzed embryos across all replicates and targeted loci**

GFP, green fluorescent protein; WT, wild-type

[Click here to download Table S2](#)

**Table S3. Guide RNAs used in this study**

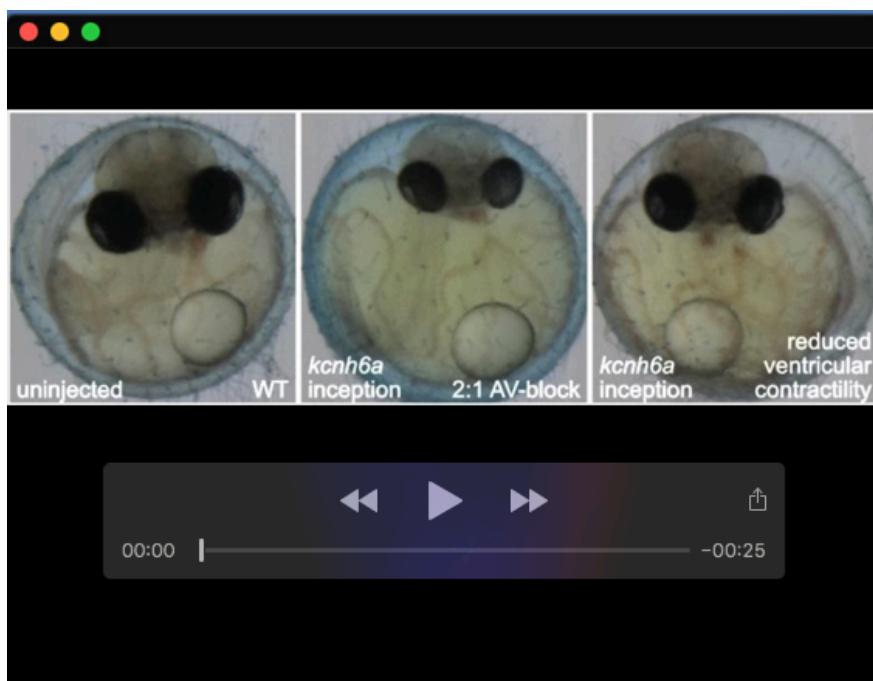
Guide RNA name	Target Site [PAM] 5'-3'	Reference
<i>oca2-step1</i> sgRNA	TTGCAGGAATCATTCTGTGT[GGG]	(Hammouda et al., 2019)
<i>oca2-step2</i> sgRNA	GGAAACCCAGGTGGCCATTG[CAG]	this paper
<i>kcnh6a-step1</i> sgRNA	TGAAGACAGCCCCACTGCTC[AGG]	(Cornean et al., 2022)
<i>kcnh6a-step2-wt</i> crRNA	TCTCATTGGATTACTGAAGA[CAG]	this paper
<i>kcnh6a-step2-adjusted</i> crRNA	TCTCATTGGATTACTGAAGg[CAG]	this paper
<i>rx3-step1</i> sgRNA	AGCAGAGCGCGCAAAGAACCC[AGG]	(Zilova et al., 2021)
<i>rx3-step2-adjusted</i> crRNA	CCGGCGCTGTGAGGAGAGCg[GAG]	this paper

**Table S4. Primers with partial Illumina adapter sequences (underscored) used in this study**

Primer name	<u>Adapter Sequence</u> , Primer sequence 5'-3'
oca2_F	<u>ACACTCTTCCCTACACGACGCTCTCCGATCTCGTTAGAG</u> TGGTATGGAGAACTGT
oca2_R	<u>GACTGGAGTTCAGACGTGTGCTCTCCGATCTATGGTCCTC</u> ACATCAGCAGC
kcnh6a_F	<u>ACACTCTTCCCTACACGACGCTCTCCGATCTAGTTGCT</u> GTGTACCTCCAGTT
kcnh6a_R	<u>GACTGGAGTTCAGACGTGTGCTCTCCGATCTATCTTCATA</u> CCGCCACACCG
rx3_F	<u>ACACTCTTCCCTACACGACGCTCTCCGATCTATGCAAAC</u> CAAGAAAGCGCC
rx3_R	<u>GACTGGAGTTCAGACGTGTGCTCTCCGATCTGGGATTTC</u> TCAAAGGCCCG

**Table S5. Primers used in this study**

Primer name	Sequence 5'-3'
oca2_F	GTTAAAACAGTTCTAAAAAGAACAGGA
oca2_R	AGCAGAAGAAATGACTCAACATTTG
kcnh6a_F	GCTTGCAAGGTATAAGCACAG
kcnh6a_R	AACGTTGCCAAAACCCACAC

**Movie 1. Exemplary heart phenotypes of *kcnh6a*-inception editants**

10 second movies of heart beat and heart morphology analysis at stage 35 of wild-type (WT, left) and *kcnh6a*-inception injected embryos. Representative phenotypes cover 2:1 atrioventricular block (middle) and reduced ventricular contractility (right). A, atrium; V, ventricle; movie plays at 10 frames per second (real time)

**Supplementary References**

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- Hammouda, O. T., Bottger, F., Wittbrodt, J. and Thumberger, T. (2019). Swift Large-scale Examination of Directed Genome Editing. *PLoS One* **14**, e0213317.
- Kluesner, M. G., Nedveck, D. A., Lahr, W. S., Garbe, J. R., Abrahante, J. E., Webber, B. R. and Moriarity, B. S. (2018). EditR: A Method to Quantify Base Editing from Sanger Sequencing. *CRISPR J* **1**, 239-250.
- Zilova, L., Weinhardt, V., Tavhelidse, T., Schlagheck, C., Thumberger, T. and Wittbrodt, J. (2021). Fish primary embryonic pluripotent cells assemble into retinal tissue mirroring *in vivo* early eye development. *Elife* **10**.