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Sequence analysis of the Hro-Twist promoter

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SEQUENCE ANALYSIS OF THE *HRO-TWIST* PROMOTER

A Thesis
Presented to
The Faculty of the Department of Biology
San Jose State University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Tara Ann Gurtler

August 2005

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A handwritten signature in cursive script, appearing to read "Julio Soto", written over a horizontal line.

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ABSTRACT

SEQUENCE ANALYSIS OF THE *HRO-TWIST* PROMOTER

By Tara Ann Gurtler

The *Twist* gene is important in mesoderm development and dorsoventral axis formation of the fly. *Twist* expression occurs earlier in the glossiphoniid leech, *Helobdella robusta*, than in other model organism. Examining the *Hro-Twist* promoter for binding sites may explain differences in *Twist* expression and function among organisms. To find putative *Hro-Twist* binding sites, the *Hro-Twist* promoter was sequenced and analyzed using two bioinformatic programs. Binding sites in the *Hro-Twist* promoter and fly *Dm-Twist* promoter were compared. The *Hro-Twist* promoter lacks dorsal clusters, but contains putative clusters for MFH-1, FoxF1, and zeste. A 1150 bp *Hro-Twist* promoter fragment was cloned into a Green Fluorescent Protein reporter, pGFP-1. The M cells of stage 6 *Helobdella robusta* embryos were injected. The embryos showed no GFP expression. The results may suggest that the TATA box identified is incorrect or that the minimal *Hro-Twist* promoter is greater than 1150 bp.

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Introduction

Homologs can have different temporal and spatial expression and varied function. Identifying promoter binding sites can therefore be a key to understanding gene regulation and developmental differences among species. *Twist* is a developmental gene that exhibits a functional discrepancy among species. In the fly, *Twist* is needed for mesoderm development, axis establishment, and somatic myogenesis (Thisse *et al.*, 1987b; Baylies and Bate, 1996). In vertebrates, however, *Twist* inhibits myogenesis (Spicer *et al.*, 1996).

Twist homologs are expressed in mesodermally-derived cells in many organisms. The mesoderm germ layer gives rise to muscles, connective tissues, and blood cells. The first *Twist* gene was identified in *Drosophila melanogaster*, and called *Dm-Twist* (Thisse *et al.*, 1987a, 1987b). *Twist* homologs identified include: frog *X-Twist* (Hopwood *et al.*, 1989), zebrafish *Dr-Twist* (Morin-Kensicki and Eisen, 1997), leech *Hro-Twist* (Soto *et al.*, 1997), mouse *M-Twist* (Gitelman, 1997), human *H-Twist* (Wang *et al.*, 1997), lancelet *Bb-Twist* (Yasui *et al.*, 1998), nematode *Ce-Twist* (Harfe *et al.*, 1998), jellyfish *Pc-Twist* (Spring *et al.*, 2000), rat *R-Twist* (Bolch-Zupan *et al.*, 2001), chicken *C-Twist* (Tavares *et al.*, 2001), and spider *At-Twist* (Yamazaki *et al.*, 2005).

The research objective of this study was to analyze the leech *Hro-Twist* promoter sequence. The difference in the timing of *Twist* homolog expression in the species studied could correlate to different protein binding sites in the *Twist* promoters.

The leech model *Helobdella robusta* (phylum Annelida) has advantages in developmental studies. The leech is a member of Lophotrochozoa, a third branch of the Bilateria. Until recently the third branch was under-represented in literature due to the limited research on developmental model organisms that belong to the Lophotrochozoa. The other two bilaterian branches, Deuterostomia (which includes vertebrates) and Ecdysozoa (which includes arthropods and nematodes), contain well established model organisms (Tessmar-Raible and Arendt, 2004). Lophotrochozoans contain ancestral genes, body plans, and modes of development (Tessmar-Raible and Arendt, 2004). The group offers a third reference point in developmental comparisons that is limited when using only the Deuterostomia and Ecdysozoa groups (Tessmar-Raible and Arendt, 2004). Additional advantages to studying *Helobdella robusta* are that *Helobdella* embryos are easily maintained in the lab, are relatively large (0.4-2 mm), and have a clear separation of ectodermal and mesodermal precursors during stage 4b (Fernández, 1980). Unlike the embryos of the larger leech, *Hirudo medicinalis*, the large yolk supply allows the *Helodbella* embryos to develop normally when removed from the parent's cocoon (Fernández, 1980; Weisblat and Huang, 2001).

Literature Review

Twist structure

Drosophila melanogaster Twist, Dm-Twist, is a basic helix-loop-helix (bHLH) protein that can act as a transcription factor. The HLH protein family is comprised of transcription factors that play a role in early development processes. These roles include muscle differentiation and neurogenesis. The amino acid sequence of the bHLH motif in Twist homologs is conserved. The bHLH region in the fly Dm-Twist and leech Hro-Twist is 78% identical; the bHLH region in the Dm-Twist and human H-Twist is 82% identical (Baylies and Castanon, 2002). Basic helix-loop-helix proteins bind to the consensus sequence CANNTG called the E box (Ephrussi *et al.*, 1985).

Another DNA region, the WR motif in the *Dm-Twist* Open Reading Frame (ORF), is found in all organisms studied except the leech (Baylies and Castanon, 2002). The function of the WR motif, a 14-amino acid region found 22 to 55 amino acids from the C-terminal end of the bHLH, is undetermined. The motif is highly conserved in vertebrates. The lack of the WR motif in Hro-Twist may correlate with *Hro-Twist* expression at an earlier stage than other *Twist* homologs.

Twist function

Dm-Twist is required for dorsoventral axis establishment, mesoderm formation, and muscle splitting. *Twist*-null fly embryos fail to form a ventral furrow and lack the mesoderm germ layer (Thisse *et al.*, 1987a, 1987b; Cripps and Olson, 1998).

Cripps and Olson (1998) examined the effect of Dm-Twist on the development of the 6 larval oblique muscles (LOMs) that develop in the dorsal thorax region of the fly. The LOMs serve as the scaffold for the dorsal longitudinal indirect flight muscles. Reducing *Dm-Twist* during the late larval stage inhibits splitting of the three LOMs into six LOMs. As a result, only three dorsal longitudinal indirect flight muscles develop.

Another gene with a similar role, which co-expresses with *Dm-Twist* in early stages, is *myocyte enhancer factor 2 (Mef-2)* (Cripps and Olson, 1998). *Mef-2* is activated by Dm-Twist (Cripps and Olson, 1998). Reduction in *Mef-2* also inhibits muscle splitting. Dm-Twist is therefore thought to be a transcriptional activator for *Mef-2*, or at least activate the gene indirectly (Cripps and Olson, 1998).

Known target genes for Dm-Twist are *snail* and *tinman*. In *Drosophila* mesoderm formation, Dm-dorsal binds to *Dm-Twist*. In turn, Dm-Twist binds to and activates *Dm-snail*. Dm-Twist also binds to enhancer regions of *tinman*, a gene needed for differentiation of visceral and cardiac muscles (Lee *et al.*, 1997; Yin *et al.*, 1997).

The importance of the bHLH motif is two-fold. The basic domain enables DNA binding (Kadesch, 1993). The HLH motif allows Dm-Twist dimers to form (Thisse and Thisse, 1992). HLH proteins are active as dimers and can form homodimers and heterodimers with other HLH proteins (Thisse and Thisse, 1992).

Dm-Twist acts as an activator or repressor depending on the dimer formed (Castanon, 2002). Castanon (2002) studied the effect of over-expressing either the fly homodimer Dm-Twist/Dm-Twist or the heterodimer Dm-Twist/Daughterless. Increasing

the homodimer produces ectopic muscle cells. Increasing the heterodimer inhibits somatic muscle development (Castanon, 2002).

H-Twist is needed for normal CBFA1/RUNX2 expression and DNA binding in osteoblasts (Yousfi *et al.*, 2003). Binding of the complex CBFA1/RUNX2 to the osteocalcin promoter is important in bone formation (Yousfi *et al.*, 2003).

Saethre-Chotzen, a rare dominant disorder, is caused by *H-Twist* mutations (Howard *et al.*, 1997). Individuals with the disease exhibit cranial deformities and digit deformities. The cranial deformity is craniosynostosis, where the skull bones fuse earlier than normal (Howard *et al.*, 1997). The digit deformities include excessively short digits (brachydactyly) and mild webbing between digits (syndactyly) (Howard *et al.*, 1997). Nonsense mutations or missense mutations in the *H-Twist* gene lead to loss of H-Twist protein in individuals with Saethre-Chotzen (El Ghouzzi *et al.*, 2000). *H-Twist* nonsense mutations results in an unstable, truncated protein (El Ghouzzi *et al.*, 2000). *H-Twist* missense mutations in the helical domain of the protein result in the inability of H-Twist/E12 dimers to form and the reduction of H-Twist protein nuclear localization (El Ghouzzi *et al.*, 2000). H-Twist missense mutations in the loop region result in the loss of dimer formation, however nuclear localization of the protein remains unchanged (El Ghouzzi *et al.*, 2000).

In mice, *M-Twist*-null mutants fail to close their neural tube in the cranial region and die 11.5 days after birth. *M-Twist*-null embryos have straight mandibular arches instead of the normal curved shape (Chen and Behringer, 1995). *M-Twist* (+/-) mice also have

limb deformities and a cranium with some bones enlarged and others underdeveloped (Lee *et al.*, 1999).

Chen and Behringer (1995) produced *M-Twist*-null/*M-Twist* chimeric mice and studied the mixing of the *M-Twist*-null and *M-Twist* cell types. *M-Twist*-null cells separate from wild type cells in the forebrain. The *Twist*-null cells in the forebrain lack a stellate shape, which is a mesenchymal characteristic. *Twist*-null mice also lack the apical ectodermal ridge (AER) that secretes fibroblast growth factors. *M-Twist* (+/-) heterozygous mice have reduced fibroblast growth factor receptor 2, FGFR2 (Rice *et al.*, 2000).

Twist also inhibits neuronal differentiation and suppresses p21 expression (Verzi *et al.*, 2002; Takahashi, 2004). N-Twist/E12 dimers sequester the E12 protein and fewer E12/Mash-1 dimers form (Verzi *et al.*, 2002). The E12/Mash-1 dimer, a transcriptional activator, is important in neurogenesis (Verzi *et al.*, 2002). H-Twist and H-snail have an additive effect of suppressing E2A-mediated p21 expression (Takahashi, 2004).

M-Twist is needed in cranial neuron establishment and osteoblast differentiation (Oshima, 2002). *M-Twist* binds to the E-box in the periostin promoter (Oshima, 2002). Both *M-periostin* and *M-Twist* are expressed in the mouse undifferentiated mesenchymal cell lines (Oshima, 2002).

Expression of *Twist* homologs

While *Twist* homolog expression is varied, expression is often present in mesodermally-derived cells. Expression of *Twist* homologs has also been detected in

ectoderm. *Dm-Twist* and *M-Twist* are also expressed in the developing central nervous system in the fly and mouse.

In the fly, *Dm-Twist* is expressed before mesoderm development. *Dm-Twist* is detected two hours after the fly egg is laid and peaks during gastrulation (Thisse *et al.*, 1987a). *Dm-Twist* appears in the early embryo at the anterior and posterior poles, and later localizes in the ventral region of the embryo (Thisse *et al.*, 1987a). *Dm-Twist* is first expressed at the cellular blastoderm in prospective mesoderm and then later as a narrow band, about half the width of the mesoderm (Thisse *et al.*, 1998; Leptin and Grunewald, 1990; Leptin, 1991). *Dm-Twist* is detected in mesoectoderm and ectodermal cells and disappears during late germ-band extension (Leptin, 1991). Mesodermal *Dm-Twist* expression, however, continues throughout germ-band extension (Leptin, 1991). Finally, fly neuronal *Twist* (*N-Twist*) is detected in the developing central nervous system (Verzi *et al.*, 2002).

In the worm, *Ce-Twist* is restricted to the mesodermal blast cell M (Harfe *et al.*, 1998). Expression occurs later in non-striated sex muscles and defecation muscles (Harfe *et al.*, 1998). Harfe and colleagues (1998) found that during muscle differentiation, the level of *Ce-Twist* decreases. *Ce-Twist* expression is also present neuron-like cells of the head (Harfe *et al.*, 1998).

In the jellyfish, *Pc-Twist* is detected in a mesoderm-like layer called the entocodon (Spring *et al.*, 2000). The entocodon differentiated into striated and smooth muscles. Expression is also present in later jellyfish bud stages where non-muscle tissue differentiated (Spring *et al.*, 2000).

In the zebrafish, *Dr-Twist* is expressed in the ventral region of the somites (Morin-Kensicki and Eisen, 1997). *Dr-Twist* is also expressed in migrating sclerotome (Morin-Kensicki and Eisen, 1997). When the sclerotome is removed from three somites in the zebrafish embryo by aspiration, *Dr-Twist* is absent from the three somites (Morin-Kensicki and Eisen, 1997).

In the frog, *X-Twist* is first expressed during early gastrulation in mesodermal cells and later in a subset of mesodermal cells (Hopwood *et al.*, 1989). *X-Twist* is detected in the notochord and lateral plate, however not in the myotome (Hopwood *et al.*, 1989). A few hours later, *X-Twist* is expressed in the neural crest (Hopwood *et al.*, 1989). The neural crest, composed of ectoderm, is located on the sides of the neural tube and develops into cranial and spinal ganglia.

In the spider *Achaearanea tepidariorum*, *At-Twist* is expressed first in cells located at the polar and equatorial areas of the embryo (Yamazaki *et al.*, 2005). When the extra-embryonic area of the embryo expands, *At-Twist* is present in epithelial cells, and cells in a layer below the epithelial cells. The deeper layer shows stripes of *At-Twist* expression (Yamazaki *et al.*, 2005). This deeper layer is most likely mesoderm (Yamazaki *et al.*, 2005).

In mice, *M-Twist* is expressed in mesodermally-derived cells and in the central nervous system (Wolf *et al.*, 1991; Verzi *et al.*, 2002). *M-Twist* expression is first detected at 7.5 embryonic days (Ang and Rossant, 1994). *M-Twist* is expressed in the cephalic and branchial mesectoderm, in limb buds, and in the mesoderm-derived

sclerotoma and somatopleura (Wolf *et al.*, 1991). When the dermamyotome and sclerotome differentiate, *M-Twist* is preferentially expressed in the sclerotome.

M-Twist is also detected in primary osteoblastic cells (Murray *et al.*, 1992). Neuronal *Twist* (*N-Twist*) is expressed in the developing central nervous system, specifically in the ventral nerve chord, midbrain, and hindbrain (Verzi *et al.*, 2002).

In humans, *H-Twist* is also found in mesodermally-derived tissues. In early development, *H-Twist* is detected in peritoneal mesothelial cells, endometrial fibroblasts, and fetal lung-derived fibroblasts (Wang *et al.*, 1997). *H-Twist* is expressed in the placenta, heart, skeletal muscles, and weakly in the kidney and pancreas (Wang *et al.*, 1997).

In the early stages of the chick embryo, *C-Twist* expression occurs in somites, limb and head mesenchyme, lateral plate mesoderm, and branchial arches (Tavares *et al.*, 2001). In later stages, *C-Twist* expression occurs in the mesenchyme of the maxillary and mandibular processes, between digits, and in the sclerotome and dermatome (Tavares *et al.*, 2001). *C-Twist* is also expressed in the developing feather, first in the mesenchyme buds (Tavares *et al.*, 2001). Expression is later restricted to mesenchyme in the proximal region of the feather filaments (Tavares *et al.*, 2001). A zone of cells located at the posterior region of the developing limb bud, called the zone of polarizing activity (ZPA), also expresses *C-Twist* (Riddle *et al.*, 1993).

***H-Twist* and cancer**

Adult *H-Twist* over-expression is linked to tumor cells. *H-Twist* over-expression occurs in breast, ovarian, prostate, bladder, and gastric tumors (Rosivatz *et al.*, 2002; Yang, 2004; Wang, 2004). *H-Twist* activates N-cadherin and represses E-cadherin (Rosivatz, 2002; Yang, 2004). Cadherins are calcium-dependent adhesion molecules located on cell surfaces. In malignant cells, E-cadherin is down-regulated (Siitonen *et al.*, 1996). In invasive lobular carcinoma, *H-Twist* is over-expressed in cell nuclei (Yang 2004). Suppressing *H-Twist* in highly metastatic mammary tumor cells inhibits metastasis to the lung (Yang 2004). The epithelial-mesenchyme transition that allows primary tumor cells to pass in and out of the blood stream is blocked when *H-Twist* is suppressed (Yang 2004).

Tumors with *H-Twist* over-expression are resistant to the two chemotherapy drugs taxol and vincristine (Wang, 2004). Taxol and vincristine function by inhibiting microtubule disassembly. Chemotherapy drugs that work by alternative mechanisms, unrelated to microtubule disassembly, are not inhibited by *H-Twist* over-expression (Wang, 2004). The specific resistance of over-expressing *H-Twist* tumor cells to taxol and vincristine suggests that *H-Twist* may maintain microtubule function (Wang, 2004).

H-Twist binds to homeobox A5 (HOXA5) in breast cancer cells and the dimer inhibits the tumor suppressor *p53* (Stasinopoulos *et al.*, 2005). *H-Twist* is an anti-apoptotic protein. HOXA5 binds to and activates the *p53* promoter. HOXA5 expression is higher in normal breast cells than in cancerous breast cells (Raman *et al.*, 2000). *H-Twist* over-expression deregulates the normal *p53* response to γ radiation (Stasinopoulos

et al., 2005). In addition, increasing HOXA5 partially restores *p53* function (Stasinopoulos *et al.*, 2005).

Overview of *Helobdella* development

Developmental stages in the leech *Helobdella* are well characterized. Leeches are hermaphroditic and can self-fertilize (Wedeen *et al.*, 1989). Internally fertilized eggs arrest in metaphase I until they are deposited in an external cocoon attached to the ventral area of the parent leech (Fernández and Olea, 1995). Leech embryos undergo spiral cleavage. To track developmental stages, a time frame was developed that starts at 0 hr when the zygotes are deposited in the cocoon. The time frame designation is called After Zygote Deposition (AZD).

Figure 1. Leech developmental stages (Soto *et al.*, 1997). The M cell of stage 6 has high *Twist* expression and was the target cell for injections. The diagram is used with the permission of Dr. J. Soto.

During stages 1 through mid-stage 7, before epiboly, the leech embryo divides and forms ten stem cells called teloblasts. In stage 1, the yolk deficient and mRNA/mitochondria-rich region of cytoplasm (called teloplasm) localizes to the animal and vegetal poles caps (Fernández *et al.* 1987; Holton *et al.*, 1994). Stage 2 consists of two cells, AB and CD. Cell CD inherits the teloplasm, from which mesoderm is derived (Fernández, 1980; Fernández *et al.*, 1987). At stage 4a, there are four cells (A, B, C, D) and four micromeres a'-d' (the micromere quartet). The micromere quartet lineage is present in the following leech structures: the neurons of the supraesophageal ganglion, the proboscis (including putative longitudinal muscle fibers and the proboscis sheath), and the provisional integument (Huang, 2002). In stage 4b, the D cell divides to forms the DM and DNOPQ cells. The DM cell is the mesoderm precursor and the DNOPQ cell is the ectodermal precursor (Bissen and Weisblat, 1989; Huang *et al.*, 2002). The DM cell cleaves in stage 4c to form a right and left M teloblast. In stage 5, the DNOPQ cell cleaves to form right and left D and NOPQ cells. In stage 6a, the N teloblast forms (cleavage of NOPQ to N and OPQ) and in stage 6b the Q teloblast forms (cleavage of OPQ to Q and OP). In early stage 7, the germinal band starts to form and the OP cell cleaves to form two OP cells. The germinal band is the collection of ten bandlets. Each bandlet forms as each teloblast produces clones.

During late-stage 7 through 11, the leech embryo undergoes epiboly and segmentation. During epiboly, the epithelium moves over the embryo. The process of epiboly dominates gastrulation in annelids and involves the movement of the germinal bands and a provisional integument (Smith *et al.*, 1996). At stage 8, the germinal bands

come together from both sides of the embryo to form the germinal plate. At stage 9, segmentation occurs, to form 32 non-renewable segments (Fernández and Stent, 1980; Irvine and Martingale, 1996). At stage 10, the body closes and the intestine and rectum form (Fernández and Stent, 1980). At stage 11, the edges of the germinal plate fuse completely and the yolk supply is depleted. The leech is considered a juvenile at the time of its first feeding (Fernández and Stent, 1980).

The AZD time for *H. robusta* embryos to reach the juvenile stage is approximately 10 days (~ 232 hrs). Stage 6a begins between 17 hrs and 18 hrs and lasts until ~ 21 hrs. Stage 6b begins at ~ 21 hrs and lasts until ~ 27 hrs. Stage 8 begin at ~ 61 hrs and lasts until ~ 94 hrs (Fernández and Stent, 1980).

Expression of Hro-Twist protein and Hro-dorsal

Hro-Twist is present in all *H. robusta* embryo stages tested, 1 through 8 (Table 1) (Nelson, 2004). Low Hro-Twist protein levels are found in the cytoplasm in stages 1 and 2. Also in stages 1 and 2, there is moderate anti-Hro-Twist staining in the teloplasm of cell CD. In stages 3 and 4, the expression level decreases, rising at stage 4b through mid-gastrulation at mid-stage 8. In stage 8, the Hro-Twist protein is nuclear (Nelson, 2004). In stage 5 and 6, the micromere cap has moderate staining. In stage 7, there is strong anti-Hro-Twist staining in the micromere cap (Nelson, 2004).

Table 1. Temporal and spatial Hro-Twist levels during early leech development (Soto, 1997; Nelson, 2004).

Gastrulation Stage	Embryo Stage	Cell(s)	Hro-Twist Level
Pre	1	zygote	moderate
Early	2	CD	low
Early	4a	Prospective micromeres, DM, and DNOPQ	low
Early	4b	DM, DNOPQ	high
Early	7	M teloblasts, m bandlets, some micromeres	high (in nuclei)
Mid-late	8	germinal plate, muscle fibers in provisional epithelium	high

Localization patterns of Hro-dorsal do not support a Hro-dorsal role in dorsoventral axis formation in the leech. Hro-dorsal antibody staining indicated that the protein is cytoplasmic until stage 6b (Goldstein *et al.*, 2001). Weak Hro-dorsal expression is detected in all teloplasm from stages 1 to 6a. At stages 6b-7, nuclear Hro-dorsal is detected in the micromeres. At stage 7, mesoderm and ectoderm blast cells show nuclear staining. By stage 9, Hro-dorsal blast cell staining disappears and strong staining is detected in the cytoplasm of cells making up segmental iterated stripes. As the striped

cells age, the expression switches from cytoplasmic to nuclear. Goldstein *et al.* (2002) suggested that Hro-dorsal in the stripes may diversify the cells within the segments. They also noted that this role is different than the Dm-dorsal role in the fly, which established the dorsoventral axis.

The pattern of the *Hro-Twist* expression level, from moderate, low, and then high could be explained if Hro-Twist is maternal. The protein level is reduced in stages 2 to 4a, until 4b when zygotic expression of *Twist* could begin. The switch from maternal expression to zygotic expression could explain why nuclear Hro-Twist is only detected in later stages. *Twist* mRNA expression continues to remain high through stage 10 (Soto *et al.*, 1997). Stage 6 shows high *Twist* expression in the M cell. *Hro-Twist* mRNA may not be activated by Hro-dorsal due to the spatial and temporal difference in expression (Goldstein *et al.*, 2001).

Nuclear Hro-dorsal expression is not detected before stage 4b, and consistent nuclei expression begins at stage 6b (Goldstein *et al.*, 2001). *Hro-Twist* mRNA is expressed earlier, in the oocyte before fertilization (Soto *et al.*, 1997).

Fly mesoderm differentiation

Dm-Twist and *Dm-snail* are necessary for fly mesoderm development (Nüsslein-Volhard *et al.*, 1984). The expression patterns of the two genes are similar (Nüsslein-Volhard *et al.*, 1984). The main expression difference is the band width in the mesoderm layer, where *Dm-snail* expression is wider (Nüsslein-Volhard *et al.*, 1984). Dm-Twist activates genes in the mesoderm and Dm-snail represses genes in the lateral/dorsal areas

of the mesoderm (Nüsslein-Volhard *et al.*, 1984). The Dm-Twist/Dm-dorsal dimer activates *Dm-snail* (Ip *et al.*, 1992; Shirokawa and Courey, 1997).

Dm-dorsal, a *rel*-domain protein, activates *Dm-Twist* and is needed for mesoderm formation and dorsoventral axis establishment (Thisse *et al.*, 1987a, 1987b, 1988). The Dm-dorsal *rel*-domain allows DNA binding and homodimerization (Govind *et al.*, 1996). *Rel*-domain proteins require a signal for nuclear localization. Dm-dorsal is translocated to the cell nuclei by the *Toll* receptor (Roth *et al.*, 1989). High nuclei concentration of Dm-dorsal is found in the ventral region of the embryo (Stewart, 1987). The Dm-dorsal concentration decreases in cells moving outward from the ventral region and no Dm-dorsal is detected in the dorsal region (Rushlow, 1989; Steward, 1989; and Roth, 1989). The ventral activation of *Dm-Twist* in the early fly embryo is dependant on Dm-dorsal homodimers binding to the *Dm-Twist* promoter (Jiang and Lavine, 1993; Thisse *et al.*, 1987, 1988).

Tinman (*tin*), regulated by Dm-dorsal, is also important in fly mesoderm differentiation (Bodmer and Frasch, 1999). *Dm-Tinman* is needed for the differentiation of mesoderm into cardiac muscles and dorsal body wall muscles (Bodmer and Frasch, 1999). Tin targets *bagpipe*, a gene needed for the formation of visceral muscles (Azpiazu and Frasch 1993; Bodmer 1993; Yin and Frasch 1998).

Other genes important in fly mesoderm development are *decapentaplegic* (*Dpp*), *wingless* (*wg*), *hedgehog* (*hh*), and *gleeful/lame duck*. *Dpp* has a role in dorsoventral axis formation and mesoderm and ectoderm induction (Irish and Gelbart, 1987; Bienz, 1997). *Dpp* activates *wg*, a segment polarity gene, needed for heart formation (Wu *et al.*, 1995).

Gleeful is needed for somatic muscle development (Furlong, 2001). Dpp and Tinman are both needed for induction of dorsal somatic muscles and midgut visceral muscles (Lee and Frasch, 2005). Wingless cooperates with Dpp/Tin during somatic muscle induction and antagonizes Dpp/Tin during visceral mesoderm induction (Lee and Frasch, 2005).

Single-minded (sim) and *myeloid ELF-1-like factor (MEF2)* are regulated by *Dm-Twist*. *Sim* is expressed at the edge of the mesoderm, in the mesectoderm. *Sim* is needed for mesectoderm differentiation (Nambu *et al.*, 1991). Fly D-mef2 expression is detected in mesodermal precursor cells before the separation of somatic and visceral muscles (Lilly *et al.*, 1994). Dm-Twist and Dm-snail are needed for D-mef2 to function (Lilly *et al.*, 1994).

Leech mesoderm differentiation

The DM cell of the leech embryo contains the precursor for the segmental mesoderm. Segmental mesoderm, which gives rise to somites and nephridia, is derived from the germinal plate (Weisblat and Huang, 2001). Lateral mesoderm gives rise to somatic muscles and visceral muscles. Leech homologs of the fly mesoderm genes include *Hro-dorsal*, *Hro-snail*, and *Hro-Twist* (Goldstein *et al.*, 2001; Soto *et al.*, 1997).

Hro-dorsal, the leech homolog to the fly mesoderm gene *Dm-dorsal*, was identified (Goldstein *et al.*, 2001). The *rel*-domain of *Hro-dorsal* and *Dm-dorsal* shares a 56% amino acid identity (Goldstein *et al.*, 2001). *Hst-dorsal*, the leech *Helobdella stagnalis* dorsal homolog, was also identified and shares a 56% amino acid identity with *Hro-dorsal* (Goldstein *et al.*, 2001). Nuclear Dm-dorsal is detected by stage 6 in the

micromeres. By stage 9, segmental stripes of Hro-dorsal appear in the cytoplasm of cells in the lateral regions of the germinal plate. As the segments age (anterior segments are older) *Hro-dorsal* expression becomes nuclear. The stripes of Hro-dorsal occur after segmentation begins, thus the protein's role is believed to specify cells within the compartments (Goldstein *et al.*, 2001). The *Hro-dorsal* expression pattern is not consistent with the *Hro-Twist* expression pattern. Therefore, Hro-dorsal is determined to be an unlikely *Hro-Twist* activator (Goldstein *et al.*, 2001). The role of Hro-dorsal is different from the role of Dm-dorsal, which is needed for mesoderm formation and dorsoventral axis establishment in the fly (Thisse *et al.*, 1987b, 1988).

Hro-sna1 and *Hro-sna2*, the leech homologs of the fly mesoderm gene *Dm-snail*, were also identified (Goldstein *et al.*, 2001). *Hro-sna1* shares a 66% sequence identity to *Dm-snail*, in an area that spans *Dm-snail* zinc fingers (Goldstein *et al.*, 2001). *Hro-sna2* shares a 77% sequence identity to the N-terminal domain of *Dm-snail* (Goldstein *et al.*, 2001). Spatial *Hro-sna1* and *Hro-sna2* expression patterns are the same as the *Hro-dorsal* expression pattern. However, temporal differences exist between *Hro-sna* and *Hro-dorsal* expression patterns. *Hro-sna* is detected after *Hro-dorsal* is detected in both blast cell nuclei and in the stripes of cells in the germinal plate. The temporal difference indicates that as in the fly, *Hro-dorsal* may activate *Hro-sna* (Rusch and Levine, 1996; Goldstein *et al.*, 2001).

Hro-Twist expression is detected before *Hro-dorsal* and *Hro-snail* expression (Soto *et al.*, 1997; Goldstein *et al.*, 2001). Similarly, spider *At-Snail* does not appear to activate *At-Twist* (Yamazaki *et al.*, 2005). *At-Snail* is not expressed in mesoderm, only in

ectoderm (Yamazaki *et al.*, 2005). The potential role of *Hro-Twist* in mesoderm differentiation is undetermined.

The muscle segment homeobox homolog, *Le-msx*, was isolated from *H. robusta* embryos (Master *et al.*, 1996). The expression pattern includes mesoderm precursors, suggesting a role in mesoderm differentiation (Master *et al.*, 1996). *Le-msx*, a maternal transcript, is expressed uniformly in the cortex of immature oocytes and later expressed in the polar regions of the oocyte (Master *et al.*, 1996). Later, *Le-msx* expression is detected in all 10 embryonic stem cells (Master *et al.*, 1996). During mid-stages of development, *Le-msx* is expressed in the expanding germinal plate. *Le-msx* expression is later confined to nephridia and the central nervous system (Master *et al.*, 1996).

***Twist* promoter studies in the fly, human, and mouse**

The *Dm-Twist* and *Drosophila virilis-Twist* (*Dv-Twist*) promoters contain two clusters of dorsal binding sites (Thisse *et al.*, 1991; Pan *et al.*, 1994). The dorsal binding motif is GGGA(A/G)A(A/T)(A/C)C(A/C) (Ip *et al.*, 1991). There are eight Dm-dorsal sites in the *Dm-Twist* promoter and seven Dv-dorsal sites in the *Dv-Twist* promoter. In the *Dm-Twist* promoter, the Dm-dorsal sites are clustered in two regions, a distal region (-1220 to -770 bp) and a proximal region (-440 to -12 bp). In the *Dv-Twist* promoter, the proximal region (ventral activation region) is relocated to intron 2 (Pan *et al.*, 1994).

Zeste binds to the *Dm-Twist* and *Dv-Twist* promoters (Benson and Pirrotta, 1988). The zeste binding consensus sequence is (T/C)GAG(T/C)G (Benson and Pirrotta, 1988). There are five zeste binding sites found in the proximal region of the *Dm-Twist* and *Dv-*

Twist promoters. Two or more *zeste* sites are needed for *zeste* to bind (Benson and Pirrotta, 1988). *Zeste* is thought to activate genes and have a role in transvection, a process where another gene can bind and regulate the first gene. One *zeste* protein can bind to two different DNA stretches at the same time, and could connect different regions of the DNA in order to regulated gene expression (Benson and Pirrotta, 1988). An enhancer or repressor located further upstream could loop and attach to a *zeste* protein that is already attached to a sequence near the promoter (Benson and Pirrotta, 1988). *Zeste* may thereby function by bringing the enhancer or repressor closer to the promoter (Benson and Pirrotta, 1988).

Several putative binding sites were identified in the *H-Twist* promoter (Wang *et al.*, 1997). The analyzed *H-Twist* promoter nucleotide sequence is 824 bp. Using the program, Macvector, Wang and colleagues (1997) identified the following *H-Twist* putative binding sites: CREB/ATF, NF-kB, engrailed, SP1 clusters, AP2, RB, and ATF. The known 463 bp of the mouse *M-Twist* promoter was aligned to the *H-Twist* promoter and high conservation was observed (Wang *et al.*, 1997). The CREB/ATF site at -70 bp, the engrailed site at -112 bp, the AP2 site at -133 bp, and one of the SP1 sites are exact matches in the *H-Twist* and *M-Twist* promoters. Since the RB site, ATF site, and two other AP2 sites are located further upstream than -463 bp, conservation of these sites is unknown (Wang *et al.*, 1997). Activating Transcription Factor (ATF) stimulates CRE-dependent transcription and acetylated histones H2b and H4 *in vitro*. *Engrailed* is a segment polarity gene expressed in stripes during fly development. *Engrailed* forms compartment boundaries in each segment (Lawrence and Morata, 1976; Kornberg, 1981).

Sp1 is a ubiquitous transcription factor commonly found in TATA-less promoters with a consensus binding sequence of GGGCGG (Kriwacki *et al.*, 1992). CREB is a cyclic AMP response element-binding protein that is activated when phosphorylated by A-kinase on a serine residue. AP2 has the consensus sequence CC(C/G)C(A/G)GGC (Mitchell *et al.*, 1987). AP2 is controlled by retinoid and important in the differentiation of epithelial cells and neural crest-derived cells (Mitchell *et al.*, 1991).

Promoter studies of other developmental genes

Dm-zen functions in dorsoventral axis establishment and germ band elongation (Wakimoto *et al.*, 1984; Rushlow and Levine, 1989). The *Dm-zen* promoter is two-tiered, containing a distal element and a proximal element (Doyle *et al.*, 1989). For wild-type *Dm-zen* expression, 1.6 kb upstream of the gene is needed (Doyle *et al.*, 1989). *Dm-zen* is expressed 2 to 4 hrs after fertilization and locates at first broadly and later is restricted to the dorsal region of the embryo (Rushlow *et al.*, 1987). The distal element represses *Dm-zen* expression in the ventral region of the embryo. The *Dm-zen* promoter proximal regulator element maintains dorsal region expression and represses dorsal-lateral region expression (Doyle *et al.*, 1989). Dm-dorsal represses lateral *Dm-zen* expression. In the *zen* promoter working model there is either a dorsal binding site on the distal ventral repressor element or Dm-dorsal interacts indirectly (Doyle *et al.*, 1989). The *Dm-zen* distal repressor placed upstream of the *hunchback* promoter represses the *hunchback* promoter (Doyle *et al.*, 1989).

The zinc finger protein Dm-snail is important in dorsoventral axis establishment. *Dm-Twist* activates genes in the mesoderm and *Dm-snail* represses genes in the mesoderm that are later expressed in the dorsal and lateral regions (Leptin, 1991). The Dm-snail homolog in the frog, *xsna*, has a 115 bp minimal promoter and is TATA-less (Hopwood *et al.*, 1989). The minimal promoter is located from -160 to -45 bp. Compared to other promoters, the minimal promoter for *xsna* is short. Like *Dm-zen*, the *xsna* promoter is two-tiered with the region from -112 to -97 bp corresponding to mesodermal expression and the region from -96 to -44 bp corresponding to ectodermal expression (Mayor *et al.*, 1993).

Use of a GFP construct in the leech *Helobdella*

A GFP/CMV promoter construct was expressed in *Helobdella robusta* embryos (Zhang and Weisblat, in press). GFP expression is detected in approximately 40% of the embryos injected in the N teloblast. The expression pattern was mosaic among cells of the same lineage, since only cells that incorporated the DNA construct into the nucleus showed expression.

Materials and Methods

Animals

Helobdella robusta embryos were used from a lab colony founded with leeches collected from a tributary of the American River in Sacramento, California. The pond snails, used to feed the leeches, were collected from the same area or from the rooftop pond of the UC Berkeley LSA building. The embryos were cultured at 23°C in *Helobdella* embryo (HL) media (4.8 mM NaCl, 1.2 mM KCl, 2mM MgCl₂, 8.0 mM CaCl₂, and 1 mM maleic acid, pH 6.6).

Sequencing the *Hro-Twist* promoter

The template DNA used for sequencing was clone 2 (8 kb). The clone was stored in sterile H₂O, for sequencing, and grown in JM109 cells. Clone 2 (8 kb) includes the *Hro-Twist* ORF and the 6 kb region upstream of the *Hro-Twist* ORF, inserted into pBluescript SKII (Soto *et al.*, 1997). Additional clone 2 (8 kb) plasmid DNA was grown and isolated using Promega Wizard Midi Preps. Sequencing was preformed at Tocoire, Inc. (Palo Alto, CA). Primer walking was employed to sequence the *Hro-Twist* promoter, using the primers listed in Table 2. The first primer attached to the known *Hro-Twist* ORF. The program Biology Workbench was used to help design primers. The reverse strand was sequenced using HrotwipF30 and HrotwipF32 (the reverse complement of HrotwipF9 and HrotwipF18, respectively). HrotwipF31 was also used (the reverse complement of HrotwipF14 minus the first two bases, G and A).

Table 2. Primers used to sequence the *Hro-Twist* promoter.

Primer Name	Sequence, 5' to 3'	Length (bp)	Tm (°C)
HroTwistTaraR1	GACGCTGTTGTTGTTGCTGT	20	60.4
HroTwistTaraR2	CGTTGCTGCTGCTGAGTTAG	20	60.0
HrotwipF3	GTTGTTGTTGCTGCTGCTGT	20	60.1
HrotwipF9	TGGTCTCAAAATAACTAGGTTTTCA	25	58.0
HrotwipF11	GCTTATTAACGTGCAAAGGTTTTTC	24	59.4
HrotwipF14	GAGAGAAGGATTTGTTTCGTAG	21	58.7
HrotwipF15	CAAGCACATTCAGTCATACCC	21	60.6
HrotwipF18	GCGAGCATTTATGAGTAATGGGG	23	62.8
HrotwipF30	TGAAAACCTAGTTATTTTGAGACCA	25	58.0
HrotwipF31	CTACGAACAAATCCTTCTC	19	55.9
HrotwipF32	CCCCATTACTCATAAATGCTCGC	23	60.6

Bioinformatic programs Genomatix and TESS

Two bioinformatic programs, TESS: Transcription Element Search System and Genomatix (MatInspector) were used to identify putative binding sites in the *Hro-Twist* promoter (Schug and Overton, 1997; Quandt *et al.*, 1995). To test the Genomatix program, the fly *Dm-Twist* promoter was inserted and 6 of the 8 known Dm-dorsal sites were recognized with a general score of ≥ 0.91 and a core similarity of ≥ 0.92 . The two sites not identified both had a string of 5 A's instead of 4. In the paper by Pan *et al.* (1991), the binding sites not recognized were labeled 5 and 6. Thus, a manual search for Dm-dorsal sites 5 and 6 was conducted when analyzing the *Hro-Twist* promoter for binding sites. The Genomatix program recognized 1 of the 4 known Dm-zeste sites. The program used the consensus T/CGAGT/CG. Other Dm-zeste sites included in the Pan *et al.* (1991) study were AGAGCG, CGAGCA, and TGAGCA. These additional sites were also manually used when searching the *Hro-Twist* promoter. The TESS program

detected 7 of the 8 Dm-dorsal sites; the site not detected was labeled 7 in the Pan *et al.* (1991) study. The program found 1 of the 4 Dm-zeste sites. The TESS program allows the user to rearrange binding site data based on each variable, such as probability, site name, or core similarity.

GFP/*Hro-Twist* promoter construct design

The PCR protocol for amplifying the 1150 bp *Hro-Twist* promoter fragment was as follows: 1X ProofStart buffer, 1.25 units of ProofStart polymerase, 0.3 mM of each dNTP, 0.5 μ M of each primer, and 56 ng of clone 2 (8 kb) DNA in a 25 μ l total volume. The PCR amplification condition were 95°C for 5 minutes, 38 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, then a final extension at 72°C for 5 minutes. The primers used to generate the 1150 bp *Hro-Twist* promoter fragment, with the restriction sites underlined, were:

*Bam*HI*twip*R2 5'-TATTAGGGATCCGATTACCGAACGAACGTTTAT-3'

34 bp, T_m = 68.2°C

*Xho*I*Hrotwip*R15 5'-TATTTCTCGAGGGGTATGACTGAATGTGCTTG-3'

32 bp, T_m = 67.2°C

The 4.2 kb peGFP-1 promoter-less vector (Clontech) was cut using *Xho*I and *Bam*HI.

The 1150 bp *Hro-Twist* promoter fragment was ligated into the promoter-less vector.

The restriction digest protocol of peGFP-1 for 1 hr at 37°C was as follows: 5 μ l peGFP-1 (at 100 ng/ μ l), 1X BSA, 0.4 μ l *Bam*HI, 0.4 μ l *Xho*I, 2 units *Bam*HI unique buffer, and

10.2 μ l sterile H₂O for a final 20 μ l volume. The Wizard SV Gel and PCR Clean-up System (Promega) was used to inactivate *Bam*HI.

The 2:1 insert to vector ligation products, made using LigaFast Rapid DNA Ligation (Promega), were screened using PCR. The ligation products were transformed into SoloPack Gold Competent Cells (Stratagene), and grown on 30 μ g/ μ l kanamycin/LB plates. Screening was performed by directly picking a portion of the colony, swirling the toothpick 30 seconds in 30 μ l sterile H₂O, and then boiling (or heating near 100°C) for 10 minutes to break open the membrane. Then 2 μ l of the DNA solution was used in a 25 μ l PCR reaction. The PCR reaction was the same as mentioned above, except the colony DNA solution replaced the clone 2 (8 kb) template. Clone 38A contained the peGFP-1/*Hro-Twist* promoter insert.

***Helobdella robusta* embryo injections and imaging**

The left M teloblasts of stage 6a and 6b *H. robusta* embryos were pressure-injected. During injections, embryos were held in place by a vacuum suction applied under a hole in a Petri dish. Needles were made by pulling capillary tubes (FHC, catalog number 30-30-0). The pulling program was: heat = 570, pull = 100, velocity = 7, and time = 200. Sixty-nine embryos were co-injected with RDA (75 mg/ml needle concentration) and the peGFP-1/*Hro-Twist* promoter construct (50 ng/ μ l or 225 ng/ μ l needle concentration). To eliminate the potential of RDA bleed through masking GFP expression, 17 embryos were injected without RDA and with only the peGFP-1/*Hro-Twist* promoter construct. Embryos were incubated 48 to 72 hrs at 23°C in HL media. Stage 8 embryos were

viewed by epifluorescence microscopy (Zeiss Axiophot). Pictures were acquired using a CCD camera (Roper Scientific, Trenton, NJ) mounted on an upright fluorescence microscope (Nikon E800) with PC control. The imaging software Metamorph (UCI, Downingtown, PA) was used.

Results

Sequencing the *Hro-Twist* promoter

A 2 kb region of the *Hro-Twist* promoter was sequenced (Figure 2). The first primer, HroTwistTaraR1, attached to the known *Hro-Twist* ORF. Primer walking was used to continue sequencing upstream. BLAST results verified that the sequence contains part of the known *Hro-Twist* ORF and an unknown upstream region. The reverse strand was also sequenced to verify the original sequence obtained. There is a 70 bp region of alternating A and T bases in the *Hro-Twist* promoter. Sequencing past the long AT repeat region was overcome by designing a primer that attached as close to the AT repeat region as possible.

***Hro-Twist* promoter elements and binding site analysis**

The 2 kb *Hro-Twist* promoter sequence was analyzed using the bioinformatic programs TESS and Genomatix. A cluster of four putative zeste sites is located from -940 to -1121 bp in the *Hro-Twist* promoter. Only one putative Hro-dorsal site was detected by the Genomatix program. The TESS program did not detect a Hro-dorsal site. A cluster of sites for the developmental proteins myosin head-1 (MFH-1) and FoxF1, also called HNF-3/fkh homologue 8 (HFH-8), is found in the *Hro-Twist* promoter. The Genomatix program recognized the MFH-1 site as FoxF2. A cluster of insulin upstream factor (IUF-1) sites, also called pancreatic and duodenal homeobox factor-1 (Pdx-1) sites, are present in the *Hro-Twist* promoter. Two other binding site clusters include F2F, a ubiquitous transcription factor, and histone H4 nuclear factor A (HiNF-A). One tailless site is also detected. The bioinformatic programs did not detect the known SuH in the *Dm-Twist* promoter, thus a manual search for the site was conducted in the *Hro-Twist* promoter. One suppressor of hairless site (SuH) was detected in the *Hro-Twist* promoter. *Hro-Twist* putative binding sites are listed in Table 3.

Table 3. Putative binding sites in *Hro-Twist* and *Dm-Twist* promoters. ETF = epidermal growth factor receptor-specific transcription factor, Hb = hunchback, HiNF-A= histone H4 nuclear factor A, Hlf = hepatic leukemia factor, IUF-1 = insulin upstream factor-1, Kr = kruppel, Sp1 = simian-virus-40-protein-1, Tll = tailless, Fox F1 = fork head box activator 1, FoxF2 = fork head box activator 2. The hyphen indicates there are no sites located. *Dm-zeste and Dm-dorsal sites are functional and SuH, although not verified by a mobility shift assay, interacted with Dm-Twist and a SuH binding site was detected (Thisse, *et al.*, 1991; Pan *et al.*, 1991; Tapanes-Castillo and Baylies, 2004).

Putative <i>Hro-Twist</i> and <i>Dm-Twist</i> Promoter Binding Sites	Number of <i>Hro-Twist</i> Putative Binding Sites	Number of <i>Dm-Twist</i> Putative Binding Sites	Location of <i>Hro-Twist</i> Putative Binding Sites	Location of <i>Dm-Twist</i> Putative Binding Sites
ATF	1	1	-1971 to -1966	-159 to -154
Dorsal	1	8*	-436 to -428	-1118 to -1108, -1105 to -1095, -1096 to -1086, -1064 to -1054, -318 to -309, -302 to -292, -272 to -262, -183 to -173
Engrailed	1	1	-266 to -258	-1031 to -1024
ETF	1	0	-331 to -325	-
FoxF1 (HFH-8)	9	0	-1913 to -1907, -1900 to -1895, -1897 to -1891, -1873 to -1867, -1855 to -1849, -1753 to -1747, -1133 to -1127, -1024 to -1018, -971 to -965, -953 to -947	-
F2F	9	1	-1273 to -1268, -1239 to -1234, -1227 to -1222, -781 to -776, -635 to -630, -261 to -256, -132 to -127, -71 to -66	-390 to -385
HINFA (H4TF-1)	6	1	-1915 to -1909, -1869 to -1863, -1093 to -1087, -1026 to -1020, -973 to -967	-1428 to -1422
Hlf	2	0	-1179 to -1172, -1138 to -1131	-
Hunchback	1	1	-1068 to -1059	-695 to -686
Ikaros -1	1	7	-1582 to -1576	-1041 to -1035, -881 to -876, -565 to -559, -440 to -435, -365 to -360, -354 to -349, -324 to -319
IUF-1 (Pdx-1)	7	2	-1647 to -1642, -1384 to -1379, -1309 to -1304, -701 to -696, -678 to -671, -658 to -653	-946 to -941, -488 to -483
Kruppel	0	1	-	-285 to -277
MFH-1 or FoxF2	6	0	-1961 to -1955, -1330 to -1324, -1251 to -1245, -1222 to -1216, -1193 to -1187, -1117 to -1111	-
Myogenin	0	1	-	-321 to -315
Sp1	1	3	-64 to -59	-1383 to -1378, -1166 to -1161, -166 to -160
SuH	1	1*	-59 to -53	-1042 to -1036
Tailless	1	1	-258 to -251	-1129 to -1121
Twist	1	1	-12 to -7	-851 to -846
WT1	0	2	-	-664 to -656, -329 to -321
Zeste	4	4*	-1121 to -1116, -1005 to -1000, -965 to -960, -946 to -940	-116 to -111, -95 to -90, -75 to -70, -28 to -23

The location of the *Hro-Twist* promoter elements are illustrated in Figure 3. The elements include the putative TATA box, +1, initiation ATG codon, binding sites found in the *Dm-Twist* promoter, and binding sites for proteins with a similar role as *Twist* homologs. A putative TATA box, TATAAA, was identified 330 bp upstream of the initiation ATG codon. The *Hro-Twist* putative TATA box was used to locate the putative transcriptional start site, 30 bp downstream of the putative TATA box.

The *Hro-Twist* promoter lacks the specific E box sequence, CATATG, present in the *Dm-Twist* promoter (Ip *et al.*, 1992). However, the general binding site for E proteins CANNTG, where *Twist* may bind to its own gene, is present (Ephrussi *et al.*, 1985). The putative E box sites CAAATG and CATCTG are present in the *Hro-Twist* promoter.

Figure 3. Putative *Hro-Twist* promoter elements previously detected in the *Dm-Twist* or *H-Twist* promoters (z = zeste, DI = dorsal, TII = tailless, Sp1 = simian-virus-40-protein-1, and SuH = suppressor of hairless) or sites for proteins detected in mesoderm (M = mesenchyme fork head-1, and F1 = fork head box activator 1). The *Hro-Twist* putative TATA box (T) is located at -30 bp. The initiation ATG codon is located at +300 bp.

***Dm-Twist* promoter binding site analysis**

The fly *Dm-Twist* promoter was re-analyzed for binding sites, after the original analysis by Thisse *et al.* (1991). The TESS and Genomatix programs, with an increased database of binding site consensus sequences, provide a tool to revisit and further analyze the *Dm-Twist* promoter. MFH-1 and Fox1 sites are not present in the *Dm-Twist* promoter. There is a putative cluster of ikaros sites in the *Dm-Twist* promoter. Ikaros-null mice lack B cell and fetal T cells (Wang *et al.*, 1996). The promoter also contains putative kruppel and myogenin sites, not found in the *Hro-Twist* promoter.

The *Dm-Twist* promoter also lacks the specific E box sequence. However, two general binding sites for E proteins, CAAATG and CAATTG, are present. The TESS program called the sequence CAAATG a Twist binding site. In addition, two Wilms tumor zinc finger protein (WT1-KTS) sites, also called WT-ZFP or WT II binding sites, were detected by the TESS program. Four WT1-KTS sites were detected by the Genomatix program.

***H-Twist* and *M-Twist* promoter binding site analysis**

The *H-Twist* and *M-Twist* promoters were re-analyzed for binding sites. The known 463 bp of the *M-Twist* promoter and 824 bp of the *H-Twist* promoter were used in the Genomatix and TESS programs. MFH-1 and FoxF1 sites are not detected. Two WT1-KTS sites are detected by the TESS program and four WT1-KTS sites are detected by the Genomatix program. The two sites have the consensus sequence AGCCCCCGC and TCGGGGGCG. One hunchback site and three HiNF-A sites are detected in the *H-Twist* promoter; however these sites are not detected in the 463 bp of the *M-Twist* promoter.

Using and injecting a GFP/*Hro-Twist* promoter construct

A 1150 bp *Hro-Twist* promoter fragment was generated with the restriction sites *Bam*HI and *Xho*I incorporated. The 1150 bp PCR product was sequenced to verify the product was the *Hro-Twist* promoter fragment. The 3' end of the fragment is at position +83 bp. The fragment was ligated into a peGFP-1 promoter-less vector (Clontech). One Shot Top 10 chemically competent *E. coli* cells (Invitrogen) were transformed with the 1150 bp peGFP-1/*Hro-Twist* promoter ligation product. PCR screening was used to identify a clone (clone 38A) with the 1150 bp *Hro-Twist* promoter fragment (Figure 4).

Figure 4. PCR screening for a clone with the 1150 bp *Hro-Twist* promoter fragment. Lane 1 = clone 38A with the 1150 bp insert. Lane M = 1 kb ladder.

A restriction digest with *Bam*HI verified that clone 38A contains the GFP/*Hro-Twist* promoter (Figure 5).

Figure 5. Restriction digest verifying that clone 38A contains the GFP/*Hro-Twist* promoter fragment insert. Lane 1 = clone 38A (1150 insert in peGFP-1) linearized with *Bam*HI. Lane 2 = peGFP-1 (no insert) linearized with *Bam*HI. Lane M = 1 kb ladder.

To test the 1150 bp *Hro-Twist* promoter fragment for promoter function, *in vivo* injections were performed. The GFP/*Hro-Twist* promoter construct was injected into the M cell of *H. robusta* embryos at stages 6a or 6b. The M cell of stage 6 embryos had *Hro-Twist* expression (Nelson, 2004). Sixty-nine embryos were injected in 5 different experiments. RDA was co-injected with the reporter construct for the first four experiments to verify the M cell was successfully injected. In the fifth experiment, the GFP/*Hro-Twist* promoter construct was injected without RDA.

GFP/*Hro-Twist* promoter construct expression

The embryos were viewed between 48 to 72 hrs post-injection, after M bandlet formation. Embryos were viewed at 4X and 10X magnifications for RDA presence and GFP expression. GFP expression was not observed (Table 4). Three embryos in the first two experiments gave a false positive for GFP expression. The three embryos were over-injected with RDA which resulted in bleed through. Over-injection of RDA can cause bleed through of RDA when viewing for GFP (Zhang, personal communication).

To detect possible weak GFP expression, potentially hidden by RDA bleed through, the GFP/*Hro-Twist* promoter construct was injected without RDA. GFP expression was not detected in these embryos (Table 4, Experiment 5).

Table 4. Total number of *H. robusta* embryos injected and expression results. The data were collected from 5 experiments. *A total of three embryos from experiments 1 and 2 showed RDA bleed through when viewed for GFP expression.

Concentration of the GFP/ <i>Hro-Twist</i> Promoter Construct in the Needle (ng/ul)	Embryo Stage Injected/Viewed	Successfully Injected Embryos Showing RDA	Embryos Injected with Only the GFP/ <i>Hro-Twist</i> Promoter Construct	Embryos Expressing GFP
50	6b/8	16	0	0*
225	6a/8	20	0	0*
225	6b/8	22	0	0
225	6b/8	11	0	0
225	6b/8	0	18	0

Discussion

Analysis of the *Hro-Twist* promoter putative binding sites

The 2 kb region of the *Hro-Twist* promoter was sequenced and analyzed for regulatory elements and binding sites. The *Hro-Twist* promoter sequence was compared with the known 1.5 kb *Dm-Twist* promoter sequence (Table 3). A comparison was also made between *Hro-Twist*, *H-Twist* and *M-Twist* promoters.

Hro-dorsal may not activate *Hro-Twist*. Eight Dm-dorsal sites, with a GAAAA core sequence, are present in the *Dm-Twist* promoter (Table 5). However, only one Hro-dorsal site is detected in the *Hro-Twist* promoter. In addition, only one of the two bioinformatic programs identified the sequence as a Hro-dorsal binding site and the sequence differed from the Dm-dorsal binding site sequence. The one Hro-dorsal consensus binding site recognized was GTGAAAATT, which differs from the known Dm-dorsal consensus binding site sequence GGGA(A/G)A(A/T)(A/C)C(A/C) (Ip *et al.*, 1991). The lack of a Hro-dorsal cluster in the *Hro-Twist* promoter is consistent with studies that show Hro-dorsal and Hro-Twist expression patterns differ (Goldstein *et al.*, 2001).

Table 5. *Dm-Twist* promoter functional binding sites Dm-dorsal, Dm-zeste, and putative SuH site. *SuH interacts with the Dm-zeste promoter and the binding site was identified, however the site was not confirmed by a mobility shift assay (Schweisguth and Posakony, 1992).

Functional <i>Dm-Twist</i> Binding Sites	Number of <i>Dm-Twist</i> Binding Sites	Location of Binding Sites
Dorsal	8	-1118 to -1108, -1105 to -1095, -1096 to -1086, -1064 to -1054, -318 to -309, -302 to -292, -272 to -262, -183 to -173
* Suppressor of Hairless	1	-1042 to -1036
Zeste	4	-116 to -111, -95 to -90, -75 to -70, -28 to -23

Both the *Dm-Twist* and *Hro-Twist* promoters contain four zeste sites. Zeste sites in the *Dm-Twist* promoter are clustered close to the transcriptional start site (Figure 6). In the *Hro-Twist* promoter, the zeste cluster is located further upstream. Zeste site clusters are not detected in the *H-Twist* and *M-Twist* promoters. However a zeste cluster may be located further upstream than the known 824 bp and 463 bp sequence of the *H-Twist* and *M-Twist* promoters. The one zeste site detected in the *H-Twist* promoter may be non-functional since more than one Dm-zeste site is needed for Dm-zeste to function. Four zeste sites are included in the 660 bp minimal *Dm-Twist* promoter (Thisse *et al.*, 1991). In the 1150 bp *Hro-Twist* promoter fragment, 3 of the 4 zeste sites are included.

Figure 6. Functional *Dm-Twist* promoter elements (Thisse *et al.*, 1991; Pan *et al.*, 1991). Dm-dorsal binding sites are located in two clusters of four sites. Dm-dorsal sites (DI) are located at: -1118 to -1108, -1105 to 1095, -1096 to -1086, -1064 to -1054, -318 to -309, -302 to -292, -272 to -262, and -183 to -173 bp. Dm-zeste sites (z) are located at: -116 to -111, -95 to -90, -75 to -70, and -28 to -23 bp. The initiation ATG codon is located at +160. The *Dm-Twist* promoter lacks a TATA box.

Clusters of FoxF1 and MFH-1 sites are unique to the *Hro-Twist* promoter compared to the *Dm-Twist*, *H-Twist*, and *M-Twist* promoters. The TESS program identified the FoxF2 site as MFH-1 (Sanchez *et al.*, 2002). In the mouse, FoxF1 and FoxF2 act as transcriptional activators (Marjo, 2002). Mouse *FoxF1* and *FoxF2* had similar expression patterns (Marjo, 2002). FoxF1 and FoxF2 mRNAs are present in teeth, and in respiratory, alimentary and urinary tract mesoderm adjacent to the epithelium (Marjo, 2002). The location suggests that the FoxF1 and FoxF2 transcription factors have a role in communication between the tissue types, which is crucial for organ development (Marjo, 2002). Unlike FoxF1, FoxF2 is also detected in the developing central nervous system, eye, ear, and limb buds (Marjo, 2002).

Mesenchyme fork head 1 (MFH-1) expression was also studied in mice (Kaestner *et al.*, 1996). MFH-1 mRNA is expressed in mice at day 10.5 and decreases after day 11.5. MFH-1 is detected in the cartilage of the tail and limb bones, head mesenchyme, and somatic and presomitic mesoderm (Kaestner *et al.*, 1996). In the adult, MFH-1 expression is detected in the kidney, brain, heart, muscle, uterus, lung, liver, ovary, and testis (Kaestner *et al.*, 1996). Interestingly, MFH-1 is mapped to mouse chromosome 8, the location of HFH-8 (a FoxF1 homolog) (Avraham *et al.*, 1995; Kaestner *et al.*, 1996).

HFH-8 (FoxF1) and the HFH-8 homologs studied in the mouse, frog, and fly are expressed in mesoderm. HFH-8 expression is detected during mouse embryogenesis in the extraembryonic and lateral mesoderm (Peterson *et al.*, 1997). The lateral mesoderm gives rise to the somatopleuric and splanchnopleuric mesoderm. The location of HFH-8

in the splanchnic mesoderm close to the visceral endoderm implies a role in mesenchymal-epithelial cell communication during gut morphogenesis (Peterson *et al.*, 1997). HFH-8 expression is also present in the lung, intestine, oral cavity, esophagus, trachea, and dorsal aorta mesenchymal cells (Peterson *et al.*, 1997). In the frog, the HFH-8 homolog, XFD-13/13, is not present during oogenesis, and zygotic transcription begins at the late gastrula stage. XFD-13/13 expression is detected in the head derived neural crest cells and in the dorsolateral plate (DLP) (Koster *et al.*, 1999). Biniou (Bin) is the fly homolog of the FoxF subfamily of forkhead domain proteins (Zaffran, 2001). The winged helix (forkhead) domain is conserved (Zaffran, 2001). *Bin* is expressed in the visceral mesoderm before *dpp* expression and the *dpp* enhancer contains bin binding sites (Zaffran, 2001; Sanchez *et al.*, 2002). The mesoderm expression pattern of the FoxF1 homologs is consistent with Twist expression patterns.

A suppressor of hairless (SuH) site is detected in both the *Dm-Twist* and *Hro-Twist* promoters. SuH represses *Dm-Twist* expression (Tapanes-Castillo and Baylies, 2004). In the fly, SuH binds to the site GTGGGAA (Tapanes-Castillo and Baylies, 2004). The SuH site in the *Hro-Twist* promoter is TGTGGGTA. In the human *H-Twist* promoter, a putative SuH site is detected, GTGGGA. A SuH site is not detected in the known 463 bp of the *M-Twist* promoter. However the presence of a SuH site further upstream is possible since the SuH site is located further upstream in the *Dm-Twist*, *Hro-Twist*, and *H-Twist* promoters. SuH functions in adult fly sensory organ development (Schweisguth and Posakony, 1992). SuH-null flies display imaginal discs containing an abnormal increase in sensory cells (Schweisguth and Posakony, 1992).

A Twist binding site, with the sequence CAAATG, was detected in both the *Hro-Twist* and *Dm-Twist* promoters. The presence of the Hro-Twist binding site suggests that *Hro-Twist* may auto-regulate expression. In the fly, Dm-Twist is needed to maintain *Dm-Twist* expression (Thisse *et al.*, 1987b).

Both the *H-Twist* and *Dm-Twist* promoters contain putative Wilms tumor (WT1) sites. WT1 induces apoptosis, independent of p53, in an osteosarcoma cell line by suppressing synthesis of epidermal growth factor receptor (Englert *et al.*, 1995). The potential interaction of WT1 and the *H-Twist* promoter could correlate with *H-Twist* over-expression in tumor cells.

Seven IUF-1 putative sites are present in the *Hro-Twist* promoter. IUF-1, also called pancreatic duodenal homeobox (PDX-1), is needed for proper differentiation of the endocrine pancreas. Human PDX-1 is expressed in the small intestine (Jepeal *et al.*, 2005). Two IUF-1 sites are present in the *Dm-Twist* promoter.

Nine F2F putative binding sites are found in the *Hro-Twist* promoter. F2F is a ubiquitous transcription factor that acts as a transcriptional repressor. The *Dm-Twist* promoter contains one putative F2F binding site. The *H-Twist* promoter lacks F2F binding sites.

In the *Hro-Twist* promoter, the one hunchback (hb) site recognized has the consensus sequence TTTTTTTTTT. The two Dm-hunchback sites have the consensus sequence TTATTTTTTTT. The leech *hunchback* homolog, *Leech Zinc Finger II* (LZF2), was identified in *Hro-Twist* (Savage and Shankland, 1996). LZF2 mRNA is detected in both mesodermal and ectodermal precursors before and during gastrulation (Iwasa,

2000). However, the LZF2 protein is detected only in ectodermal precursors (Iwasa, 2000). The lack of the LZF2 protein in mesoderm precursors does not support the idea of the *hb* homolog as an early activator of *Hro-Twist*.

The cluster of binding sites from -1910 to -1750 bp and -1320 to -940 bp suggests that the *Hro-Twist* promoter may be two-tiered, like the *Dm-zen* promoter, where different promoter regions are needed for different expression patterns.

Kruppel and myogenin binding sites are found in the *Dm-Twist* promoter, although not in the *Hro-Twist* promoter. The kruppel binding site consensus sequence is AAAAC/GGGGTAA (Rosenberg, 1986; Pankratz, 1989). The Dm-Kruppel site recognized by the bioinformatic programs is ATAACCCAA. The myogenin consensus sequence is CCAGGCA. Kruppel, a gap gene, is needed for development of the central segments in the fly (Wieschaus *et al.*, 1984). Kruppel is first detected in the center of the unfertilized egg and then along the anterior/posterior axis (Knipple *et al.*, 1985). Myogenin is needed for the differentiation of myoblasts (Brunetti and Goldfine, 1990).

A cluster of seven putative ikaros-1 sites is detected in the *Dm-Twist* promoter. Only one ikaros-1 site is detected in the *Hro-Twist* promoter. The ikaros-1 consensus sequence detected by TESS is TGGGAAT. Ikaros-1 can increase or decrease transcription. Ikaros-1-null mice lack B cells and have delayed T cell development (Wang *et al.*, 1996). In mice, ikaros-1 is detected in the early lymphocytes, early fetal liver, mature T cells, spleen, and thymus (Molnar and Georgopoulos, 1994).

An engrailed binding site is recognized in the *Hro-Twist*, *Dm-Twist*, and *H-Twist* promoters. The engrailed sites in the human, fly, and leech promoter have the sequences

CCTATAAAA, CTAATTAG, and TCAATTAAA, respectively. *Engrailed* is first expressed in alternating segments and then expressed in every segment (Morata and Lawrence, 1975). *Engrailed* levels peak during morphogenesis. In the fly, embryonic expression of *engrailed* also occurs in the central nervous system, epidermis, and antennal segments. The expression pattern of *Htr-engrailed* in the leech *Helobdella triserialis*, is not consistent with *Hro-Twist* expression.

Binding sites detected by bioinformatic programs may be non-functional. Putative binding sites need to be verified empirically. The probability that a site is functional increases as the number of sites recognized increases, for example in a cluster. Therefore, functionality of the zeste, MFH-1, FoxF1, F2F, and IUF-1 sites is highly probable. The probability also increases as the number of bases in the consensus binding site increases.

Table 6 shows common putative binding sites in the *Hro-Twist*, *Dm-Twist*, *H-Twist*, and *M-Twist* promoters based on available promoter sequences.

Table 6. Common putative binding sites in the *Hro-Twist*, *Dm-Twist*, *H-Twist*, and *M-Twist* promoters. The size of the promoter sequenced is in parentheses for each *Twist* homolog.

Putative Binding Sites in Common	<i>Hro-Twist</i> Binding Sites (2000 bp)	<i>Dm-Twist</i> Binding Sites (1500 bp)	<i>H-Twist</i> Binding Sites (824 bp)	<i>M-Twist</i> Binding Sites (463 bp)
ATF	1	1	1	1
En	1	1	1	1
Hb	1	1	1	0
HiNF-A	6	1	3	0
Sp1	1	3	19	8
SuH	1	1	1	0
zeste	4	4	1	0

The *Hro-Twist* promoter contains a putative TATA box. The *Dm-Twist* promoter lacks a TATA box. The *M-Twist* promoter contains two putative TATA boxes and the *H-Twist* promoter contains a functional TATA box at -32 bp (Wolf *et al.*, 1991).

Explanations for the absence of GFP expression

One possible reason for the lack of GFP expression is that the transcriptional start site was omitted from the promoter fragment generated. If the TATA box identified was incorrect, the transcriptional start site may be further downstream than the +83 bp end of the 1150 bp *Hro-Twist* promoter fragment. In *Dm-Twist*, *X-Twist*, and *M-Twist* the distance between +1 and the initiation ATG codon is shorter than the putative 300 bp distance in the *Hro-Twist* promoter. In the *Dm-Twist*, *X-Twist*, and *M-Twist* genes, the transcriptional start sites are 160, 152, and 167 bp upstream from ATG initiation codon,

respectively. However, in the *H-Twist* promoter, the 310 bp distance from the transcriptional start site and the ATG initiation codon is longer than the putative 300 bp distance in the *Hro-Twist* promoter. Therefore, the TATA box located and putative +1 site in the *Hro-Twist* promoter is within the range of one *Twist* homolog.

A second possible reason for the lack of GFP expression is that the minimal *Hro-Twist* promoter is greater than 1150 bp. The minimal *Dm-Twist* promoter is 660 bp (Thisse *et al.*, 1991). The minimal promoter for other *Twist* homologs is undetermined.

Conclusions

Several conclusions can be drawn from the *Hro-Twist* promoter data. First, there is one putative Hro-dorsal binding site in the *Hro-Twist* promoter as compared to the eight Dm-dorsal sites in the *Dm-Twist* promoter. Consistent with previous leech studies, Hro-dorsal does not appear to activate *Hro-Twist*. Second, like the *Dm-Twist* promoter, the *Hro-Twist* promoter contains a putative cluster of four zeste sites. The putative Hro-zeste sites are located further upstream in the *Hro-Twist* promoter than in the *Dm-Twist* promoter. Third, putative binding site clusters for fork head box activator 1 (FoxF1) and mesenchymal fork head-1 (MFH-1) sites are located in the *Hro-Twist* promoter. Fourth, although hunchback is a maternal protein and one putative site was detected in the *Hro-Twist* promoter, the location of the hunchback homolog protein expression in the leech makes hunchback an unlikely early activator of *Hro-Twist*. Fifth, the absence of GFP expression when the 1150 bp promoter fragment/GFP construct was injected indicates that the TATA box identified is incorrect or more than 1150 bp are needed. Sixth, the location of the repetitive binding sites in two clusters may indicate that the *Hro-Twist* promoter is two-tiered, similar to other promoters such as *Dm-zen*.

Future Investigations

Results of the *Hro-Twist* promoter study lead to new questions and ideas for future investigations. To answer why the 1150 bp promoter construct did not express GFP, future *Hro-Twist* promoter studies could include verifying the transcriptional start site and generating a longer promoter fragment to test for activity. Primer extension could be employed to verify the transcriptional start site. Verifying the transcriptional start site would help indicate if the putative TATA box located is functional. A *Hro-Twist* promoter fragment longer than 1150 bp, including the region downstream of the ATG initiation codon, could be tested for GFP expression. Eliminating binding sites through smaller fragment generation and testing for expression in GFP constructs could also be conducted. Another step is to search for the leech homologs of *MHF-1*, *FoxF1*, and *zeste* using degenerate primers. Determining if the leech MHF-1 homolog is expressed as early as *Hro-Twist* expression would be important to determine if Hro-MHF-1 is an early activator of *Hro-Twist*. Gel mobility shift assays could be used to test the functionality of the putative binding sites. Identifying the function of *Hro-Twist* using antisense morpholino oligos in leech embryos is another future investigation.

Twist homolog promoter work is still limited. Only the fly *Dm-Twist* and *Dv-Twist* promoters were analyzed and tested for binding site functionality. Dm-dorsal and Dm-zeste sites were functional, and SuH interacted with *Dm-Twist*. In the fly, a 1500 bp region of *Dm-Twist* promoter was sequenced. For the *H-Twist* promoter, an 800 bp region was sequenced; however functionality of the putative binding sites is

undetermined (Wang *et al.*, 1997). In the mouse, a 611 bp region of the *M-Twist* promoter was sequenced and, as in the *H-Twist* promoter, binding site functionality is undetermined (Wolf *et al.*, 1991). Further promoter sequencing and testing for binding site functionality would enable a more thorough comparison between *Dm-Twist*, *Hro-Twist*, *H-Twist*, and *M-Twist* promoters. Detecting additional H-Twist dimer partners in adult tumor cells with *H-Twist* over-expression may also give insight into the mechanism of *H-Twist* in tumors.

References

- Ang, S.L., and Rossant, J., 1994. HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* 78 (4), 561-574.
- Avraham, K.B., Fletcher, C., Overdier, D.G., Clevidence, D.E., Lai, E., Costa, R.H., Jenkins, N.A., and Copeland, N.G., 1995. Murine chromosomal location of eight members of the hepatocyte nuclear factor 3/fork head winged helix family of transcription factors. *Genomics* 25 (2), 388-393.
- Azpiazu, N., and Frasch, M., 1993. Tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* 7 (7B), 1325-1340.
- Baylies, M. and Bate, M., 1996. Twist: A myogenic switch in *Drosophila*. *Science* 272, 1481-1484.
- Benson, M. and Pirrotta, V., 1988. The *Drosophila zeste* protein binds cooperatively to sites in many gene regulatory regions: implications for transvection and gene regulation. *EMBO J.* 7 (12), 3907-3915.
- Bienz M., 1997. Endoderm induction in *Drosophila*: the nuclear targets of the inducing signals. *Curr. Opin. Genet. Dev.* 7 (5), 683-688.
- Blair, S.S. and Weisblat, D.A., 1984. Cell interactions in the developing epidermis of the leech *Helobdella triserialis*. *Dev. Biol.* 101, 318-325.
- Bodmer, R., 1993. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development* 118, (3), 719-729.
- Bodmer, R. and Frasch, M. 1999. Genetic Determination of *Drosophila* Heart Development. In: Harrey RP, Rosenthal N, eds. *Heart Development*. San Diego, CA; Academic Press 65-90.
- Bolch-Zupan, A., Hunter, N., Manthey, A., and Gibbins, J., 2001. *R-twist* gene expression during rat palatogenesis. *Int. J. Dev. Biol.* 45 (2), 397-404.
- Brunetti, A. and Goldfine, I.D., 1990. Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. *J. Biol. Chem.* 265 (11), 5960-5963.
- Castanon, I. and Baylies, M.K., 2002. A Twist in fate: evolutionary comparison of Twist structure and function. *Gene* 287, 11-22.

- Chen, Z.F., and Behringer, R.R., 1995. Twist is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* 9, 686-699.
- Cripps, R., and Olson, E., 1998. Twist is required form muscle template splitting during adult *Drosophila* myogenesis. *Dev. Biol.* 203, 106 -115.
- DiNardo, S., Kuner, J.M., Theis, J., and O'Farrell, P.H., 1985. Development of embryonic pattern in *D. melanogaster* as revealed by accumulation of the nuclear engrailed protein. *Cell* 43 (1), 59-69.
- Doyle, H.J., Rraut, R., and Lavine, M., 1989. Spatial regulation of *zerknüllt*: a dorsal-ventral patterning gene in *Drosophila*. *Genes Dev.* 3, 1518-1533.
- El Ghouzzi, V., Legeai-Mallet, L., Aresta, S., Benoist, C., Munnich, A., de Gunzburg, J., and Bonaventure, J., 2000. Saethre-Chotzen mutations cause TWIST protein degradation or impaired nuclear location. *Hum. Molec. Genetic.* 9 (5), 813-819.
- Englert, C., Hou, X., Maheswaran, S., Bennett, P., Ngwu, C., Re, G.G., Garvin, A.J., Rosner, M.R., and Haber, D.A., 1995. WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. *Embo. J.* 14, 4662-4675.
- Ephrussi, A., Church, G., Tonegawa, S., and Gilber, W., 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. *Science* 250, 1104-1111.
- Fernández, J., 1980. Embryonic development of the glossiphoniid leech *Theromyzon rude*: Characterization of developmental stages. *Dev. Biol.* 76, 245-262.
- Fernández, J., and Stent, G.S., 1980. Embryonic development of the glossiphoniid leech *Theromyzon rude*: structure and development of the germinal bands. *Dev. Biol.* 78 (2), 407-434.
- Fernández, J., Olea, N., and Matte, C., 1987. Structure and development of the egg in the glossiphoniid leech *Theromyzon rude*; characterization of the developmental stages and structure of the early uncleaved egg. *Development* 100, 211-225.
- Fernández, J., and Olea, N., 1995. Formation of the female pronucleus and reorganization and disassembly of the first interphase cytoskeleton in the egg of the glossiphoniid leech *Theromyzon rude*. *Dev. Biol.* 171, 541-553.
- Georgopoulos, K., Bigby, M., Wang, J., Molnar, A., Winandy, W., and Sharpe, A., 1994. The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 79, 143-156.

- Gitelman, I., 1997. Twist protein in mouse embryogenesis. *Dev. Biol.* 189, 205-214.
- Goldstein, B., Leviten, M.W., and Weisblat, D.A., 2001. Dorsal and snail homologs in leech development. *Dev. Genes Evol.* 211 (7), 329-37.
- Govind, S., Drier, E., Huang, L.H., and Steward, R., 1996. Regulated nuclear import of the *Drosophila* rel protein dorsal: structure-function analysis. *Mol. Cell Biol.* 16 (3), 1103-1114.
- Harfe, B.D., Vaz Gomes, A., Kenyon, C., Liu, J., Krause, M., and Fire, A., 1998. Analysis of a *Caenorhabditis elegans* Twist homolog identifies conserved and divergent aspects of mesodermal patterning. *Genes Dev.* 12 (16), 2623-2635.
- Holton, B., Wedeen, C.J., Astrow, S.H., and Weisblat, D.A., 1994. Localization of polyadenylated RNAs during teloplasm formation and cleavage in leech embryos. *Roux's Arch. Dev. Biol.* 204, 46-53.
- Hopwood, N.D., Pluck, A., and Gurdon, J.B., 1989. A *Xenopus* mRNA related to *Drosophila* Twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* 59, 893-903.
- Howard, T.D., Paznekas, W.A., Green, E.D., Chiang, L.G., Ma, N., Ortiz de Luna, R.I., Delgado, C.G., Gonzales-Ramos, M., Kline, A., and Jabs, E.W., 1997. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. *Nature Genet.* 15, 36-41.
- Huang, F.Z., Kang, D., Ramirez-Weber, F.A., Bissen, S.T., and Weisblat, D.A., 2002. Micromere lineages in the glossiphoniid leech *Helobdella*. *Development* 129, 719-732.
- Ip, Y.T., Kraut, R., Levine, M., and Rushlow, C.A., 1991. The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* 64, 439-446.
- Iwasa, J.H., Suver, D.W., and Savage, R.M., 2000. The leech hunchback protein is expressed in the epithelium and CNS but not in the segmental precursor lineages. *Dev. Genes Evol.* 210 (6), 277-288.
- Irish, V.F., Gelbart, W.M., 1987. The decapentaplegic gene is required for dorsal-ventral patterning of the *Drosophila* embryo. *Genes Dev.* 1 (8), 868-879.
- Irvine, S.M., and Martindale, M.Q., 1996. Cellular and molecular mechanisms of segmentation in annelids. *Semin. Cell Dev. Biol.* 7, 593-604.

- Jackson, S.M., Keech, C.A., Williamson, D.J., and Gutierrez-Hartmann, A., 1992. Interaction of basal positive and negative transcription elements controls repression of the proximal rat prolactin promoter in nonpituitary cells. *Mol. Cell. Biol.* 12, 2708- 2719.
- Jepeal, L.I., Fujitani, Y., Boylan, M.O., Wilson, C.N., Wright, C.V., Wolfe, M.M., 2005. Cell-specific expression of glucose-dependent-insulinotropic polypeptide is regulated by the transcription factor PDX-1. *Endocrinology* 146, 383-391.
- Jiang, J., Kosman, D., Ip, Y.T., and Levine, M., 1991. The dorsal morphogen gradient regulates the mesoderm determinant twist in early *Drosophila* embryos. *Genes & Development* 5, 1881-1891.
- Jiang, J. and Lavine, M., 1993. Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. *Cell* 72 (5), 741-752.
- Kadesch, T., 1993. Consequences of heteromeric interactions among helix-loop-helix proteins. *Cell Growth Differ.* 4 (1), 49-55.
- Kaestner, K.H., Bleckmann, S.C., Monaghan, A.P., Schlondorff, J., Mincheva, A., Lichter, P., and Schutz, G., 1996. Clustered arrangement of winged helix genes fkh-6 and MFH-1: possible implications for mesoderm development. *Development* 122 (6), 1751-1758.
- Knipple, D.C., Seifert E., Rosenberg U.B., Preiss A., and Jackle H., 1985. Spatial and temporal patterns of *Kruppel* gene expression in early *Drosophila* embryos. *Nature* 317, 40-44.
- Kornberg, T., 1981. *Engrailed*: a gene controlling compartment and segment formation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 78, 1095 -1099.
- Koster, M., Dillinger, K., and Knochel, W., 1999. Genomic structure and embryonic expression of the *Xenopus* winged helix factors XFD-13/13'. *Mech. Dev.* 1, 89-93.
- Kriwacki , R.W., Schultz, S.C., Steitz, T.A., and Caradonna, J.P., 1992, Sequence-specific recognition of DNA by zinc-finger peptides derived from the transcription factor Sp1. *Proc. Natl. Acad. Sci. USA* 89 (20), 9759-9763.
- Lawrence, P. A. and Morata, G., 1976. Compartments in the wing of *Drosophila*: a study of the engrailed gene. *Dev. Biol.* 50, 321-337.
- Lee, Y.M., Park, T., Schulz, R.A., and Kim, Y., 1997. Twist-mediated activation of the

- NK-4 homeobox gene in the visceral mesoderm of *Drosophila* requires two distinct clusters of E-box regulatory elements. *J. Biol. Chem.* 272 (28), 17531-17541.
- Lee, M.S., Lowe, G.N., Strong, D.D., Wergedal, J.E., and Glackin, C.A., 1999. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. *J. Cell. Biochem.* 75 (4), 566-577.
- Leptin, M., and Grunewald, B., 1990. Cell shape changes during gastrulation in *Drosophila*. *Development* 110 (1), 73-84.
- Leptin, M., 1991. Twist and snail as positive and negative regulators during *Drosophila* mesoderm development. *Genes & Development* 5, 1568-1576.
- Lilly, B., Galewsky, S., Firulli, A.B., Schulz, R.A., and Olson, E.N., 1994. D-MEF2: a MADS box transcription factor expressed in differentiating mesoderm and muscle cell lineages during *Drosophila* embryogenesis. *Proc. Natl. Acad. Sci. USA* 1 (12), 5662-5666.
- Marjo, A., 2002. Developmental expression of transcription factors, Tampere University Press Series, Acta Universitatis Tamperensis; 894 ISBM, 951-44-5492-8, electronic series Acta Electronica Universitatis Tamperensis; 212, ISBN of the electronic version 951-44-5493-6.
- Master, V.A, Kourakis, M.J., and Martindale, M.Q., 1996. Isolation, characterization, and expression of *Le-msx*, a maternally expressed member of the *msx* gene family from the glossiphoniid leech, *Helobdella*. *Dev. Dyn.* 207 (4), 404-419.
- Mayor, R., Essex, J.L., Bennett, F.M., and Sargent, G.M., 1993. Distinct elements of the *xsna* promoter are required for mesodermal and ectodermal expression. *Development* 119, 661-671.
- Mitchell, P.J., Wang, C., and Tjian, R., 1987. Positive and negative regulation of transcription *in vitro*: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50, 847-861.
- Mitchell, P.J., Timmons, P.M., Hébert, J.M., Rigby, P.W. and Tjian, R., 1991. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. *Genes Dev.* 5, 105-119.
- Molnar, A., and Georgopoulos, K., 1994. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol. Cell. Biol.* 14, 8292-8303.
- Morata, G., and Lawrence, P.A., 1975. Control of compartment development by the

- engrailed gene in *Drosophila*. *Nature* 255 (5510), 614-617.
- Morin-Kensicki, E.M. and Eisen, J.S., 1997. Sclerotome development and peripheral nervous system segmentation in embryonic zebrafish. *Development* 124, 159-167.
- Murray, S.S., Glackin, C.A., Winters, K.A., Gazit, D., Kahn, A.J., and Murray, E.J., 1992. Expression of helix-loop-helix regulatory genes during differentiation of mouse osteoblastic cells. *J. Bone Miner Res.* 7 (10), 1131-1138.
- Nambu, J.R., Lewis, J.O, Wharton, K.A. Jr., and Crews, S.T., 1991. The *Drosophila* single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS development. *Cell* 67, 1157-1167.
- Nelson, B.H., and Weisblat, D.A., 1992. Cytoplasmic and cortical determinants interact to specify ectoderm and mesoderm in the leech embryo. *Development* 115, 103-115.
- Nelson, C.L., 2004. Expression of H-Twi protein in the *Helobdella* leech embryos [master's thesis] San Jose, (CA): San Jose State University.
- Nüsslein-Volhard, C., Wieschaus, E., and Kluding, H., 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol.* 193, 267-282.
- Olson, E.N., and Klein, W.H., 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* 8, 1-8.
- Oshima, A., Tanabe, H., Yan, T., Lowe, G.N., Glackin, C.A, and Kudo, A., 2002. A novel mechanism for the regulation of osteoblast differentiation: transcription of periostin, a member of the pasciclin I family, is regulated by the bHLH transcription factor, twist. *Journal of Cellular Biochemistry* 86, 792-804.
- Pan, D., Huang, J., and Courey, A.J., 1991. Functional analysis of the *Drosophila Twist* promoter reveals a dorsal-binding ventral activator region. *Genes Dev.* 5, 1892-1901.
- Pan, D., Valentine, S.A., and Courey, J.A., 1994. The bipartite *D. melanogaster Twist* promoter is reorganized in *D. virilis*, *Mech. Dev.* 46, 41-53.
- Pankratz, M.J., Hoch, M., Seifert, E., and Jackle, H., 1989. Kruppel requirement for knirps enhancement reflects overlapping gap gene activities in the *Drosophila* embryo. *Nature* 341 (6240), 337-340.
- Peterson, R.S., Lim, L., Ye, H., Zhou, H., Overdier, D.G., and Costa, R.H., 1997. The winged helix transcriptional activator HFH-8 is expressed in the mesoderm of the primitive streak stage of mouse embryos and its cellular derivatives. *Mech. Dev.* 69

(1-2), 53-69.

- Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T., 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* 23, 4878-4884.
- Rice, D.P., Aberg, T., Chan, Y., Tang, Z., Kettunen, P.J., Pakarinen, L., Maxson, R.E., and Thesleff, I., 2000. Integration of FGF and TWIST in calvarial bone and suture development. *Development* 127 (9), 1845-1855.
- Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75 (7), 1401-1416.
- Rosenberg, U.B., Schröder, C., Preiss, A., Kienlin, A., Cote, S., Riede, I. and Jäckle, H., 1986. Structural homology of the product of the *Drosophila Kruppel* gene with *Xenopus* transcription factor IIIA. *Nature* 319, 336 -339.
- Rosivatz, E., Becker, I., Specht, K., Fricke, E., Lubert, B., Busch, R., Hofler, H., and Becker, K.F., 2002. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and Twist in gastric cancer. *Am. J. Pathol.* 161 (5), 1881-1891.
- Roth, S., Stein, D., and Nusslein-Volhard, C., 1989. A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* 59, 1189-1202.
- Rusch, J., and Levine, M., 1996. Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Dev.* 6 (4), 416-423.
- Rushlow, C., Frasch, M., Doyle, H., and Levine, M., 1987. Maternal regulation of *zerknüllt*: a homoeobox gene controlling differentiation of dorsal tissues in *Drosophila*. *Nature* 330 (6148), 583-586.
- Rushlow, C.A., Han, K., Manley, J.L., and Levine, M., 1989. The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell* 59 (6), 1165-1177.
- Sanchez, C., Casas-Tinto, S., Sanchez, L., Rey-Campos, J, and Granadino B. 2002. DmFoxF, a novel *Drosophila* fork head factor expressed in visceral mesoderm. *Mech. Dev.* 111 (1-2), 163-166.
- Savage, R.M., and Shankland, M., 1996. Identification and characterization of a hunchback orthologue, *Lzf2*, and its expression during leech embryogenesis. *Dev. Biol.* 175, 205-217.

- Schug, J., and Overton, G.C., 1997. TESS: transcription element search software on the www', technical report CBIL-TR-1997-1001-v0.0, Computational Biology and Informatics Laboratory School of Medicine, University of Pennsylvania, URL: <http://www.cbil.upenn.edu/tess>
- Schweisguth, F., and Posakony, J.W., 1992. Suppressor of Hairless, the *Drosophila* homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell* 69 (7), 1199-1212.
- Shirokawa, J.M., and Courey, A.J., 1997. A direct contact between the Dorsal Rel homology domain and Twist may mediate transcriptional synergy. *Mol. Cell. Biol.* 17, 3345-3355.
- Sitonen, S.M., Kononen, J.T., Helin, H.J., Rantala, I.S., Holli, K.A., and Isola, J.J., 1996. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am. J. Clin. Pathol.* 105 (4), 394-402.
- Smith, C.M., Lans, D., and Weisblat, D.A., 1996. Cellular mechanisms of epiboly in leech embryos. *Development* 122 (6), 1885-1894.
- Soto, J.G., Nelson, B.H., and Weisblat, D.A., 1997. A leech homolog of *Twist*: evidence for its inheritance as a maternal mRNA. *Gene* 199, 31-37.
- Spicer, D.B., Rhee, J., Cheung, W.L., and Lassar, A.B., 1996. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. *Science* 272, 1476-1480.
- Spring, J., Yanze, N., Middel, A.M., Stierwald, M., Groger, H., and Schmid, V., 2000. The mesoderm specification factor Twist in the life cycle of jellyfish. *Dev. Biol.* 28, 363-375.
- Stasinopoulos, I.A., Mironchik, Y., Raman, A., Wildes, F., Winnard, P. Jr., Raman, V., 2005. HOXA5-twist interaction alters p53 homeostasis in breast cancer cells. *J. Biol. Chem.* 280 (3), 2294-2299.
- Stent, G.S., Kristan, W.B., Torrence, S.A., French, K.A., and Weisblat, D.A., 1992. Development of the leech nervous system. *Int. Rev. Neurobiol.* 33, 109-193.
- Steward, R., 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* 59, 1179-1188.
- Takahashi, E., Funato, N., Higashihori, N., Hata, Y., Grindley, T., and Nakamura, M., 2004. Snail regulates p21 (WAF/CIP1) expression in cooperation with E2A and Twist.

- Biochem. Biophys. Res. Com. 325, 1136-1144.
- Tapanes-Castillo, A. and Baylies, M., 2004. Notch signaling patterns *Drosophila* mesodermal segments by regulating the bHLH transcription factor *twist*. *Development* 131, 2359-2372.
- Tavares, T.A., Izpisua-Belmonte, C.J., and Rodríguez-León, J., 2001. Developmental expression of chick *Twist* and its regulation during limb patterning. *Int. J. Dev. Biol.* 45, 707-713.
- Tessmar-Raible, K. and Arendt, D., 2003. Emerging systems: between vertebrates and arthropods, the Lophotrochozoa. *Curr Opin Genet Dev* 13, 331-340.
- Thisse, B., Stoetzel, C., El Massal, M., and Perrin-Schmitt, F., 1987a. Genes of the *Drosophila* maternal dorsal group control the specific expression of the zygotic gene *twist* in presumptive mesodermal cells. *Genes Dev.* 1, 709-714.
- Thisse, B., El Massal, M., and Perrin-Schmitt, F., 1987b. The *twist* gene: isolation of a *Drosophila* zygotic gene necessary for the establishment of dorso-ventral pattern. *Nucleic Acids Res.* 15, 3439-3453.
- Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., and Perrin-Schmitt, F., 1988. Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J.* 7, 2175-2183.
- Thisse, C., Perrin-Schmitt, F., Stoetzel, C., and Thisse, B., 1991. Sequence-specific transactivation of the *Drosophila twist* gene by the dorsal gene product. *Cell* 65, 1191-1201.
- Thisse, C., and Thisse, B., 1992. Dorsoventral development of the *Drosophila* embryo is controlled by a cascade of transcriptional regulators. *Dev. Suppl.* 173-181.
- Verzi, M.P., Anderson, J.P., Evdokia, D., Kelly, K.K., Green, S.B., North, B.J., Cripps, R.M., and Black, B.L., 2002. N-Twist, an evolutionarily conserved bHLH protein expressed in the developing CNS, functions as a transcriptional inhibitor. *Dev. Biol.* 249, 174-190.
- Wang, J., Nichogiannopoulou, A., Wu, L., Sun, L., Sharpe, A., Bigby, M., and Georgopoulos, K., 1996. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 5 (6), 537-549.
- Wang, S.M., Coljee, V.W., Pignolo, R.J., Rotenberg, M.O., and Cristofalo, V.J., 1997. Cloning of the human *Twist* gene: its expression is retained in adult mesodermally-derived tissues. *Gene* 187 (1), 83-92.

- Wang, X., Ling, M.T., Guan, X.Y., Tsao, S.W., Cheung, H.W., Lee, D.T., and Wong, Y.C., 2004. Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. *Oncogene* 23 (2), 474-482.
- Wakimoto, B.T., Turner, F.R., Kaufman, T.C., 1984. Defects in embryogenesis in mutants associated with the antennapedia gene complex of *Drosophila melanogaster*. *Dev. Biol.* 102 (1), 147-172.
- Wedeer, C.J., Price, D.J., and Weisblat, D.A., 1989. Analysis of the life cycle, genome and homeo box genes of the leech, *Helobdella triserialis*. *The Cellular and Molecular Biology of Pattern Formation*. Oxford University Press. 145-167.
- Weisblat, D.A. and Huang, F.Z., 2001. An overview of Glossiphoniid leech development. *Can. J. Zool.* 79, 218-232.
- Wieschaus, E., Nusslein-Volhard, C., and Kluding, H., 1984. Kruppel, a gene whose activity is required early in the zygotic genome for normal embryonic segmentation. *Dev. Biol.* 104 (1), 172-186.
- Wingender, E., Chen, X., Fricke, E., Geffers, R., Hehl, R., Liebich, I., Krull, M., Matys, V., Michael, H., Ohnhäuser, R., Pruss, M., Schacherer, F., Thiele, S., and Urbach, S., 2001. The TRANSFAC system on gene expression regulation. *Nucleic Acids Res.* 29, 281-283.
- Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., and Perrin-Schmitt, F., 1991. The *M-Twist* gene of *Mus* is expressed in subsets of mesodermal cells and is closely related to *Xenopus X-twi* and the *Drosophila Twist* genes. *Dev. Biol.* 143, 363-373.
- Wu, X., Golden, K., and Bodmer, R., 1995. Heart development in *Drosophila* requires the segment polarity gene wingless. *Dev. Biol.* 169 (2), 619-628.
- Yamazaki, K., Akiyama-Oda, Y., and Oda, H., 2005. Expression patterns of a *twist*-related gene in embryos of the spider *Achaearanea tepidariorum* reveal divergent aspects of mesoderm development in the fly and spider. *Zoolog. Sci.* 22 (2), 177-185.
- Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A., 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 117 (7), 927-939.
- Yao, Y., Dupont, B.R., Ghosh, S., Fang, Y., Leach, R.J., and Seto, E., 1998. Cloning, chromosomal localization and promoter analysis of the human transcription factor

- YY1. *Nucleic Acids Res.* 26 (16), 3776-3783.
- Yasui, K., Zhang, S.C., Uemura, M., Aizawa, S., and Ueki, T., 1998.
Expression of a twist-related gene, *Bbtwist*, during the development of a lancelet species and its relation to cephalochordate anterior structures.
Dev. Biol. 195 (1), 49-59.
- Yin Z., Xu, X-L., and Frasch M., 1997. Regulation of the Twist target gene *tinman* by modular cis-regulatory elements during early mesoderm development. *Development* 124, 4971-4982.
- Yousfi, M., Lasmoles, F., and Marie, P. J., 2003. TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. *Biochem. Biophys. Res. Commun.* 297 (3), 641-644.
- Zaffran S., Kuchler, A., Lee, H.H., and Frasch, M., 2001. Biniou (FoxF), a central component in a regulatory network controlling visceral mesoderm development and midgut morphogenesis in *Drosophila*. *Genes Dev.* 15 (21), 2900-2915.
- Zhang, S., and Weisblat, D., 2005. Applications of mRNA injections for analyzing cell lineage and asymmetric cell divisions during segmentation in the leech *Helobdella robusta*. *Development*, in press.

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Graduate Studies and Research

To whom it may concern,

I am giving my graduate student (Tara Gurtler) permission to use Fig.1 on her MS Thesis (Leech embryonic development). I created the original image for one of my publications.

Sincerely,

Julio G. Soto, Ph.D.

Associate Professor of Biology and Science Education