Supplementary Methods

In situ hybridization

A two-step polymerase chain reaction (PCR) strategy was used to amplify DNA as template for antisense mRNA probe synthesis. To detect complementary mRNA in subsequent ISH, the DNA template was equipped with T7 promoter. To amplify DNA of the gene of interest, first a standard PCR (denaturation at 94°C, annealing at 57°C, elongation at 72°C, 35 cycles) using cDNA as template was performed. A second PCR was performed using only one of the gene specific primers (3’ for sense/5’ for antisense) and one T7 universal primer that contains the T7 promoter sequence. The universal primer binds to a specific sequence which is attached to the 5’ end of the gene specific primer (T7 linker) and was amplified during the first PCR. Cycling parameters for the second PCR reaction are the same as for the first PCR. The amplicon from the second PCR is used as template for RNA probe synthesis.

To label the probe during synthesis UTP-Digoxigenin (DIG) (Roche) was used. 3 μl of the amplicon from the second PCR were mixed with 7 μl of T7 master mix (3.75 μl RNase-free H2O, 0.25 μl RNase inhibitor, 1 μl 10x T7 reaction buffer, 1 μl DIG labelling mix, 1 μl T7 polymerase; all from Roche) and incubated for 4 hours at 37°C. The reaction was stopped by adding 15 μl H2O and 25μl 2x Stop solution (0.2M NaAc, 1% HAc; pH 6.0). For precipitation 2.5 μl tRNA (20 mg/ml; Roche), 5 μl LiCl (4M) and 150 μl 100% EtOH were added. The probes were incubated for at least 30 minutes at -20°C followed by a full speed centrifugation at room temperature for 30 minutes. The RNA pellet was dried for 5 minutes before it was resuspended in 40 μl 50% formamide, 2xSSC. The probes were stored at -20°C.

Tribolium eggs were rinsed from dirt and left over flour with tap water. For dechorionization the eggs were put for 2 min in 50% DanKlorix (Colgate-Palmolive). The chlorine was then thoroughly washed away with tap water. To fix the embryos, they were incubated in fixation solution (2 ml 10% formaldehyde, 3 ml PBS, 5 ml heptane), shaking at 120 rpm, for 45 min to 1.5 h. The embryos were devitellinized by methanol shock or by hand dissection. Embryos were transferred into PBST (first 2:1, then 1:2, methanol : PBST), washed with PBST (2 times, 5 min each), fixed in 5% PFA/PBST for 30 min. The PFA was washed away with PBST (four times, 5 min each). The embryos were transferred into hybridization solution I (25 ml formamide, 12.5 ml 20x SSC, 50 μl heparin (50 mg/ml), 250 μl 20% Tween, fill up to 50 ml with millipore H2O ) (first 1:1 hyb sol I : PBST for 10 min, then neat hyb sol I for 10 min) and then prehybridized in hybridization solution II (25 ml formamide, 12.5 ml 20x SSC, 50 μl heparin (50 mg/ml), 250 μl 20% Tween, 500 μl salmon sperm DNA (100 μg/ml), filled up to 50 ml with millipore H2O) at 59°C for 1 h. 3 μl ISH probe, specific for the mRNA of interest, was diluted in 150 μl hybridization solution II and incubated with the embryos overnight at 61 °C while rotating. The probe was washed away by washing four times for 5 min, and four times for 30 min with hybridization solution I at 61 °C. The embryos were then transferred into PBST (first 2:1, then 1:2, hyb sol I : PBST, at 61 °C) and washed for 10 min with PBST (room
temperature). For blocking, embryos were incubated two times for 30 min in PBST that contains bovine serum albumin (BSA, 100 µl/ml) and normal goat serum (NGS, 30 µl/ml). Then, antidiigoxigenin antibody coupled with alkaline phosphatase was added in a final concentration of 1:4000 and incubated with the embryos overnight at 4 °C while rotating. The next day the embryos were rinsed by washing three times for 5 min and three times for 15 min with PBST and three times for 5 min with AP-buffer (1 ml 1 M TRIS HCl pH 9.5, 500 µl 1 M MgCl2, 200 µl 5 M NaCl, ≤50 µl 20% Tween, fill up to 10 ml with millipore H2O). For the color reaction 8 µl NBT/BCIP (nitro blue tetrazolium chloride/5brome-4chlor-3indolyl-phosphate; Roche) solution in 400 µl AP-buffer were added to the embryos. The color reaction was observed and stopped by washing three times with PBST when background staining started to develop. The staining was then fixed by incubating the embryos for 30 min in 5% PFA in PBST. Background staining was in some cases removed by incubating the embryos for ≥30 min in pure EtOH. The EtOH was washed away by two washes with PBST. The embryos were embedded into VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

For fluorescent in situ hybridization (Tc-vasa) we used a 1:200 dilution of the anti-DIG-POD antibody by Roche (Cat. no. 00000011426346910) and TSA™ Kit #2 by Invitrogen (Cat. no. 20912).

**DAPI staining**

Dechorionated, fixed embryos were transferred from methanol to PBST (PBS supplemented with 1 % Tween) and washed three times for 5 min with PBST. Then, the embryos were embedded in VECTASHIELD Mounting Medium with DAPI (4′,6-Diamidin-2-phenylindol) (Vector Labs).

**Phalloidin staining**

Dechorionated, fixed embryos (not treated with methanol) were devitellinized by hand and washed in PBST (3 times, 5 min each). The embryos were postfixied in 350 µl 5% PFA/PBST for 20 min. The PFA was removed by five 5 min washes with PBST. After 1 h incubation in 500 µl blocking solution (100 µl BSA (100 µg/ml), 250 µl NGS (30 µl/ml), 5 µl 20% Tween, 5 ml 1xPBS), Alexa Fluor 555/568 Phalloidin (Molecular Probes, life technologies) was added (1:50 in blocking solution) and the embryos were incubated over night at 4°C. Phalloidin was removed by washes in PBST (5 times, 10 min each). The embryos were covered with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).
RNA isolation

Using standard protocols or kits (TRIzol reagent, Invitrogen; AmbionRNAeasy mini kit, Invitrogen; RNAqueous Micro Kit, Ambion; some kit from Zymo Research) for isolating Tribolium RNA, did not yield high quality RNA. The 260/230 values, which indicate salt, phenol or similar contaminations, differed a lot between the samples and were rarely above 1.8, but sometimes as low as 0.4. The contaminations were unlikely salt or phenol contaminations, but either some yolk component or polysaccharide contaminations from the flour sticking to the beetles with biochemical abilities quiet similar to those of RNA. The following protocol is partially based on protocols for extracting RNA form polysaccharide rich specimens (Logemann et al., 1987; Wang et al., 2012).

1. Separate eggs from flour, wash thoroughly with water, bleach in 50% Klorix for 2 min, wash again with water.
2. Transfer eggs into 1.5 ml reaction tube. Do not take to many eggs. The eggs should have a volume like 25 µl to 50 µl water at the bottom of the reaction tube. Flash freeze in liquid nitrogen and store at –80 °C for later processing or add directly 100 µl TRIzol reagent. It is essential to use fresh TRIzol. Old TRIzol that works fine for other samples, e.g. Nasonia eggs, Zebrafish tissues, may perform bad on Tribolium eggs.
3. Homogenize eggs in TRIzol using a pestle. Spin down quickly and apply some more strokes with the pestle. Work on ice.
4. Centrifuge for 10 min, 12,000g, at 4 °C. Transfer the supernatant into a fresh tube and add 400 µl TRIzol (total volume 500 µl).
5. Incubate the sample for 5 min at room temperature.
6. Add 100 µl room temperature chloroform. Keep chloroform stock in the dark.
7. Shake tube thoroughly by hand for 15 sec.
8. Incubate sample at room temperature for 2 min to 3 min.
9. Centrifuge sample for 15 min with 12,000g, at 4 °C. Spin longer if the phases do not build a sharp border.
10. Transfer upper aqueous phase into a new reaction vessel. Do not take any material from the interphase. Sometimes, white cloudy stuff is present at the lower part of the upper phase.
11. Precipitate by adding 40 µl 3 M NaAc pH 5.2 and 800 µl room temperature EtOH, invert 2 to 3 times and incubate for at least 30 min on –80 °C.
12. Centrifuge at full speed for 20 min at 4 °C. The pellet might be hard to see. Take of fluid. Sometimes two pellets can form, one covering the other. One pellet is pure RNA, the other one is highly contaminated. Separate pellets and put each into an own reaction vessel. Do the rest of the protocol in parallel for both pellets.
13. Wash by adding 500 µl 3 M NaAc pH 5.2, shake lightly for 5 min.
14. Centrifuge for 20 min at full speed. Pellet might be hard to see. Take of fluid.
15. Wash by adding 800 µl room temperature 70% EtOH.
16. Centrifuge for 10 min at full speed. The pellet should now be better visible than before. Take of all fluid.
17. Repeat steps 13 to 16.
18. Let dry for 5 min and resuspend pellet in nuclease free water.

Another protocol that worked well, but is longer and causes more sample loss was described by G. Wang et al. (Wang et al., 2012).
Figure S1: qRT-PCR evaluation confirms kd efficiency

(A - G) Examples of kd validation by qRT-PCR for individual RNA samples of each condition. The graphs show average loss or gain in expression as compared to wild type and dsRed RNAi embryos (normalized to 0). (A) Tc-Toll expression in Tc-Toll RNAi embryos. (B) Tc-sog expression in Tc-sog RNAi embryos. (C) Tc-dpp expression in Tc-dpp RNAi embryos. (D) Tc-twist expression in Tc-twist RNAi embryos. (E) Tc-twist expression is reduced in Tc-Toll RNAi embryos, as expected since Tc-twist is downstream of Tc-Toll. (F) Tc-doc expression is slightly increased in Tc-sog RNAi embryos. (G) Tc-doc expression is decreased Tc-dpp RNAi embryos.

The changes in transcript levels observed by qRT-PCR correspond to alterations of the expression pattern observed by whole mount ISH. (H) Wild type. Tc-twist is expressed in the future mesoderm. (I) Tc-Toll RNAi. Tc-twist is just expressed in a patch at the posterior pit indicating that Tc-Toll is essential for most of Tc-twist’s expression. (J) Wild type. Tc-doc is expressed in the dorsal serosa. (K) Tc-sog kd. Tc-doc expression is expanded. However, the Tc-doc expression levels within the expanded domain are slightly decreased compared to wild type. This might explain why the qRT-PCR data (F) show only a mild increase in total Tc-doc expression levels upon Tc-sog kd. (L) Tc-dpp kd. Tc-doc expression is lost.

(H, I) modified from (Nunes da Fonseca et al., 2008). (I – L) modified from (van der Zee et al., 2006).
Figure S2: Cardioblasts are lost upon *Tc-twist* kd

(A, A’’’) Untreated embryos of the enhancer trap line G04609. The cardioblast cell row (the presumptive heart) of this enhancer trap line expresses EFGP (green) (Trauner et al., 2009; Koelzer et al., 2014). (B, B’’’) *Tc-twist* kd embryos. The cardioblast cells, which are mesodermal derivatives, are lost upon down of *Tc-twist*. The segmental blocks of cells expressing EGFP were previously proposed to be body wall muscle cells (Koelzer et al., 2014). This is highly unlikely since EGFP expression is still present after *Tc-twist* kd. However, the body wall muscles marked in the pBA19 line are lost upon *Tc-twist* kd (see Figure 2 O, P). Further, we did live imaging of wild type and *Tc-twist* RNAi G04609 embryos and looked for muscle twitching, as described by (Koelzer et al., 2014). We observed the twitching in 100% (n = 21) of the wild type embryos, while we observed no twitching in 22 of 23 *Tc-twist* kd embryos. In all embryos that did not twitch, no EGFP expression was observed in the cardioblast cell row. All pictures were taken from fixed embryos, counterstained with DAPI. Embryos in A and B were in addition stained with phalloidin (red) to visualize muscles (see A’’ and B’’ for single channel). Phalloidin stains muscle and non-muscle actin. The massive reduction of phalloidin-labeled structures in B’’ compared to A’’ correlates with the loss of muscles upon *Tc-twist* kd. (A’’’, B’’’) DAPI staining of the respective embryos. (C) Wild type cuticle. (D) *Tc-twist* kd cuticle. The arrow points to the rotated hindgut.
Figure S3: Marker gene expression after RNAi for genes differentially regulated upon Tc-sog and Tc-dpp knockdown


Expression patterns in all kd embryos are wildtype -like with the following exception: (L) The expression of Tc-achaete-scute homolog is only visible in the head and the posterior end of elongated germ band embryos upon TC014658/Tc-tartan kd. All panels show ISHs and ventral surface views of embryos with the anterior pole pointing to the left. exp - expression; kd - knock down; Tc-ash - Tc-achaete-scute homolog; Tc-gsb - Tc-gooseberry; Tc-en - Tc-engrailed.
Figure S4: Marker gene expression after RNAi for genes differentially regulated upon Tc-Toll and Tc-twist knockdown.


Expression patterns in all kd embryos are wild type-like with following exception: (O) Tc-twist expression is missing from a large portion of the trunk in TC011114/Tc-zfh1 kd embryos. All panels show ISHs and ventral surface views of embryos with the anterior pole pointing to the left with the exception of (O) showing a lateral view. exp - expression; kd - knock down; Tc-ash - Tc-achaete-scute homolog; Tc-gsb - Tc-gooseberry; Tc-en - Tc-engrailed.
Figure S5: Knockdown of Tc-LanB1 leads to loss of mesodermal derived tissues.

(A – D) pBA19 enhancer trap line which expresses EGFP in muscles (blue counterstaining is DAPI) (Lorenzen et al., 2003a). (A) Wild type pBA19 embryo. The body wall muscles are labeled by EGFP. (B – D) Tc-LanB1 knockdown. (B) Mild phenotype showing partial loss and irregular shape of the muscles. (C) Intermediate Tc-LanB1 kd phenotype. The number of muscles fibers is strongly reduced, remaining muscles have a more spherical shape compared to wild type. (D) Strong Tc-LanB1 kd phenotype. The muscles are almost completely lost. (E – H) Embryos of the enhancer trap line G04609. The cardioblast cell row (the presumptive heart) expresses EFGP (blue counterstaining is DAPI) (Trauner et al., 2009; Koelzer et al., 2014). (E) Wild type embryo of the G04609 line. (F – H) Different stages of G04609 embryos upon Tc-LanB1 kd (F = youngest embryo, before serosal rupture; G = oldest embryo, after serosal rupture) show loss of the cardioblast cell row. kd: knockdown DAPI: 4’,6-diamidino-2-phenylindole for nuclear staining.
Table S1: Number of differentially expressed genes overlap substantially between different analysis methods

Two algorithms, DESeq2 and edgeR were used to test for differentially expressed genes (Anders and Huber, 2010; Robinson et al., 2010). The table shows the number of significantly differentially expressed genes detected by the two algorithms with a FDR of 1%. In all cases >85% of the genes determined by edgeR were also identified by DESeq2. Only genes found by both methods were further considered. Transcriptomes of A) Tc-twist RNAi embryos, B) Tc-Toll RNAi embryos, C) Tc-sog RNAi embryos and D) Tc-dpp RNAi embryos were compared to the transcriptome of control embryos. ctrl - control embryos; kd - knockdown; FDR - false discovery rate; up - upregulated; down - downregulated.

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Table S2: Differentially expressed genes upon Tc-dpp RNAi compared to control

The Table shows all genes which appeared as significantly differentially expressed upon Tc-dpp kd compared to the control. The list contains all up- and downregulated genes detected with a false positive rate of 1% (FDR01) and 5% (FDR05).

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Table S3: Differentially expressed genes upon *Tc-sog* RNAi compared to control

The Table shows all genes which appeared as significantly differentially expressed upon *Tc-sog* kd compared to the control. The list contains all up- and downregulated genes detected with a false positive rate of 1% (FDR01) and 5% (FDR05).

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Table S4: Differentially expressed genes upon *Tc-Toll* RNAi compared to control

The Table shows all genes which appeared as significantly differentially expressed upon *Tc-Toll* kd compared to the control. The list contains all up- and downregulated genes detected with a false positive rate of 1% (FDR01) and 5% (FDR05).

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Table S5: Differentially expressed genes upon \textit{Tc-twist} RNAi compared to control

The Table shows all genes which appeared as significantly differentially expressed upon \textit{Tc-twist} \textit{kd} compared to the control. The list contains all up- and downregulated genes detected with a false positive rate of 1\% (FDR01) and 5\% (FDR05).

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Table S6: Raw data of differentially expressed genes corresponding to the Venn Diagram

All genes which are differentially expressed in more than one knockdown condition compared to the control (overlapping fields of the Venn diagram). The upregulated genes are labelled in green and the downregulated genes in red. The numbers indicate the fold change.

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Table S7: Genes differentially regulated upon Tc-dpp kd and Tc-sog kd

Genes detected as up- or downregulated upon Tc-dpp kd and Tc-sog kd with a false positive rate of 1% (FDR01) and 5% (FDR05). The list contains the official TC-identifier, information about up- or downregulation in the different knockdown conditions and Drosophila orthologs of the respective genes.

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Table S8: Genes differentially regulated upon Tc-Toll kd and Tc-twist kd

Genes detected as up- or downregulated upon Tc-Toll kd and Tc-twist kd with a false positive rate of 1% (FDR01) and 5% (FDR05). The list contains the official TC-identifier, information about up- or downregulation in the different knockdown conditions and Drosophila orthologs of the respective genes.

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Table S9: Primerlist

The list contains the primer sequences of all genes relevant for this paper. Primers containing the linker sequences and both T7 universal primers where used for RNA-probe synthesis and generation of dsRNA for injections. Primers without linker sequences where used for qRT-PCR.

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Table S10: Off-target analysis of primers and dsRNA-fragments used for pRNAi

The specificity of the primers as well as the fragments used for dsRNA synthesis was tested using ViroBLAST and the official Annotation of the Tribolium castaneum genome based on Assembly Tcas 3.0 (Ref). The red numbers and text indicate the primary Blast hits. The results of the analysis can be checked on [http://bioinf.uni-greifswald.de/gb2/gbrowse/tribolium/](http://bioinf.uni-greifswald.de/gb2/gbrowse/tribolium/)

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**Supplementary Movies**

**Movie 1:** EFGP expression (white) in the wild type nGFP strain embryo (Sarrazin et al., 2012) was filmed from early uniform blastoderm stage to extended germ band stage at 30 °C. Z-stacks were made every 17 minutes.
**Movie 2:** *Tc-twist* kd in the nGFP strain (Sarrazin et al., 2012). EFGP expression (white) was filmed from uniform blastoderm stage to extended germ band stage at 30 °C. Z-stacks were made every 8 minutes.
**Movie 3:** *Tc-twist* *kd* in the nGFP strain (Sarrazin et al., 2012). EFbP expression (white) was filmed from uniform blastoderm stage to extended germ band stage at 30 °C. Z-stacks were made every 17 minutes.