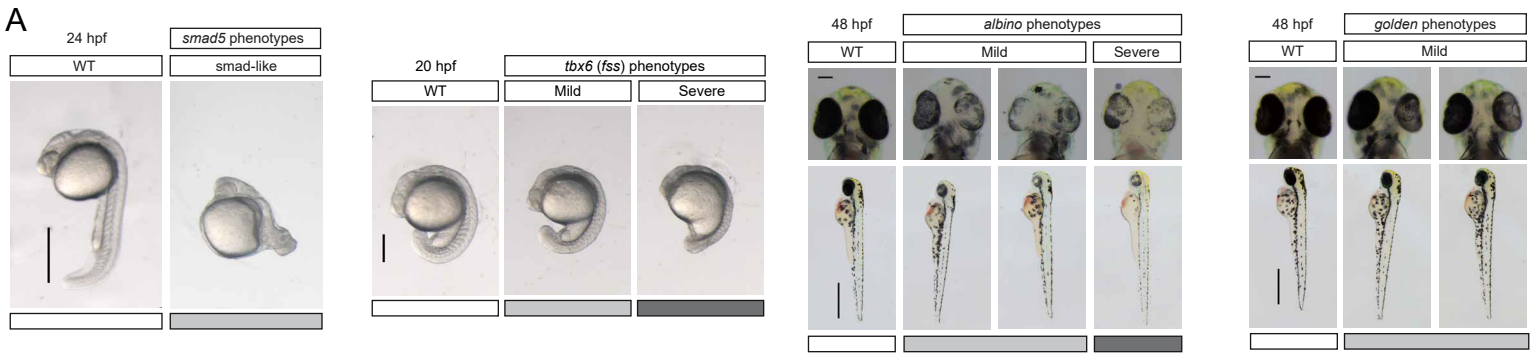
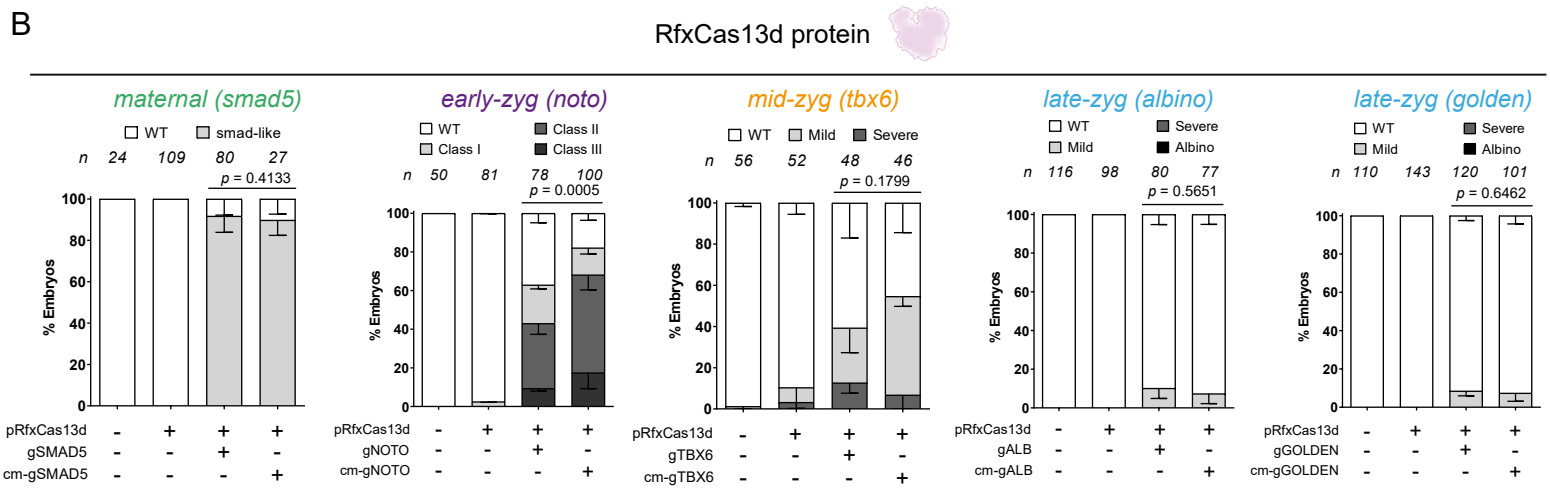
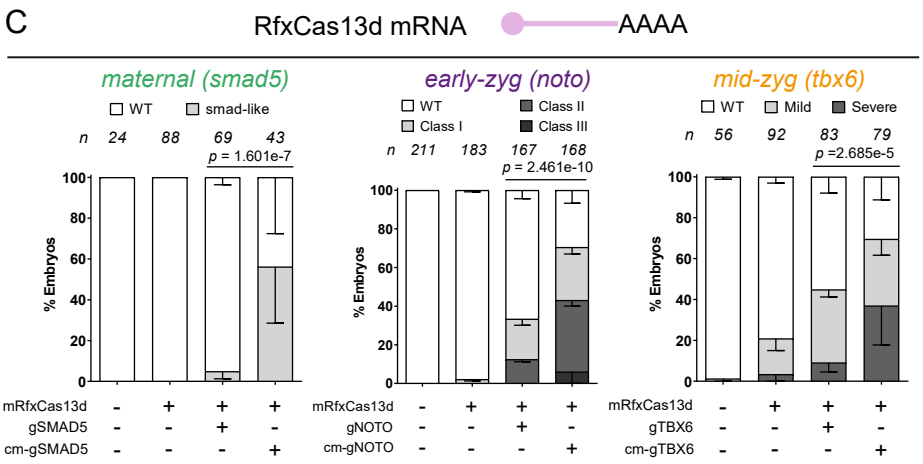
Supplementary Information

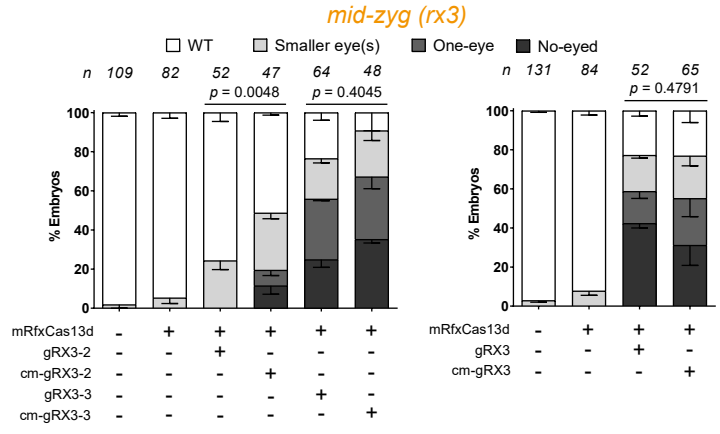
Enhanced RNA-targeting CRISPR-Cas technology in zebrafish

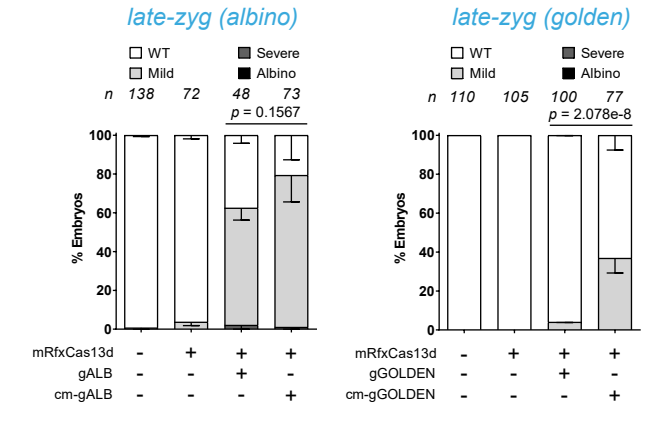
Ismael Moreno-Sánchez, Luis Hernández-Huertas, Daniel Nahón-Cano, Pedro Manuel Martínez-García, Anthony J. Treichel, Carlos Gómez-Marín, Laura Tomás-Gallardo, Gabriel da Silva Pescador, Gopal Kushawah, Rhonda Egidy, Anoja Perera, Alejandro Díaz-Moscoso, Alejandra Cano-Ruiz, John A. Walker II, Manuel J. Muñoz, Kevin Holden, Joan Galcerán, M. Ángela Nieto, Ariel A. Bazzini, Miguel A. Moreno-Mateos

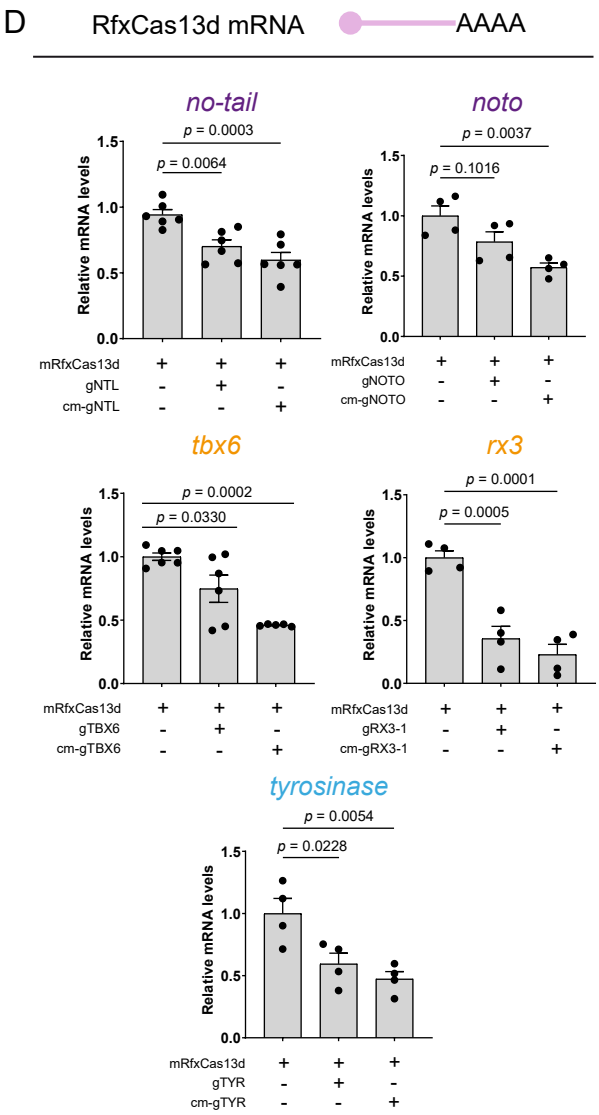

A
24 hpf
smad5 phenotypes
WT
smad-like
20 hpf
tbx6 (fss) phenotypes
WT
Mild
Severe
48 hpf
albino phenotypes
WT
Mild
Severe
48 hpf
golden phenotypes
WT
Mild


B
RfxCas13d protein
maternal (smad5)
WT
smad-like
n 24 109 80 27
p = 0.4133
% Embryos
pRfxCas13d - + + +
gSMAD5 - - + -
cm-gSMAD5 - - - +
early-zyg (noto)
WT
Class I
Class II
Class III
n 50 81 78 100
p = 0.0005
% Embryos
pRfxCas13d - + + +
gNOTO - - + -
cm-gNOTO - - - +
mid-zyg (tbx6)
WT
Mild
Severe
n 56 52 48 46
p = 0.1799
% Embryos
pRfxCas13d - + + +
gTBX6 - - + -
cm-gTBX6 - - - +
late-zyg (albino)
WT
Mild
Severe
Albino
n 116 98 80 77
p = 0.5651
% Embryos
pRfxCas13d - + + +
gALB - - + -
cm-gALB - - - +
late-zyg (golden)
WT
Mild
Severe
Albino
n 110 143 120 101
p = 0.6462
% Embryos
pRfxCas13d - + + +
gGOLDEN - - + -
cm-gGOLDEN - - - +


C
RfxCas13d mRNA
AAAA
maternal (smad5)
WT
smad-like
n 24 88 69 43
p = 1.601e-7
% Embryos
mRfxCas13d - + + +
gSMAD5 - - + -
cm-gSMAD5 - - - +
early-zyg (noto)
WT
Class I
Class II
Class III
n 211 183 167 168
p = 2.461e-10
% Embryos
mRfxCas13d - + + +
gNOTO - - + -
cm-gNOTO - - - +
mid-zyg (tbx6)
WT
Mild
Severe
n 56 92 83 79
p =2.685e-5
% Embryos
mRfxCas13d - + + +
gTBX6 - - + -
cm-gTBX6 - - - +


mid-zyg (rx3)
WT
Smaller eye(s)
One-eye
No-eyed
n 109 82 52 47 64 48
p = 0.0048
p = 0.4045
% Embryos
mRfxCas13d - + + + + +
gRX3-2 - - + - - -
cm-gRX3-2 - - - + - -
gRX3-3 - - - - + -
cm-gRX3-3 - - - - - +
n 131 84 52 65
p = 0.4791
% Embryos
mRfxCas13d - + + +
gRX3 - - + -
cm-gRX3 - - - +


late-zyg (albino)
WT
Mild
Severe
Albino
n 138 72 48 73
p = 0.1567
% Embryos
mRfxCas13d - + + +
gALB - - + -
cm-gALB - - - +
late-zyg (golden)
WT
Mild
Severe
Albino
n 110 105 100 77
p = 2.078e-8
% Embryos
mRfxCas13d - + + +
gGOLDEN - - + -
cm-gGOLDEN - - - +


D
RfxCas13d mRNA
AAAA
no-tail
p = 0.0003
p = 0.0064
Relative mRNA levels
mRfxCas13d + + +
gNTL - + -
cm-gNTL - - +
noto
p = 0.0037
p = 0.1016
Relative mRNA levels
mRfxCas13d + + +
gNOTO - + -
cm-gNOTO - - +
tbx6
p = 0.0002
p = 0.0330
Relative mRNA levels
mRfxCas13d + + +
gTBX6 - + -
cm-gTBX6 - - +
rx3
p = 0.0001
p = 0.0005
Relative mRNA levels
mRfxCas13d + + +
gRX3-1 - + -
cm-gRX3-1 - - +
tyrosinase
p = 0.0054
p = 0.0228
Relative mRNA levels
mRfxCas13d + + +
gTYR - + -
cm-gTYR - - +

Supplementary Figure 1. RfxCas13d protein is less efficient than *RfxCas13d* mRNA when targeting mid- and late-zygotically expressed mRNAs.

A) Representative images of the phenotypes obtained after the injection of the mRNA-gRNAs or ribonucleoprotein (RNP) complexes targeting maternally provided *smad5*, and zygotically-expressed *tbx6 – fussed-somites* (*fss*), *slc45a2 – albino*, and *slc24a5 – golden*. *Smad5* loss-of-function phenotypes were evaluated at 24 hpf (scale bar 0.5 mm). *Tbx6* (*fss*) loss-of-function phenotypes were evaluated at 20 hpf (scale bar 0.25 mm). Loss-of-function phenotypes of *albino* or *golden* (reduced or lack of pigmentation) were evaluated at 48 hpf (scale bar, 0.75 mm for lateral views and 0.1 mm for insets). See Methods for a detailed description of loss-of-function phenotypes.

B) Stacked barplots showing percentage of observed phenotypes in injected embryos with standard or chemically modified gRNAs (cm-gRNAs) targeting *smad5*, *noto*, *tbx6*, *albino* and *golden* (gSMAD5, gNOTO, gTBX6, gALB and gGOLDEN, respectively) together with RfxCas13d protein (pRfxCas13d).

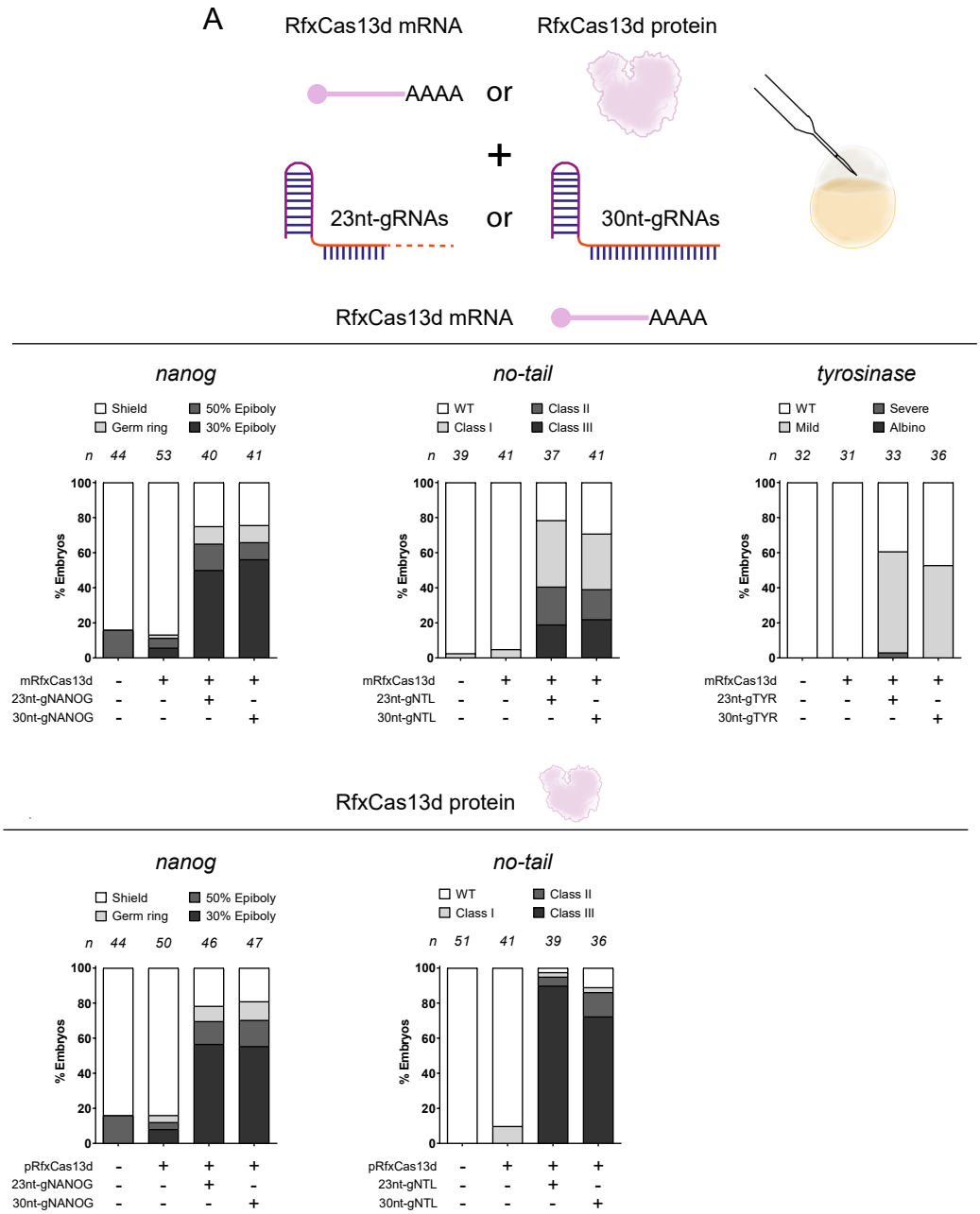
C) Stacked barplots showing percentage of observed phenotypes in injected embryos with standard or cm-gRNAs targeting *smad5*, *noto*, *tbx6*, *rx3*, *albino* and *golden* (1 ng of a mix of three gRNAs – gSMAD5, gNOTO, gTBX6, gRX3, gALB and gGOLDEN; 1 ng of single gRNAs – gRX3-2 and gRX3-3) together with RfxCas13d mRNA (300 pg of mRfxCas13d).

(n) total number of injected embryos is displayed for each condition. The results are shown as the averages ± standard deviation of the mean of each phenotypic category (**B** and **C**).

D) Barplots representing mRNA relative levels analyzed by qRT-PCR at 6 hpf (*no-tail* and *noto*) or 20 hpf (*rx3*, *tbx6* and *tyrosinase*) from injected embryos with mRfxCas13d together with gNTL, gNOTO, gRX3-1, gTBX6 or gTYR. Results are shown as the averages ± standard deviation of the mean. *taf15* (for *no-tail* and *noto*) or *ef1α* (for *rx3*, *tyrosinase* and *tbx6*) mRNA was used as a normalization control.

p-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.

Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
RfxCas13d mRNA
RfxCas13d protein
AAAA
or
+
23nt-gRNAs
or
30nt-gRNAs
B
RfxCas13d mRNA
AAAA
nanog
Shield
50% Epiboly
Germ ring
30% Epiboly
n 44 53 40 41
% Embryos
mRfxCas13d - + + +
23nt-gNANOG - - + -
30nt-gNANOG - - - +
no-tail
WT
Class II
Class I
Class III
n 39 41 37 41
mRfxCas13d - + + +
23nt-gNTL - - + -
30nt-gNTL - - - +
tyrosinase
WT
Severe
Mild
Albino
n 32 31 33 36
mRfxCas13d - + + +
23nt-gTYR - - + -
30nt-gTYR - - - +
C
RfxCas13d protein
nanog
Shield
50% Epiboly
Germ ring
30% Epiboly
n 44 50 46 47
% Embryos
pRfxCas13d - + + +
23nt-gNANOG - - + -
30nt-gNANOG - - - +
no-tail
WT
Class II
Class I
Class III
n 51 41 39 36
pRfxCas13d - + + +
23nt-gNTL - - + -
30nt-gNTL - - - +

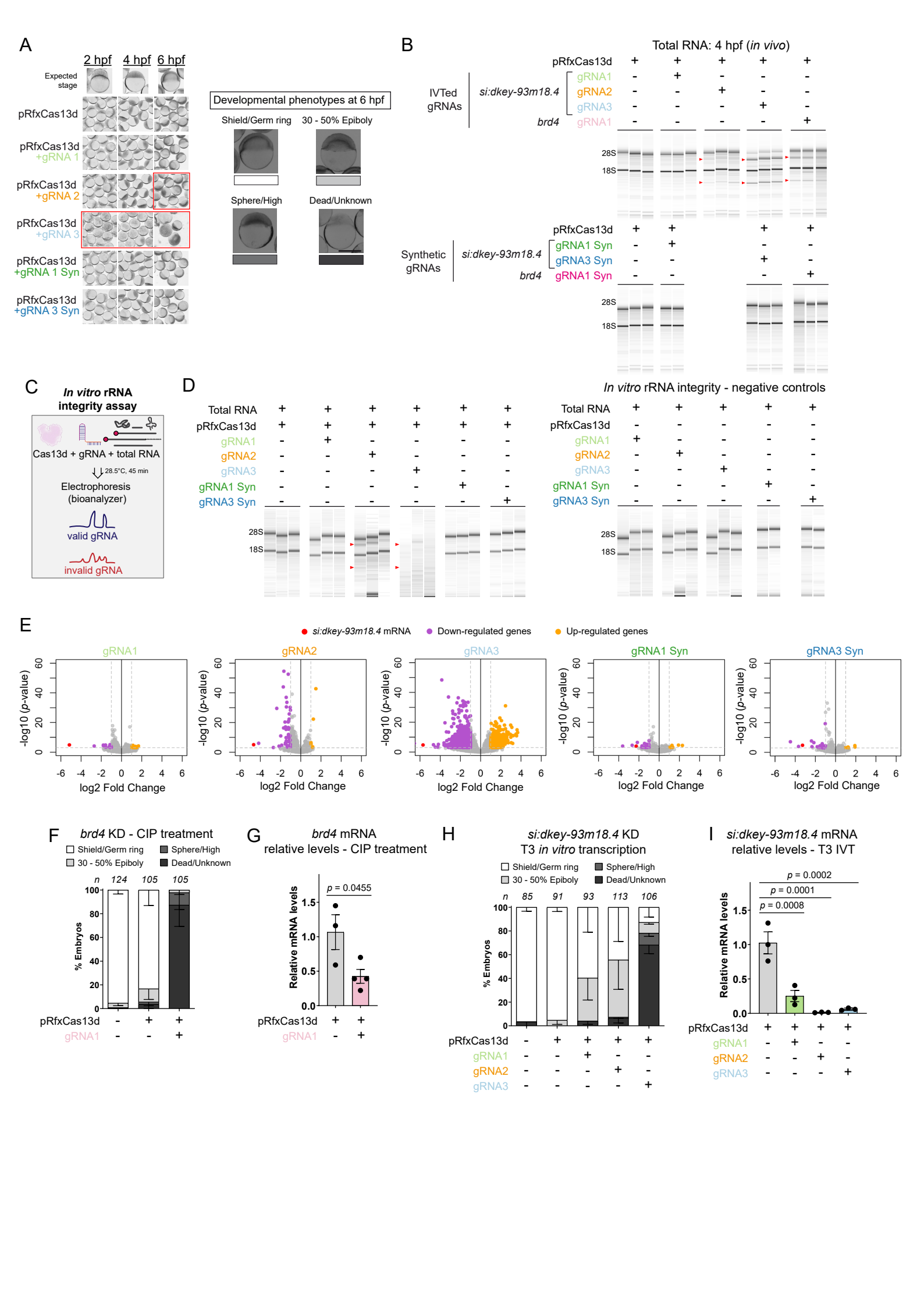
Supplementary Figure 2. Increasing the length of gRNA spacers does not improve CRISPR-RfxCas13d activity in zebrafish embryos.

A) Schematic illustration of the experimental setup used to compare 23 and 30 nt long spacers for CRISPR-RfxCas13d gRNAs. 300 pg of *RfxCas13d* mRNA (mRfxCas13d) or 3 ng of RfxCas13d protein (pRfxCas13d) were injected together with 1 ng of a mix of three gRNAs (~300 pg/gRNA) into one-cell stage zebrafish embryos.

B, C) Stacked barplots showing percentage of observed phenotypes using mRfxCas13d (**B**) or pRfxCas13d (**C**) targeting *nanog*, *no-tail* and *tyrosinase* (gNANOG, gNTL, and gTYR, respectively). (n) total number of embryos is indicated for each condition. One independent experiment. Representative images of phenotypes are displayed in **Fig. 1C**.

Source data is provided as a Source Data file.

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A
2 hpf
4 hpf
6 hpf
Expected stage
pRfxCas13d
pRfxCas13d +gRNA 1
pRfxCas13d +gRNA 2
pRfxCas13d +gRNA 3
pRfxCas13d +gRNA 1 Syn
pRfxCas13d +gRNA 3 Syn
Developmental phenotypes at 6 hpf
Shield/Germ ring
30 - 50% Epiboly
Sphere/High
Dead/Unknown
B
Total RNA: 4 hpf (in vivo)
IVTed gRNAs
si:dkey-93m18.4
brd4
gRNA1
gRNA2
gRNA3
Synthetic gRNAs
gRNA1 Syn
gRNA3 Syn
28S
18S
C
In vitro rRNA integrity assay
Cas13d + gRNA + total RNA
28.5°C, 45 min
Electrophoresis (bioanalyzer)
valid gRNA
invalid gRNA
D
In vitro rRNA integrity - negative controls
Total RNA
pRfxCas13d
E
si:dkey-93m18.4 mRNA
Down-regulated genes
Up-regulated genes
-log10 (p-value)
log2 Fold Change
F
brd4 KD - CIP treatment
n 124 105 105
% Embryos
G
brd4 mRNA relative levels - CIP treatment
p = 0.0455
Relative mRNA levels
H
si:dkey-93m18.4 KD T3 in vitro transcription
n 85 91 93 113 106
I
si:dkey-93m18.4 mRNA relative levels - T3 IVT
p = 0.0002
p = 0.0001
p = 0.0008

Supplementary Figure 3. *In vitro* transcribed gRNAs can induce toxicity

A) Representative images of RfxCas13d-mediated *si:dkey-93m18.4* KD and control embryos at 2, 4, and 6 hpf. Single embryos in top row show expected stage. Red boxes denote abnormal development (left set of images). Representative images of developmental stages and dead embryos evaluated at ~6 hpf: sphere/high, 30% – 50% epiboly, and germ ring/shield stages correspond to 3.3 – 4, 4.6 – 5.3, 5.7 – 6 hpf in non-injected embryos growing in standard conditions, respectively (scale bar, 0.5 mm) (right set of images).

B) RNA integrity images from bioanalyzer of total RNA from RfxCas13d mediated *si:dkey-93m18.4* and *brd4* KD and control embryos at 4 hpf. Red arrows denote bands from 28S rRNA cleavage.

C) Schematic representation of *in vitro* rRNA integrity assay (see Methods for further details).

D) RNA integrity images from at least two replicates of bioanalyzer electrophoresis of the *in vitro* rRNA integrity assay. Red arrows denote bands from 28S rRNA cleavage. Negative controls are *in vitro* rRNA integrity assays without RfxCas13d protein.

E) Volcano plots showing down- (purple) and up-regulated (orange) genes upon *si:dkey-93m18.4* KD with RfxCas13d *in vitro* transcribed gRNAs (IVTed, gRNAs 1-3) or chemically synthetized (Syn) gRNAs (400 pg/embryo) measured by RNAseq. *si:dkey-93m18.4* levels are depicted in red. Fold-Change of 2 and *p*-value < 0.001 (dashed lines) were set to determine deregulated genes.

F) Stacked barplots showing percentage of developmental effect of *brd4* KD at 6 hpf using an *in vitro* transcribed gRNA (IVTed, gRNA 1) after CIP treatment (300 pg/embryo).

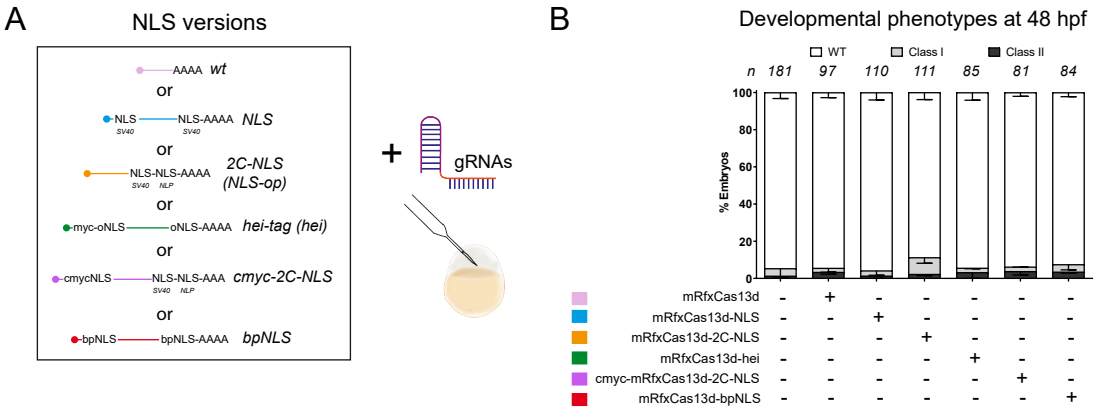
G) Barplot showing relative *brd4* mRNA levels measured by qRT-PCR at 4 hpf from injected embryos in **F**. *taf15* mRNA was used as a normalization control.

H) Stacked barplots showing percentage of developmental effect of *si:dkey-93m18.4* KD at 6 hpf using T3 *in vitro* transcribed gRNAs (IVTed, gRNAs 1-3) (400 pg/embryo).

I) Barplot showing relative *si:dkey-93m18.4* mRNA levels measured by qRT-PCR at 4 hpf from injected embryos in **H**. *cdk2ap2* mRNA was used as a normalization control.

(n) total number of injected embryos is shown for each condition. The results are shown as the averages ± standard deviation of the mean of each developmental stage (**F** and **H**). mRNA relative levels measured by qRT-PCR are shown as the averages ± standard deviation of the mean (**G** and **I**). *p*-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.

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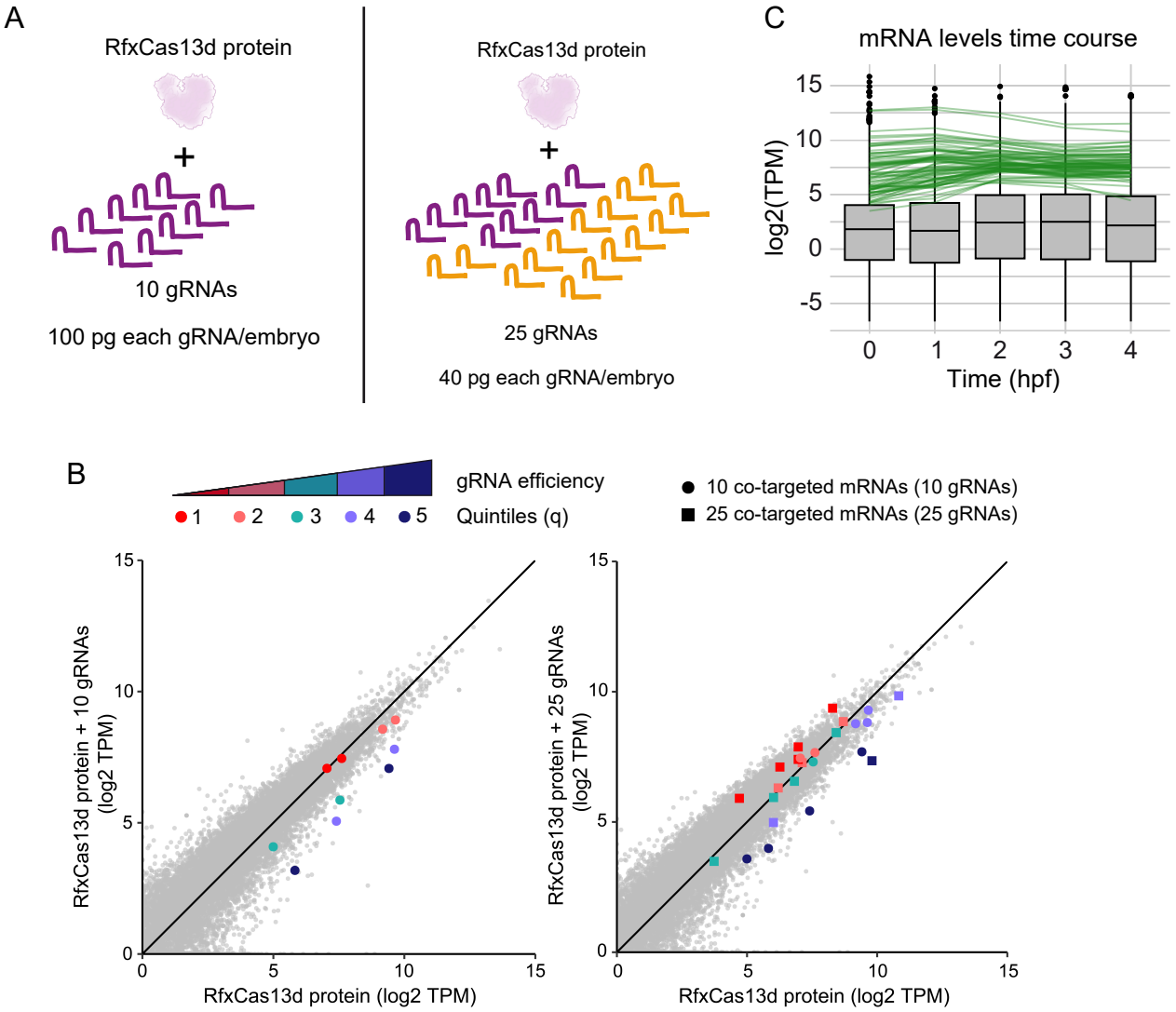

A
NLS versions
AAAA wt
or
NLS SV40 NLS-AAAA SV40 NLS
or
NLS-NLS-AAAA SV40 NLP 2C-NLS (NLS-op)
or
myc-oNLS oNLS-AAAA hei-tag (hei)
or
cmycNLS NLS-NLS-AAA SV40 NLP cmyc-2C-NLS
or
bpNLS bpNLS-AAAA bpNLS
+
gRNAs
B
Developmental phenotypes at 48 hpf
WT
Class I
Class II
n 181 97 110 111 85 81 84
% Embryos
100
80
60
40
20
0
mRfxCas13d - + - - - - -
mRfxCas13d-NLS - - + - - - -
mRfxCas13d-2C-NLS - - - + - - -
mRfxCas13d-hei - - - - + - -
cmyc-mRfxCas13d-2C-NLS - - - - - + -
mRfxCas13d-bpNLS - - - - - - +

Supplementary Figure 4. Optimization of NLS versions to increase CRISPR-RfxCas13d nuclear RNA-targeting

A) Diagram illustrating the cytoplasmic (pink) and five different NLS versions of RfxCas13d: NLS (SV40 NLS, blue), 2C-NLS or optimized NLS (SV40 and nucleoplasmin long-NLP NLS, orange; Liu et al., 2019[55]), hei-tag (oNLS, green; Thumberger et al., 2022[58]), cmyc-2C-NLS (cmyc, SV40 and nucleoplasmin long-NLP NLS, purple; Wu et al., 2019[56]) and bpNLS (bipartite NLS, red; Liang et al., 2022[57]).

B) Stacked barplots showing percentage of observed phenotypes at 48 hpf (**Fig. 3A**) when injecting each mRNA alone (300 pg/embryo). (n) total number of embryos is indicated for each condition. The results are shown as the averages ± standard deviation of the mean of each developmental stage from two or three (mRfxCas13d and mRfxCas13d-NLS) independent experiments.

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A
RfxCas13d protein
+
10 gRNAs
100 pg each gRNA/embryo
RfxCas13d protein
+
25 gRNAs
40 pg each gRNA/embryo
C
mRNA levels time course
log2(TPM)
Time (hpf)
B
gRNA efficiency
Quintiles (q)
10 co-targeted mRNAs (10 gRNAs)
25 co-targeted mRNAs (25 gRNAs)
RfxCas13d protein + 10 gRNAs (log2 TPM)
RfxCas13d protein + 25 gRNAs (log2 TPM)
RfxCas13d protein (log2 TPM)

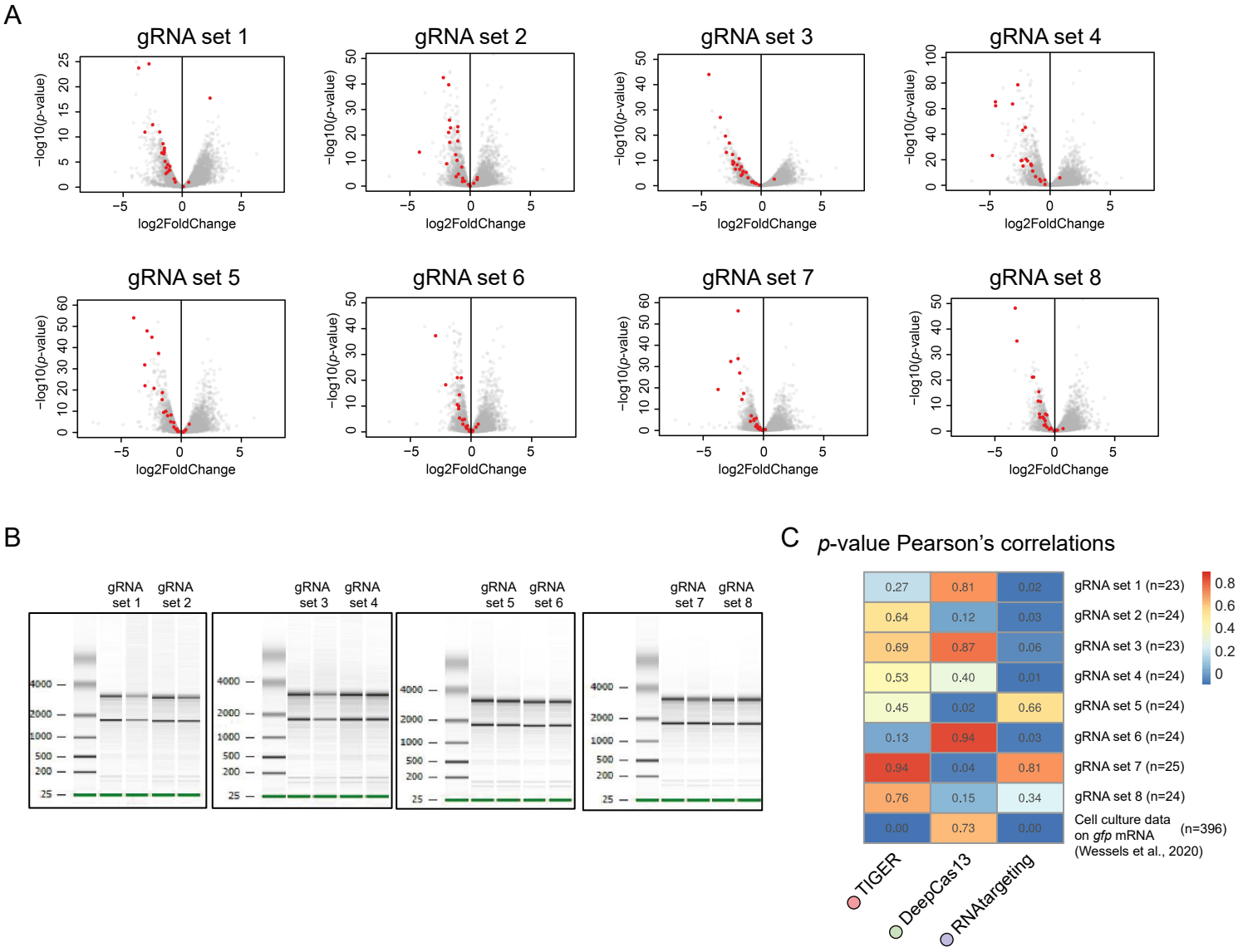
Supplementary Figure 5. Highly efficient CRISPR-RfxCas13d gRNAs can be identified when injecting up to 25 gRNAs together.

A) Schematic representation of multiplexing with CRISPR-RfxCas13d using 10 gRNAs (100 pg each gRNA – purple) or 25 gRNAs (40 pg each gRNA – orange).

B) Scatterplot showing mRNA level (RNA-seq) of zebrafish embryos injected with RfxCas13d protein (3 ng/embryo) together with 10 or 25 gRNAs compared with embryos injected only with RfxCas13d protein at 4 hpf. gRNAs are color-coded in quintiles (q). The first 10 targeted mRNAs are depicted in circles and 15 mRNAs up to 25 are indicated in squares in their respective panels.

C) Boxplots showing the expression levels of polyadenylated RNAs during the first 4 hours of zebrafish embryo development (log2 TPM: Transcript per million). The 75 mRNAs targeted in **Fig. 4** and **Supplementary Fig. 6** are depicted in green. Data from Medina-Muñoz et al., 2021[62].

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A
gRNA set 1
gRNA set 2
gRNA set 3
gRNA set 4
gRNA set 5
gRNA set 6
gRNA set 7
gRNA set 8
−log10(p-value)
log2FoldChange
B
gRNA set 1
gRNA set 2
gRNA set 3
gRNA set 4
gRNA set 5
gRNA set 6
gRNA set 7
gRNA set 8
4000
2000
1000
500
200
25
C
p-value Pearson's correlations
0.27 0.81 0.02 gRNA set 1 (n=23)
0.64 0.12 0.03 gRNA set 2 (n=24)
0.69 0.87 0.06 gRNA set 3 (n=23)
0.53 0.40 0.01 gRNA set 4 (n=24)
0.45 0.02 0.66 gRNA set 5 (n=24)
0.13 0.94 0.03 gRNA set 6 (n=24)
0.94 0.04 0.81 gRNA set 7 (n=25)
0.76 0.15 0.34 gRNA set 8 (n=24)
0.00 0.73 0.00 Cell culture data on gfp mRNA (n=396) (Wessels et al., 2020)
0.8
0.6
0.4
0.2
0
TIGER
DeepCas13
RNAtargeting
D
TIGER (Wessels et al., 2024)
p = 0.0081
E
DeepCas13 (Cheng et al., 2023)
p = 0.0146
F
RNAtargeting (Wei et al., 2023)
p = 0.0003
Score prediction
1.00
0.75
0.50
0.25
0
High
Low
Activity
gRNA set
1
2
3
4
5
6
7
8

Supplementary Figure 6. *In vivo* activity of CRISPR-RfxCas13d gRNAs activity predicted by *ex vivo* computational models.

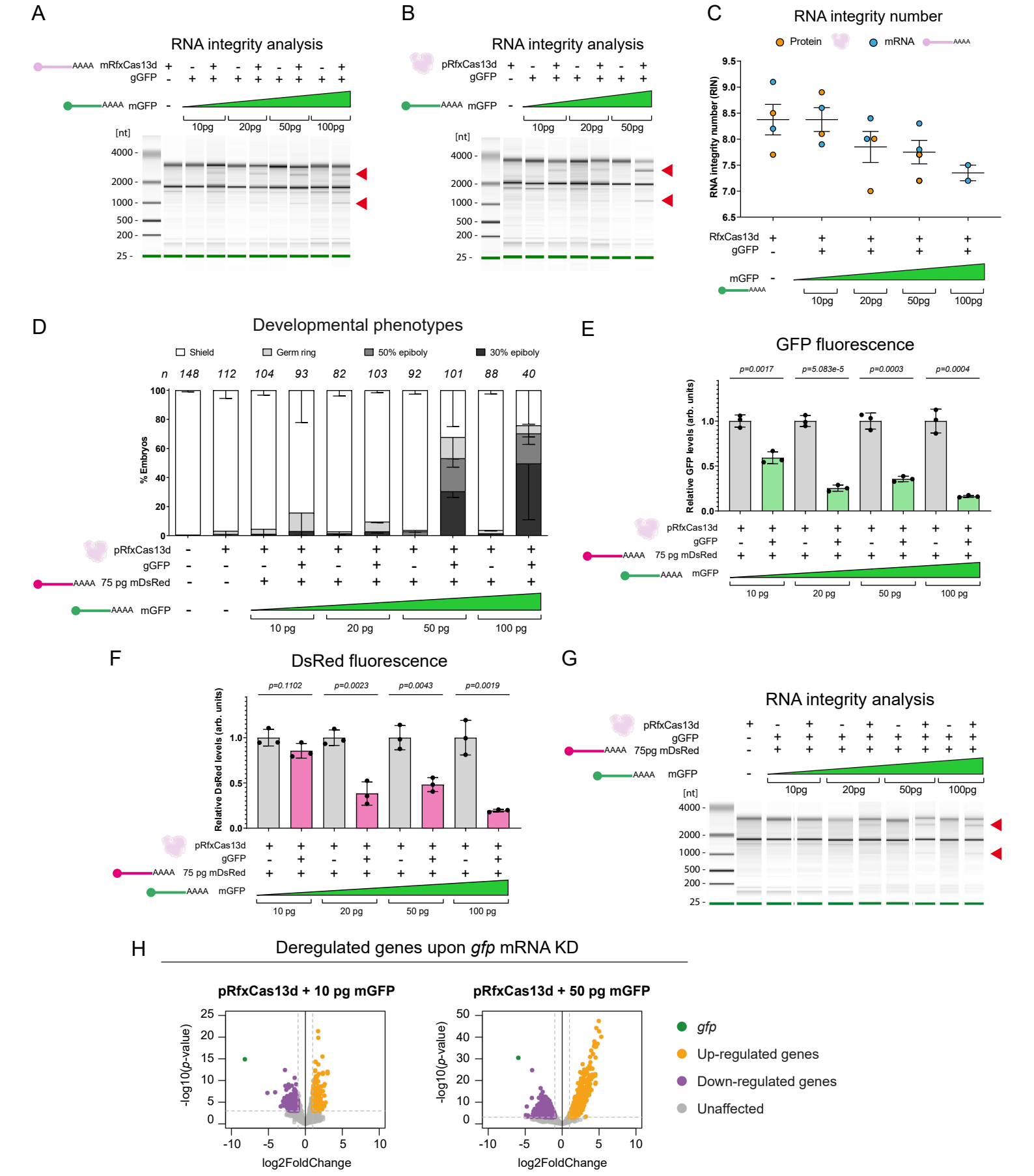
A) Volcano plots showing mRNAs targeted (red) with each set of 25 gRNAs co-injected with RfxCas13d protein in one-cell stage embryos and measured at 4 hpf by RNAseq.

B) RNA integrity images from Bioanalyzer analysis of the indicated samples. RNA samples purified at 4 hpf from injected embryos with each set of 25 gRNAs and RfxCas13d protein.

C) Heatmap of *p*-values obtained from Pearson's correlations performed with each gRNA set and prediction scores by TIGER (Wessels et al., 2024[24]), DeepCas13 (Cheng et al., 2023[25]) and RNAtargeting (Wei et al., 2023[26]) represented in **Fig. 4B**. Correlation with cell culture data from *gfp* mRNA from 396 gRNAs (Wessels et al., 2020[23]) was used as a control.

D, E, F) Violin-plots representing the score of the five highest (High) and five lowest (Low) active gRNAs per set (indicated in colors) predicted by TIGER[24] (**D**), DeepCas13[25] (**E**) and RNAtargeting[26] (**F**). The median and first and third quartiles of the 40 gRNAs score are represented as dashed and dotted lines, respectively. Data shown here is the same as represented in **Fig. 4C-E**.

p-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.


A
RNA integrity analysis
AAAA mRfxCas13d
gGFP
AAAA mGFP
10pg
20pg
50pg
100pg
[nt]
4000
2000
1000
500
200
25
B
RNA integrity analysis
pRfxCas13d
gGFP
AAAA mGFP
10pg
20pg
50pg
[nt]
4000
2000
1000
500
200
25
C
RNA integrity number
Protein
mRNA
AAAA
RNA integrity number (RIN)
9.5
9.0
8.5
8.0
7.5
7.0
6.5
RfxCas13d
gGFP
mGFP
AAAA
10pg
20pg
50pg
100pg
D
Developmental phenotypes
Shield
Germ ring
50% epiboly
30% epiboly
n 148 112 104 93 82 103 92 101 88 40
% Embryos
100
80
60
40
20
0
pRfxCas13d
gGFP
AAAA 75 pg mDsRed
AAAA mGFP
10 pg
20 pg
50 pg
100 pg
E
GFP fluorescence
p=0.0017
p=5.083e-5
p=0.0003
p=0.0004
Relative GFP levels (arb. units)
1.0
0.5
0.0
pRfxCas13d
gGFP
AAAA 75 pg mDsRed
AAAA mGFP
10 pg
20 pg
50 pg
100 pg
F
DsRed fluorescence
p=0.1102
p=0.0023
p=0.0043
p=0.0019
Relative DsRed levels (arb. units)
1.0
0.5
0.0
pRfxCas13d
gGFP
AAAA 75 pg mDsRed
AAAA mGFP
10 pg
20 pg
50 pg
100 pg
G
RNA integrity analysis
pRfxCas13d
gGFP
AAAA 75pg mDsRed
AAAA mGFP
10pg
20pg
50pg
100pg
[nt]
4000
2000
1000
500
200
25
H
Deregulated genes upon gfp mRNA KD
pRfxCas13d + 10 pg mGFP
pRfxCas13d + 50 pg mGFP
-log10(p-value)
log2FoldChange
gfp
Up-regulated genes
Down-regulated genes
Unaffected

Supplementary Figure 7. Highly abundant *dsRed* mRNA partially buffers the collateral activity triggered by CRISPR-RfxCas13d.

A, B) RNA integrity images from Bioanalyzer analysis of the indicated samples. RNA samples purified at 6 hpf from injected embryos with 300 pg *RfxCas13d* mRNA (mRfxCas13d, **A**) or 3 ng RfxCas13d protein (pRfxCas13d, **B**) together with 1 ng of a mix of three gRNAs targeting *gfp* (gGFP) and 10, 20, 50 or 100 pg of ectopic *gfp* mRNA (mGFP). Red arrowheads indicate major and minor bands of degradation of 28S ribosomal subunit.

C) Dot-blot displaying RNA integrity number calculated by Bioanalyzer from indicated samples shown in **A** and **B**. Results are shown as the averages ± standard deviation of the mean from two biological replicates. Data from samples injected with mRfxCas13d or pRfxCas13d are depicted in blue or orange, respectively.

D) Stacked barplots representing developmental phenotypes (epiboly stages) of injected embryos with pRfxCas13d together with gGFP, 75 pg of *dsRed* mRNA (mDsRed) and 10, 20 or 50 pg of mGFP. Representative images of indicated epiboly stages are shown in **Fig. 1C**. (n) total number of embryos is indicated for each condition. The results are shown as the averages ± standard deviation of the mean of each developmental stage.

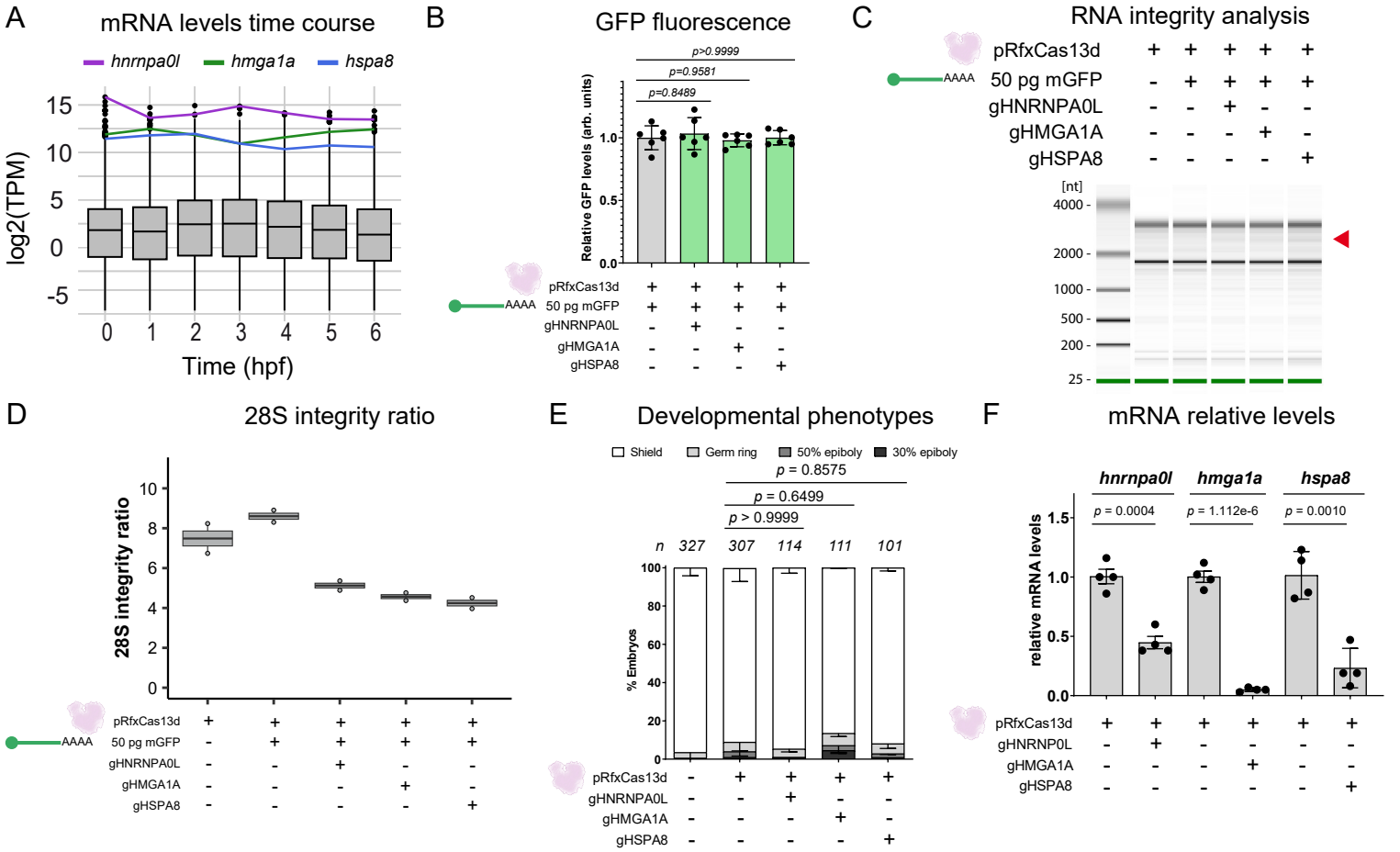
E, F) Barplots displaying GFP (**E**) and DsRed (**F**) fluorescence levels of injected embryos with pRfxCas13d together with gGFP, mDsRed and 10, 20 or 50 pg of mGFP. Results are shown as the averages ± standard deviation of the mean. *p*-values are indicated above.

G) RNA integrity analysis of the indicated samples by Bioanalyzer. RNA samples purified at 6 hpf from injected embryos with pRfxCas13d together with gGFP, mDsRed and 10, 20 or 50 pg of mGFP. Red arrowhead indicates degradation of 28S ribosomal subunit.

H) Volcano plot showing down- (purple) and upregulated (orange) genes upon *gfp* mRNA KD (10 or 50 pg) with pRfxCas13d. Fold-Change of 3 and *p*-value < 0.001 (dashed lines) were set to determine deregulated genes.

Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.

Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
mRNA levels time course
hnrnpa0l
hmga1a
hspa8
log2(TPM)
Time (hpf)
B
GFP fluorescence
p>0.9999
p=0.9581
p=0.8489
Relative GFP levels (arb. units)
pRfxCas13d
50 pg mGFP
gHNRNPA0L
gHMGA1A
gHSPA8
AAAA
C
RNA integrity analysis
pRfxCas13d
50 pg mGFP
gHNRNPA0L
gHMGA1A
gHSPA8
[nt]
4000 -
2000 -
1000 -
500 -
200 -
25 -
AAAA
D
28S integrity ratio
28S integrity ratio
pRfxCas13d
50 pg mGFP
gHNRNPA0L
gHMGA1A
gHSPA8
AAAA
E
Developmental phenotypes
Shield
Germ ring
50% epiboly
30% epiboly
p = 0.8575
p = 0.6499
p > 0.9999
n 327 307 114 111 101
% Embryos
pRfxCas13d
gHNRNPA0L
gHMGA1A
gHSPA8
F
mRNA relative levels
hnrnpa0l
hmga1a
hspa8
p = 0.0004
p = 1.112e-6
p = 0.0010
relative mRNA levels
pRfxCas13d
gHNRNP0L
gHMGA1A
gHSPA8
G
RNA integrity analysis
hnrnpa0l
hmga1a
hspa8
pRfxCas13d
gHNRNPA0L
gHMGA1A
gHSPA8
[nt]
4000 -
2000 -
1000 -
500 -
200 -
25 -

Supplementary Figure 8. CRISPR-RfxCas13d does not induce a physiologically relevant collateral activity when targeting highly expressed endogenous mRNAs.

A) Boxplots showing the expression levels of all polyadenylated RNAs during the first 4 hours of zebrafish embryo development. *hnrnpa0l*, *hmga1a* and *hspa8* mRNAs are depicted in purple, green and blue, respectively. Data from Medina-Muñoz et al., 2021[62].

B) Barplots exhibiting GFP fluorescence relative levels of injected embryos with pRfxCas13d together with gRNAs targeting endogenous *hnrnpa0l*, *hmga1a* and *hspa8* transcripts (gHNRNPA0L, gHMGA1A and gHSPA8, respectively) and 50 pg of *gfp* (mGFP) as a collateral activity reporter control. Results are shown as the averages ± standard deviation of the mean.

C) RNA integrity analysis by Bioanalyzer of RNA samples at 6 hpf from injected embryos with pRfxCas13d together with gHNRNPA0L, gHMGA1A or gHSPA8 and 50 pg mGFP. Red arrowhead indicates degradation of 28S ribosomal subunit.

D) Boxplot of 28S integrity ratio *in vivo* at 6 hpf from injected embryos with pRfxCas13d together with gHNRNPA0L, gHMGA1A or gHSPA8 and 50 pg mGFP. Individual values are represented by points. The mean, first and third quartile are represented for each condition. Vertical lines indicate the variability outside the upper and lower quartiles.

E) Stacked barplots representing developmental phenotypes (epiboly stages) of injected embryos with pRfxCas13d together with gHNRNPA0L, gHMGA1A or gHSPA8. Representative images of indicated epiboly stages are shown in **Fig. 1C**. (n) total number of embryos is indicated for each condition. The results are shown as the averages ± standard deviation of the mean of each developmental stage.

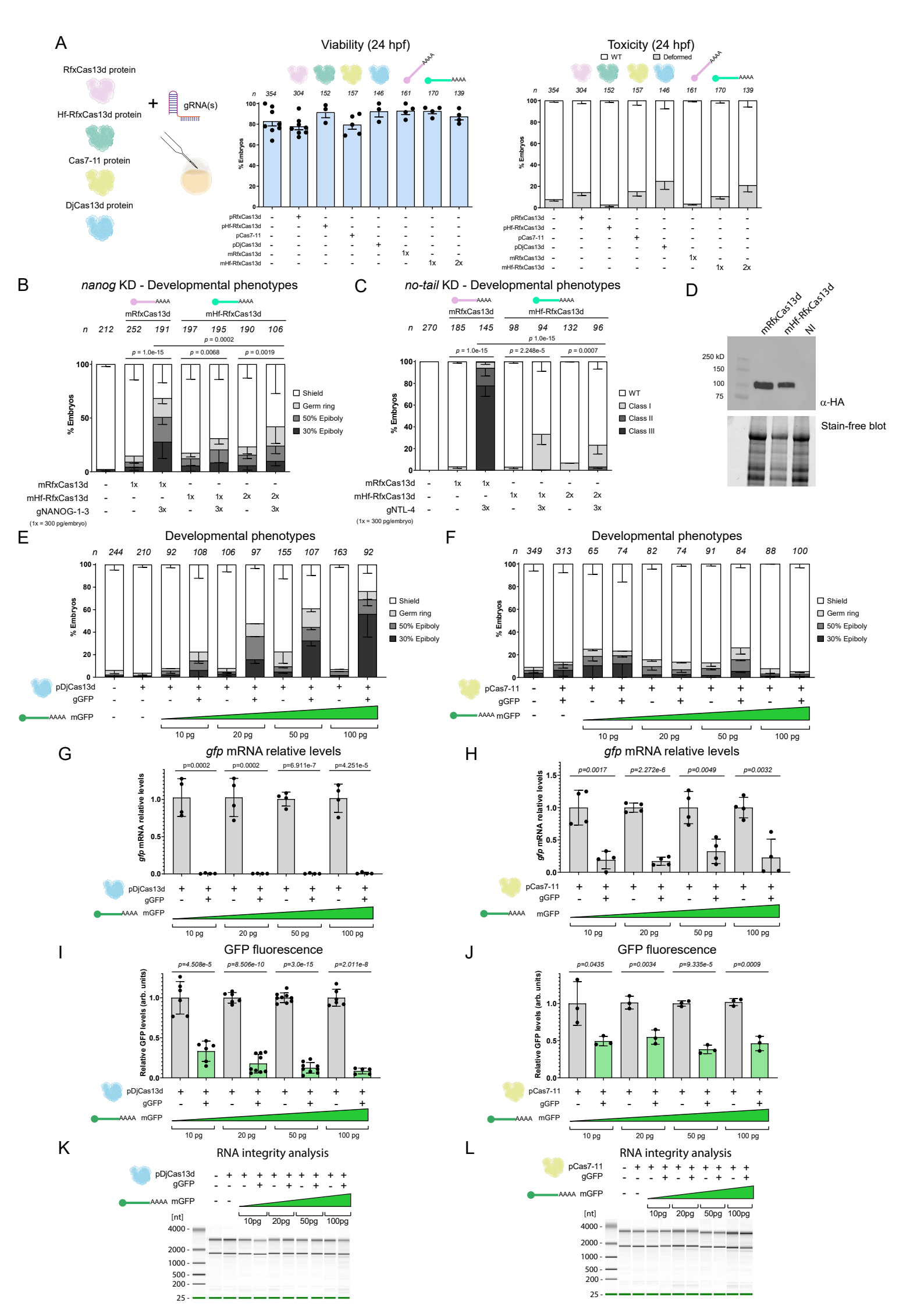
F) Barplots indicating mRNA relative levels analyzed by qRT-PCR of injected embryos with pRfxCas13d together with gHNRNPA0L, gHMGA1A or gHSPA8. Results are shown as the averages ± standard deviation of the mean. *taf15* mRNA was used as a normalization control.

G) RNA integrity analysis of the *hnrnpa0l*, *hmga1a* and *hspa8* mRNA KD by Bioanalyzer. RNA samples purified at 6 hpf from injected embryos with

pRfxCas13d together with gHNRNPA0L, gHMGA1A or gHSPA8. Red arrowhead indicates degradation of 28S ribosomal subunit.

p-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data isa provided as a Source Data file.

Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
RfxCas13d protein
Hf-RfxCas13d protein
Cas7-11 protein
DjCas13d protein
gRNA(s)
Viability (24 hpf)
Toxicity (24 hpf)
WT
Deformed
% Embryos
pRfxCas13d
pHf-RfxCas13d
pCas7-11
pDjCas13d
mRfxCas13d
mHf-RfxCas13d
B
nanog KD - Developmental phenotypes
Shield
Germ ring
50% Epiboly
30% Epiboly
gNANOG-1-3
(1x = 300 pg/embryo)
C
no-tail KD - Developmental phenotypes
WT
Class I
Class II
Class III
gNTL-4
D
mRfxCas13d
mHf-RfxCas13d
NI
250 kD
150
100
75
α-HA
Stain-free blot
E
Developmental phenotypes
pDjCas13d
gGFP
mGFP
10 pg
20 pg
50 pg
100 pg
F
Developmental phenotypes
pCas7-11
G
gfp mRNA relative levels
H
gfp mRNA relative levels
I
GFP fluorescence
Relative GFP levels (arb. units)
J
GFP fluorescence
K
RNA integrity analysis
[nt]
L
RNA integrity analysis

Supplementary Figure 9. CRISPR-Cas7-11 RNP complexes do not exhibit collateral activity.

A) Schematic illustration of the experimental setup used to compare alternative and high-fidelity CRISPR-Cas RNA targeting systems (Hf-RfxCas13d, DjCas13d and Cas7-11) with CRISPR-RfxCas13d (left). Barplots showing viability (mid) and toxicity (right) at 24 hpf from embryos injected with RfxCas13d, Hf-RfxCas13d, DjCas13d and Cas7-11 proteins, and *RfxCas13d* and *Hf-RfxCas13d* mRNAs. 3 ng of each purified protein were injected in one-cell stage zebrafish embryos. 1x indicates 300 pg of mRNA per embryo.

B, C) Stacked barplots showing the percentage of observed phenotypes of injected embryos with *RfxCas13d* or *Hf-RfxCas13d* mRNA together with 1 ng of a mix of three gRNAs targeting *nanog* (**A**, gNANOG1-3) and 1 ng of one gRNA targeting *no-tail* (**B**, gNTL-4). 1x indicates 300 pg of mRNA or gRNA per embryo.

D) Western blot of RfxCas13d and Hf-RfxCas13d protein samples from 6 hpf zebrafish embryos injected with 300 pg of each mRNA (detected by α-HA primary antibody). Stain-free blot image is shown as loading control.

E, F) Stacked barplots representing developmental phenotypes (epiboly stages) of injected embryos with DjCas13d (**E**) or Cas7-11 (**F**) together with 1 ng of a mix of three gRNAs (gGFP) targeting 10, 20, 50 or 100 pg of ectopic *gfp* mRNA (mGFP).

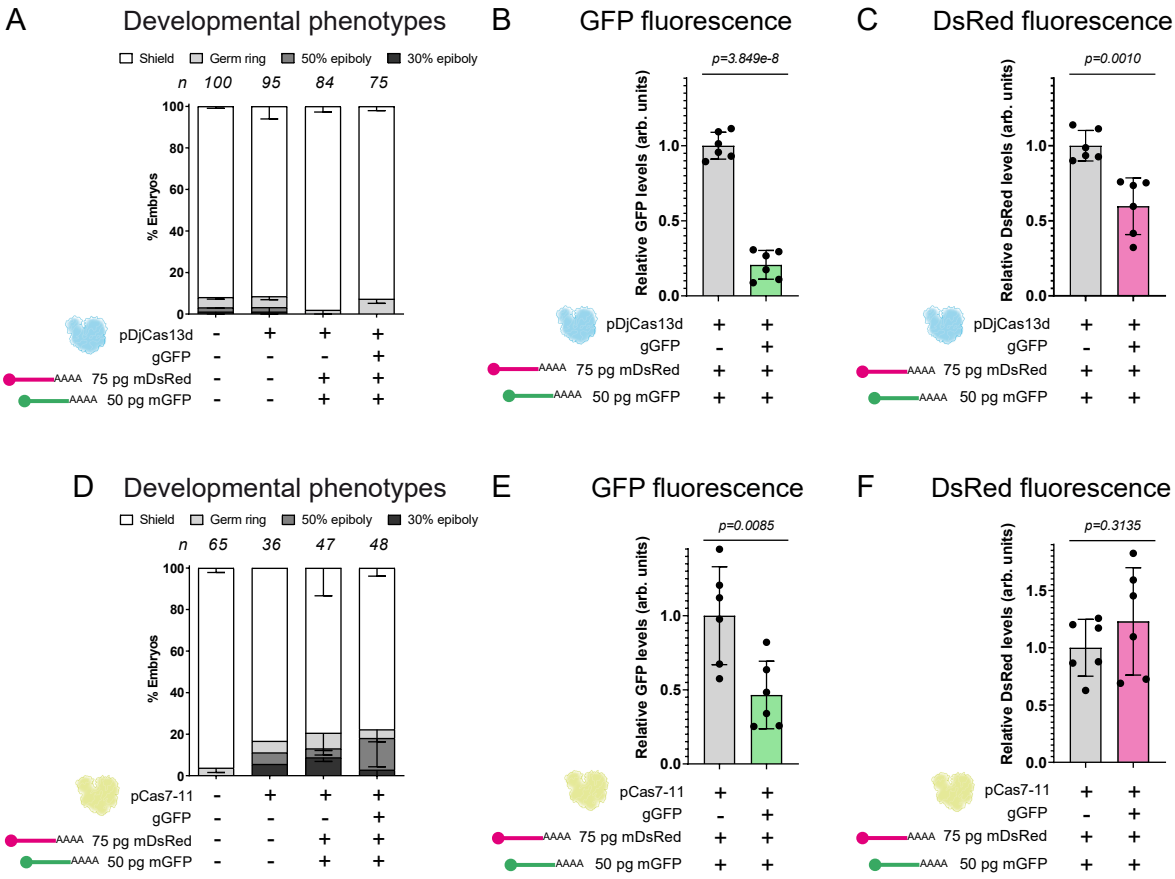
G, H) Barplots showing *gfp* mRNA relative levels measured by qPCR of injected embryos with DjCas13d (**G**) or Cas7-11 (**H**) together with gGFP targeting 10, 20, 50 or 100 pg of mGFP. *taf15* mRNA was used as a normalization control.

I, J) Barplots showing GFP fluorescence levels of injected embryos with DjCas13d (**I**) or Cas7-11 (**J**) together with gGFP targeting 10, 20, 50 or 100 pg of mGFP.

K, L) RNA integrity analysis of the indicated samples by Bioanalyzer. RNA samples purified at 6 hpf from injected embryos with DjCas13d (**K**) or Cas7-11 (**L**) together with gGFP and 10, 20, 50 or 100 pg of mGFP.

Representative images of epiboly stages and *no-tail* phenotypes are exhibited in **Fig. 1C**. (n) total number of injected embryos is indicated for each condition. The viability, toxicity and phenotypic results are shown as the averages ± standard deviation of the mean of each developmental stage (**A**, **B**, **C**, **D** and **F**). mRNA relative levels measured by qRT-PCR (**G** and **H**) and GFP fluorescence levels (**I**

and **J**) are shown as the averages ± standard deviation of the mean. *p*-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.
Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
Developmental phenotypes
Shield
Germ ring
50% epiboly
30% epiboly
n 100 95 84 75
% Embryos
pDjCas13d - + + +
gGFP - - - +
AAAA 75 pg mDsRed - - + +
AAAA 50 pg mGFP - - + +
B
GFP fluorescence
p=3.849e-8
Relative GFP levels (arb. units)
pDjCas13d + +
gGFP - +
AAAA 75 pg mDsRed + +
AAAA 50 pg mGFP + +
C
DsRed fluorescence
p=0.0010
Relative DsRed levels (arb. units)
pDjCas13d + +
gGFP - +
AAAA 75 pg mDsRed + +
AAAA 50 pg mGFP + +
D
Developmental phenotypes
Shield
Germ ring
50% epiboly
30% epiboly
n 65 36 47 48
% Embryos
pCas7-11 - + + +
gGFP - - - +
AAAA 75 pg mDsRed - - + +
AAAA 50 pg mGFP - - + +
E
GFP fluorescence
p=0.0085
Relative GFP levels (arb. units)
pCas7-11 + +
gGFP - +
AAAA 75 pg mDsRed + +
AAAA 50 pg mGFP + +
F
DsRed fluorescence
p=0.3135
Relative DsRed levels (arb. units)
pCas7-11 + +
gGFP - +
AAAA 75 pg mDsRed + +
AAAA 50 pg mGFP + +

Supplementary Figure 10. CRISPR-DjCas13d RNP complexes induce less unspecific RNA degradation and collateral activity than CRISPR-RfxCas13d.

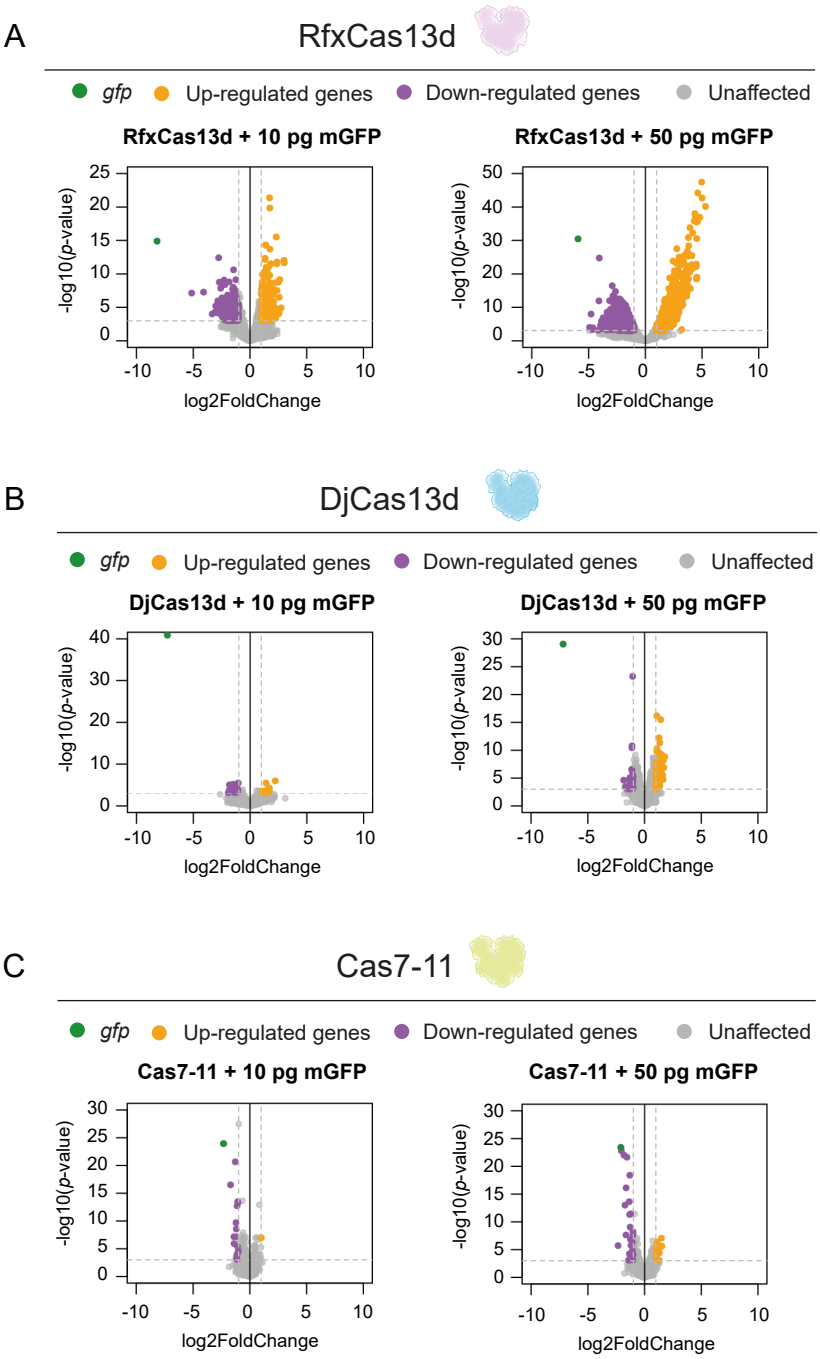
A, D) Stacked barplots representing developmental phenotypes (epiboly stages) of injected embryos with DjCas13d (**A**) or Cas7-11 (**D**) together with gGFP, 50 pg of mGFP and 75 pg of ectopic *dsRed* mRNA (mDsRed). Representative images of indicated epiboly stages are shown in **Fig. 1C**. (n) total number of embryos is indicated for each condition. The results are shown as the averages ± standard deviation of the mean of each developmental stage.

B, E) Barplots showing GFP fluorescence levels of injected embryos with DjCas13d (**B**) or Cas7-11 (**E**) together with gGFP, 50 pg of mGFP and 75 pg of mDsRed.

C, F) Barplots showing DsRed fluorescence levels of injected embryos with DjCas13d (**C**) or Cas7-11 (**F**) together with gGFP, 50 pg of mGFP and 75 pg of mDsRed.

GFP (**B** and **E**) and DsRed (**C** and **F**) fluorescence levels are shown as the averages ± standard deviation of the mean. *p*-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.

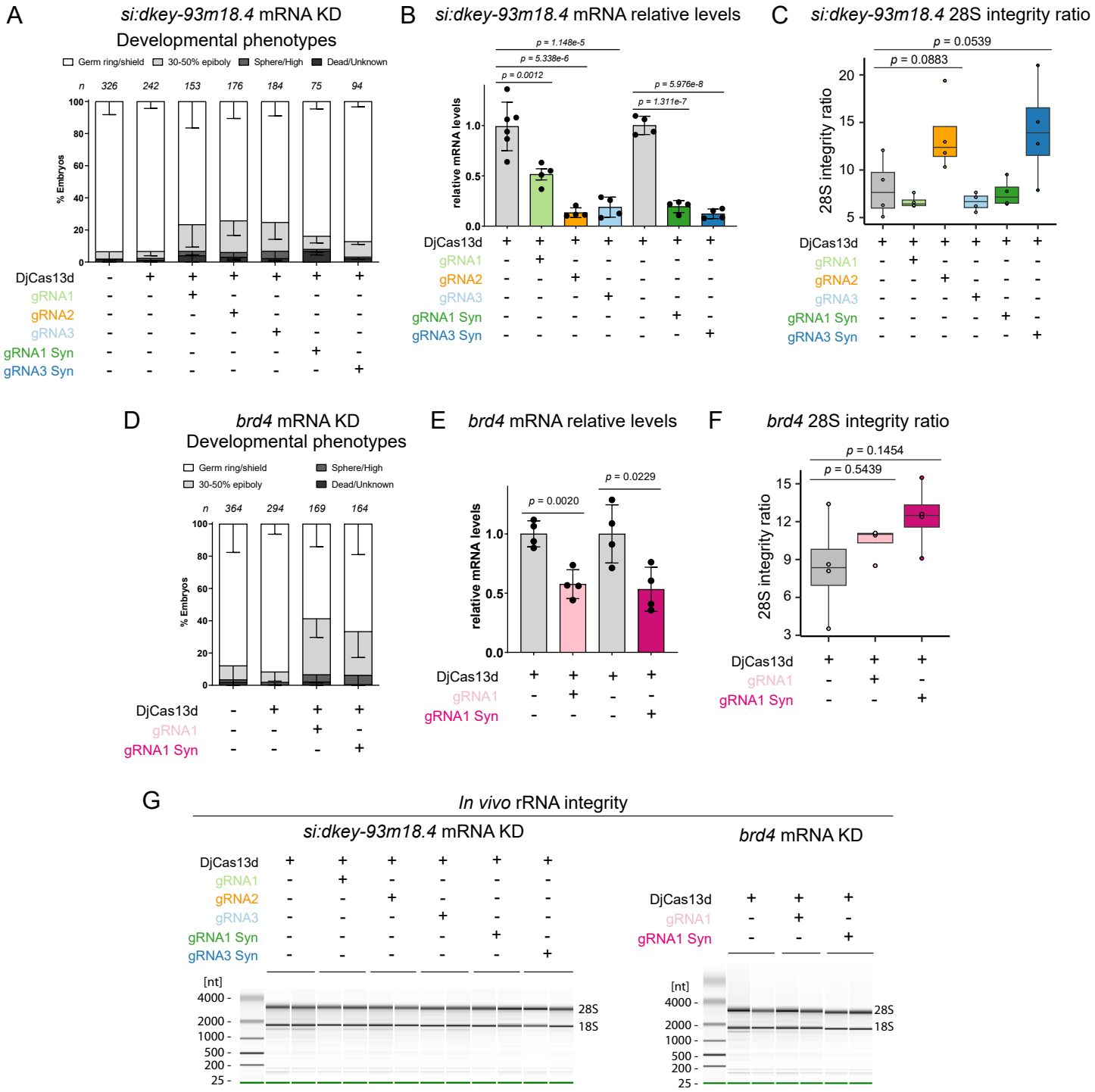
Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
RfxCas13d
gfp
Up-regulated genes
Down-regulated genes
Unaffected
RfxCas13d + 10 pg mGFP
RfxCas13d + 50 pg mGFP
-log10(p-value)
log2FoldChange
B
DjCas13d
DjCas13d + 10 pg mGFP
DjCas13d + 50 pg mGFP
C
Cas7-11
Cas7-11 + 10 pg mGFP
Cas7-11 + 50 pg mGFP

Supplementary Figure 11. DjCas13d did not lead to massive deregulation upon collateral activity.

A, B, C) Volcano plots showing down- (purple) and upregulated (orange) genes upon 10 or 50 pg mGFP KD with RfxCas13d (**A**, same data from **Supplementary Fig. 7H**), DjCas13d (**B**) and Cas7-11 (**C**) measured by RNAseq. *gfp* levels are depicted in green. Fold-Change of 2 and *p*-value < 0.001 (dashed lines) were set to determine deregulated genes. Statistical information is shown in **Supplementary Data 1**.

Schematics adapted from Developmental Cell, Volume 54, Issue 6, Gopal Kushawah et al. CRISPR-Cas13d Induces Efficient mRNA Knockdown in Animal Embryos, 805-817.e7, Copyright (2020), with permission from Elsevier.


A
si:dkey-93m18.4 mRNA KD
Developmental phenotypes
Germ ring/shield
30-50% epiboly
Sphere/High
Dead/Unknown
n 326 242 153 176 184 75 94
% Embryos
DjCas13d
gRNA1
gRNA2
gRNA3
gRNA1 Syn
gRNA3 Syn
B
si:dkey-93m18.4 mRNA relative levels
p = 1.148e-5
p = 5.338e-6
p = 0.0012
p = 5.976e-8
p = 1.311e-7
relative mRNA levels
C
si:dkey-93m18.4 28S integrity ratio
p = 0.0539
p = 0.0883
28S integrity ratio
D
brd4 mRNA KD
Developmental phenotypes
n 364 294 169 164
E
brd4 mRNA relative levels
p = 0.0020
p = 0.0229
F
brd4 28S integrity ratio
p = 0.1454
p = 0.5439
G
In vivo rRNA integrity
si:dkey-93m18.4 mRNA KD
brd4 mRNA KD
[nt]
4000
2000
1000
500
200
25
28S
18S

Supplementary Figure 12. DjCas13d *in vitro* transcribed gRNAs do not induce toxicity

A) Stacked barplots showing percentage of developmental effect of *si:dkey-93m18.4* KD with DjCas13d at 6 hpf using *in vitro* transcribed gRNAs (IVTed, gRNAs 1-3) or chemically synthetized (Syn) gRNAs (200-300 pg/embryo).

B) Barplot showing relative *si:dkey-93m18.4* mRNA levels measured by qRT-PCR at 4 hpf from injected embryos in **A**. *cdk2ap2* mRNA was used as a normalization control.

C) Boxplot of 28S integrity ratio *in vivo* at 4 hpf from DjCas13d mediated *si:dkey-93m18.4* KD and control embryos.

D) Stacked barplots showing percentage of developmental effect of *brd4* KD at 6 hpf using an *in vitro* transcribed or chemically synthetized gRNA (IVTed, gRNA1 and Syn, respectively; 300 pg of gRNA/embryo).

E) Barplot showing relative *brd4* mRNA levels measured by qRT-PCR at 4 hpf from injected embryos in **D**. *taf15* mRNA was used as a normalization control.

F) Boxplot of 28S integrity ratio *in vivo* at 4 hpf from RfxCas13d mediated *brd4* KD and control embryos.

G) RNA integrity images from bioanalyzer of total RNA from DjCas13d mediated *si:dkey-93m18.4* and *brd4* KD and control embryos at 4 hpf.

(n) total number of injected embryos is shown for each condition. The phenotypic results are shown as the averages ± standard deviation of the mean of each developmental stage (**A** and **D**). mRNA relative levels measured by qRT-PCR (**B** and **E**) are shown as the averages ± standard deviation of the mean. Individual values are represented by points in boxplots (**C** and **F**). The mean, first and third quartile are represented for each condition. Vertical lines indicate the variability outside the upper and lower quartiles. *p*-values are indicated above. Statistical information is shown in **Supplementary Data 1**. Source data is provided as a Source Data file.