



Supporting Online Material for

Target Protectors Reveal Dampening and Balancing of Nodal Agonist and Antagonist by miR-430

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SUPPORTING ONLINE MATERIAL

Target Protectors Reveal Dampening and Balancing of Nodal Agonist and Antagonist by miR-430

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Materials and Methods

GFP reporter and mRNA misexpression constructs

GFP reporter: The 3' UTR of the gene of interest was amplified by PCR from a 0-24 hour post-fertilization (hpf) cDNA library. The primers used for *sqt*-3'UTR, *lft2*-3'UTR, and *lft1*-3'UTR were:

sqt 5'-AAACTCGAGGGCTGCCACTGATTCTTCA-3',
5'-AAATCTAGACTATTTACAGATAAGGCCAAACACG-3';
lft2 5'-AAACTCGAGAGTGTGGTGTCTGAATAGTTTGCT-3',
5'-CAATCTAGATACTTTATTTTTCAAACATCACATCTC-3';
lft1 5'-AAAGTCGACGTATAAATAGCCTGTTTGTATCGA-3',
5'-AAATCTAGAACCCGTGCTATATGCTCA-3'.

PCR fragments were digested and cloned into pCS2+GFP between Xho-Xba.

Mutant GFP reporter: The 3' UTR of the gene of interest was amplified in two fragments. The two fragments had a ~40 nt overlap in the mutant region. The mutation in the miR-430 target site was included in the bottom primer of the 5' fragment and the top primer of the 3' fragment. The mismatch primers for *sqt*, *lft2*, and *lft1* were:

sqt 5'-AGGAACTCAACTCTAGGTCTTTGGATATGCTCCTTGACCCC-3',
5'-GAGCATATCCAAAGACCTAGAGTTGAGTTCCTTTGGGG-3';
lft2 5'-CCAAATCCCCACTGAGGTCTTAAAAACAAAGTATTTATGAAT-3',
5'-ACTTTGTTTTTCAAGACCTCAGTGGGGATTTGGGG-3';
lft1 5'-TACTAGATACCTCGAGGTCTTTGTATAGACTTGTAATAGA-3',
5'-ACAAGTCTATACAAAGACCTCGAGGTATCTAGTAAGGGTT-3'.

The full-length 3' UTR was obtained by PCR across the mismatch fragments with the 3'UTR primers described above.

sqt/miR-1 GFP reporter: The miR-1 target site was added to the *sqt*-GFP reporter by ligation of a fragment containing an imperfect miR-1 site (5'-ctagctcgagaTACATACTTCTaatCATTCCAta-3', 5'-ctagtaTGGAATGattAGAAGTATGTAtctcgag-3').

sqt, *lft2* and *lft1* full-length mRNA constructs: The gene of interest, including its 3'UTR, was amplified by PCR from a 0-24 hpf cDNA library using the top primers:

sqt 5'-AAACTCGAGCCTTGATTGACATGTTTTCC-3'

lft2 5'-AAACTCGAGGGCACGAGCATGGCTCTG-3'

lft1 5'-AAACTCGAGCACCTTGAAAAGATGACTTCA-3'.

Bottom primers were the same as for amplifying the 3'UTRs. The mismatches in the miR-430 target site were introduced as described for the GFP reporters.

mRNA synthesis, target validation, miRNA duplexes and injection

mRNA for injection was generated using the Message machine kit according to manufacturer's instructions (Ambion). 1 nL of a 0.2 ng/nl GFP reporter mRNA solution was injected into wild-type or *MZdicer* embryos at the one-cell stage. DsRed control mRNA was co-injected with the GFP reporter mRNA at 0.1 ng/nl.

miRNA duplex: The miRNA duplexes were purchased from IDT and resuspended in the manufacturer's buffer to a concentration of 100 μ M. Working aliquots were prepared in RNase-free water at 10 μ M and stored at -80° C. miR-430 rescue experiments were performed by injecting 2 nl of 10 μ M miR-430a (*I*). miR-430a, miR-430b and miR-430c share the same seed region. Injection of miR-430a, miR-430b or miR-430c alone has the same ability to rescue *MZdicer* mutants as a combination of all three miRNAs, suggesting that they are likely to function redundantly (*I*).

Misexpression analysis

sqt misexpression: Embryos were fixed when uninjected wild-type embryos reached 50% epiboly and analyzed for *fas* or *gsc* expression by in situ hybridization. "Increased Nodal signaling" was scored as *gsc* staining covering greater than 50% of the animal pole.

lft misexpression: Embryos were scored at \sim 30 hpf for the extent of *lft* overexpression phenotype. Embryos were considered to have "reduced Nodal signaling" when lacking trunk mesoderm and exhibiting cyclopia. *fas* in situs were performed on embryos fixed at 50% epiboly. 15-25 embryos were scored for overexpression phenotypes at each concentration.

Target protector (TP) nomenclature

The convention for future naming of target protectors is: [gene name]-TP^{miR-x}

In case of multiple target sites for one miRNA in one 3' UTR, each target protector for successive target sites should be numbered accordingly. For example, a target protector for the 2nd target site of miR-17 in *Myc*, would be named, *Myc*-TP^{miR-17 (2)}.

Target protector and AUG morpholino injections

Target protectors used were:

sqt-TP^{miR-430} 5'-AAAGTGCTAGAGTTGAGTTCCTTTG-3'

sqt-TP^{control} 5'-TTCTTAAATACATATTTTTGGGGTC-3'

sqt-TP^{miR-1} 5'-ATGGAATGATTAGAAGTATGTAT-3'

lft2-TP^{miR-430} 5'-TAAATACTTTGTTTTCAAGTGCTC-3'

lft2-TP^{control} 5'-CCATTTCTAAAGCTTACATTACATA-3'

lft1-TP^{miR-430} 5'-AAAGTGCTCGAGGTATCTAGTAAGG-3'

Target protectors used in this study were 25-nucleotide long morpholinos designed to bind to the region of the target mRNA complementary to the miRNA seed region (6-8 nt miRNA target site) and to flanking sequences in the 3'UTR. One way to optimize the specificity of TPs is to design the 3' end of the TP to bind to the target site while the 5' region of the TP binds to the unique downstream flanking sequences in the 3'UTR. Alternatively, TPs can be designed to have their 5' end bind to the target site while the 3' region of the TP binds the upstream flanking sequences in the 3'UTR. Sequence specificity should be confirmed using whole genome sequence alignment programs such as BLAST. All morpholinos and target protector oligonucleotides were obtained from Gene Tools. 1 ng of each target protector was injected at the one-cell stage for target protection experiments. Rescue experiments were performed by injecting 2 ng of *sqt*-AUG or *lft*-AUG morpholinos that block translation.

AUG morpholinos sequences were:

sqt-AUG MO (2)

5'-ATGTCAAATCAAGGTAATAATCCAC-3'

lft-AUG MO (3)

5'-AGCTGGATGAACAGAGCCATGCT-3'

In situ hybridization and cell counting

Embryos were staged by morphology as described (4), correcting for the developmental delay of *MZdicer* embryos. Probe preparation and in situ hybridization were performed as described (5). *Sox17*-stained endodermal and forerunner cells were counted from images of deyolked and filleted embryos (6).

Image acquisition

Embryos were analyzed with Leica MZ16F, Zeiss AxioImager.Z1 and Zeiss Discovery.V12 microscopes and photographed with Optronics MicroFire 2.2 or AxioCam MRc digital cameras. Images were processed with Zeiss AxioVision 4.4, PictureFrame 2.2 and Adobe Photoshop CS software. The levels of the images were adjusted in Photoshop, and identical modifications were applied to all the images in the same experiment.

Fish strains

MZdicer embryos were generated as described (1,7). *MZdicer* mutant embryos were *dicer*^{hu715/hu715}, *dicer*^{hu896/hu896} or *dicer*^{hu896/hu715} (8). The *sqt* mutant embryos carried the *sqt*^{cz35} allele (9, 10).

Fig. S1. Nodal signaling components are targeted by miR-430.

(A) Nodal signaling pathway. The Nodals Squint and Cyclops are antagonized by Lefty1 and Lefty2. Nodal signaling activates target genes including *gsc*, *fas*, and *sox17*. The agonist Squint (green), antagonists Lefty1 and Lefty2 (red), and Smad2 (orange) contain putative miR-430 target sites. (B) Schematic of expression patterns of Nodal target genes at 50% epiboly. *sqt*, *lft*, and *fas* are expressed all around the margin while *gsc* is

expressed only at the dorsal margin (animal view). (C) Conservation of miR-430 target sites in *squint/nodal*, *lefty* and *smad2*.

Fig. S2. Regulation of *lft1* by miR-430.

(A) The *lft2* 3'UTR is more strongly repressed by miR-430 than the *lft1* 3'UTR as shown by comparison of GFP reporters with wild-type or mutated 3'UTRs. (B) The *lft2* 3'UTR is more strongly repressed by miR-430 than the *lft1* 3'UTR as shown by comparison of GFP reporters in wild-type or *MZdicer* mutant embryos. (C) $lft1\text{-TP}^{\text{miR-430}}$ prevents repression of *lft1*-GFP reporter. Repression of *lft1*-GFP reporter is not affected by $lft2\text{-TP}^{\text{miR-430}}$. (D) Wild-type embryos injected with increasing amounts of wild-type *lft1* or *lft1*^{mut-3'UTR} mRNA. (E) Percentage of embryos with reduced Nodal signaling (cyclopia and loss of trunk mesoderm) at increasing concentrations of wild-type *lft1* or *lft1*^{mut-3'UTR} mRNA. (F) *lft1* expression in wild-type and *MZdicer* embryos at 50% epiboly.

Fig. S3. Concentration-dependence of $sqt\text{-TP}^{\text{miR-430}}$ and $lft2\text{-TP}^{\text{miR-430}}$.

$sqt\text{-TP}^{\text{miR-430}}$ and $lft2\text{-TP}^{\text{miR-430}}$ prevent GFP repression in wild-type embryos.

Fig. S4. Binding to 3'UTR is not sufficient for protection

$Sqt\text{-TP}^{\text{control}}$ is a morpholino designed to bind the *sqt* 3'UTR downstream of $sqt\text{-TP}^{\text{miR-430}}$. $Sqt\text{-TP}^{\text{control}}$ does not affect *sqt*-GFP reporter repression.

Fig. S5. Specificity of $\text{TP}^{\text{miR-430}}$

$Sqt\text{-TP}^{\text{miR-430}}$, $lft2\text{-TP}^{\text{miR-430}}$, and $lft1\text{-TP}^{\text{miR-430}}$ prevent GFP repression in wild-type embryos (left) but do not further increase GFP expression in *MZdicer* embryos (right). Mutation of the miR-430 target site does not affect GFP expression in *MZdicer* embryos.

Fig. S6. $sqt\text{-TP}^{\text{miR-430}}$ and $lft2\text{-TP}^{\text{miR-430}}$ modulate Nodal signaling.

Extended version of Fig. 3C and D and 4B. (A-H) *gsc* expression domain marks dorsal mesoderm at the onset of gastrulation. (A' and B') Lateral view of embryo at onset of gastrulation. (A'' and B'') Embryonic shield marks dorsal mesoderm (outlined) at 6 hpf. (B, B' and B'') Ectopic *gsc* induction corresponds to expanded shield (outlined). (C) *sqt*-AUG morpholino suppresses the ectopic *gsc* induction phenotype caused by $sqt\text{-TP}^{\text{miR-430}}$. (D) *gsc* induction is reduced by *sqt*-AUG morpholino. (E') Frontal view of a 30hpf wild type embryo; arrowheads indicate the distance between the retinas. (F and F') Reduced *gsc* induction and cyclopia caused by $lft2\text{-TP}^{\text{miR-430}}$. (G and G') Reduced *gsc* induction and cyclopia caused by $lft2\text{-TP}^{\text{miR-430}}$ are suppressed by *lft2*-AUG morpholino. (H and H') *gsc* induction and embryonic phenotype in *lft2*-AUG MO injected embryos.

Fig. S7. Effects of $sqt\text{-TP}^{\text{miR-430}}$ are caused by protection of zygotic *sqt*.

(A) *gsc* induction is increased in $sqt\text{-TP}^{\text{miR-430}}$ -injected wild-type embryos compared to $sqt\text{-TP}^{\text{control}}$ -injected wild-type embryos (onset of gastrulation 5-6 hpf). (B and C) Embryonic mRNAs are provided both maternally and zygotically i.e. by both the mother and the embryo (11, 12). MiR-430 represses a large number of maternal mRNAs (7). Since *lft1* and *lft2* are only expressed zygotically, our results show that miR-430 can also target zygotically expressed genes. *Sqt* mRNA is provided both maternally and zygotically (13-16). To determine whether the effect of $sqt\text{-TP}^{\text{miR-430}}$ was caused by its

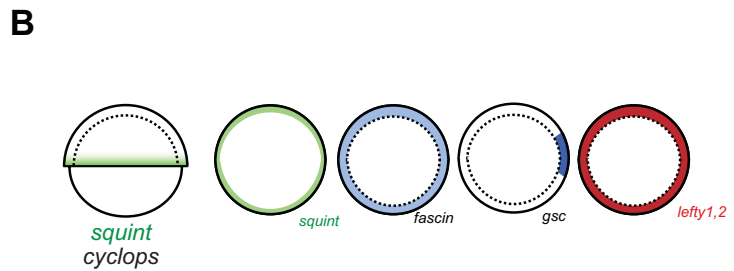
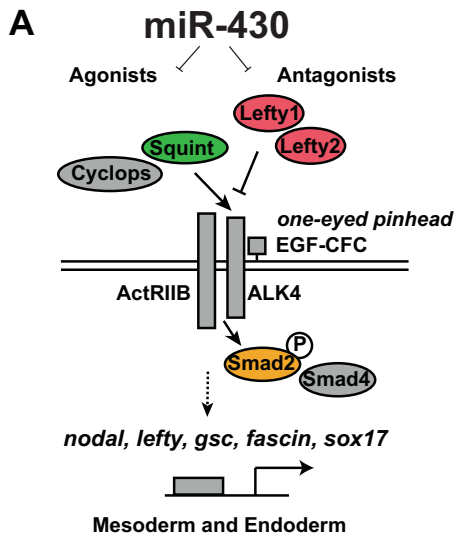
effects on maternal or zygotic *sqt* mRNA, we injected $sqt\text{-TP}^{\text{miR-430}}$ into embryos that contain only maternal *sqt* mRNA (zygotic *sqt* mutants, *Zsqt*) or only zygotic *sqt* mRNA (maternal *sqt* mutants, *Msqt*) (15, 17). (B) Expansion of *gsc* induction and higher levels of *sqt* in $sqt\text{-TP}^{\text{miR-430}}$ -injected *Msqt* embryos. (C) Levels of *gsc* induction and *sqt* expression are unaffected in $sqt\text{-TP}^{\text{miR-430}}$ -injected *Zsqt* embryos compared to $sqt\text{-TP}^{\text{control}}$ -injected embryos.

Fig. S8. Target protector function and applications.

(A) Target protectors enable the study of the physiological function of specific miRNA-mRNA target pairs that cannot be uncovered in miRNA knockouts. (B-D) Potential therapeutic applications of target protectors. (B) miRNA normally regulates mRNA X. Decreased production of gene product as a result of haploinsufficiency, regulatory mutation, or hypomorphic allele results in disease. Target protection can increase production of gene product to relieve the diseased state. (C) mRNA X is not normally regulated by a miRNA. Misregulation of mRNA X by a miRNA leads to disease. Target protection can prevent this misregulation. Analogously, target mRNAs can be protected from overexpression of a miRNA that normally regulates X. (D) Target protectors may be used to increase the levels of a beneficial gene product X in the wild-type context by blocking miRNA-induced repression.

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C **Squint/Nodal**

Danio rerio
 Tetraodon nigroviridis
 Takifugu rubripes
 Oryzias latipes
 Gasterosteus aculeatus
 Xenopus tropicalis xnr3-A
 Xenopus laevis xnr5-14
 Xenopus laevis xnr3-A
 Ciona savignyi
 Mus musculus

CCCCAAAGGAACUCAACUCUAGCACUUUGGAUAUGCUCUUGACCCCA
 CGCGGGGGUUCGGACCCGAGGCACUUGAUAGAUGUUUCGGUUUGAA
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 GGAUCCACAUUUUACAGAGCAGCACUUUCUUGUCCACUAUUUAUUGUG
 UUAUAGAUAUUUCUUUUUCUUUGCACUUUCAAGAAUAUUAUUUUUA
 UGCCUUUGACCAGAGAGGUGGACACUUGUCAAGAGGGGACUGGCCAU

Lefty

Danio rerio lefty2
 Danio rerio lefty1
 Takifugu rubripes-1
 Takifugu rubripes-2
 Tetraodon nigroviridis-1
 Tetraodon nigroviridis-2
 Xenopus laevis lefty-A
 Xenopus laevis lefty-B
 Mus musculus lefty1
 Mus musculus lefty2
 Homo sapiens lefty1
 Homo sapiens lefty2

GACUCCCCAAAUCCCCACUGAGCACUUGAAAAACAAGUAUUUAUGAA
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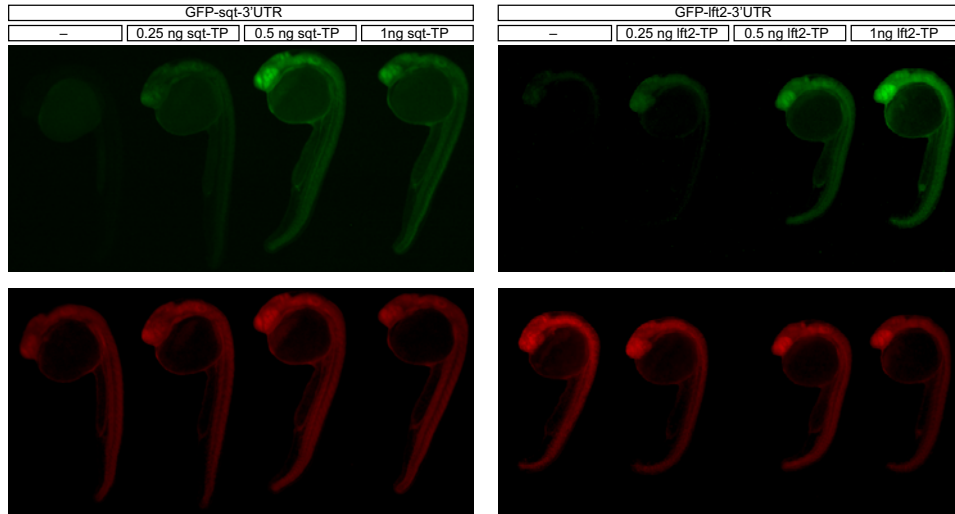
Smad2

Danio rerio smad2
 Xenopus tropicalis
 Takifugu rubripes
 Tetraodon nigroviridis
 Mus musculus (ORF)
 Homo sapiens (ORF)

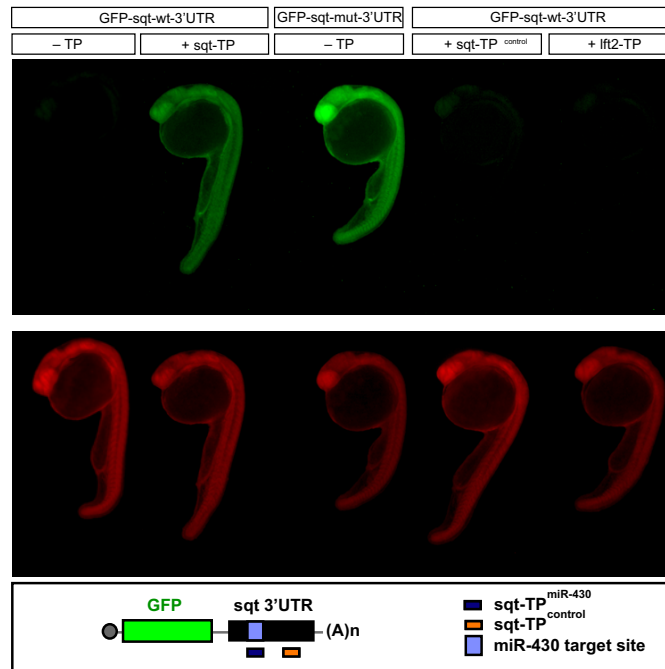
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miR-430a
 miR-430b
 miR-430c

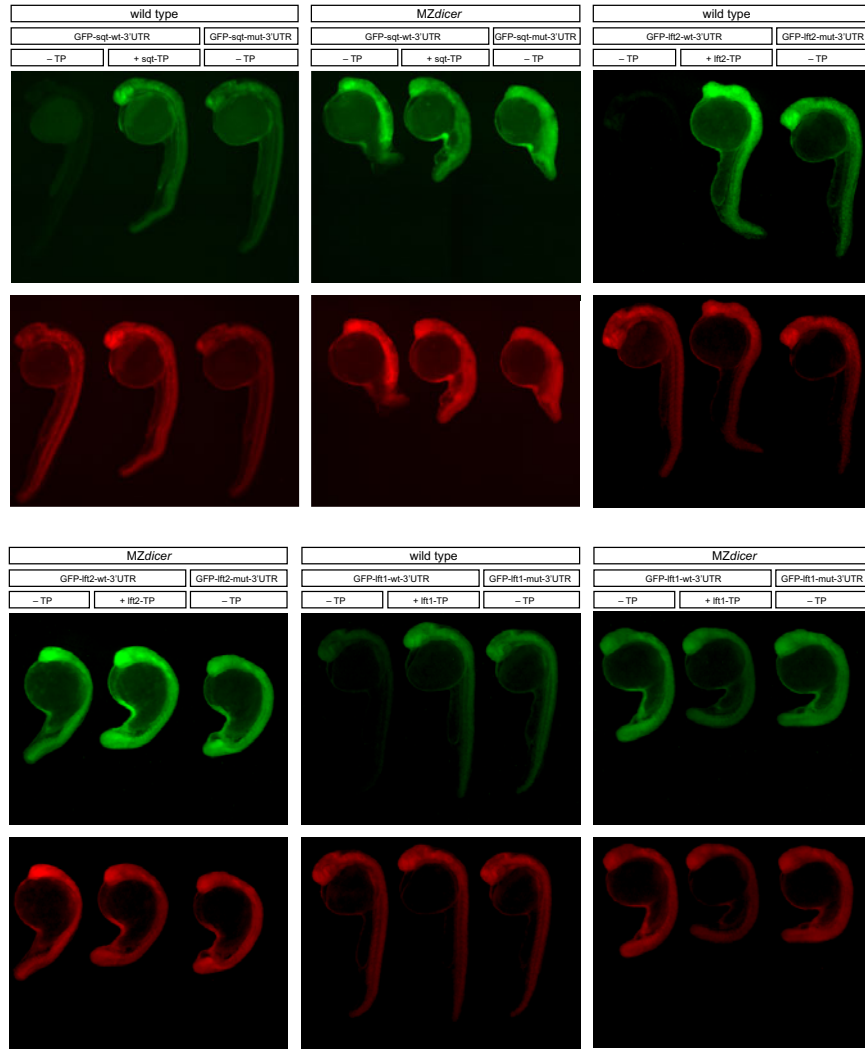
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 3' GAUGGGGUUCUCUUCGUGAAU 5'



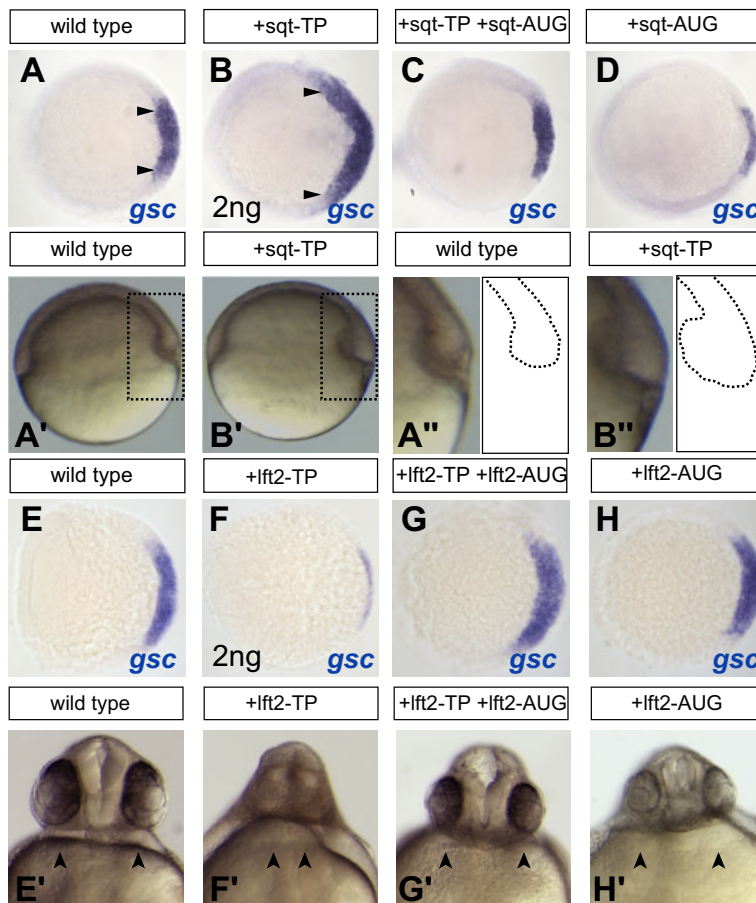
Choi et al, Figure S3



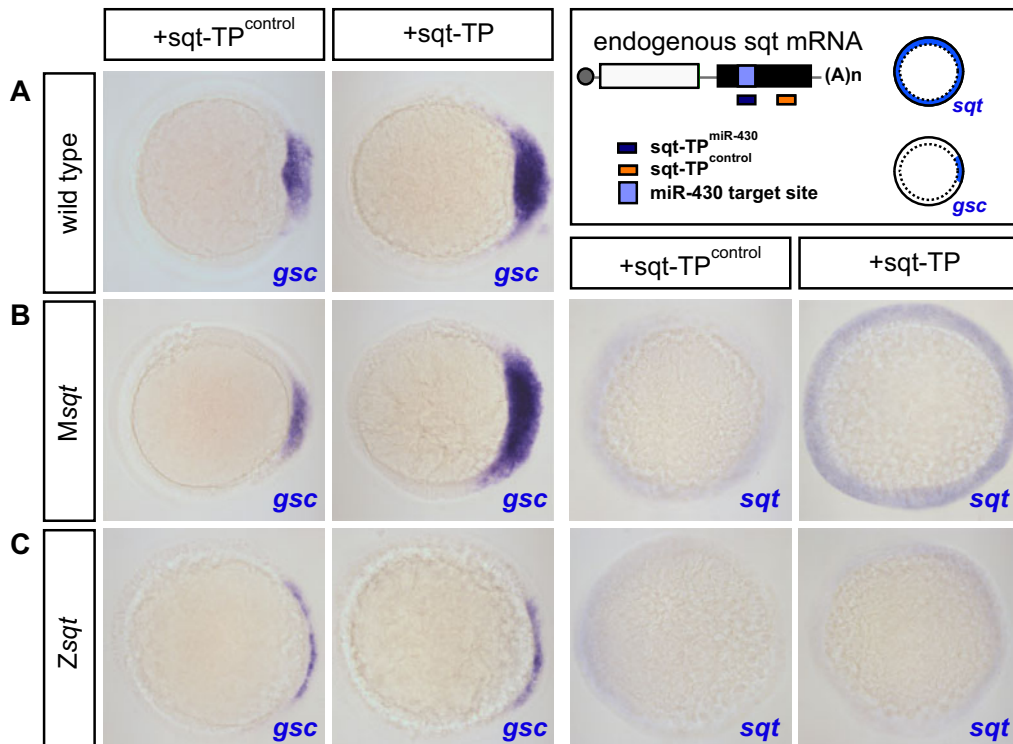
Choi et al, Figure S4



Choi et al, Figure S5



Choi et al, Figure S6



Choi et al, Figure S7

