

Online appendix - Supplemental Information

Spatial Environment Affects *HNF4A* Mutation-Specific Proteome Signatures and Cellular Morphology in hiPSC-Derived β -Like Cells

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Suppl Table 1 Clinical data

Parameter	N904-1	N904-2	N904-6	N904-7
Mutation status	NM	NM	NM	NN
Age (years)*	23	51	26	31
Sex	F	M	M	M
Age at diabetes diagnosis (years)	19	36	NA	NA
Weight (kg)*	60	86	78	80
Height (cm)*	165	180	186	176
F-glucose (mM)*	9,0	NA	4,7	NA
2h-glucose, OGTT (mM)*	20,0	NA	NA	NA
HbA1C (%)*	6,4	6,9	5,7	5,4
HbA1C (mmol/mol)+	34	49	36	NA
Total cholesterol (mM)+	4.1	3.4	NA	NA
LDL-cholesterol (mM)+	2.4	2.2	NA	NA
HDL-cholesterol (mM)+	1.3	1.1	NA	NA
Triglycerides (mM)+	0.8	0.53	NA	NA
Treatment diabetes*	Diet	Glimepirid 6 mg	None	NA
Treatment diabetes+	Diet	Glimepirid 6 mg	None	NA
	(Insulin during pregnancy)	Metformin 500 mg x2 Sitagliptin 100 mg x 1		
Complications diabetes	None	None	None	NA

Data are presented as means \pm SD. P values were calculated using unpaired t-tests. *At fibroblast sampling date. + At clinical follow-up seven years after fibroblast sampling. NA, Not available/applicable

Legend supplementary figures

Suppl Figure 1. iPSC colony morphology for the three mutated clones (6A, 6D, 6F) from N904-1 and four corrected clones based on N904-1 iPSCs (E75, D13, D94 and E87). Note the classic morphology (ref Bjørlykke et al, Stem Cell International, 2019) in all these clones.

Suppl Figure 2. Chromatograms of three additional corrected clones: D13, D94 and E87. Note the restoration of the ATC-ATC pattern. The chromatogram of the fourth additional corrected clone, E75, is shown in Fig 1E. Specifically, we used an online tool (<http://crispr.mit.edu>) to design a guide RNA (gRNA: CCT GGG TCA AAG AAG ATT GA) targeting the Cas9 to cut exactly at the location of the mutation c.811dupA, using single-stranded donor oligonucleotide (ssODN) to serve as the HDR template (GCCCTTCCAGGAGCTGCAGATCGATGACAATGAGTATGCCTACCTCAAAGCTATCATCTTCTTTGACCCAGgtacagtgcacacctctaagccatccc). The PAM site on the ssODN template was altered with a synonymous substitution (GCC->GCT), and hence we also removed a BccI restriction enzyme site, which facilitated the subsequent screening using restriction fragment length polymorphism assays. This screening revealed that six out of 96 clones had been successfully corrected as confirmed by DNA Sanger sequencing, of which four of these corrected lines (E75,D13,D94,E87) were subsequently included in the subsequent studies. The top six most probable *in silico* detected off-target sites (one site in a coding region and six sites in non-coding regions) were unaltered as demonstrated by Sanger sequencing (Suppl Fig 3).

Suppl Figure 3. Sequence data of off-targets for the six *in silico* detected off target sites 1 (Chromosome 1), 2 (Chromosome X), 3 (Chromosome 8), 4 (Chromosome 2), 5 (Chromosome 15) and 6 (Chromosome 10). The sequencing was done for one mutated clone (6D), one sham-corrected clone (6D-SHAM) and four corrected clones (E75, D13, D94 and E87).

Suppl Figure 4. iPSC colony staining of the pluripotency markers SSAE4 and SOX2 by immunofluorescence. The staining was done for three mutated iPSC clones (6A, 6D, 6F) and four corrected clones (E75, D13, D94 and E87).

Suppl Figure 5. iPSC colony staining of the pluripotency markers OCT4 and NANOG by immunofluorescence and corresponding FACS quantification, performed in a second lab (C.W, H.S). The analyses were done for one mutated (*HNF4A*^{+/-c811dupA} = Hnf4a (+/-)) iPSC clone (6D) and two corrected (*HNF4A*^{+/-corrected} = Hnf4a (+/+)) clones (D13 and D94).

Suppl Figure 6. Embryoid bodies (day 4) demonstrating pluripotency potential by immunofluorescence markers for each of the three germ layers: ectoderm (OTX2), endoderm (SOX17) and mesoderm (Brachyuri). The assay was done for three mutated clones (6A, 6D, 6F), one sham-corrected clone (6D-SHAM) and four corrected clones (E75, D13, D94 and E87)

Suppl Figure 7. Chromosome analysis of the fixed cell suspensions at passage 20 from three mutated iPSC clones (6A, 6D, 6F) and four corrected clones (E75, D13, D94 and E87). 6A and 6D showed Trisomy 1 in all 20 lines investigated, whereas 6F showed Trisomy 1 in 1/20 lines and isochromosome 1q (break p10) in the remaining 19/20 lines. Trisomy 1 (47,XY,+1) and isochromosome 1q have been reported as a recurrent chromosomal abnormality in stem cell lines. E75 showed 46,XY,del (18)(q11.2) in 15/20 lines and 45,X0 with normal

chromosome 18 in the remaining 5/20 lines. D13 showed 46,XY,del (18)(q11.2) in 14/20 lines and 45,X0 with normal chromosome 18 in the remaining 6/20 lines. D94 showed 46,XY,del (18)(q11.2) in 17/20 lines and 45,X0 with normal chromosome 18 in the remaining 3/20 lines. E87 showed 46,XY,del (18)(q11.2) in 16/20 lines and 45,X0 with normal chromosome 18 in the remaining 4/20 lines. The extent to which these genomic alterations potentially perturb the experimental readouts is unclear as most of the chromosomes remained intact. However, the information of chromosomal aberrations must be kept in mind when interpreting the results.

Suppl Figure 8. Details of quality control of differentiation for mutated (*HNF4A*^{+/-c811dupA} = Hnf4a (+/-)), corrected (*HNF4A*^{+/-corrected} = Hnf4a (+/+)) and control (hESC) lines.

- (A) Flow cytometric analysis of Stage 1 cells immunostained for SOX17. hES-1 cell line used as positive control, undifferentiated iPS cells were used as negative control in blue color.
- (B) Flow cytometric analysis of Stage 4 cells immunostained for PDX1 and NKX6.1.
- (C) Flow cytometric analysis of Stage 5 cells immunostained for PDX1 and NKX6.1.
- (D) Immunostaining of Stage 5 cells stained for the indicated markers. hES-1 cell line differentiated cells at Stage 4 were used as process control. Scale bar = 50 μ m.
- (E) Flow cytometric analysis of Stage 7 cells immunostained for INSULIN. hES-1 cell line used as positive control, undifferentiated iPS cells were used as negative control in blue color.

Suppl Figure 9 A) The relative protein abundance of pancreatic hormones and selected β -cell markers for mutated (*(HNF4A*^{+/-c811dupA}); 6D) and corrected (*(HNF4A*^{+/-corrected}); E75) cells differentiated in the 3D cell aggregate format (first row), the 3D alginate bead environment (second row). **B)** Analysis of pathways and upstream regulators (using Ingenuity Pathway Analysis (IPA) generated top canonical pathways (z-score > 2) in the class a DAPs analysis of alginate-encapsulated cells. C) IPA-generated top disease annotations and the most differentially abundant protein in class a DAPs analysis of alginate-encapsulated cells, the PSMB9 (a proteasome protein). Significant differences are shown as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Student's t-test. D) The abundance level of ALDOA, TPI1, PGK1 and ENO1 in 2D.

Suppl Figure 10. Rescue of HNF4A protein shown by FACS of HNF4A, evidenced by higher HNF4A counts (left panel) in the corrected clone (*(HNF4A*^{+/-corrected} = Hnf4a (+/+); D13) compared to the mutated clone (*(HNF4A*^{+/-c811dupA} = Hnf4a (+/-); D6). Right panel shows HNF4A immunoblotting of different mutated and corrected clones and corresponding quantification normalized by GAPDH expression, with hepatocyte expression as a positive control.

Suppl Figure 11. RNA-seq data. **A).** Aggrewell RNA-seq data. Violin plots showing normalized RNA-seq-based expression levels and the corresponding comparative original proteome-based protein levels for the four differentially expressed proteins shown in Figure 4D. The mutated cell lines (red) include 6a (6A), 6d (6D) and 6f (6F) and the corrected cell lines (green) include d13 (D13), d94 (D94), e75 (E75) and e87 (E87). Note the partial correspondence, in particular the direction, between protein and mRNA levels. Complete correspondence cannot be anticipated since the dynamic quantitative levels of proteins and nRNAs, respectively, are results of different processes with discrepancies related to different half-lives between transcripts and proteins, miRNA intervention, delays between transcription and translation as well as other forms posttranscriptional regulation. **B).** Alginate RNA-seq data. Overview of mRNA expression levels and the corresponding comparative original proteome-

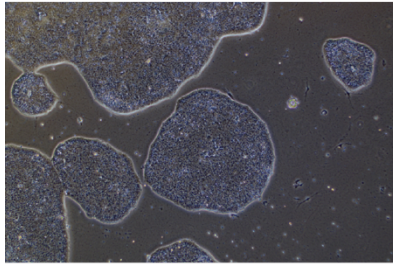
based protein levels for the differentially expressed glycolytic proteins shown in Figure 3C and detailed comparative violinplots for each of these proteins (Hexokinase = *HK1* and *HK2*; Phosphofructokinase = *PFKM*, *PFKL*, *PFKP*; Aldolase = *ALDOA*, Triphosphate isomerase = *TPII*; Glyceraldehyde 3 phosphate = *GAPDH*; Phosphoglycerate kinase = *PGK1*; Phosphomutase = *PGAM1*; Enolase = *ENOA* and *ENOB*; pyruvate kinase = *PKM*). We also provide comparative analysis of proteins and corresponding mRNA levels for stearate biosynthesis genes (*HSD17B10*, *SLC27A3*, *ACOT1*, *FASN*, *ACSL1*, *DHCR24*, *MBOAT7*, *ZADH2*). The mutated cell lines (red) include 6a (6A), 6d (6D) and 6f (6F) and the corrected cell lines (green) include d13 (D13), d94 (D94), e75 (E75) and e87 (E87). Note the very low to even zero levels of mRNA in alginate-encapsulated cells (but not in 2D cells), disallowing a valid comparison with protein levels and probably reflecting the challenges with robust mRNA retrieval from alginate-encapsulated cells.

Suppl Figure 12. Light microscopy images of ST7 cells in Aggrewell for three mutated clones (6A, 6D, 6F) and four corrected clones (E75, D13, D94 and E87). Note the “aggregated” morphology in the mutated clones, which is restored to normal “non-aggregated” morphology in the corrected clones.

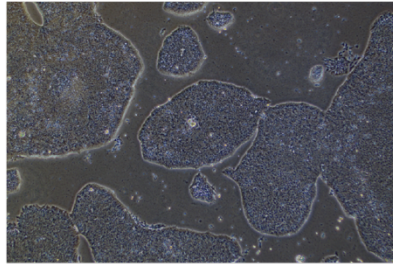
Suppl Figure 13. Functional studies of ST7 cells in the 2D and aggregated 3D context. A) Insulin secretion levels at low glucose (2 nM), high glucose (20 nM) and KCl stimulation during static incubation of mutated clones ((*HNF4A*^{+/-c811dupA} = Hnf4a (+/-)); 6A) vs corrected clones (*HNF4A*^{+/-corrected} = Hnf4a (+/+)); D94) at 2D or 3D (Aggrewell conditions). B) The corresponding insulin secretion indices for high vs low glucose levels. C) The corresponding insulin release fold change for KCl vs high glucose levels. Note that 3D conditions restore KCl-stimulated insulin secretion in corrected clones (in contrast to Glucose-stimulated insulin secretion). D) Dynamic glucose-stimulated insulin secretion of mutated clones (*HNF4A* +/-; 6A) vs corrected clones (*HNF4A* +/+; D94) at 2D or 3D (Aggrewell conditions). E) Oxygen consumption rates (OCR) at baseline, high glucose condition (20 mM) , Oligomycin stimulation, CCCP stimulation and Rotenone stimulation of mutated clones (*HNF4A* +/-; 6A) vs corrected clones (*HNF4A* +/+; D94) at 2D or 3D (Aggrewell conditions). F, The corresponding AUCs of the OCR curve at baseline, high glucose and CCCP stimulation. Note the significant higher levels at baseline and high glucose conditions in corrected clones at 3D conditions (Aggrewell).

Supplementary Figure 1

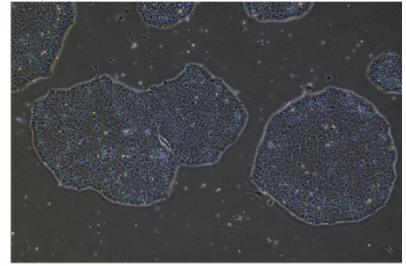
MUTATED CLONES



6A

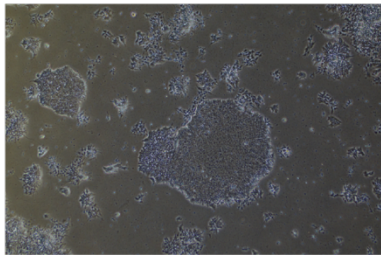


6D

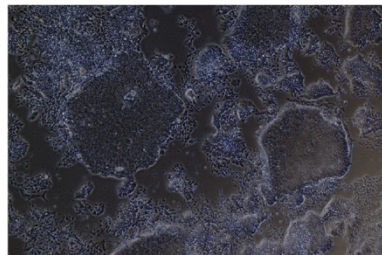


6F

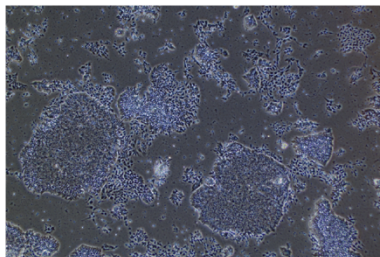
CORRECTED CLONES



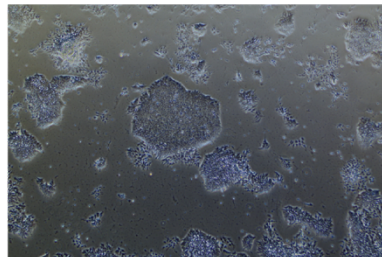
E75



D13



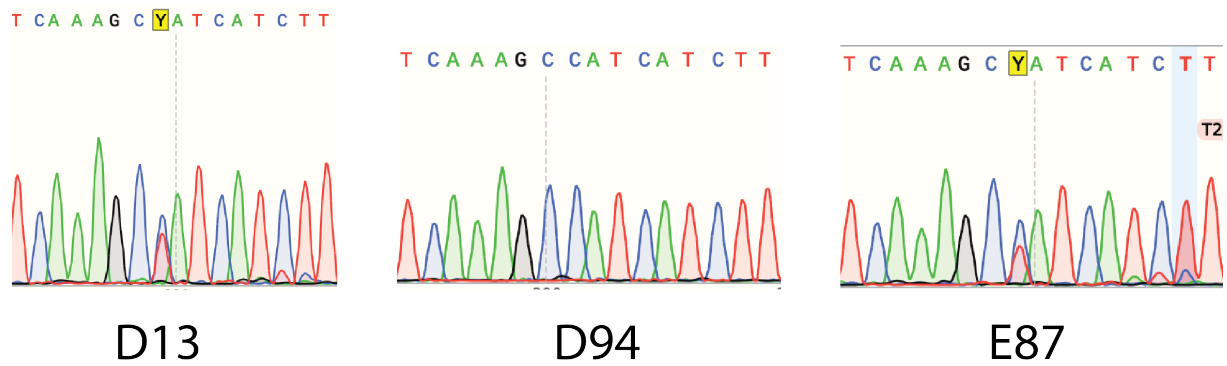
D94



E87

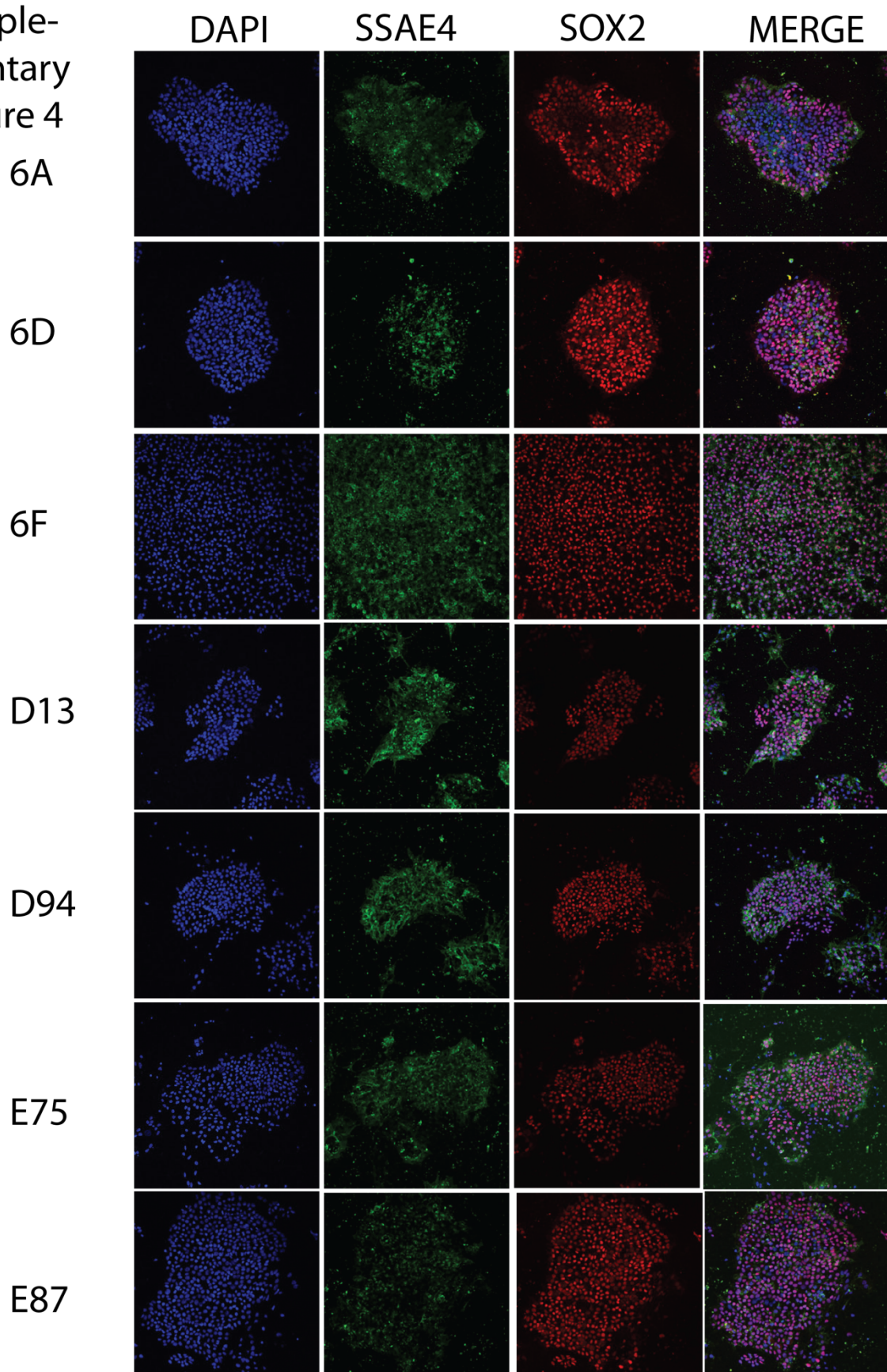
Supplementary Figure 2

CORRECTED CLONES

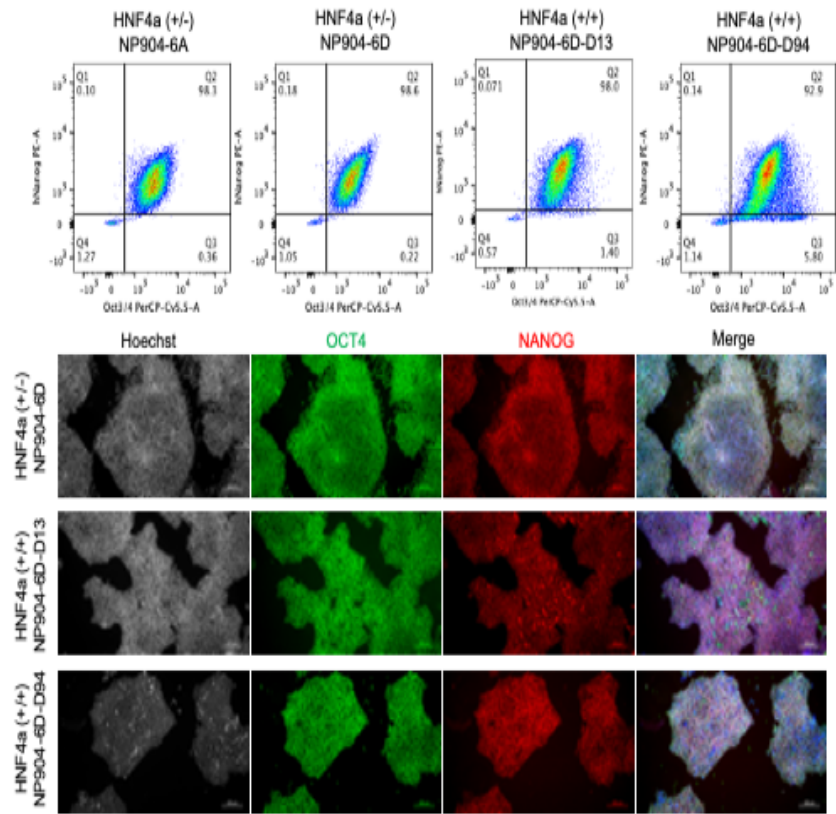


Supplementary Figure 3: Please see the end of this file

Supple-
mentary
Figure 4
6A

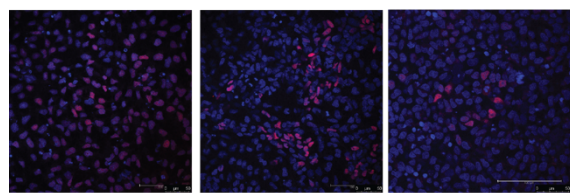


Supplementary Figure 5

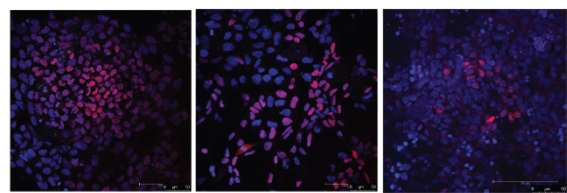


Supplementary Figure 6

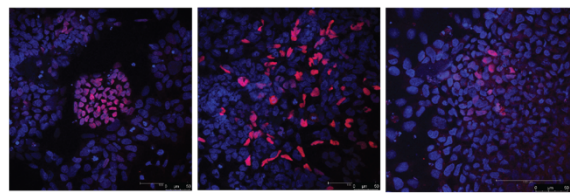
MUTATED CLONES



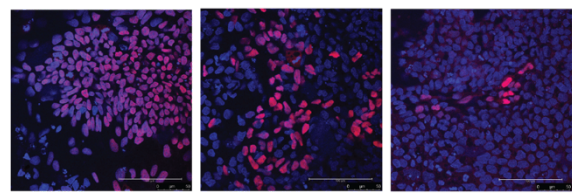
OTX2 SOX17 BRACHYURI
6A



OTX2 SOX17 BRACHYURI
6D

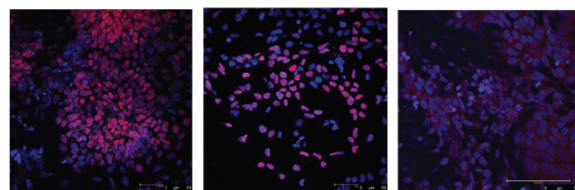


OTX2 SOX17 BRACHYURI
6F

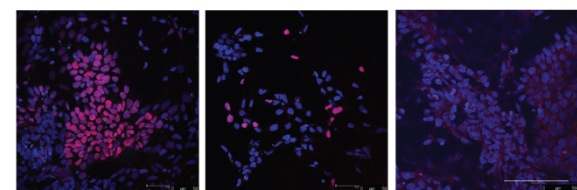


OTX2 SOX17 BRACHYURI
6D-SHAM

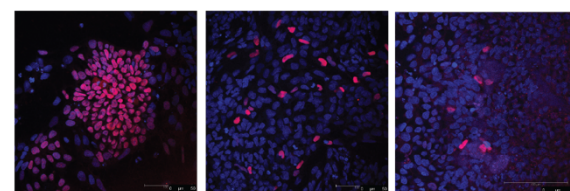
CORRECTED CLONES



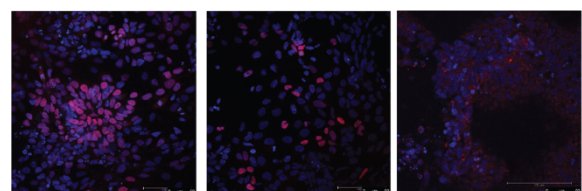
OTX2 SOX17 BRACHYURI
E75



OTX2 SOX17 BRACHYURI
D13

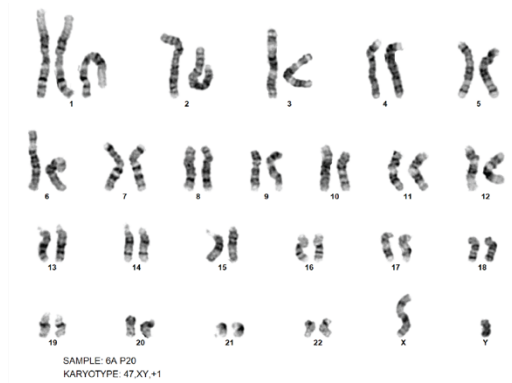


OTX2 SOX17 BRACHYURI
D94



OTX2 SOX17 BRACHYURI
E87

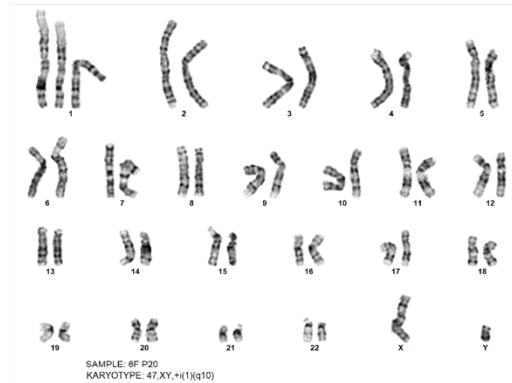
Supple- mentary Figure 7



6A: 47,XY,+1



6D: 47, XY,+1



6F:47,XY,+i(1)(q10)



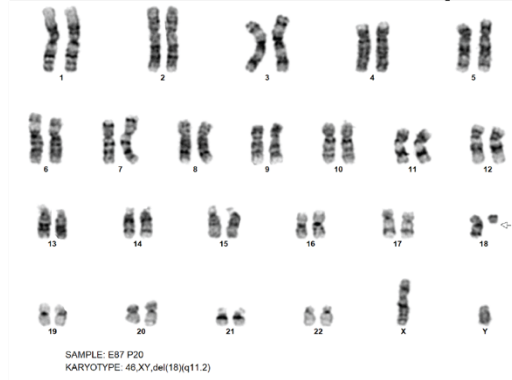
E75: 46,XY,del(18)(q11.2)



D13: 46,XY,del(18)(q11.2)

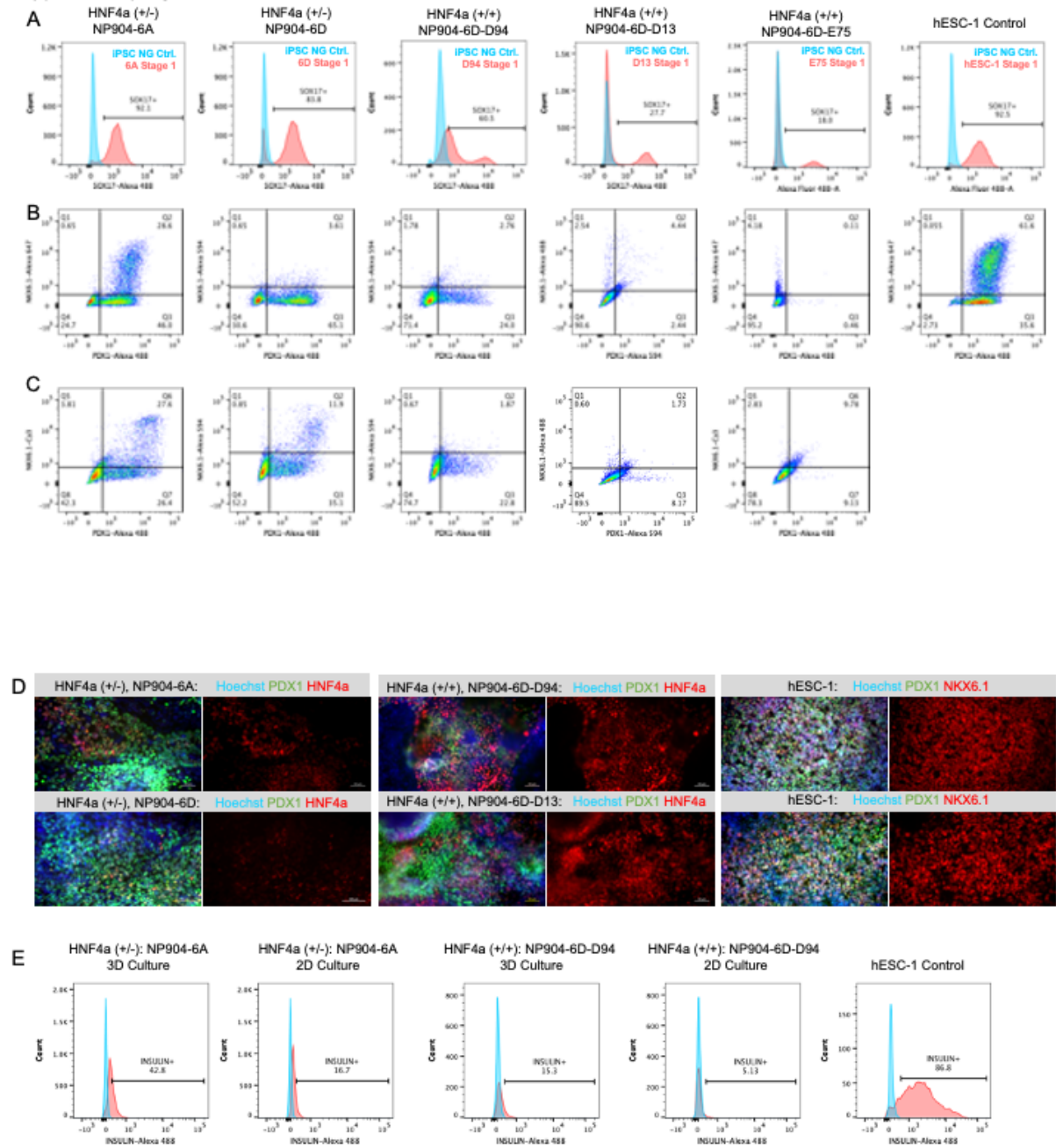


D94: 46,XY,del(18)(q11.2)

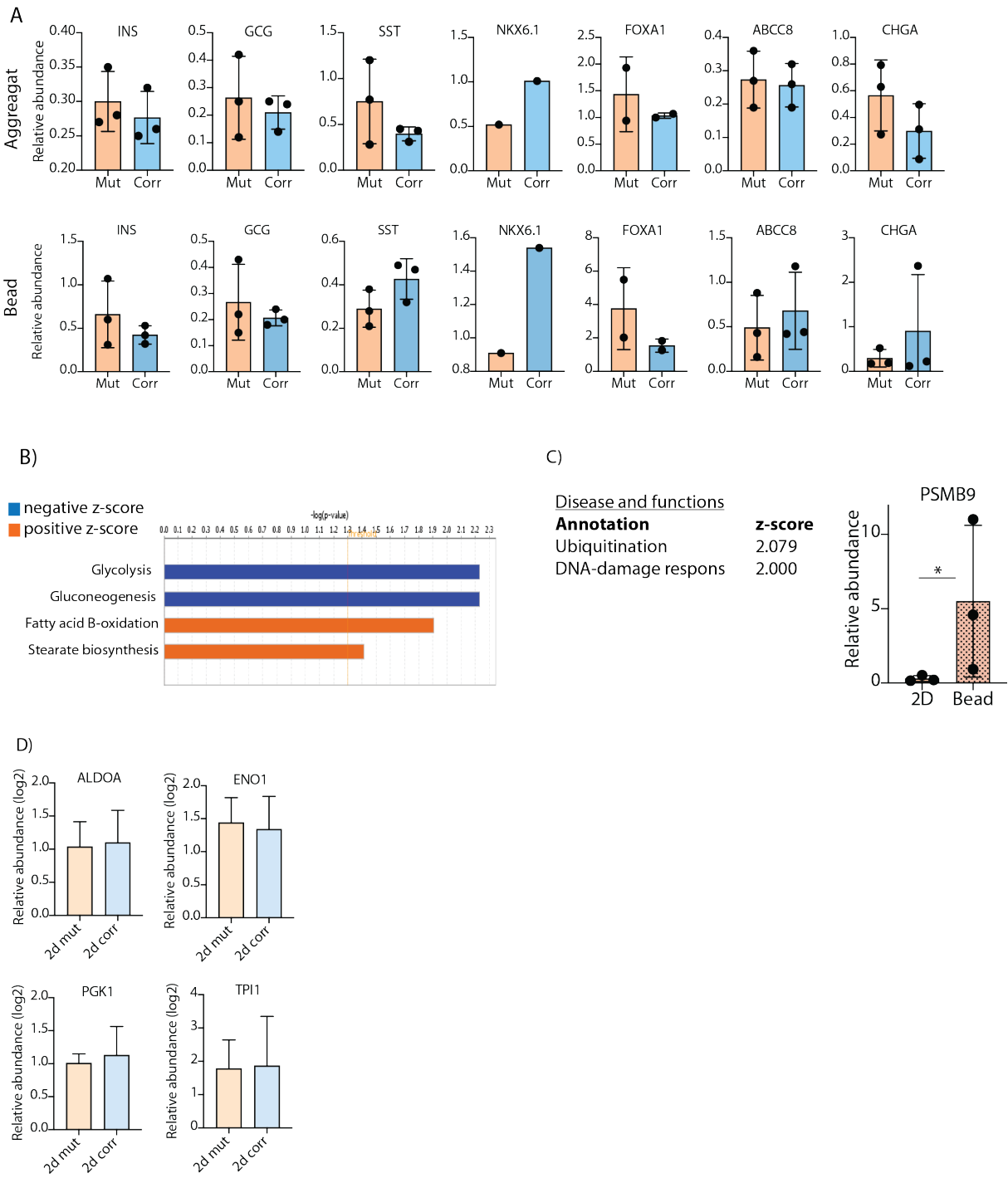


E87: 46,XY,del(18)(q11.2)

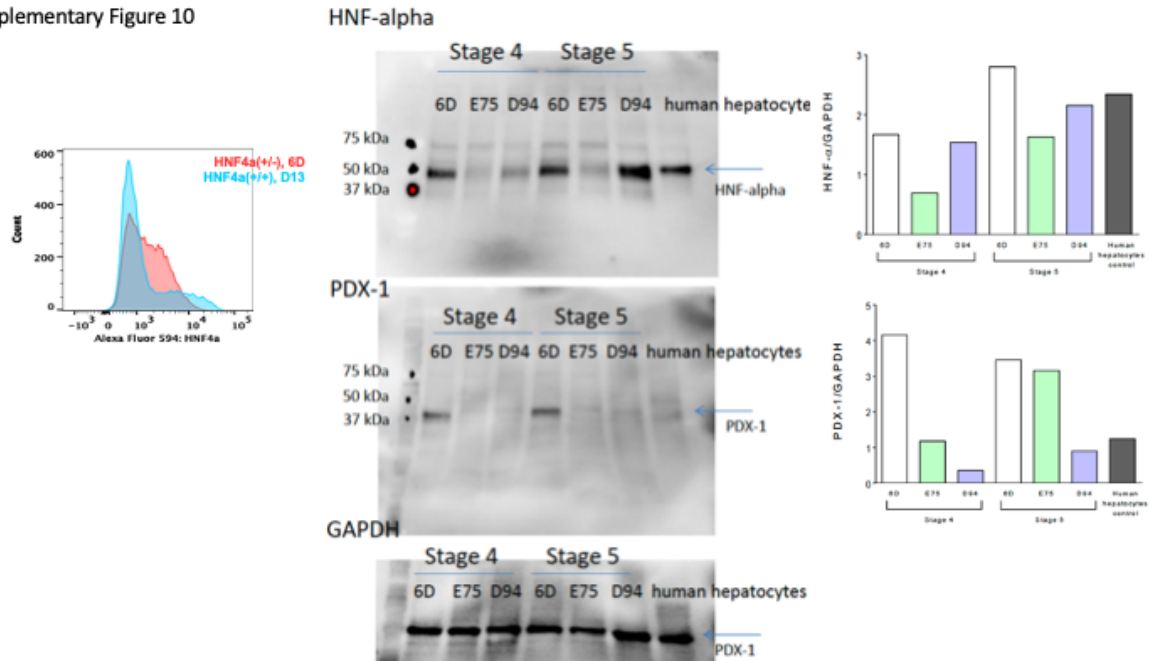
Supplementary Figure 8



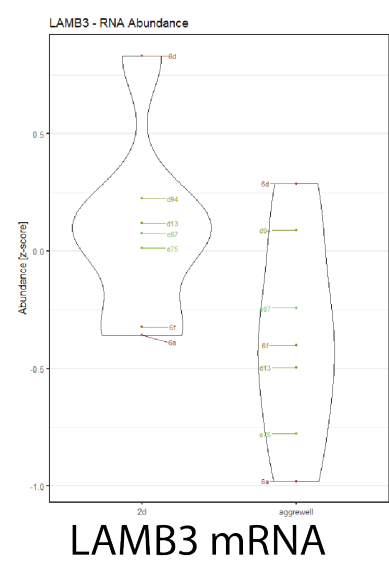
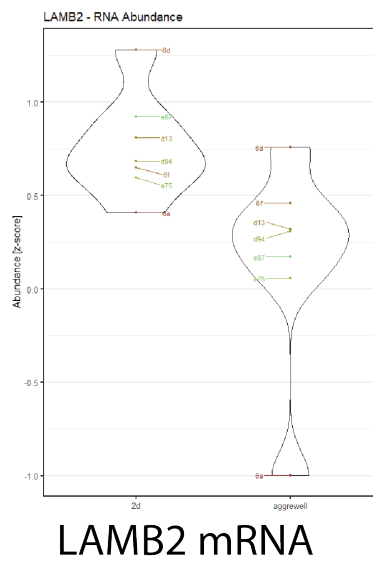
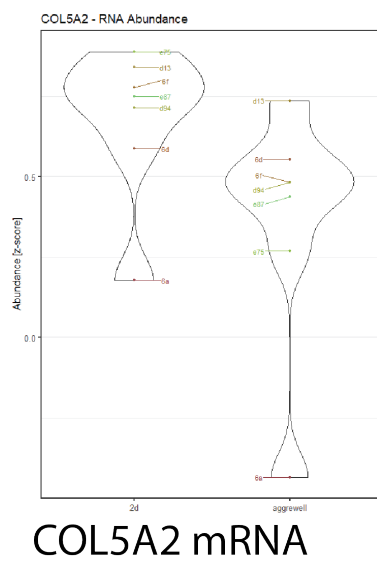
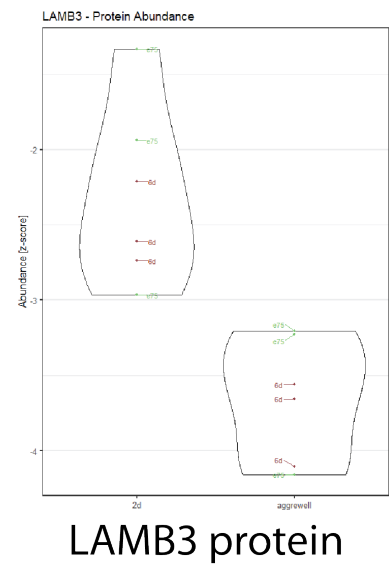
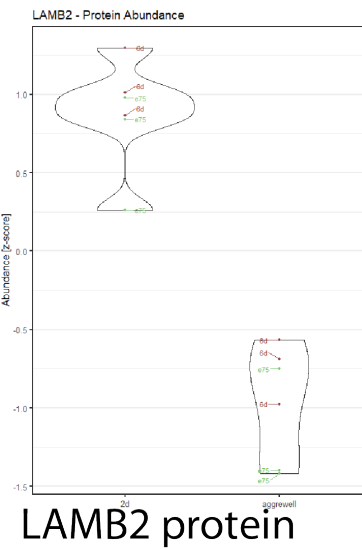
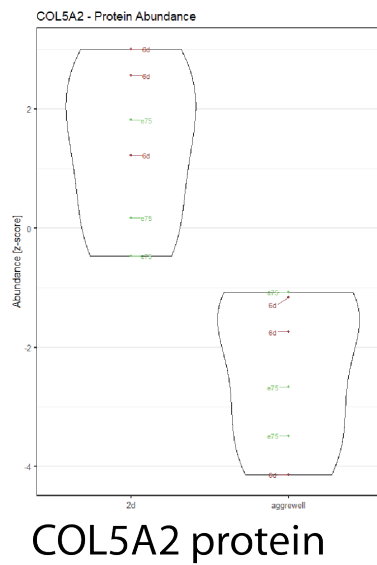
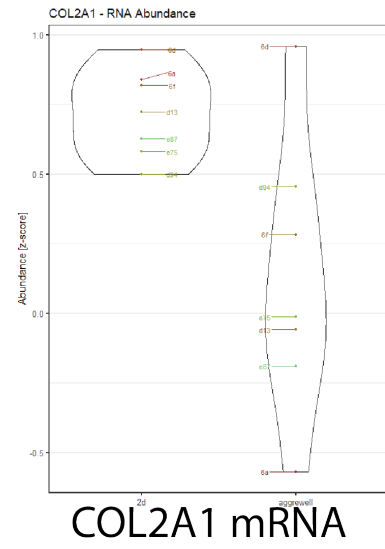
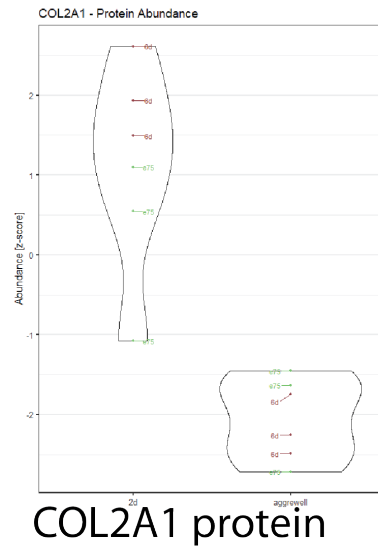
Supplementary Figure 9



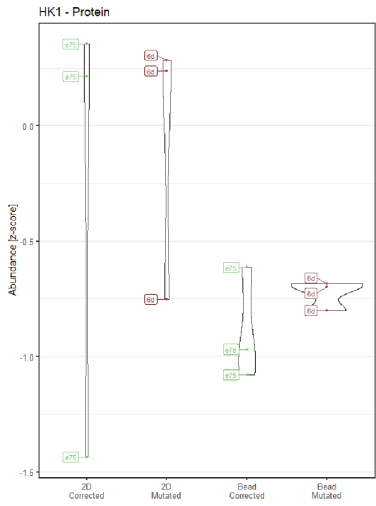
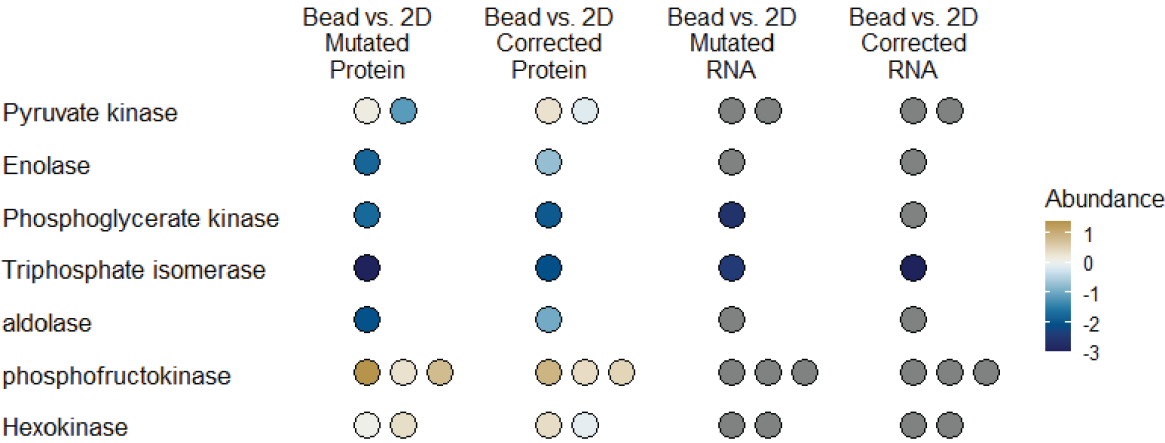
Supplementary Figure 10



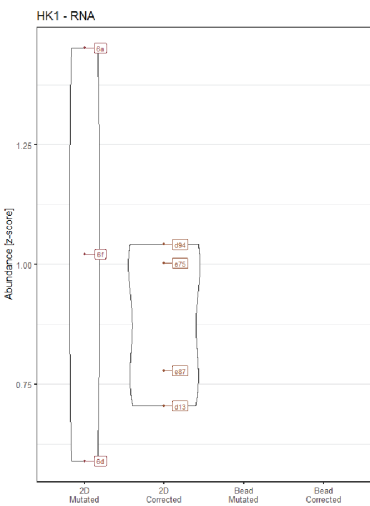
Supple- mentary Figure 11A



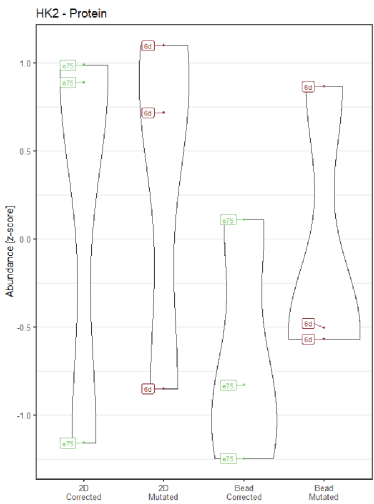
Supplementary Figure 11B



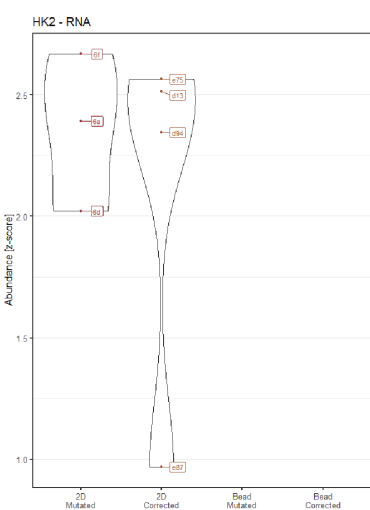
HK1 protein



HK1 mRNA

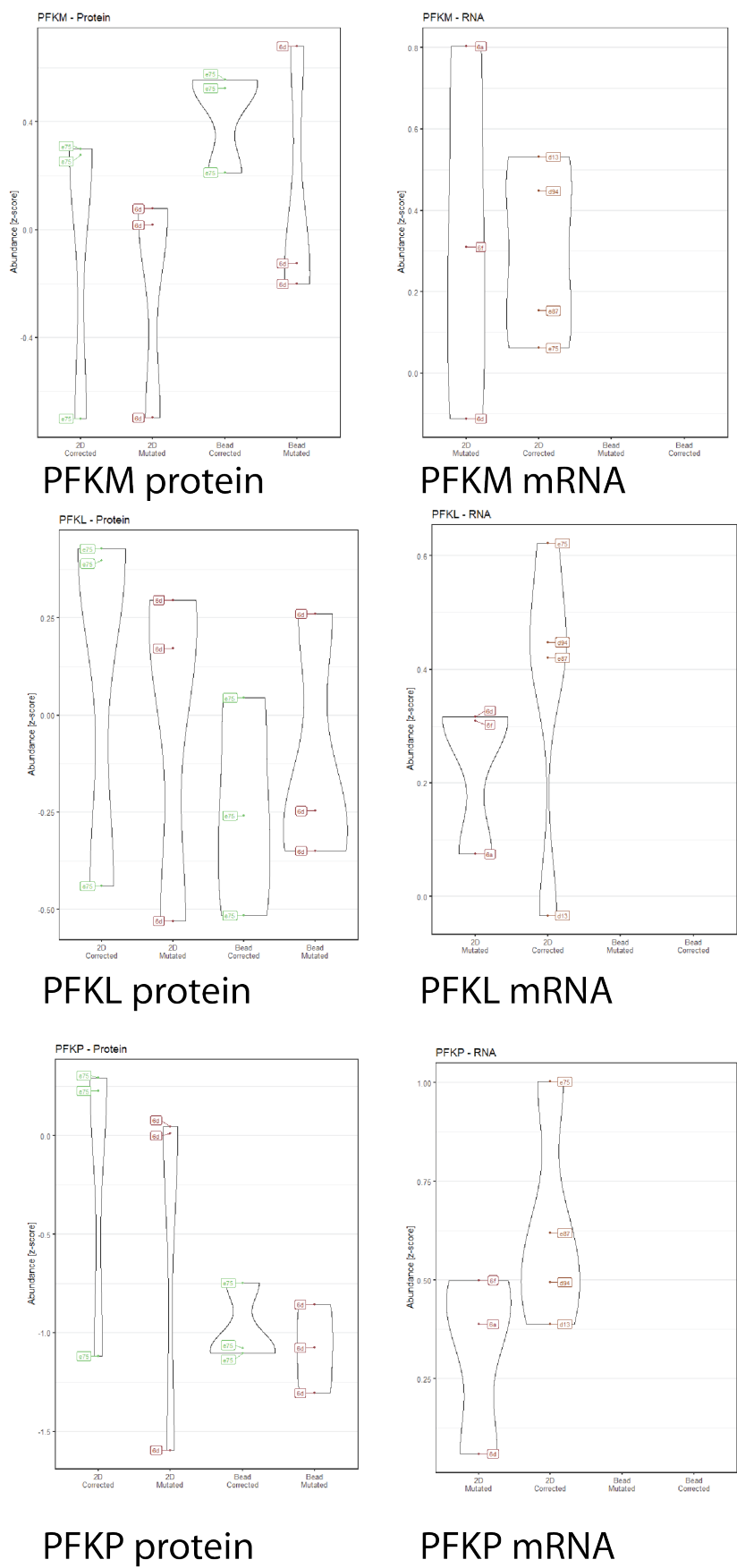


HK2 protein

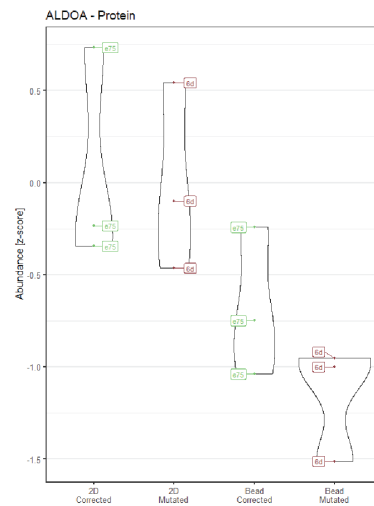


HK2 mRNA

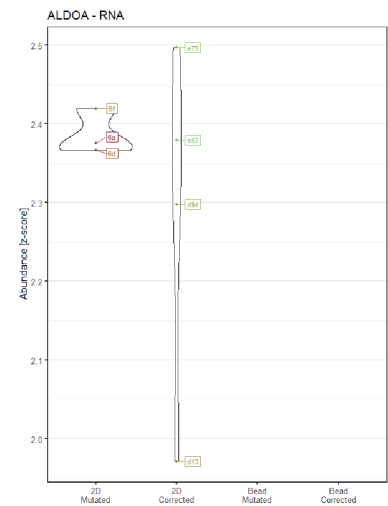
Supplementary
Figure 11B con-
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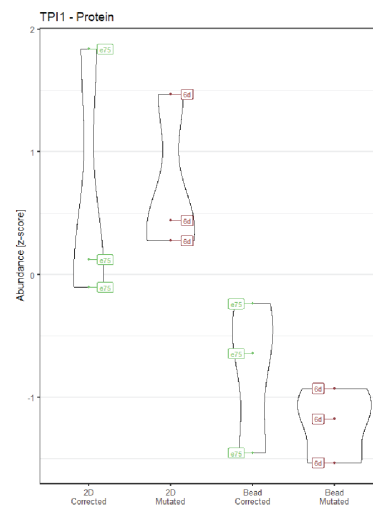
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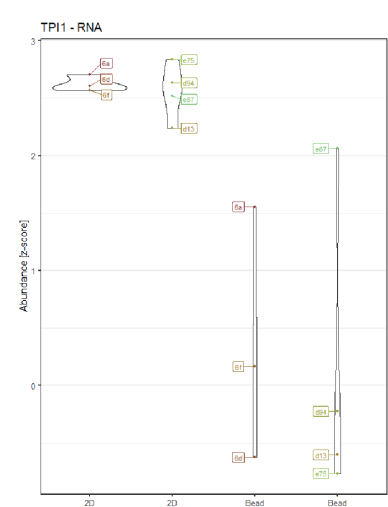
ALDOA protein



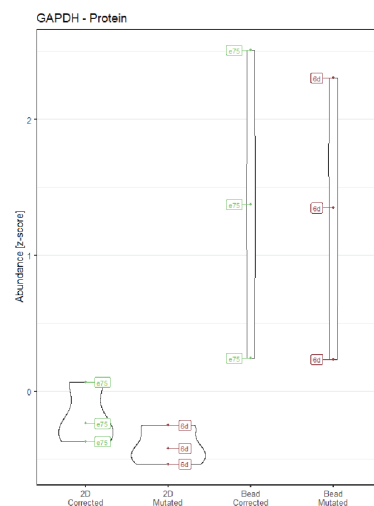
ALDOA mRNA



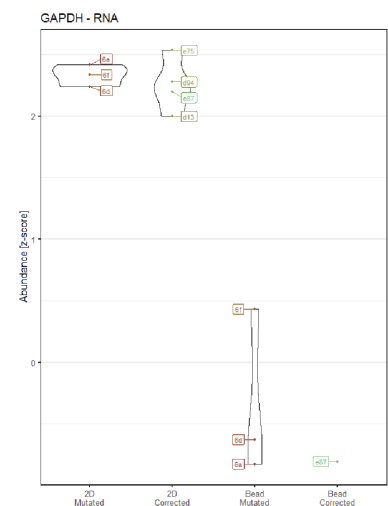
TPI1 protein



TPI1 mRNA

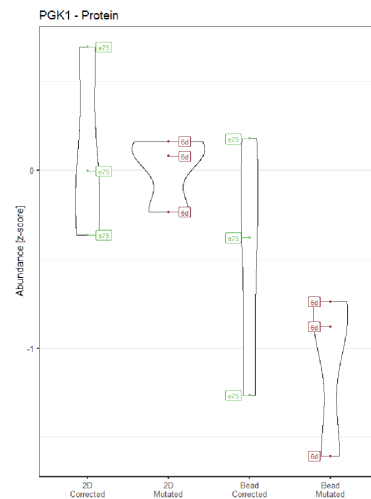


GAPDH protein

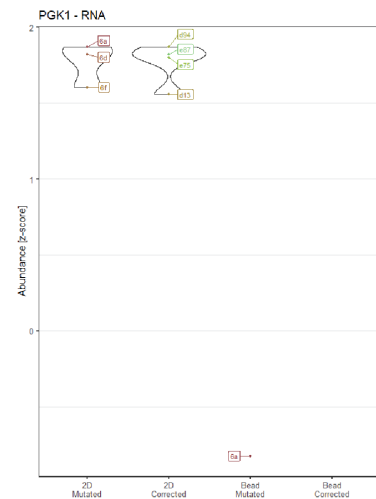


GAPDH mRNA

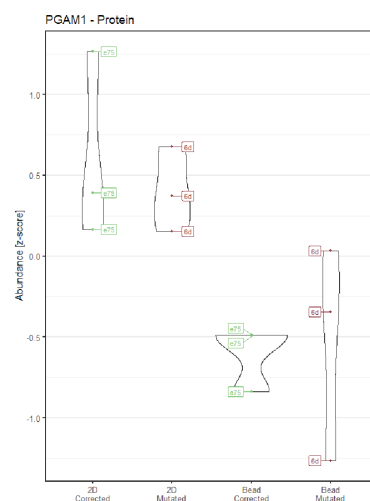
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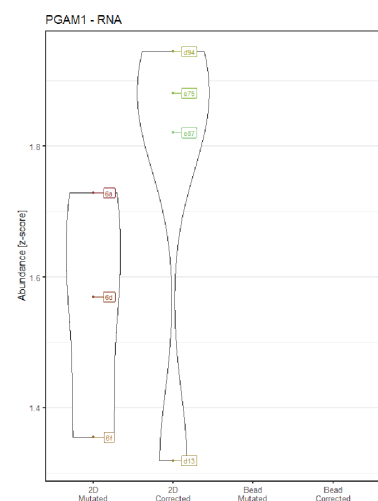
PGK1 protein



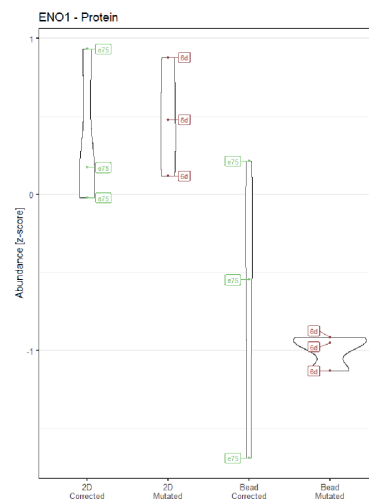
PGK1 mRNA



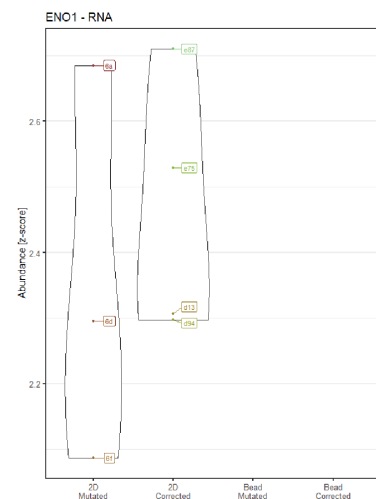
PGAM1 protein



PGAM1 mRNA



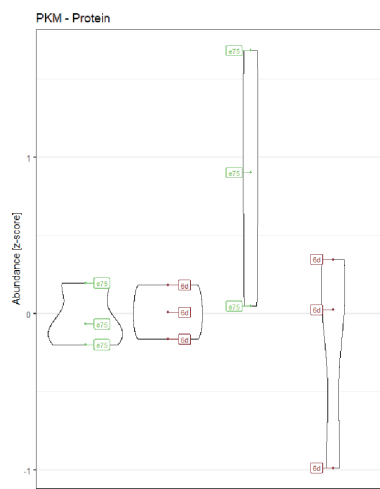
ENO1 protein



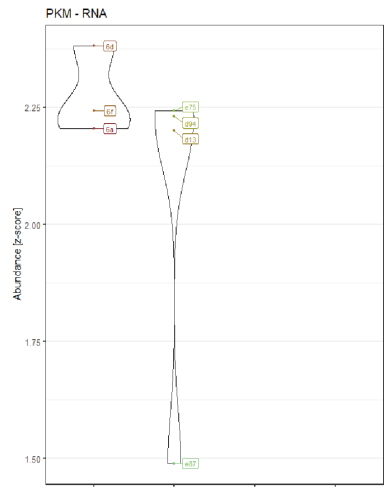
ENO1 mRNA

Supplementary
Figure 11B con-
tinued

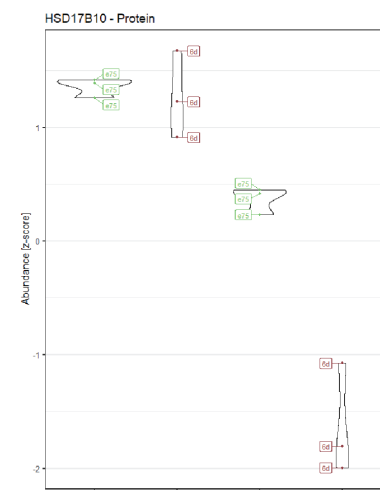
Stearate biosyn-
thesis
proteins/mRNAs:



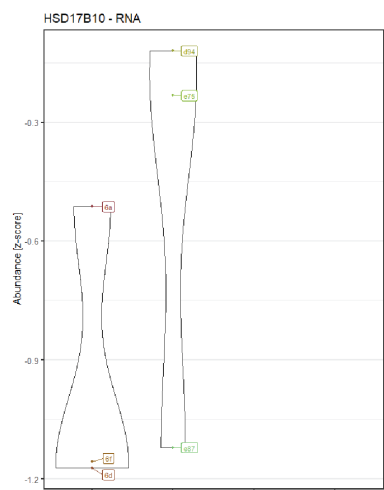
PKM protein



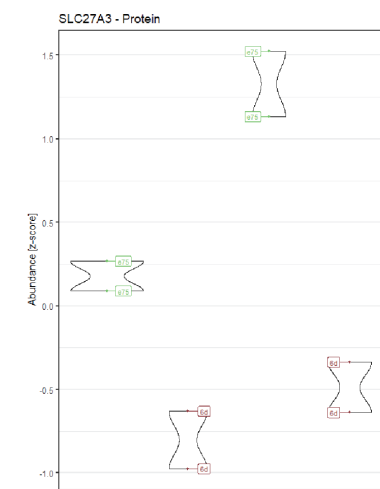
PKM mRNA



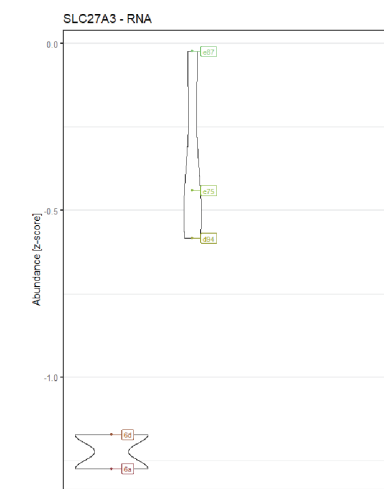
HSD17B10 protein



HSD17B10 mRNA

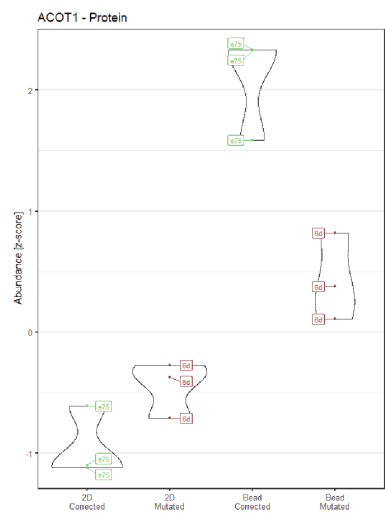


SLC27A3 protein

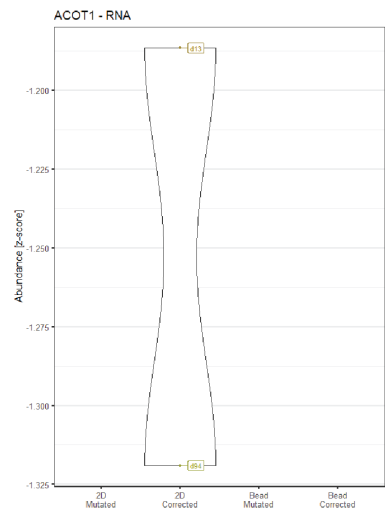


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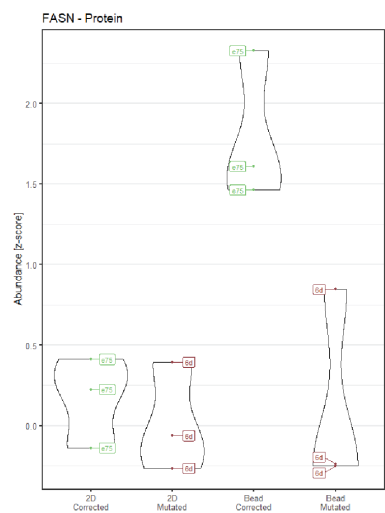
Supplementary
Figure 11B con-
tinued



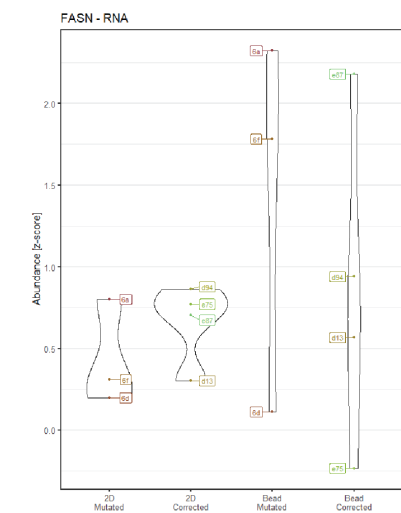
ACOT1 protein



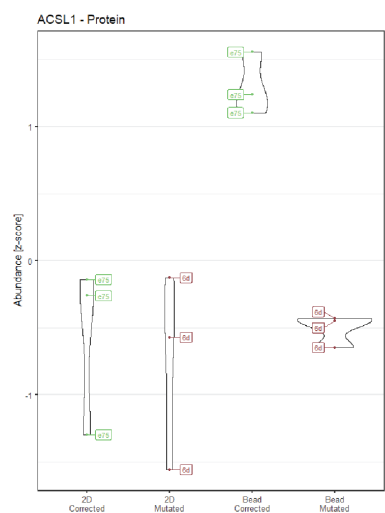
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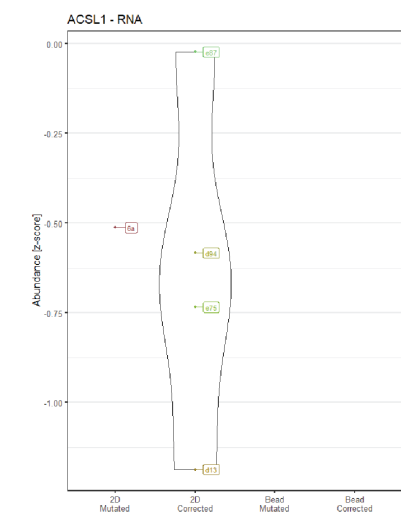
FASN protein



FASN mRNA

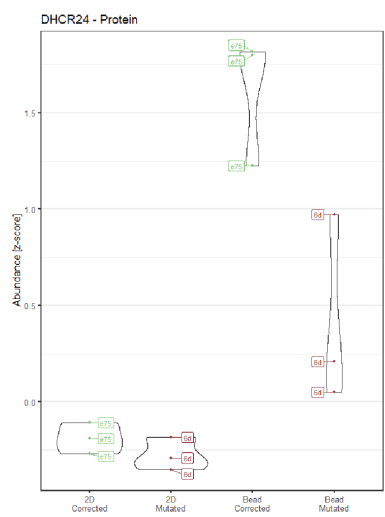


ACSL1 protein

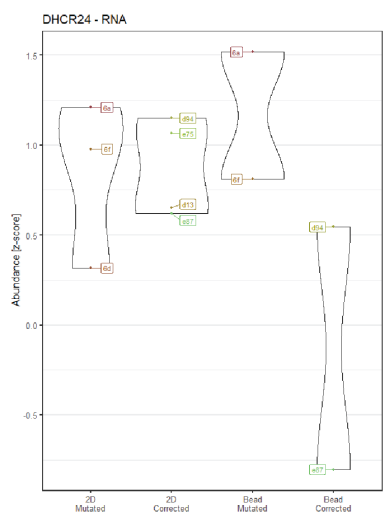


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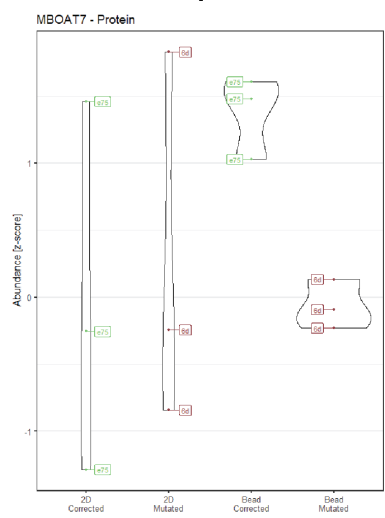
Supplementary
Figure 11B con-
tinued



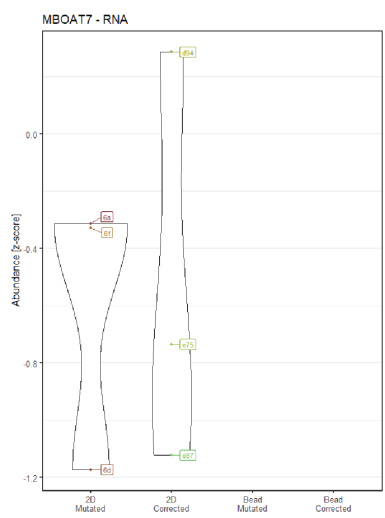
DHCR24 protein



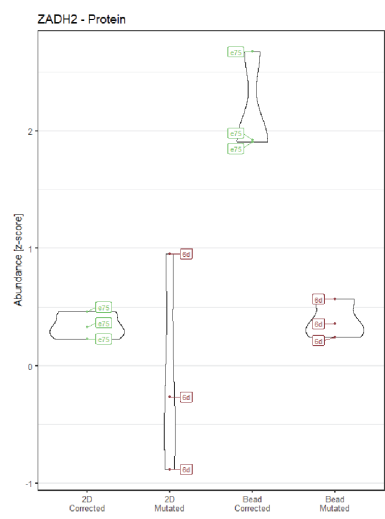
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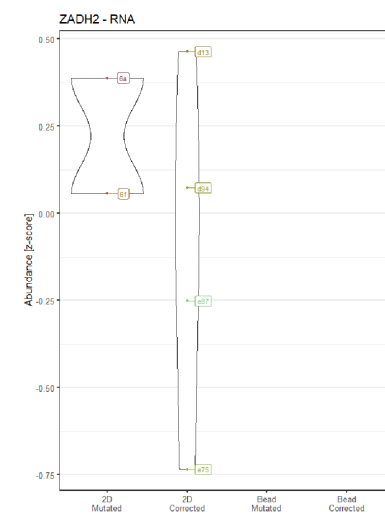
MBOAT7 protein



MBOAT7 mRNA



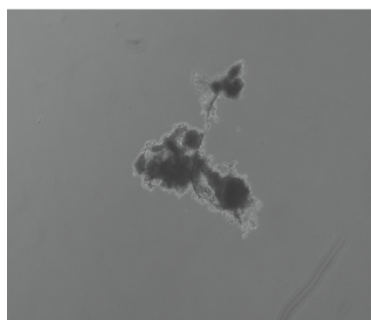
ZADH2 protein



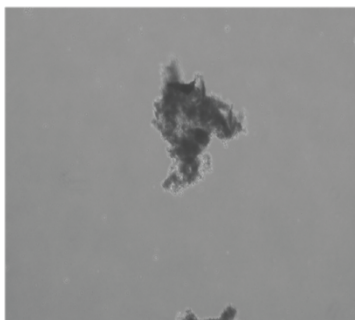
ZADH2 mRNA

Supplementary Figure 12

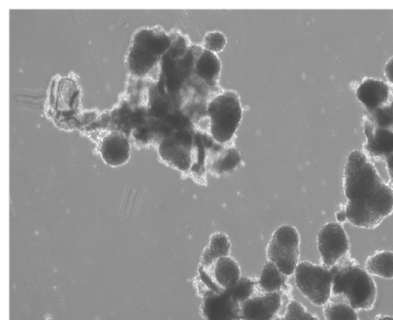
MUTATED CLONES



6A

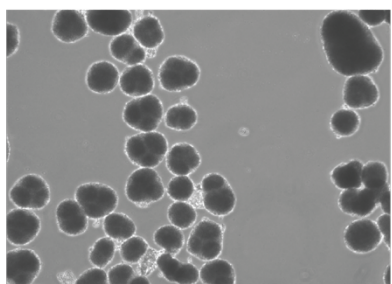


6D

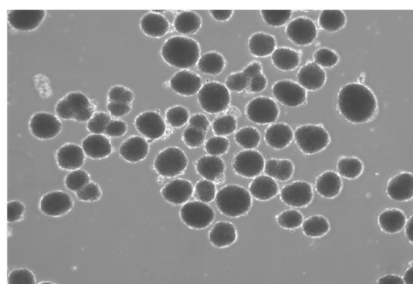


6F

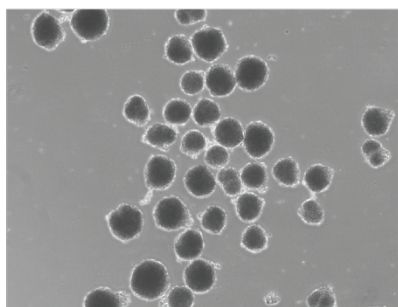
CORRECTED CLONES



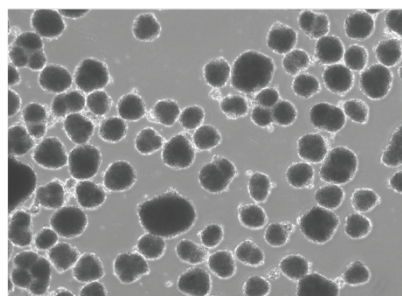
E75



D13



D94



E87

Supplementary Figure 3

A

Insulin (μ U/60 clusters)

Low High KCl

HNF4a (+/-)

Low High KCl

HNF4a (+/-) 3D

HNF4a (+/-) 2D

B

Insulin secretion index (20mM/2mM)

Low High KCl

HNF4a (+/-)

Low High KCl

HNF4a (+/-) 3D

HNF4a (+/-) 2D

C

Insulin releases fold change (KCl/20mM)

3D 2D

HNF4a (+/-)

3D 2D

HNF4a (+/-)

D

Dynamic GSIS

Insulin (μ U/ml) per 60 clusters

Time (minutes)

1.67mM Glu 20mM Glu 1.67mM Glu

HNF4a (+/-) 3D

HNF4a (+/-) 2D

HNF4a (+/-) 3D

HNF4a (+/-) 2D

E

Oxygen consumption rate

OCR (% Baseline)

Time (minutes)

20mM Glucose

Oligo

CCCP

Rot.

HNF4a (+/-) 3D

HNF4a (+/-) 2D

HNF4a (+/-) 3D

HNF4a (+/-) 2D

F

AUC-Baseline

AUC-20mM Glu Stimuli

AUC-CCCP

Area Under Curve of OCR

3D 2D

HNF4a (+/-)

3D 2D

HNF4a (+/-)

3D 2D

HNF4a (+/-)

3D 2D

HNF4a (+/-)

Off-target site nr. 1: GCTAGCTCAAAGAAGATTGAGAG score 1.5 3mm (1:4:6) **locus** chr1:+62261902
ENST00000371158.2

```
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GCTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCT
ATTGGAGGGAGATCAGTGTGT
>6Do
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CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
TTGGAGGGAGATCAGTGTGT
>6Dshao
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>D13o
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CLUSTAL O(1.2.4) multiple sequence alignment

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6Do       AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
6Dshao    AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
D13o      AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
D94o      AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
E87o      AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
E7511o    AAGCACATTTTCATGACTCTTGGCTTTAAAAGCCTTATGGGGAAAGTTGAAATAYTTACAG
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Off1      CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
6Do       CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
6Dshao    CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
D13o      CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
D94o      CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
E87o      CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
E7511o    CTAGCTCAAAGAAGATTGAGAGAGGAAGCCTATTGGTTCTGATAGACTTCAGGAATTGGG
          *****

Off1      CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
6Do       CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
6Dshao    CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
D13o      CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
D94o      CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
E87o      CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
E7511o    CTTGACATAGATTAAGAATACTGTTCTTGCTCACACTTTTTCAGAAAGCTAGAAGACCTA
          *****

Off1      TTGGAGGGAGATCAGTGTGT
6Do       TTGGAGGGAGATCAGTGTGT
6Dshao    TTGGAGGGAGATCAGTGTGT
D13o      TTGGAGGGAGATCAGTGTGT
D94o      TTGGAGGGAGATCAGTGTGT
E87o      TTGGAGGGAGATCAGTGTGT
E7511o    TTGGAGGGAGATCAGTGTGT
          *****
```

Off-target site nr. 2: ACTTAGTCAGAGAAGATTGACAG score 1.3 4mm (1:4:5:10) **locus** chrx:-4100552

```
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CLUSTAL O(1.2.4) multiple sequence alignment

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6Do       TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
6Dshao    TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
D13o      TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
D94o      TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
E87o      TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
E7511o    TCTAGGTTCTGTGTCCTGTTAAGGAGTGAAAACCTTAGAGAAGATTGACAGATTGCAAGGT
*****
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```
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6Dshao    TTTCATTACAGTTTCTGTTGTTTGCTTTTGCTTTAATATTACAGTAAGTTGGTTTCATACA
D13o      TTTCATTACAGTTTCTGTTGTTTGCTTTTGCTTTAATATTACAGTAAGTTGGTTTCATACA
D94o      TTTCATTACAGTTTCTGTTGTTTGCTTTTGCTTTAATATTACAGTAAGTTGGTTTCATACA
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E7511o    TTTCATTACAGTTTCTGTTGTTTGCTTTTGCTTTAATATTACAGTAAGTTGGTTTCATACA
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D13o      TCCGAGTGACTGAGGATTCCCAATAATGTCAGGAAACCCACGCAATATCTGTGTTTGA
D94o      TCCGAGTGACTGAGGATTCCCAATAATGTCAGGAAACCCACGCAATATCTGTGTTTGA
E87o      TCCGAGTGACTGAGGATTCCCAATAATGTCAGGAAACCCACGCAATATCTGTGTTTGA
E7511o    TCCGAGTGACTGAGGATTCCCAATAATGTCAGGAAACCCACGCAATATCTGTGTTTGA
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```

```
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6Do       AACCTTCTTTGTCCAGAGCTCCATTTGGG
6Dshao    AACCTTCTTTGTCCAGAGCTCCATTTGGG
D13o      AACCTTCTTTGTCCAGAGCTCCATTTGGG
D94o      AACCTTCTTTGTCCAGAGCTCCATTTGGG
E87o      AACCTTCTTTGTCCAGAGCTCCATTTGGG
E7511o    AACCTTCTTTGTCCAGAGCTCCATTTGGG
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Off-target site nr. 3: CTTCAGTCATAGAAGATTGATAG score 1.3 4mm (2:4:5:10) **locus** chr8:-122232999

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>6Do
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AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT
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CLUSTAL O(1.2.4) multiple sequence alignment

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6Dshao	TCAGATGATAGAGTTGCTGTAGAGCAATGAGGGAAGAGATATTCTTGCAGCTATATTAGG	60
D13o	TCAGATGATAGAGTTGCTGTAGAGCAATGAGGGAAGAGATATTCTTGCAGCTATATTAGG	60
D94o	TCAGATGATAGAGTTGCTGTAGAGCAATGAGGGAAGAGATATTCTTGCAGCTATATTAGG	60
E87o	TCAGATGATAGAGTTGCTGTAGAGCAATGAGGGAAGAGATATTCTTGCAGCTATATTAGG	60
E7511o	TCAGATGATAGAGTTGCTGTAGAGCAATGAGGGAAGAGATATTCTTGCAGCTATATTAGG	60

Off3	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
6Do	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
6Dshao	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
D13o	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
D94o	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
E87o	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120
E7511o	AAGTCTATCTTCCACATAGAGATCCATTCTTATGAATTTGCCAGTCACGCTTGCCTCTGA	120

Off3	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
6Do	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
6Dshao	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
D13o	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
D94o	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
E87o	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168
E7511o	AATTACCCTCTGATTGATATTTCTGCTCAAGTCCTCAACATCATTGTT	168

Off-target site nr. 4: TGTGGGTCAAAGAAGATTAAGGG score 1.1 3mm (1:2:19) **locus** chr2:+15179576
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>6Do

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>6Dshao

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>D94o

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>E87o

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CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTGTTGGGGGAGCTAACAGAAGG

>E7511o

AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
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GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTGTTGGGGGAGCTAACAGAAGG

CLUSTAL O(1.2.4) multiple sequence alignment

Off4	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
6Do	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
6Dshao	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
D13o	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
D94o	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
E87o	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA
E7511o	AGCTTCCTGTGCTAACAACCTCTAAGGCGCCTTCAGCTCAGAAACACCCTATGATTCAAA

Off4	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
6Do	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
6Dshao	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
D13o	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
D94o	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
E87o	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA
E7511o	ATCCTCATAGAAAAAAGTCAGGGAAGGATTTAAAGAAATTGTTCCATATGATTTTTTCACA

Off4	GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
6Do	GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
6Dshao	GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
D13o	GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
D94o	GGCTCGTCAGTGACTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC


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E87o      GGCTCGTCAGTGA CTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
E7511o    GGCTCGTCAGTGA CTCTGTGTGGGTCAAAGAAGATTAAGGGCCTCTGGAAATTCAAAGGC
*****
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Off4      TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
6Do       TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
6Dshao    TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
D13o      TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
D94o      TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
E87o      TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
E7511o    TGATTACATCAAATCCTATCCCAGGAGCCAAGAAGGGAGTGTGCACCACAGCTCAATATT
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Off4      CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
6Do       CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
6Dshao    CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
D13o      CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
D94o      CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
E87o      CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
E7511o    CTATGCACAAGCCAGGAGCTTTTAGATAAGCATTTTGGGGGAGCTAACAGAAGG
*****
```

Off-target site nr. 5: CATGGGGCCAAGAAGATTGACAG score 1.1 3mm (2:7:9) **locus** chr15:-48163869

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>Off5
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ATAGAGAAAAC TTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAAC TGCACCATG
GTTAGATGG
>6Do
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ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
ATAGAGAAAAC TTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAAC TGCACCATG
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GTTAGATGG
>D13o
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GTTAGATGG
>E7511o
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GTTAGATGG
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CLUSTAL O(1.2.4) multiple sequence alignment

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Off5      ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
6Do       ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
6Dshao    ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
D13o      ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
D94o      ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
E87o      ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
E7511o    ATGTCACAGAGATGAAGGGTGCTGAGATGCTGGTGGGAAGTAGTTCATGGGGCCAAGAAG
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Off5      ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
6Do       ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
```

6Dshao ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
D13o ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
D94o ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
E87o ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG
E7511o ATTGACAGAACGATCTGAGGTTTCTTTTAAGCTTTGCTGGCATGTCACTGTCATCACAGG

Off5 ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
6Do ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
6Dshao ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
D13o ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
D94o ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
E87o ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG
E7511o ATAGAGAAAACCTGCATTACACGTCCTATCAGACTGGAGCTAGGAAAACCTGTCACCATG

Off5 GTTAGATGG
6Do GTTAGATGG
6Dshao GTTAGATGG
D13o GTTAGATGG
D94o GTTAGATGG
E87o GTTAGATGG
E7511o GTTAGATGG

Off-target site nr. 6: CCTGGGTAAGAGAAGAGTGACAG score 1.0 3mm (8:10:17) **locus** chr10:+3236153

>Off6
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CATTTGACTGGACTACCTTCTAGCTATAGTTTGTGACAAACCACTTTTGTGTAGAAGGGC
TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA
AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTTCTTAGTGGACGCCATGGACATGG
TGTCCATGAGCACCACAGAGTAAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA
CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA
>6Do
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TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA
AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTTCTTAGTGGACGCCATGGACATGG
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TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA
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TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA
AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTTCTTAGTGGACGCCATGGACATGG
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>D94o
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TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA
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>E7511o
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AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTTCTTAGTGGACGCCATGGACATGG
TGTCCATGAGCACCACAGAGTAAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA
CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA

CLUSTAL O(1.2.4) multiple sequence alignment

Off6	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
6Do	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
6Dshao	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
D13o	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
D94o	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
E87o	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60
E7511o	AGCTGGTTTCTTTCACTTGCTGCCCCAACTCCTGAAGATATAAAAGTCAAAGGCAAATGC	60

Off6	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
6Do	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
6Dshao	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
D13o	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
D94o	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
E87o	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120
E7511o	CATTTGACTGGACTACCTTCTAGCTATAGTTTTGACAAACCACTTTTTGTGTAGAAGGGC	120

Off6	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
6Do	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
6Dshao	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
D13o	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
D94o	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
E87o	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180
E7511o	TACCCCTCCAGTAACCTTCCCTCACTTTGTAGTTTTCGGGTGCCTGGTGTGTCCTGGGTA	180

Off6	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
6Do	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
6Dshao	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
D13o	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
D94o	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
E87o	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240
E7511o	AGAGAAGAGTGACAGCAGCTGGTTTGAATGACGCTTCTTAGTGGACGCCATGGACATGG	240

Off6	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
6Do	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
6Dshao	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
D13o	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
D94o	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
E87o	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300
E7511o	TGTCCATGAGCACCACAGAGTAACTAAGGGACCTCTCATGTCCACCCAAAGGTGTGTTA	300

Off6	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
6Do	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
6Dshao	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
D13o	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
D94o	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
E87o	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
E7511o	CTGCACAGAGGTTTATGCAATGCGGCTTTGCATCACCA 338	
