Dorsal activity of maternal sqint is mediated by a non-coding function of the RNA

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SUMMARY
Despite extensive study, the earliest steps of vertebrate axis formation are only beginning to be elucidated. We previously showed that asymmetric localization of maternal transcripts of the conserved zebrafish TGFβ factor Squint (Sqt) in 4-cell stage embryos predicts dorsal, preceding nuclear accumulation of β-catenin. Cell ablations and antisense oligonucleotides that deplete Sqt lead to dorsal deficiencies, suggesting that localized maternal sqt functions in dorsal specification. However, based upon analysis of sqt and Nodal signaling mutants, the function and mechanism of maternal sqt was debated. Here, we show that sqt RNA may function independently of Sqt protein in dorsal specification. sqt insertion mutants express localized maternal sqt RNA. Overexpression of mutant/non-coding sqt RNA and, particularly, the sqt 3’UTR, leads to ectopic nuclear β-catenin accumulation and expands dorsal gene expression. Dorsal activity of sqt RNA requires Wnt/β-catenin but not Oep-dependent Nodal signaling. Unexpectedly, sqt ATG morpholinos block both sqt RNA localization and translation and abolish nuclear β-catenin, providing a mechanism for the loss of dorsal identity in sqt morphants and placing maternal sqt RNA upstream of β-catenin. The loss of early dorsal gene expression can be rescued by the sqt 3’UTR. Our findings identify new non-coding functions for the Nodal genes and support a model wherein sqt RNA acts as a scaffold to bind and deliver/sequester maternal factors to future embryonic dorsal.

KEY WORDS: 3’UTR, Axis formation, Dorsal localization, Dorsal expansion, Maternal factors, Non-coding RNA, Nodal, RNA localization, Squint (Nodal-related 1), Zebrafish

INTRODUCTION
The specification of the embryonic axes in many organisms is initiated by maternally deposited factors that activate various signaling pathways to establish the body plan. In Drosophila, frogs and fish, asymmetric localization of maternal factors in oocytes and eggs is crucial for correct embryonic patterning (St Johnston, 1995; Grunert and St Johnston, 1996; Mowry and Cote, 1999; Pelegri, 2003; Heasman, 2006; Abrams and Mullins, 2009; Nojima et al., 2010; Lu et al., 2011). In Xenopus, wnt11 RNA is localized in dorsal vegetal cells, and blocking maternal Wnt and β-catenin functions leads to dorsal deficiencies (Heasman et al., 1994; Wylie et al., 1996; Schroeder et al., 1999; Heasman et al., 2000; Kofron et al., 2001; Tao et al., 2005). Recent work has also implicated vegetally localized Trim36 ubiquitin ligase in microtubule-dependent cortical rotation and axis formation (Cuykendall and Houston, 2009).

In zebrafish, several experiments have suggested that dorsal determinants are vegetally localized, and are translocated via microtubules to trigger dorsal axis specification (Jesuthasan and Strahle, 1997; Mizuno et al., 1999; Ober and Schulte-Merker, 1999). The molecular nature of the maternal factors is just beginning to be elucidated (Abrams and Mullins, 2009). We previously showed that maternal RNA encoding the Nodal morphogen Squint [Sqt; Nodal-related 1 (Ndr1) – Zebrafish Information Network] asymmetrically localizes in two cells of 4-cell stage embryos (Gore and Sampath, 2002; Gore et al., 2005), and predicts embryonic dorsal prior to the nuclear accumulation of β-catenin in dorsal cells. Removal of sqt RNA-containing (sqt+) cells and knockdown by antisense morpholino oligonucleotides leads to dorsal deficiencies, suggesting that asymmetrically localized maternal sqt functions in dorsal specification.

Paradoxically, females homozygous for the sqt insertion mutations sqtcz35 and sqthi975 (Heisenberg and Nusslein-Volhard, 1997; Erter et al., 1998; Feldman et al., 1998; Amsterdam et al., 2004) produce embryos with mild dorsal deficiencies and do not manifest the loss of anterior and dorsal structures observed upon depletion of sqt, either by antisense morpholinos or embryological dissections of sqt+ cells (Aoki et al., 2002; Bennett et al., 2007; Pei et al., 2007). Similarly, maternal and zygotic mutations affecting the Nodal co-receptor one-eyed pinhead (oei) (Gritsan et al., 1999) result in embryos similar to zygotic cye:sqt double mutants (cye is also known as ndr2 – Zebrafish Information Network) (Feldman et al., 1998; Dougan et al., 2003), but these embryos do not manifest complete loss of anterior or dorsal structures. Therefore, the function of maternal sqt has been a matter of debate (Bennett et al., 2007; Gore et al., 2007).

Using several mutations that disrupt Sqt, we find that sqt RNA has functions independent of Sqt protein in dorsal initiation. Furthermore, our analysis shows that the sqt235 and sqtphi975 insertion alleles, on which the genetic analysis was based, still express and localize maternal sqt transcripts, similar to wild-type embryos. We demonstrate that mutant RNAs still have dorsal-inducing activity in embryos, and show that this activity is a non-coding function of sqt RNA. Interestingly, the non-coding, dorsal-inducing activity of sqt RNA is dependent on sequences within its 3’UTR. We also revisited the activity of sqt morpholinos, and find that sqt translation initiation site sequences are also required for sqt
RNA localization. The dorsal-inducing activity of sqt RNA is independent of Sqt/Nodal signaling, but requires functional maternal Wnt/β-catenin signaling. Our findings identify novel non-coding functions for the Nodal genes and reveal new roles for non-coding RNAs in the maternal control of axis specification.

**MATERIALS AND METHODS**

**Generation of constructs**

pcS2+sqtSTOP, pcS2-FLAGsqt and pcS2+FLAGsqtSTOP were generated by PCR-based site-directed mutagenesis. pcS2-sqtSTOP and pcS2+FLAGsqt were generated using pcS2-sqt (Gore et al., 2005) as PCR template, whereas pcS2-FLAGsqtSTOP was generated from pcS2-sqtSTOP. pcS2-sqtSTOP was generated by amplifying two overlapping fragments from MZsqtE135 cDNA, followed by overlap extension PCR and subcloning into pcS2+. pcS2-T-sqt was generated using pcS2+sqtE35 as PCR template, followed by subcloning into pcS2+. Primers are listed in supplementary material Table S1.

**Zebrafish strains**

Wild-type zebrafish, MZsqtE135, MZsqtA075, MZdicER715, MZoepl75 and homozygous ich+ mutant fish were maintained at 28.5°C and embryos were obtained by natural mating using standard procedures, in accordance with institutional animal care regulations (Westerfield, 2007). The genotype of MZsqtE135 embryos was determined as described (Feldman et al., 1998). Homozygous ich mutant mothers that yielded 100% radialized embryos were used and identified as described (Kelly et al., 2000; Bellipanni et al., 2006).

**Quantitative real-time RT-PCR**

Total RNA was extracted from embryos using TRIzol reagent (Invitrogen). 250 ng RNA from WT, MZsqtA075 and MZsqtE135 embryos and 250 ng RNA from lacZ:glo or lacZ:sqt RNA-injected embryos was used for cDNA synthesis. 1 μl first-strand cDNA was used in 10 μl PCR reactions. Genomic DNA contamination was checked by PCR to detect acth2 (act), sqt, dharma (dha; bozozok), vox and vent. Primers are listed in supplementary material Table S1. RT-PCR was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using the comparative Ct method. Control experiments to measure changes in Ct with template dilutions were performed to test whether amplification efficiencies of target (sqt, dha, vox and vent) and control (act) primers were similar. All results were normalized to act.

**Capped mRNA synthesis, injections and in situ hybridizations**

Capped mRNA was synthesized from linearized plasmids (Notl, NEB) using the SP6 mMessage mMachine Kit (Ambion), and 25 pg aliquots were injected into 1-cell stage embryos. Fluorescent Alexa 488-labeled RNA was synthesized, injected at the 1-cell stage (Gore et al., 2005; Gilligan et al., 2011) and RNA localization scored visually (Gilligan et al., 2011) by two individuals independently.

To target maternal sqt RNA prior to the formation of sqt RNA aggregates that develop upon egg activation (Gore and Sampath, 2002), 10 ng aliquots of control or sqt morpholinos were injected into squeezed wild-type eggs and fertilized with wild-type sperm (Gore et al., 2005; Gore et al., 2007). Injected embryos were fixed in 4% paraformaldehyde/PBS at oblong, sphere, dome, 30% epiboly, 40% epiboly, 50% epiboly, 60% epiboly and at 24 hours post-fertilization (hpf), and processed for whole-mount in situ hybridization to detect goosecoid, chordin, gata2 and no tail (Sampath et al., 1998). Localization of sqt RNA was detected by in situ hybridization using full-length cDNA probes.

**In vitro translation and protein detection**

The TNT® SP6 Coupled Rabbit Reticulocyte Lysate System (Promega) was used to transcribe and translate from the following plasmid templates: pcS2+, pcS2-sqtFL:sqt, pcS2+sqtSTOP, pcS2+sqtSTOP, pcS2+FLAGsqt, pcS2+FLAGsqtSTOP and pcS2-T-sqt. 1 μg of plasmid DNA was used in 50 μl reactions according to the manufacturer’s instructions (Promega). Biotin-labeled protein products were separated by SDS-PAGE and transferred onto Hybond-C Extra membranes (GE Healthcare). Immunoblotting was performed using avidin and biotinylated HRP (1:200 dilution) (Ultra-sensitive ABC Peroxidase Rabbit IgG Staining Kit, Pierce). Proteins were detected by Kodak Biomax MS film using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). FLAG epitope-tagged peptides were detected with anti-FLAG M2 mouse monoclonal primary antibody (1:2500, Sigma) and HRP-conjugated anti-mouse IgG secondary antibody (1:5000, DAKO). To detect nuclear β-catenin, 512-cell stage embryos were fixed in 4% paraformaldehyde/PBS and processed for fluorescence immunohistochemistry using a rabbit polyclonal anti-β-catenin antibody (C2206, Sigma) and Alexa 488-conjugated goat anti-rabbit secondary antibodies (Molecular Probes).

To compare translation efficiencies of Sqt:glo UTR and Sqt:sqt UTR, 1-cell stage wild-type embryos were injected with 20 pg sqt-GFP:glo or sqt-GFP:sqt. Approximately 40-50 injected embryos were manually dechorionated and lysed at 50% epiboly in RIPA buffer. Whole embryo lysates (50 μg/lane) were separated by SDS-PAGE and transferred onto Hybond-C Extra membranes. Proteins were detected on film as described above. Sqt-GFP was immunoblotted using rabbit polyclonal anti-GFP antibodies (1:2500, Abcam), followed by HRP-conjugated anti-rabbit IgG secondary antibodies (1:5000, DAKO). Tubulin was detected using mouse monoclonal anti-tubulin antibodies (1:2500, Sigma), followed by HRP-conjugated anti-mouse IgG secondary antibodies.

**Microscopy**

Live embryos injected with fluorescent RNAs or expressing Sqt-GFP fusion protein were manually dechorionated, mounted in 2.5% methylcellulose (Sigma) and visualized using a Zeiss Axioplan2 microscope with a CoolSNAP HQ camera (Photometrics). MetaMorph (Universal Imaging Corporation) and ImageJ (NIH) software packages were used to acquire and process images. Stained embryos from in situ hybridization and immunohistochemistry experiments were mounted in 100% glycerol and imaged using a Zeiss Axioplan2 microscope equipped with a Nikon DXM1200 color camera. Images were acquired using ACT-1 software (Nikon) and cropped using Adobe Photoshop.

For β-catenin- and DAPI-stained embryos, images were acquired using a Zeiss LSM 5 Exciter upright confocal microscope. To quantify β-catenin-positive nuclei, 15-25 optical sections at 1.76 μm intervals starting from the yolk syncytial layer nuclei were examined per embryo. We detected β-catenin-positive nuclei in many sections. However, owing to intense membrane and cytoplasmic β-catenin staining that obscured nuclear staining upon z-projection of all sections obtained, three serial confocal sections for each embryo were selected, 2-projected using LSM Image Browser software, and cropped using Adobe Photoshop.

**Measurement of expression domains**

Animal pole view images of embryos stained for gsc were used. In ImageJ, we drew a best-fit circle for the circumference of the embryo using the Circle tool. Using the Angle and Measure tools the angle of expression was determined.

**RESULTS**

**Mutant sqt RNA expands dorsal gene expression in early embryos**

To examine whether the insertion mutants had any sqt activity, we first determined if the RNA and protein are expressed. We previously showed that embryos from fish homozygous for the sqtSTOP and sqtPIRD insertions express maternal sqt RNA (Gore et al., 2007). Whole-mount in situ hybridizations show that, similar to wild-type embryos, mutant sqt RNA is localized to two cells at the 4- and 8-cell stage in MZsqt mutant embryos (Fig. 1A-C; genotypes confirmed by PCR, Fig. 1C). Quantitative PCR show that MZsqt mutant and wild-type embryos have similar levels of maternal sqt RNA at the 1-cell and 4-cell stages, and that a decrease in sqt RNA levels occurs in MZsqt mutant embryos later during gastrulation (Fig. 1D). Similar to wild-type embryos, we
detect non-polyadenylated mutant sqt RNA that is spliced as well as unspliced in pd(N)₆-primed cDNA from 4-cell stage embryos, whereas only spliced sqt RNA is detected using oligo(dT)-primed cDNA (supplementary material Fig. S1A,B). These results show that mutant maternal sqt RNA is expressed and localized to future dorsal cells in MZ sqt mutant embryos at levels similar to wild-type embryos. Therefore, the sqt insertion alleles are not maternal transcript nulls, and maternal sqt RNA levels in mutant embryos are similar to those in wild-type embryos.

To test whether the sqtcz35 insertion RNA generates any Sqt protein, we expressed sqtcz35:sqt RNA in a rabbit reticulocyte lysate system. The insertion RNA is predicted to encode a 17 kDa C-terminally truncated peptide lacking any functional ligand. We find that protein expressed from sqtcz35:sqt RNA is the predicted 17 kDa peptide (Feldman et al., 1998). We also tested synthetic sqt RNA with a stop codon in the first exon (TTG>TAG, which results in Leu11>STOP; sqtSTOP:sqt), a truncated sqt RNA (T-sqt:sqt) (Bennett et al., 2007), and FLAG epitope-tagged versions of wild-type Sqt and SqtSTOP (Fig. 1E).

Translation from control and FLAG-tagged sqt RNA showed the expected 44 kDa proteins, whereas translation from sqtcz35:sqt RNA yielded an N-terminally truncated peptide shafted from utilization of an ATG downstream of the engineered stop. We then tested whether sqtcz35 mutant RNA has any activity in embryos. One-cell stage wild-type embryos were injected with synthetic capped sqtcz35 RNA, sqtSTOP or T-sqt RNA, and nuclear accumulation of β-catenin and dorsal expression of goosecoid (gsc) and chordin (chd) was examined at the onset of gastrulation (Fig. 2A-L). Mutant sqt RNA-injected embryos reached developmental landmarks at the same time as control-injected embryos (Fig. 2J,K,Q,R; data not shown), suggesting that morphogenesis or development is not generally delayed.

Surprisingly, embryos injected with mutant sqt RNAs (sqtmutsqt) showed expansion of nuclear β-catenin expression at the 512-cell stage, with ~16 β-catenin-positive nuclei (n=18 embryos), as compared with control lacZ:glo RNA-injected embryos (glo, Xenopus globin 3’ UTR) that showed about five positive nuclei (n=10 embryos; Fig. 2A-D and Table 1). We detected at least 15 β-catenin-positive nuclei in more than 50% of sqtmutsqt RNA-injected embryos, and three embryos showed more than 25 β-catenin-positive nuclei (n=18; Table 1 and supplementary material Table S2). The lower borders of the first tier blastoderm cells are not visible (Fig. 2A,C), consistent with yolk syncytial layer (YSL) formation at the tenth mitosis (512-cell to 1000-cell stage) (Kimmel and Law, 1985). Increased numbers of β-catenin-positive nuclei were observed both in the blastoderm (red arrows, Fig. 2A-D) and dorsal YSL of sqtmutsqt-injected embryos (yellow arrows, Fig. 2C,D), whereas in wild-type and control-injected embryos YSL expression of β-catenin is not detected until the 1000-cell stage (supplementary material Fig. S2) (Dougan et al., 2003). Thus, injected mutant sqt RNA can substantially increase the dorsal accumulation of nuclear β-catenin.
The sqtmu:sqt-injected embryos show expanded gsc expression at 30% and 40% epiboly (Fig. 2L; data not shown). Expansion of gsc is only observed along the margin and does not extend animally. By contrast, control embryos injected with lacZ:glo RNA did not show expanded gsc expression at comparable stages (Fig. 2E; data not shown). Dorsal expansion by sqtmu:sqt RNAs was transient, and by 60% epiboly gsc expression was indistinguishable from that of control lacZ:glo RNA-injected embryos and uninjected embryos (Fig. 2I,P; data not shown). To quantify dorsal expansion, we measured the angle of gsc expression around the gastrula margin in injected embryos (Fig. 2S). In wild-type embryos and control lacZ:glo-injected embryos, the gsc angle is ~70°, whereas in embryos injected with sqtmu:sqt RNAs the arc of gsc expression is much broader, resulting in angles ranging between 70° and 150°, with a mean exceeding 95° (Fig. 2T). Similarly, chd expression at 40% epiboly also expanded significantly (Fig. 2M). Control-injected embryos did not show expanded gsc or chd. We also observed expanded no tail (ntl) expression around the entire margin (Fig. 2G,N), and reduced ventral gene expression of gata2 (Fig. 2H,O) and eve1 (not shown), in comparison to control embryos.

### Table 1. Quantification of β-catenin-positive nuclei in injected embryos

<table>
<thead>
<tr>
<th>Injected RNA/MO</th>
<th>0-2</th>
<th>3-5</th>
<th>6-9</th>
<th>10-15</th>
<th>&gt;15</th>
<th>Total (N)</th>
</tr>
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<tr>
<td>lacZ:glo</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>sqtmu:sqt</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
<td>33.3</td>
<td>50</td>
<td>18</td>
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<tr>
<td>Con MO</td>
<td>0</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>sqt MO</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>12</td>
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Shown is the percentage of embryos that exhibit particular numbers of β-catenin-positive nuclei. In lacZ:glo RNA- and control MO-injected embryos, we typically detect four to five β-catenin-positive nuclei, and only one embryo showed six positive nuclei. In sqtmu:sqt RNA-injected embryos, we observed substantially increased numbers of β-catenin-positive nuclei, whereas sqt MO-injected embryos have reduced or no β-catenin-positive nuclei.

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Fig. 2. Mutant sqt RNAs expand the dorsal domain in early zebrafish embryos. (A-D) Embryos injected with capped lacZ:glo mRNA show β-catenin in nuclei of about five cells at the 512-cell stage (A,B), in comparison to mutant sqt RNA-injected embryos which show ~11 β-catenin-positive nuclei (C,D). Red arrows indicate β-catenin-positive nuclei in the blastoderm, whereas yellow arrows show β-catenin-positive nuclei in the yolk syncytial layer (YSL). DAPI staining (B,D) shows all nuclei in blastoderm and YSL. (E-R) Normal expression of gsc (E), chd (F) and ntl (G) in lacZ:glo-injected embryos at 30% epiboly, 40% epiboly and 50% epiboly, respectively, as compared with expanded gsc (L), chd (M) and ntl (N) in mutant sqtmu:sqt UTR (sqtmu:sqt)-injected embryos. Expression domain of the ventral marker gata2 (brackets in H,O) is reduced in sqtmu:sqt RNA-injected embryos (O), in comparison to controls (H). At 60% epiboly, gsc expression is similar in lacZ:glo-injected (I) and mutant sqtmu:sqt-injected (P) embryos. Embryos injected with sqtmu:sqt RNA reach developmental landmarks such as sphere (J,Q) and 60% epiboly (K,R) at the same time as control-injected embryos (J,K). Red arrowheads (E,F,I,M) mark the extent of gsc and chd expression; yellow asterisks (K,R) mark the shield. (S) Schematic showing measurement of gsc angle (θ). The best-fit circle is indicated in red, green squares mark x and y coordinates, and the magenta ‘X’ marks the center. (T) Angle of gsc expression in sqtmu:sqt RNA-injected or control lacZ:glo-injected embryos at 30% epiboly. Each blue dot represents a single embryo. N indicates the number of injected embryos for each RNA and μ(°) shows the mean gsc angles. A-D, dorsal views; E-I,K-P,R,S, animal pole views with dorsal to the right; I,Q, lateral view. Scale bars: 25 μm in A; 100 μm in E.
We then examined whether the sqt 3'UTR is sufficient for dorsal expansion. Embryos were injected with RNA encoding the fluorescent protein Venus fused with either sqt 3'UTR (venus:sqt) or glb3 3'UTR (venus:glo). Consistent with the evidence that the sqt 3'UTR is required for dorsal expansion, venus:sqt RNA-injected embryos showed transient expansion of gsc ranging up to 160° and with a mean of 96°. Control venus:glo-injected embryos showed a ~72° gsc angle (Fig. 3J), similar to un.injected embryos. Quantitative real-time RT-PCRs to detect expression of sqt, dha, vox and vent in lacZ:sqt-injected embryos showed that endogenous sqt and dha transcript levels increase transiently at sphere stages and revert to normal levels by 30% epiboly (supplementary material Fig. S3), as compared with control lacZ:glo-injected embryos. Conversely, vox and vent levels are transiently reduced initially and subsequently revert to normal (supplementary material Fig. S3). Thus, the sqt 3'UTR is both necessary and sufficient to transiently expand dorsal and reduce ventral gene expression.

**Activity of sqt 3'UTR in dorsal is independent of Sqt protein activity**

Overexpressing sqt 3'UTR expands dorsal gene expression, but only transiently, raising the question of whether its dorsalizing activity is developmentally relevant to the embryo. To address this, we used ichabod (ich) mutant embryos, which lack all dorsal structures (Kelly et al., 2000). Embryos from homozygous ich mothers can be rescued by sqt RNA injections (Kelly et al., 2000; Gore et al., 2005). We compared the effect of sqt versus glb3 3'UTR sequences on the ability of Sqt to rescue ich mutant embryos. Capped synthetic mRNA encoding Sqt fused to either sqt (sqt:sqt) or to glb3 (sqt:glo) 3'UTR was injected into ich embryos. In these experiments, Sqt protein would be generated from both RNAs, with the UTRs providing the only difference in activity.

Interestingly, we find that sqt:sqt induces a complete axis and rescues more efficiently than sqt:glo at comparable doses (Fig. 4A-G). Nearly 70% of sqt:sqt-injected embryos show rescue of dorsal structures to varying extents (15% show complete rescue and 53% partial rescue; n=100 embryos; Fig. 4G) (Gore et al., 2005). By contrast, only 37% of sqt:glo-injected ich embryos show any rescue of dorsal structures. Furthermore, ~55% of sqt:glo-injected ich embryos (n=103 embryos) manifest early gastrula arrest, as compared with ~30% of sqt:sqt injections (n=100 embryos), which is likely to be due to unregulated Sqt signaling from mislocalized sqt:glo in contrast to localized Sqt from sqt:sqt. SDS-PAGE to detect Sqt protein in whole embryo lysates showed that the expression levels of Sqt from sqt:sqt versus sqt:glo are similar (Fig. 4H-I), indicating comparable translation efficiencies. These results show that the sqt 3'UTR conveys more efficient activity to sqt in forming dorsal structures. Therefore, the sqt 3'UTR has biological activity in dorsal specification that is distinct from any activity of Sqt protein.

**Dorsal activity of sqt RNA is dependent on sequences in the 3'UTR**

Since localization of sqt RNA to dorsal progenitors depends on sequences in its 3'UTR, we tested whether dorsal expansion by overexpression of sqt RNA also requires the 3'UTR. Embryos injected with sqt STOPmutant RNA fused to sqt 3'UTR (sqtSTOP:sqt) were compared with those injected with mutant RNA fused with Xenopus glb3 3'UTR (sqtSTOP:glo; Fig. 3A). Whereas sqtSTOP:sqt RNA injection expanded gsc expression at 30% epiboly (Fig. 3C), injection of sqtSTOP:glo had no discernible effect (Fig. 3B,J), and injected embryos were indistinguishable from control or uninjected embryos. Expanded gsc expression in sqtSTOP:sqt RNA-injected embryos was transient and by 60% epiboly all injected embryos were similar to uninjected (not shown) or control-injected embryos (Fig. 3F-I). Therefore, the 3'UTR is required for transient dorsal expansion by the sqt RNA.

Thus, mutant sqt RNA that is incapable of supporting functional Sqt protein synthesis or generating the classical ligands can still expand dorsal and reduce ventral gene expression.

**Dorsal activity of sqt RNA is dependent on sequences in the 3'UTR**

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100°, with a mean of ~88°, in comparison to control lacZ:glo-injected embryos that show gsc expression ranging from 63°-70°, with a mean of ~66°. Therefore, expansion of dorsal by sqtRNA is Oep-independent, and this function of sqt does not require Nodal signaling.

We then tested the requirement of maternal Wnt/β-catenin signaling, which is known to be essential for dorsal specification in frogs and fish (Kelly et al., 2000; Tao et al., 2005). To test whether dorsal activity of sqt RNA is mediated via canonical Wnt signaling, we injected mutant sqt RNAs into ich embryos and examined gsc expression at early gastrula. Expression of gsc is not expanded in ich embryos injected with sqt mut:sqt RNA (Fig. 5F-J,Q), in contrast to injected wild-type embryos (Fig. 5K-O,Q). Thus, expansion of dorsal by the sqt 3’UTR requires Wnt/β-catenin signaling.

**Antisense morpholinos that target sqt ATG sequences block sqt RNA localization**

The dorsal activity of maternal sqt RNA appears to be mediated by the 3’UTR and is independent of Sqt protein or Nodal signaling. However, these findings raise the question of how a sqt ATG-targeting morpholino [sqtMO1 (Gore et al., 2005)] leads to loss of dorsal structures (Gore et al., 2005). sqtMO1 spans the translational start site sequence (Feldman and Stemple, 2001) and is presumed to block Sqt protein synthesis. Indeed, co-injection of sqtMO1 with mRNA encoding Sqt-GFP fusion protein leads to substantially reduced expression of Sqt-GFP in the blastoderm (38%, n=97), as compared with co-injection of Sqt-GFP with a sqt ATG mismatch control morpholino (ConMO, 76%, n=114; Fig. 6B-E). Thus, Sqt protein expression is disrupted by the ATG morpholino, but not by control morpholinos. Interestingly, we found that a substantial number of sqtMO1-injected embryos showed Sqt-GFP fluorescence in the yolk (~62% yolk expression, n=97; arrowhead in Fig. 6D), suggesting a localization defect. So, we revisited our sqt morpholino injection experiments (Gore et al., 2005) and tested whether sqtMO1 also affects other functions pertaining to sqt, such as RNA localization and/or maintenance. To examine RNA levels, we performed RT-PCRs and found that sqt RNA levels are unchanged in sqtMO1-injected embryos at least until the 8-cell stage (supplementary material Fig. S4). Therefore, injection of the sqt ATG morpholino does not lead to degradation of sqt RNA at these stages.
To test whether sqtMO1 affects sqt RNA localization, we coinjected fluorescently labeled sqt RNA with either sqtMO1 or ConMO, and examined the embryos for localization at the 4-cell stage (Gore et al., 2005; Gilligan et al., 2011). Remarkably, sqtMO1 nearly abolishes sqt RNA localization at the 4-cell stage (90%, n=127), and in ~35% of the embryos the injected fluorescent sqt RNA was detected as aggregates in the yolk (Fig. 6G,I,J). Similarly, sqtMO2 (which spans exon 2/intron 2; see Fig. 6A) also affects sqt RNA localization (data not shown). By comparison, ConMO and control target protector morpholinos [TPcontrol (Giraldez et al., 2005)] do not significantly affect localization (Fig. 6F,H,J), whereas a morpholino targeting the dorsal localization element (DLE MO; see Fig. 6A) reduces sqt localization (Fig. 6J) (Gilligan et al., 2011). These results suggest that sequences surrounding the translational start site and splice junctions are also required for sqt RNA localization. Therefore, in addition to disrupting the translation/splicing of sqt RNA, the sqt morpholinos also unexpectedly affect sqt RNA localization.

Disruption of sqt RNA localization leads to ectopic dorsal expansion

We find that blocking the translational start site disrupts sqt RNA localization (Fig. 6). However, sqt RNA localization is also dependent on sequences in the 3′UTR (Gore et al., 2005; Gilligan et al., 2011), and dorsal expansion also requires the 3′UTR. We therefore tested whether dorsal expansion by the 3′UTR depends on sqt RNA localization. We injected DLE MO, which disrupts sqt RNA localization (Gilligan et al., 2011). Morpholinos targeting a different region of the sqt 3′UTR (TPcontrol MO) and miR430 target protector morpholinos [TPmiR430 MO (Giraldez et al., 2005)] were used as controls. Embryos injected with the DLE MO showed lateral expansion of dorsal gsc expression even at low doses (2 ng; Fig. 7B,E), similar to embryos injected with non-coding sqt RNA (see Fig. 2F and Fig. 3C,E). At higher doses (10 ng), gsc expression extended further towards the animal pole (Fig. 7C,E) or even covered the entire blastoderm (Fig. 7D,E). By contrast, TPcontrol MO-injected and TPmiR430 MO-injected embryos did not manifest gsc expansion at 2 ng doses, and only a few TPmiR430 MO-injected embryos showed mild expansion of gsc at 10 ng (Fig. 7E). These experiments show that blocking the dorsal localization element in endogenous sqt RNA with DLE MO leads to expanded or ectopic gsc expression. Thus, dorsal expansion by sqt RNA is not dependent on the DLE in the sqt 3′UTR.

We also examined the expression of early dorsal markers in sqt ATG morphant embryos. Injection of sqt morpholinos into eggs to target maternal sqt RNA prior to the formation of aggregates in the yolk upon egg activation (Gore and Sampath, 2002) causes loss of dorsal specification. We find that gsc expression is not detected in these embryos, consistent with the loss of dorsal structures (Fig. 7G). This is in contrast to MZsqt and MZoeq mutant embryos, in which early gsc expression is detected at comparable stages (Fig. 7H,I and Fig. 5A). Furthermore, β-catenin fails to accumulate in dorsal nuclei of sqtMO1-injected embryos, in contrast to ConMO-injected embryos (Fig. 7N-Q, Table 1 and supplementary material Table S2). Ventral markers such as gata2 are concomitantly expanded in sqtMO1-injected embryos (Fig. 7J-M). Therefore, the sequences spanning the translational start site in sqt RNA are required for localization, and disruption of endogenous sqt function by sqtMO1 leads to loss of dorsal gene expression, probably by blocking both maternal sqt RNA localization and translation. These results also show that maternal sqt RNA functions prior to nuclear β-catenin accumulation.

The sqt 3′UTR rescues anterior and dorsal structures in sqt morphant embryos

We then tested whether the sqt 3′UTR is capable of restoring early dorsal gsc expression in sqt morphant embryos. In comparison to control morpholino-injected embryos (Fig. 8A), MZsqt and MZoeq embryos (Fig. 7H,I and Fig. 5A), injection of the sqt morpholinos into eggs results in embryos with severe dorsal deficiencies (Gore et al., 2005; Gore et al., 2007) and loss
Co-injection of lacZ:sqt or sqtSTOP:sqt RNA with sqtMO1 or sqtMO2 rescued early gsc expression (Fig. 8E and supplementary material Fig. S5). At prim-5 stages, sqt morpholino-injected embryos manifest loss of anterior and dorsal structures, whereas co-injection of the sqtMOs with sqtmut RNA or lacZ:sqt RNA produces phenotypes that are strikingly similar to that of MZ sqtcz35 mutant embryos (Fig. 8D,F,H,I). The sqt morpholinos prevent endogenous Sqt protein translation and endogenous sqt RNA localization, but cannot target lacZ:sqt. Therefore, the rescue by the sqt 3′UTR sequences is very significant. Thus, sqt RNA, and specifically the sqt3′/H11032 UTR, is sufficient to initiate dorsal gene expression in early embryos. These findings indicate that the biological activity of maternal sqt RNA in dorsal specification is likely to reside within its 3′UTR.

**DISCUSSION**

Asymmetric localization of sqt RNA in presumptive dorsal cells shows that dorsoventral asymmetry in the blastoderm is established prior to zygotic transcription, during cleavage stages. Based upon cell ablations and antisense morpholino injections, we proposed that asymmetrically localized sqt RNA and associated factors specify dorsal identity (Gore et al., 2005). However, studies using the insertion mutants sqtcz35 and sqthi975 suggested that early specification of the dorsoventral axis might not require the activity of maternal Sqt (Aoki et al., 2002; Bennett et al., 2007; Pei et al., 2007). Therefore, the function of maternal sqt was unclear. We find that sqt RNA has functions that are independent of Sqt protein in dorsal initiation. Furthermore, we show that the sqt<sup>e235</sup> and sqt<sup>h975</sup> insertion alleles are not maternal transcript nulls: sqt<sup>e235</sup> RNA is expressed in MZ sqt at similar levels to sqt in wild-type embryos at early stages and also localizes to two cells in 4- and 8-cell stage embryos. We observed a reduction in sqt RNA levels during...
gastrulation, consistent with a previous report of reduced sqt mutant transcripts in late blastula embryos (Bennett et al., 2007). However, Bennett et al. (Bennett et al., 2007) also stated that MZ sqtcz35 embryos at the 8-cell stage contain no detectable sqt RNA using sqt-specific RT-PCR primers, and Pei et al. (Pei et al., 2007) reported that sqt RNA is reduced or absent in MZ sqthi975 embryos based on RT-PCR using oligo(dT)-primed cDNA. By RNA sequencing and RT-PCR, we find that maternally deposited sqt RNA is non-polyadenylated and unspliced, and that sqt mRNA is detected during cleavage stages (supplementary material Fig. S1A,B) (Gore et al., 2007), providing an explanation for the Pei et al. (Pei et al., 2007) observation. However, our findings differ from the lack of maternal sqt RNA reported by Bennett et al. (Bennett et al., 2007), which we detect in early MZ sqt embryos by RT-PCR, quantitative PCR and whole-mount in situ hybridization.

We previously reported that injection of sqt splice-blocking morpholinos into wild-type eggs leads to aberrantly spliced sqt RNA (Gore et al., 2007). The presence of pre-mRNAs has been reported in oocytes in other metazoans as well (Hachet and Ephrussi, 2004). In Xenopus eggs, many maternal pre-mRNAs are also known to be non-polyadenylated or have very short poly(A) tails, which presumably prevent precocious protein translation (Sagata et al., 1980; McGrew et al., 1989; Paris and Philippe, 1990; Varnum and Wormington, 1990). Our findings are also consistent with the recent genome-wide transcriptome analysis in zebrafish by Aanes et al. (Aanes et al., 2011), who showed by RNA sequencing that a large cohort of maternal transcripts in zebrafish eggs are non-polyadenylated, and become polyadenylated during early embryogenesis (Aanes et al., 2011). Thus, the sqt insertion alleles are not maternal transcript nulls and not bona fide functional null alleles.

Mutant sqt RNA can expand dorsal gene expression in wild-type embryos, with a concomitant reduction in ventral gene expression. The transient gsc expansion by sqt\textsuperscript{mut} sqt RNAs and the sqt 3’ UTR is unlikely to be due to a morphological delay as the embryos are not generally delayed. Moreover, the gsc expansion is restricted to the gastrula margin and does not extend animally, in contrast to the broad gsc expression domain reported in early embryos (Schulte-Merker et al., 1994). We also observed expansion of chd and substantially increased numbers of β-catenin-positive nuclei in embryos injected with non-coding sqt RNA. Furthermore, dha and endogenous sqt transcript levels transiently increase, with a concomitant reduction in vox and vent transcripts, providing the basis for the transient increase in chd, gsc and ntl and reduction in ventral expression of gata2 (Hammerschmidt et al., 1996; Erter et al., 1998; Rebagliati et al., 1998; Yamanaka et al., 1998; Imai et al., 2001; Gilardelli et al., 2004). Thus, sqt RNA, and specifically the sqt\textsuperscript{3}/H11032 UTR, can nucleate a complex of factors that are sufficient to expand dorsal gene expression. We also find that mutant/non-coding sqt RNA injections into sqt morphants can phenocopy MZsqt mutant embryos. Taken together, these findings suggest that it is the

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**Fig. 7.** The sqt DLE MO and ATG MO differentially affect dorsal gene expression. (A-E) gsc expression expands upon injection of the DLE MO even at low doses, in comparison to injections of Tpmir430 MO or Tpcontrol MO. (E) The percentage and number of embryos and the extent of gsc expression (classes I-IV, A-D) in injected embryos at sphere stages. (F-I) Expression of gsc is abolished in sqtMO1-injected embryos at the sphere stage (G), in comparison to control wild-type (F), MZsqt (H) or MZoesp (I) embryos. (J-M) Expression of the ventral marker gene gata2 is expanded to varying extents in sqtMO1-injected embryos (K-M), in comparison to control embryos (J). (N-Q) Nuclear β-catenin (N,P) in dorsal cells is not detected in sqtMO1-injected embryos at the 512-cell stage (P,Q), in contrast to ConMO-injected embryos (arrows, N,O). DAPI staining (O,Q) shows the presence of nuclei. (A-D,F-M) Animal pole views; (N-Q) dorsal views. Scale bars: 100 μm in A; 25 μm in N.
activity of the mutant sqt transcripts in the maternal sqt insertion mutants that leads to initial dorsal expression in mutant embryos, and MZsqt mutants do not exhibit the severe loss of dorsal identity manifested by sqt morphants in which maternal sqt RNA localization and activity are both disrupted.

Interestingly, we never detected any ectopic sites of gsc or chd in the mutant sqt RNA-injected embryos, unlike those injected with wild-type sqt RNA (Erter et al., 1998; Rebagliati et al., 1998). Thus, although mutant sqt RNAs and the sqt 3'UTR can expand the endogenous dorsal domain, these sqt sequences do not induce dorsal at ectopic locations, presumably because these RNAs harbor the dorsal localization element. By contrast, injection of sqt DLE MO to target endogenous sqt RNA leads to ectopic gsc expression, probably owing to mislocalization and misexpression of endogenous Sqt.

Mutant sqt RNAs transiently expand the dorsal domain in wild-type embryos, but this expansion is not sustained, and by mid-gastrula stages the embryos appear to have regulated dorsal gene expression levels back to that observed in control embryos. This transient expansion is consistent with the normal gsc expression observed at 70% epiboly by Bennett et al. (Bennett et al., 2007) in T-sqt-injected embryos. Similar changes in early patterning with no overt later consequences have also been observed in other contexts/organisms. For example, extra copies of bicoid in Drosophila lead to transient oversized head regions in embryos, which develop into apparently normal adults (Berleth et al., 1988).

Dorsal gsc expression is also expanded in MZoep embryos, which are presumed to lack all Nodal signaling (Gritsman et al., 1999). However, the expansion is not sustained. Initiation of dorsal gsc expression and its subsequent loss has been reported previously in cyp/sqt compound mutant embryos (Dougan et al., 2003). Thus, maintenance of gsc expression during gastrulation requires Nodal signaling. Although sqt RNA can initiate and expand dorsal gene expression independently of Sqt protein or Oep-dependent Nodal signaling, the sustained expression of dorsal genes requires the signaling functions of Sqt mediated via Oep.

So how does sqt RNA function to initiate and expand dorsal? This is likely to be a non-coding function of sqt, as the sqt 3'UTR even when fused to heterologous reporter genes (lacZ or venus) can expand dorsal gene expression in wild-type embryos and rescues early dorsal gene expression in sqt morphants. Furthermore, coding sqt sequences fused to the sqt 3'UTR rescue ich embryos more efficiently than sqt fused to globin UTR sequences, demonstrating that the activity of the sqt 3'UTR is distinct from the Sqt coding sequences. Taken together, these findings suggest that sqt RNA, and specifically the sqt 3'UTR, functions in dorsal specification.

Our current evidence is based upon overexpression and knockdown strategies, which have their limitations (Robu et al., 2007; Eisen and Smith, 2008). Although it is imperative to determine whether a sqt mutant that lacks maternal sqt RNA expression also lacks dorsal identity, such a mutant is not yet available despite our attempts using zinc-finger nuclease (ZFN) (Doyon et al., 2008; Meng et al., 2008) technology.

The sqt 3'UTR harbors microRNA (miRNA) target sites (Giraldez et al., 2006; Choi et al., 2007), raising the possibility that dorsalinization by the sqt 3'UTR might be mediated via miRNAs. However, this seems unlikely because sqt 3'UTR with mutations in target site sequences of three predicted miRNAs still expands the dorsal domain, as does sqt RNA injection into MZdicer embryos (supplementary material Fig. S6). Although it remains possible that there are other unidentified dicer-independent small RNA targets in the sqt 3'UTR, our experiments show that the sqt 3'UTR functions independently of the miRNAs tested and of dicer. Rather, we surmise that sqt RNA might translocate some factor(s)/protein(s) that bind to the UTRs to the future dorsal side. In Drosophila, oskar RNA has
functions that are independent of Oskar protein in early oogenesis (Jenny et al., 2006). Similarly, *Xenopus* veg-T RNA has a scaffolding function that is independent of the later functions of Veg-T protein in germ layer specification (Zhang and King, 1996; Kloc et al., 2005). Since sqt RNA is normally present in limiting amounts in early embryos (Rebagliati et al., 1998; Gore et al., 2005), even small increases in the amount of sqt UTR could lead to an amplified effect of any UTR-bound factors (see model in Fig. 9) in the future embryonic dorsal side. These factor(s) might function via the canonical Wnt/β-catenin pathway, as the sqt 3'UTR by itself is unable to expand the dorsal domain in the context of ich embryos, which are deficient in β-catenin signaling. It is conceivable that the factor(s) binding to the sqt 3'UTR are components of the Wnt/β-catenin pathway.

In *Xenopus*, RNA encoding Xwnt11, which is required for dorsal specification and functions via β-catenin (Tao et al., 2005), is localized to dorsal vegetal cells of early embryos. The factors that bind and localize Xwnt11 RNA are not known. In zebrafish, embryos from ich mutant mothers show that maternal β-catenin function is required for dorsal specification, and maternal wnt8a RNA is likely to activate Wnt/β-catenin signaling (Lu et al., 2011). Asymmetric localization of sqt RNA in the blastoderm by the 4-cell stage precedes dorsal accumulation of nuclear β-catenin at the 128-cell stage (Dougan et al., 2003; Gore et al., 2005).

The sqt 5'UTR and 3'UTR both affect sqt RNA localization, but somewhat differently. Whereas the sqt ATG morpholino results in sqt RNA being mislocalized diffusely or stuck in the yolk as aggregates, the DLE MO causes sqt RNA to be mislocalized diffusely in the blastoderm. The phenotypes caused by the morpholinos targeting these two regions are also distinct in that the ATG morpholino causes loss of nuclear β-catenin and loss of gsc expression, whereas the 3'UTR DLE MO causes mislocalized and ectopic gsc, probably owing to mislocalized endogenous Sqt in the blastoderm. This suggests that the sqt 5'UTR and 3'UTR sequences might function in distinct steps of sqt RNA localization and activity. Moreover, our finding that the sqt ATG morpholino disrupts both sqt RNA localization and translation suggests that the sqt 5'UTR and 3'UTR sequences might interact with each other, perhaps via binding of a protein complex, to regulate sqt RNA localization (see model in Fig. 9). The sqt morpholinos cause loss of dorsal nuclear β-catenin and gsc expression, which might underlie the severe loss of dorsal structures in the morphants. Our results suggest that localized maternal sqt RNA functions prior to the accumulation of nuclear β-catenin, and raise the possibility that sqt RNA acts as a scaffold to bind and deliver/sequester maternal factors, which are likely to be intracellular component(s) of the Wnt pathway, to future dorsal. Our findings identify novel functions for the Nodal genes and suggest a new role for non-coding RNAs in the control of axis specification.
Acknowledgements

We thank members of the K.S. laboratory, the Singapore fish community, Tom Carney, Bill Chia, Steve Cohen, Ray Dunn, Aniket Gore, Masahiko Hibi, Greg Jedd, Mithilesh Mishra and Mohan Balasubramanian for discussions and suggestions; Shingo Maegawa and Eric Weinberg for ich fish; and the Temasek Life Sciences Laboratory sequencing and fish facilities.

Funding

S.L. was supported by a pre-doctoral fellowship from the Singapore Millennium Foundation and Temasek Life Sciences Laboratory; S.M. is supported by the Genome Institute of Singapore; work in the laboratory of K.S. is supported by Temasek Life Sciences Laboratory.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077081/-/DC1

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