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Supplemental Information

Innate Immune Response and Off-Target Mis-splicing Are Common Morpholino-Induced Side Effects in *Xenopus*

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Legends for Supplemental Figures

Figure S1. Generation of the Double Heterozygous Line for *Brachyury* paralogues *t* and *t2*, Related to Figure 1

(A) Scheme to generate the $t^{e1.2D/+}t2^{e3.7D/+}$ ($t^{-/+}t2^{-/+}$) *X. tropicalis* line. (B,F) TALEN design for *t* or *t2* mutagenesis and positions of MOs blocking donor splice site (MO_{splice}) or translation initiation site (MO_{transl}) of the corresponding transcript. (C,G) TALEN-induced mutation rate at the targeted *Sac*I or *EcoR*I site as estimated by the partial restriction digest of specific PCR amplicons. (D) Sanger sequencing summary of generated indels in exon 1 of *t*. (E) Morphological defects caused by TALEN-induced *t* mutations at late tailbud stage. Scale bar, 0.5 mm. (H) Western blot of injected wild-type and mutant *t* or *t2* constructs tagged either N- or C-terminally with *HA*. The detection of exogenous myc (as part of the injected *fam83g-myc* mRNA) and endogenous α-tubulin were used as controls for injection/translation efficiency and gel electrophoresis loading, respectively. (I) Mutant *Brachyury* constructs failed to disrupt gastrulation. Scale bar, 0.25 mm.

Figure S2. *Brachyury* KO and KD Embryos Are Morphologically Very Similar, Related to Figure 2

(A) Gross morphological comparison between different conditions of the KD and KO experiment at the indicated tailbud stages. Uninjected (uni) and control MO (cMO)-injected embryos were controls for the *t/t2* morphants (*t/t2* MO). Wild-type (wt) embryos were siblings of the mutant embryos (hetero- and homozygous for the mutant *t* and *t2* allele, *t/t2* het and *t/t2* KO). Scale bar, 0.5 mm. (B) Comparison of low fragment count gene transcripts to estimate minimal fragment count required for calling reliable fold changes. Genes that averaged <7 fragments between cMO-injected and uninjected and heterozygous and wild-type embryos over tailbud stage 26 and 34 were excluded due to a higher degree of spurious fold changes.

Figure S3. Increased Transcription of *tp53* Depending on GC Content of MO Does Not Cause More Apoptosis, Related to Figure 3 and 4

(A) TUNEL assay on morphants and sibling embryos from double heterozygous $t^{-/+}t2^{+/-}$ parents. DNase-treated wild-type embryos were used as positive controls. (B) Single WMISH for *tp53 and* multi-probe WMISH for various mesoderm cell lineage and

derivative markers (*cav1*, notochord; *hoxd8*, pronephros; *myh6*, heart; *tal1*, ventral blood island; *tbx6*, paraxial mesoderm) of late tailbud embryos injected with single MOs or tracer sulforhodamine-dextran. Scale bar, 0.5 mm.

Figure S4. Specific MOs of the *t/t2* MO Cocktail Cause Off-Target Splicing Defects, Related to Figure 5

(A,C) Superimposed Sashimi plot of *abi1* and *bloc1s4* transcripts whose splicing was perturbed by the injected *t/t2* MO mix. Canonical and alternative splicing are shown with solid and dashed lines, respectively. Blocked splice sites containing matches of ≥8 consecutive bases with a specific MO are shown as alignments. Canonical Watson-Crick and non-canonical wobble base pairing are marked as vertical bar and colon, respectively. (B,D) RT-qPCR (n = 4, mean ± SD) confirmed that specific MOs of the *t/t2* MO mix were responsible for mis-splicing. The fold change (log_2 scale) of transcript levels and alternative splicing between exon 7 and 11 (*abi1*) and exon 4 and 6 (*bloc1s4*) are shown as filled and solid bars, respectively. Two-tailed t-test: *, p ≤0.1; **, p ≤0.01. See Figure 3D and Key Resources Table for the design of RT-qPCR primers.

Figure S5. Temperature and MO Dosage Effects on *Brachyury* Phenotype and Immune Response Related Gene Transcription, Related to Figure 7

(A) WMISH for *tp53* of late tailbud embryos injected with 4.5 or 18 ng of the *t/t2* MO mix and developed at 22 ºC or 28.5ºC. *, Remark: Increasing incubation temperature also slightly up-regulated *tp53* in the absence of any MO (confirmed by RT-qPCR, data not shown). (B) Multi-probe WMISH for various mesoderm cell lineage and derivative markers (*cav1*, notochord; *hoxd8*, pronephros; *myh6*, heart; *tal1*, ventral blood island; *tbx6*, paraxial mesoderm) and single WMISH for *tp53, tp53inp1* and *c3ar1* of mid-tailbud (stage 26) and late tailbud embryos (stage 34) injected with 6 or 18 ng of the *t/t2* MO mix. White arrowheads point to the expression domains of *tbx6* and *cav1* that were not maintained in embryos without functional Brachyury. (C) WMISH for *tp53inp1* on wild-type (or *t/t2* heterozygous) and *t/t2* null mutant embryos as well as embryos injected with 1 and 3 ng MO. The embryos were developed to late tailbud stage 34 at 25-26ºC. Scale bar, 0.5 mm.

Figure S1

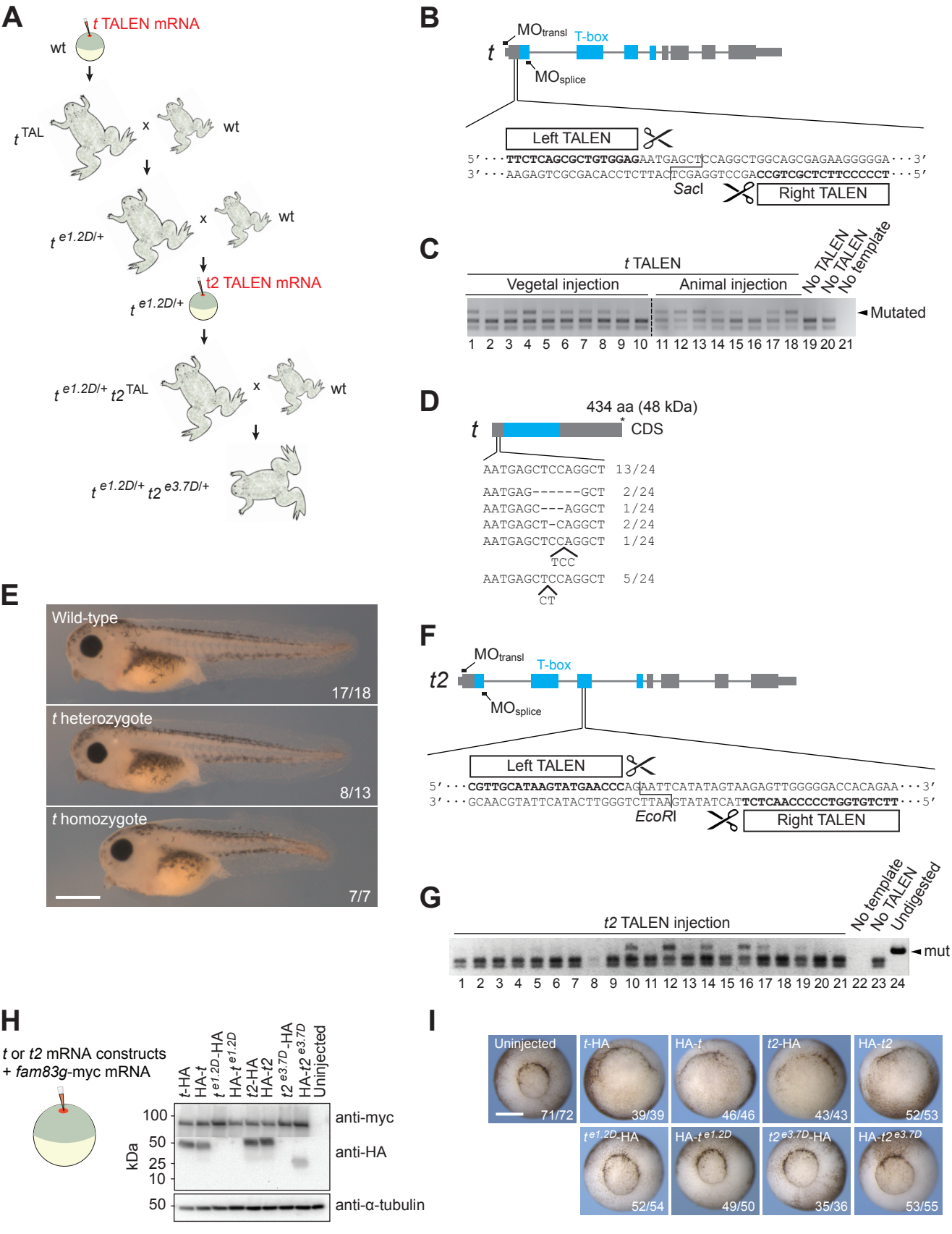
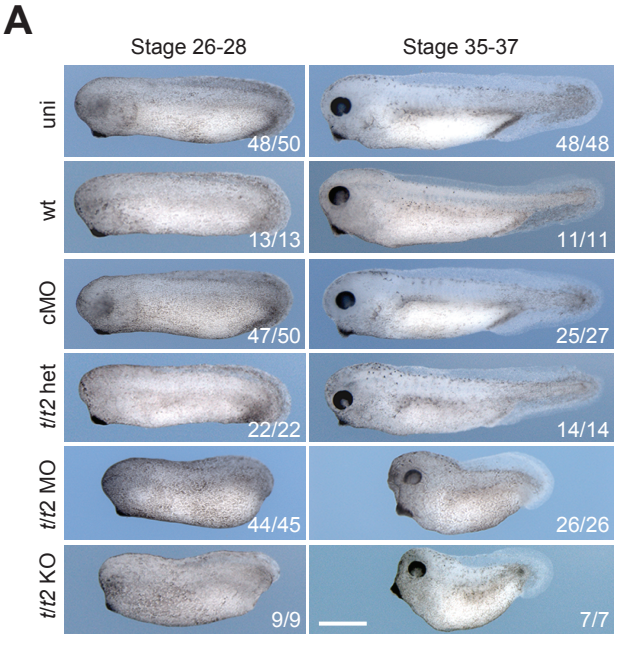

A
t TALEN mRNA
wt
t TAL
x
wt
t e1.2D/+
x
wt
t2 TALEN mRNA
t e1.2D/+
t e1.2D/+ t2 TAL
x
wt
t e1.2D/+ t2 e3.7D/+
B
MOtransl
T-box
t
MOsplice
Left TALEN
5′···TTCTCAGCGCTGTGGAGAATGAGCTCCAGGCTGGCAGCGAGAAGGGGGA···3′
3′···AAGAGTCGCGACACCTCTTACTCGAGGTCCGACCGTCGCTCTTCCCCCT···5′
SacI
Right TALEN
C
t TALEN
Vegetal injection
Animal injection
No TALEN
No TALEN
No template
Mutated
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D
434 aa (48 kDa)
t
CDS
AATGAGCTCCAGGCT 13/24
AATGAG------GCT 2/24
AATGAGC---AGGCT 1/24
AATGAGCT-CAGGCT 2/24
AATGAGCTCCAGGCT 1/24
TCC
AATGAGCTCCAGGCT 5/24
CT
E
Wild-type
17/18
t heterozygote
8/13
t homozygote
7/7
F
MOtransl
T-box
t2
MOsplice
Left TALEN
5′···CGTTGCATAAGTATGAACCCAGAATTCATATAGTAAGAGTTGGGGGACCACAGAA···3′
3′···GCAACGTATTCATACTTGGGTCTTAAGTATATCATTCTCAACCCCCTGGTGTCTT···5′
EcoRI
Right TALEN
G
t2 TALEN injection
No template
No TALEN
Undigested
mut
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
H
t or t2 mRNA constructs
+ fam83g-myc mRNA
t-HA
HA-t
t e1.2D-HA
HA-t e1.2D
t2-HA
HA-t2
t2 e3.7D-HA
HA-t2 e3.7D
Uninjected
kDa
100
50
25
10
50
anti-myc
anti-HA
anti-α-tubulin
I
Uninjected
71/72
t-HA
39/39
HA-t
46/46
t2-HA
43/43
HA-t2
52/53
t e1.2D-HA
52/54
HA-t e1.2D
49/50
t2 e3.7D-HA
35/36
HA-t2 e3.7D
53/55

Figure S2


A
Stage 26-28
Stage 35-37
uni
wt
cMO
t/t2 het
t/t2 MO
t/t2 KO
48/50
48/48
13/13
11/11
47/50
25/27
22/22
14/14
44/45
26/26
9/9
7/7

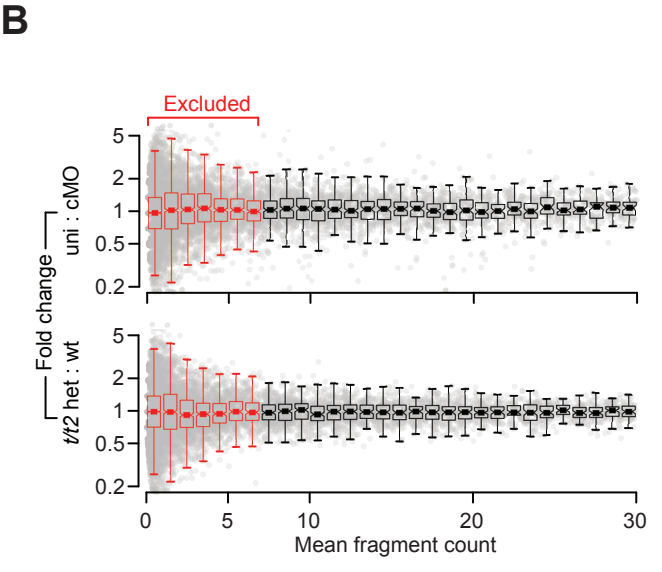

B
Excluded
Fold change
uni : cMO
t/t2 het : wt
5
2
1
0.5
0.2
0
5
10
20
30
Mean fragment count

Figure S3

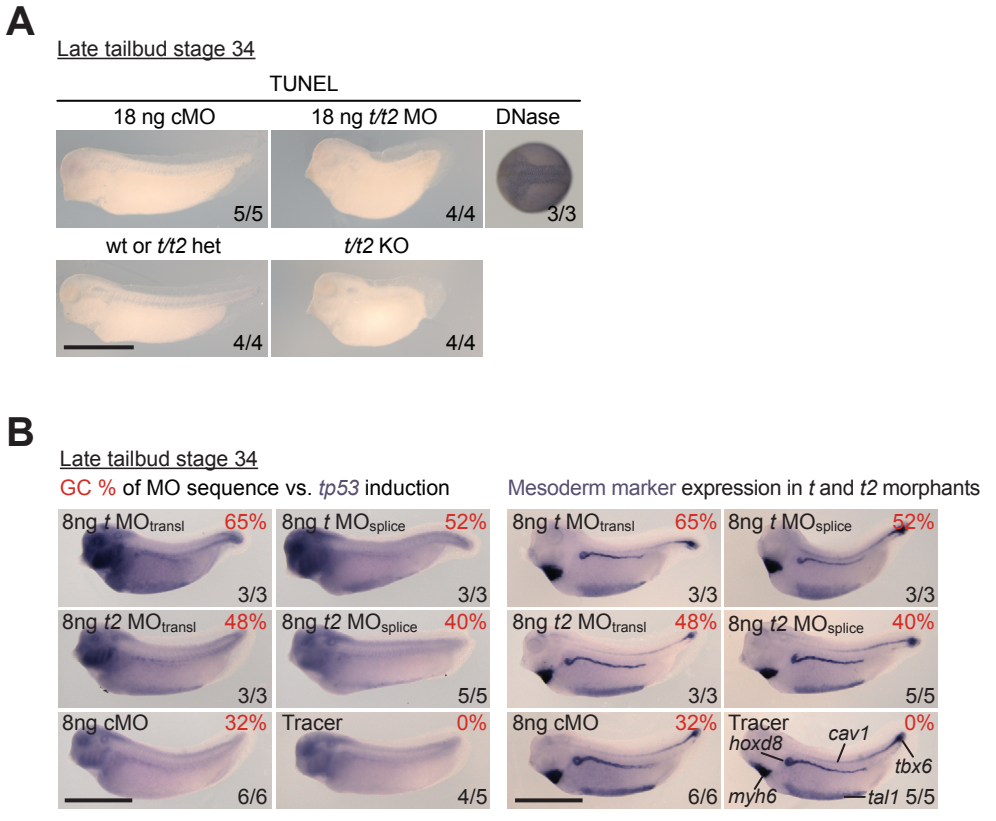

A
Late tailbud stage 34
TUNEL
18 ng cMO
18 ng t/t2 MO
DNase
5/5
4/4
3/3
wt or t/t2 het
t/t2 KO
4/4
4/4
B
Late tailbud stage 34
GC % of MO sequence vs. tp53 induction
Mesoderm marker expression in t and t2 morphants
8ng t MOtransl 65%
3/3
8ng t MOsplice 52%
3/3
8ng t2 MOtransl 48%
3/3
8ng t2 MOsplice 40%
5/5
8ng cMO 32%
6/6
Tracer 0%
4/5
8ng t MOtransl 65%
3/3
8ng t MOsplice 52%
3/3
8ng t2 MOtransl 48%
3/3
8ng t2 MOsplice 40%
5/5
8ng cMO 32%
6/6
Tracer 0%
hoxd8
cav1
tbx6
myh6
tal1 5/5

Figure S4

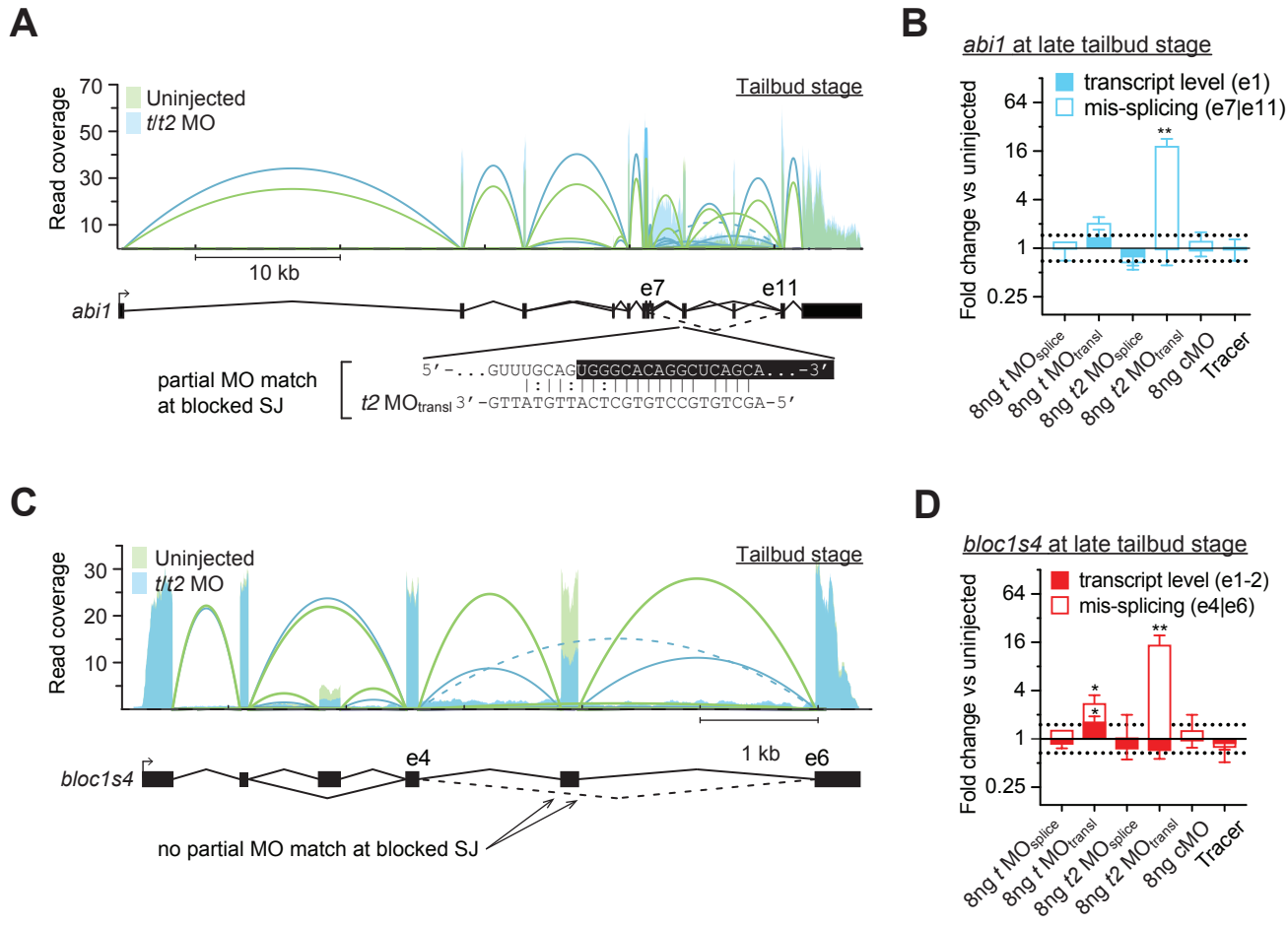
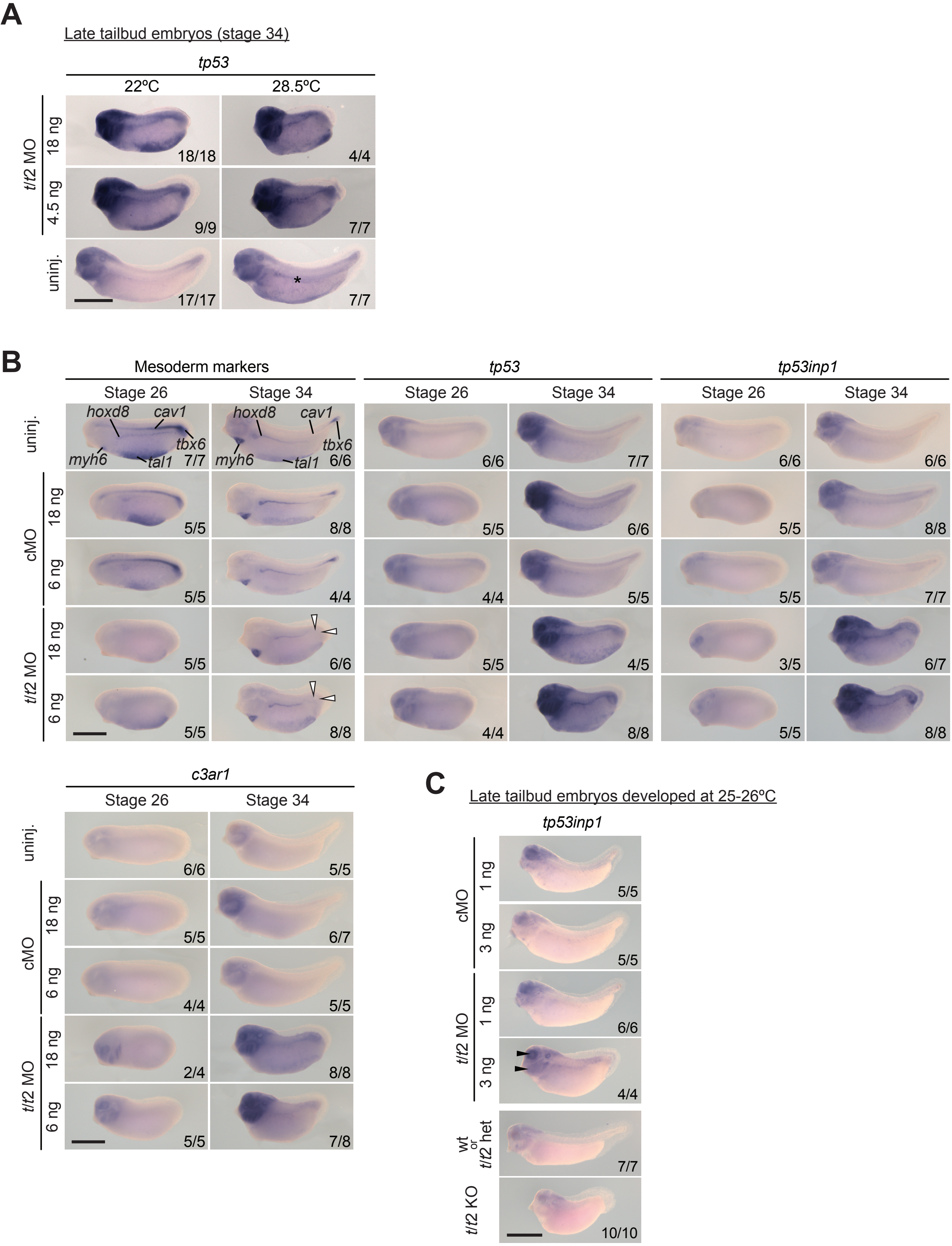

A
Uninjected
t/t2 MO
Tailbud stage
Read coverage
70
50
30
10
10 kb
abi1
e7
e11
partial MO match
at blocked SJ
5′-...GUUUGCAGUGGGCACAGGCUCAGCA...-3′
t2 MOtransl 3′-GTTATGTTACTCGTGTCCGTGTCGA-5′
B
abi1 at late tailbud stage
transcript level (e1)
mis-splicing (e7|e11)
Fold change vs uninjected
64
16
4
1
0.25
8ng t MOsplice
8ng t MOtransl
8ng t2 MOsplice
8ng t2 MOtransl
8ng cMO
Tracer
C
Uninjected
t/t2 MO
Tailbud stage
Read coverage
30
20
10
bloc1s4
e4
e6
1 kb
no partial MO match at blocked SJ
D
bloc1s4 at late tailbud stage
transcript level (e1-2)
mis-splicing (e4|e6)
Fold change vs uninjected
64
16
4
1
0.25
8ng t MOsplice
8ng t MOtransl
8ng t2 MOsplice
8ng t2 MOtransl
8ng cMO
Tracer

Figure S5



A
Late tailbud embryos (stage 34)
tp53
22ºC
28.5ºC
t/t2 MO
18 ng
4.5 ng
uninj.
18/18
4/4
9/9
7/7
17/17
7/7
*
B
Mesoderm markers
tp53
tp53inp1
Stage 26
Stage 34
hoxd8
cav1
tbx6
myh6
tal1
uninj.
cMO
t/t2 MO
18 ng
6 ng
7/7
6/6
6/6
7/7
6/6
6/6
5/5
8/8
5/5
6/6
5/5
8/8
5/5
4/4
4/4
5/5
5/5
7/7
5/5
6/6
5/5
4/5
3/5
6/7
5/5
8/8
4/4
8/8
5/5
8/8
c3ar1
Stage 26
Stage 34
6/6
5/5
5/5
6/7
4/4
5/5
2/4
8/8
5/5
7/8
C
Late tailbud embryos developed at 25-26ºC
tp53inp1
cMO
t/t2 MO
1 ng
3 ng
5/5
5/5
6/6
4/4
wt or t/t2 het
7/7
t/t2 KO
10/10