twist: A Myogenic Switch in *Drosophila*

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Somatic muscle is derived from a subset of embryonic mesoderm. In *Drosophila*, Twist (Twi), a basic helix-loop-helix transcription factor, is a candidate regulator of mesodermal differentiation and myogenesis. Altering amounts of Twist after gastrulation revealed that high levels of Twist are required for somatic myogenesis and block the formation of other mesodermal derivatives. Expression of *twist* in the ectoderm drives these cells into myogenesis. Thus, after an initial role in gastrulation, *twist* regulates mesodermal differentiation and propels a specific subset of mesodermal cells into somatic myogenesis. Vertebrate homologs of *twist* may also participate in the subdivision of mesoderm.

Differentiation of skeletal muscle requires a coordinated program of gene expression, which initiates the segregation of myoblasts from mesoderm and leads to myoblast fusion and assembly of the contractile apparatus. In vertebrates, this program depends on a number of key regulatory genes of the myogenic basic helix-loop-helix (bHLH) family, including MyoD, myogenin, Myf5, and MRF4 (*1*). In *Drosophila*, the single MyoD homolog *nautilus* is expressed in a number of muscle precursors but is not required for myogenesis in general (*2*, *3*). A candidate gene for regulating entry into somatic myogenesis in the fly is *twist*. During embryogenesis, *twist* is expressed at high levels in cells that give rise to somatic muscles and is required for the expression of mesodermal genes, including those involved in somatic myogenesis (*4*–*8*). Yet, *twist* is necessary for gastrulation, without which no mesoderm is formed (*7*, *9*), and this requirement has hindered systematic analysis of *twist* function in the myogenic pathway. Here, we report the results of a genetic and molecular analysis of *twist* function in *Drosophila* myogenesis.

The pattern of expression of *twist* suggests two ways in which it could function in somatic myogenesis (Fig. 1). Early in embryogenesis, *twist* is expressed in a modulated fashion in which progenitors of somatic muscles contain relatively large amounts, whereas relatively small amounts are expressed in progenitors of other derivatives, such as visceral mesoderm and heart (Fig. 1, A through C) (*5*). Later, *twist* expression is lost from cells as they differentiate to form larval muscles but persists in cells that will proliferate to form the adult myoblasts (Fig. 1, D and E) (*4*, *10*). These two features of *twist* expression, namely early modulation and late persistence in cells that represent a pool of adult myoblasts, suggest that two alternative models of *twist* function in the mesoderm. The gene *twist* could have an early function in the subdivision of the mesoderm into different derivatives, with relatively high levels of Twist propelling cells into the somatic myogenic pathway (model 1) or a late function in switching cells between proliferative and differentiating states in the somatic myogenic lineage (model 2) (*11*).

To test these models for *twist* function, we used the Gal4 targeted expression system (*12*) to alter *twist* expression during development. First, we tested the role of *twist* in switching cells from the proliferative to the differentiated state. We used the Gal4 line, *24BGAL4*, to promote *twist* expression throughout the mesoderm before, during, and after the normal period of somatic muscle formation (*13*). If loss of Twist is a prerequisite for myoblast differentiation into muscle, then maintained Twist might prevent muscle formation and perhaps result in a large population of *twist*-expressing myoblasts, like those that in normal embryos form the adult muscles. We found that maintained *twist* expression in embryonic myoblasts did not prevent somatic myogenesis. Larval muscles formed normally, even with continued *twist* expression, and adult myoblasts segregated normally as a distinct pattern of unfused cells expressing Twist (Fig. 2) (*14*, *15*). Thus, maintained *twist* expression alone is insufficient to retain myoblasts in the proliferative state that precedes muscle assembly.

To test for possible roles of *twist* in the subdivision of mesoderm, we used a *twist-GAL4* construct to promote *twist* expression in the early mesoderm and monitored segregation of mesodermal derivatives. In such embryos, the modulation of *twist* expression that occurs after gastrulation was prevented (Fig. 2, D and E). Cells normally expressing Twist at relatively low levels now expressed relatively high levels (*16*, *17*). In such embryos, differentiation of mesoderm was deranged. Whereas the development of somatic muscles was normal, the development of visceral mesoderm and heart was disturbed (Fig. 3).

Thus, increasing the amount of Twist selectively affected differentiation of cells from regions that normally have low Twist expression. Specifically, the number of visceral mesoderm progenitors was reduced, and cells that constitute the heart (cardial and pericardial cells) were missing or deranged (Fig. 3, A, B, E, and F). Dorsally, cells were detected that appeared to fuse and form spindle-shaped, myosin-expressing syncytia along the midline where the heart would normally form. If the amount of Twist was increased by addition of copies of the constructs (*18*), cells contributing to the visceral mesoderm and heart were further reduced (Fig. 3C). In addition, somatic

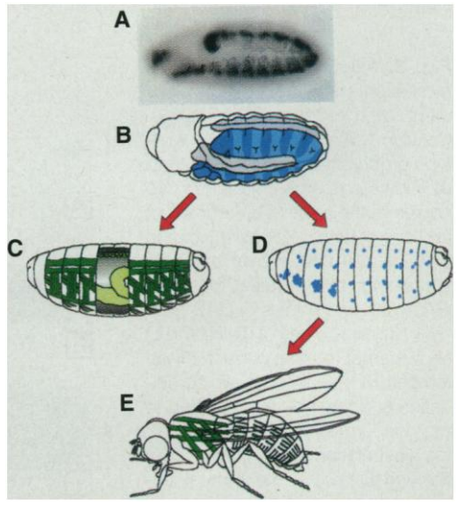

Fig. 1. Patterns of *twist* expression after gastrulation. (**A**) Wild-type stage 10 embryo, showing the modulated pattern of *twist* protein expression. (**B**) Cartoon of an embryo of approximately the same age, showing the modulated pattern of *twist* expression with high levels of *twist* expression in dark blue and low levels in light blue. (**C**) Cartoon of stage 16 embryo, showing the derivatives of the mesoderm: the heart, visceral mesoderm, and muscles are shown in shades of green. Expression of *twist* has disappeared from these derivatives. The somatic muscles are in dark green as they arise from cells that originate in the region with large amounts of *twist* [dark blue in (B)]. The visceral mesoderm and heart (lighter green shading) arise from the regions with low levels of *twist* [light blue regions in (B)]. Model 1 suggests that *twist* function is required for the subdivision of the mesoderm, with high levels of *twist* propelling cells into somatic myogenesis. (**D**) Cartoon of a stage 16 embryo, showing the persistent *twist* expression in the progenitors of the adult muscles (dark blue). (**E**) Cartoon of an adult fly with a selection of muscles in dark green. The persistent *twist* cells [dark blue in (D)] turn off *twist* during pupation, and adult muscle development proceeds. Model 2 suggests that removal of *twist* expression in the embryo (larval muscles) and, later, in the pupa (adult muscles) is necessary for somatic muscle differentiation.

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muscle–like structures formed on the gut where cells of the visceral mesoderm would normally form mononucleate visceral muscle fibers (Fig. 4A). We also detected cells expressing somatic muscle markers where the heart would normally develop (*19*).

Together, these results suggest that *twist* functions in the subdivision of the mesoderm. High levels of Twist propel cells into the somatic muscle pathway, whereas low levels permit cells to differentiate into other mesodermal derivatives. This model suggests that reducing the amount of Twist should affect somatic myogenesis but should not prevent formation of other mesodermal derivatives. A straightforward test of this prediction is complicated by the requirement for *twist* in gastrulation (*7*, *9*). To assay the effects of reduced *twist* function after gastrulation, we used a temperature-sensitive allelic combination, $twist^{ts50}/twist^{ry50}$, which reduced the activity of *twist* protein at restrictive temperatures (*20*). The activity of *twist* was reduced by shifting embryos to the restrictive temperature after gastrulation, and development of mesodermal derivatives was monitored. Visceral mesoderm and heart developed normally (*21*), whereas differentiation of somatic muscles was deranged (Fig. 3, D, H, and L). Thus, reducing *twist* activity after gastrulation selectively affected the differentiation of cells from the domain of high *twist* expression.

The effects of increasing and reducing expression of *twist* suggest that *twist* plays a pivotal role in regulating the switch into somatic myogenesis. Early ectopic *twist* expression might therefore be sufficient to convert nonmesodermal cells to the myogenic program. To test this, we used a *daughterless-GAL4* line to drive expression of *twist* in the ectoderm. In these embryos, ectodermal derivatives (nervous system and the epidermis) failed to develop and epidermal genes, such as *Distalless*, were not expressed (*22*, *23*). External cells (normally epidermis) expressed myosin, and neighboring cells fused to form bi- or trinucleate syncytia (Fig. 4B).

We conclude that levels of *twist* expression are critical determinants of mesodermal differentiation in the early embryo, with high levels of Twist switching cells into somatic myogenesis during subdivision of the mesoderm. Increasing *twist* expression in mesodermal domains where Twist is usually low blocks formation of other mesodermal derivatives and may lead to formation of ectopic muscle. Expression of Twist at low levels interferes with somatic myogenesis but permits the development of other derivatives. Because *twist* is required throughout the mesoderm for gastrulation, it is the patterned reduction of *twist* expression in the mesodermal primordium that limits the size of the muscle-forming population. We expect that the modulation of *twist* expression during normal development depends on a combination of factors that are intrinsic and extrinsic to the mesoderm. These include the activity of segmentation genes, such as *even-skipped* and *fushi tarazu* within the mesoderm, as well as the influence of signals, such as *wingless* and *decapentaplegic*, emanating from the overlying ectoderm (*24*).

The general requirement to subdivide the mesoderm and assign cells to form mesodermal derivatives is common to all animal embryos. In *Drosophila*, a key element is *twist*, modulation of which provides a mechanism whereby different derivatives can arise from the mesoderm. In vertebrates, subdivision of mesoderm may also depend on Twist homologs such as Xtwist, Mtwist, Meso-1, and bHLH-EC2 (*25*). Meso-1 and

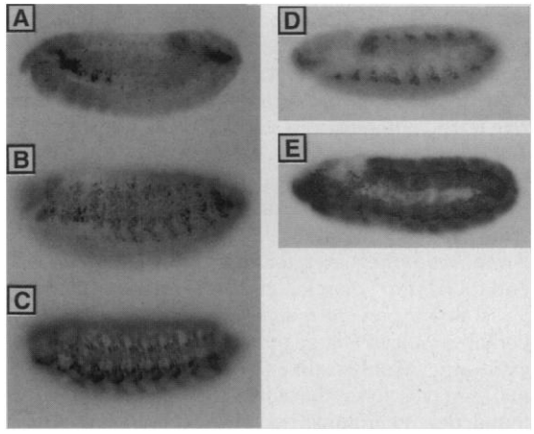

Fig. 2. Prolonged expression of *twist* in developing myoblasts does not prevent somatic myogenesis. (**A**) Late Twist pattern in wild-type stage 13 embryo represents those cells that will produce the adult muscles. Expression of Twist is no longer found in the larval muscles. (**B**) Twist expression in a stage 16 embryo in which *UAS-twist* is driven by *24BGAL4* (*9*, *10*). Twist is found in all formed muscles. (**C**) Myosin staining in a stage 16 *24BGAL4;UAS-twist* embryo. All muscles form normally (*14*, *15*). (**D** and **E**) Twist expression under the control of *twist-GAL4* eliminates the normally modulated pattern of *twist* expression in the *Drosophila* embryo. In (D), wild-type embryo stage 11 was stained with antibody to Twist. In (E), a similarly staged embryo was stained with antibody to Twist but carried the *twist-GAL4;UAS-twist* constructs.

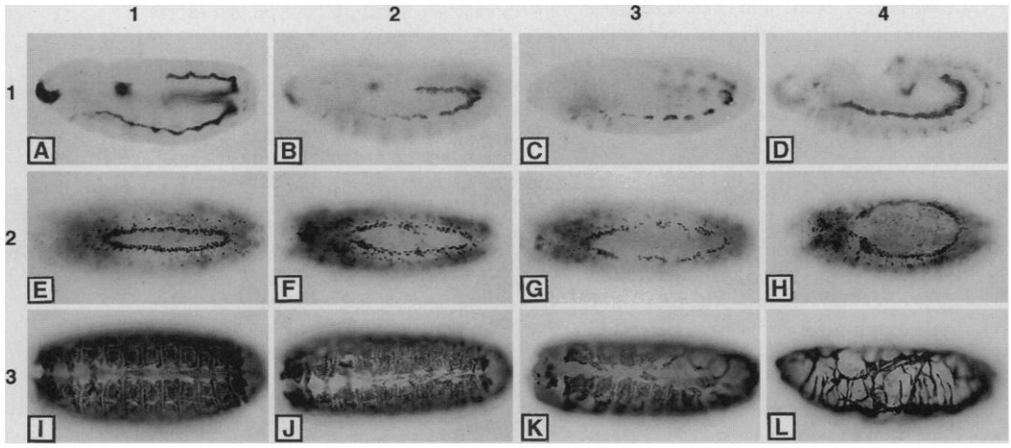

Fig. 3. Alterations in the level of *twist* expression affect the development of mesodermal derivatives. Column 1 (**A**, **E**, and **I**) shows wild-type embryos; column 2 (**B**, **F**, and **J**) shows embryos containing two copies of the *twist-GAL4* construct and one copy of *UAS-twist*; column 3 (**C**, **G**, and **K**) shows embryos containing two copies of *twist-GAL4* and two copies of *UAS-twist* (*16–18*); and column 4 (**D**, **H**, and **L**) shows embryos that carry the *twist* temperature-sensitive allelic combination and that had been shifted to the nonpermissive temperature after gastrulation (*20*). Row 1 [(A) through (D)] shows the visceral mesoderm progenitors (anti-fasciclin III). Note the reduction in the number of progenitors with increasing dose of *twist*. Reduction of *twist* (D) has no effect. Row 2 [(E) through (H)] shows cardial and pericardial cells of the heart (anti-Zfh1). Note the reduction in number with increasing dose of *twist*. Reduction in the amount of *twist* (H) has no effect. Row 3 [(I) through (L)] shows somatic muscles, with a dorsal view in (I), (J), and (K) and a lateral view in (L) (anti-myosin). Myogenesis proceeds normally in the presence of elevated amounts of *twist* (apart from minor distortions of embryos) but is deranged when the amount of *twist* is reduced (L). Panels (I), (J), and (K) include the heart which, as in row 2, is deranged with increasing amounts of *twist*.

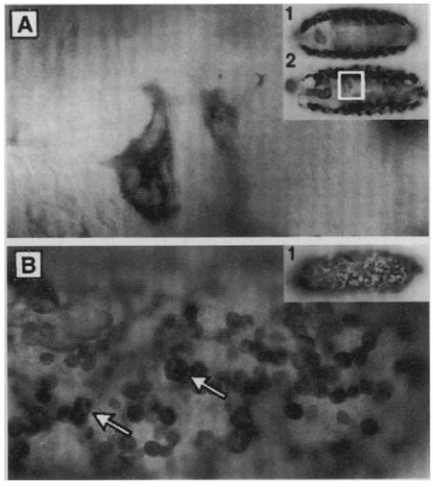

Fig. 4. High levels of Twist can propel cells into the somatic myogenesis. (**A**) Inset 1 shows a wild-type embryo at stage 14 stained with an antibody to myosin. The visceral muscles form a uniform sheet of muscles around the midgut endoderm. Inset 2 shows a similarly staged embryo in which the levels of *twist* have been elevated (*18*). The white box highlights one region of the midgut where visceral muscle is missing and an ectopic somatic muscle has been formed. Panel (A) is a higher magnification of the multinucleated ectopic muscle that expresses myosin, extending growth cone–like processes over the midgut endoderm (*4–8*). (**B**) Inset 1 shows a myosin-stained embryo in which *UAS-twist* is expressed under the control of *daughterless-GAL4* (*22*). In addition to expression within the internal mesodermal cells, myosin is found in the most external cells of the embryos where it is never normally expressed. Panel (B) is a higher magnification of the most external cells of this embryo, showing fused di- and trinucleated myosin-expressing cells (white arrows), which are characteristic of the initial steps of somatic myogenesis. No ectodermal derivatives can be found in these embryos (*22, 23*).

its human homolog, bHLH-EC2, are expressed in a pattern reminiscent of *twist*: first generally in the presomitic mesoderm, then in cells giving rise to skeletal myoblasts, and finally, like *twist*, disappearing before muscle differentiation. Meso-1 is expressed before expression of the MyoD family, and injection of Meso-1 into *Xenopus* embryos results in the formation of ectopic skeletal muscle (*3, 25*). Expression patterns and functional studies for Xtwist and Mtwist indicate that these proteins, unlike *twist* itself, are not involved in the determination of the skeletal muscle lineage, although they may be implicated in the formation of other mesodermal derivatives, namely notochord and sclerotome. Indeed, ectopic expression of Mtwist in cell lines has been shown to prevent skeletal muscle formation (*26*), much as *twist* prevents visceral mesoderm differentiation in *Drosophila*. Thus, there is an analogy in the function of the *twist* family of proteins in vertebrates and in flies, which is reflected in restricted patterns of expression in the mesoderm and an ability to promote the formation of some derivatives and block the formation of others.

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13. *24BGAL4* drives expression of a reporter gene at midstage 10 and continues throughout the mesoderm until the end of embryogenesis (*12, 27*) [A. M. Michelson, *Development* **120**, 755 (1994)].
14. Immunocytochemistry was performed as described (*28*). Antibody dilutions were as follows: anti-myosin, 1:500; anti-fasciclin III, 1:100; anti-Zfh1, 1:10,000; and anti-Twist, 1:500. Two *UAS-twist* constructs were made for these experiments. One is a *UAS-twist* cDNA construct, where a full-length *twist* cDNA was subcloned as a blunt-ended 2.2-kb Hind III–Not I fragment into the blunt-ended Xho I site of pUAST (*12*). The second construct is a *UAS-twist* genomic construct, where a 606–base pair Bam HI fragment from the 5′ end of the *twist* cDNA was used as a probe to screen a LambdaGEM-11 *Drosophila* embryonic library (Promega). Plaques (600,000) were screened at high stringency, and a number of hybridizing clones were recovered. Restriction mapping and Southern (DNA) analysis revealed the *twist* promoter and coding sequences as described (*29–31*). A 5.2-kb Cla I fragment that encompasses the entire *twist* coding sequence was subcloned into pBluescript SK (Stratagene). Subsequently, a correctly oriented Not I–Kpn I fragment was isolated and subcloned into pUAST (*12*). Transformants were made as described [M. K. Baylies, L. B. Vosshall, A. Sehgal, M. W. Young, *Neuron* **9**, 575 (1992)]. The *UAS-twist*–containing independent fly strains were tested by driving expression of the construct in the ectoderm and assaying for the induction of mesodermal gene expression. We could induce expression of *tinman* [N. Azpiazu and M. Frasch, *Genes Dev.* **7**, 1325 (1993); R. Bodmer, *Development* **118**, 719 (1993)] and *DMEF2* (*6*). The *UAS-twist* cDNA construct worked consistently better than the genomic construct and was used for all further experiments.
15. When the level of *twist* was raised by driving additional copies of the *UAS-twist* constructs [see below (*18*)] by *24BGAL4*, we found that somatic myogenesis occurred normally, although some patterning defects were noted. No free myoblasts that would indicate a block in muscle differentiation were seen. Many of these embryos move and hatch into larvae. Expression of one or two copies of the *UAS-twist* construct with *24BGAL4* does lead, however, to minor defects in the differentiation of the visceral mesoderm and heart. For example, the gastric ceca, which form by the interaction of the visceral mesoderm and the endoderm, do not form properly in these experimental embryos. We reason that the relatively minor effects on the differentiation of the visceral mesoderm and heart compared with those described below are due to the time in which *twist* is expressed under the control of *24BGAL4*.
16. The *twist-GAL4* construct was made as follows: A 1.4-kb Hind III–Cla I fragment from the *twist* genomic clone, containing the enhancer and promoter elements necessary for proper *twist* expression (*30, 31*) was cloned into pBlueScript SK (Stratagene) and released with Bam HI for cloning into pGATB (*12*). Subsequently, a 5.1-kb Kpn I–Not I fragment, which encompasses the *twist* promoter and Gal4 sequences, was then cloned into the transformation vector pW8 [R. Klemenz, U. Weber, W. J. Gehring, *Nucleic Acids Res.* **15**, 3947 (1987)]. This Gal4 construct, when transformed in flies, drove expression of β-galactosidase in a pattern similar to that of *twist* (*28*): Expression of *lacZ* can be detected first at gastrulation (whereas *twist* itself is detected before gastrulation) and continues in the mesoderm as it spreads along the inner face of the ectoderm. Expression is maintained generally in the mesoderm until late stage 11, whereupon it fades in the visceral mesoderm but continues in the heart and somatic muscles. Expression in the heart and somatic muscles fades by the end of embryogenesis. Whereas *twist* is expressed early (before gastrulation) in mesectoderm and then fades, *twist-GAL4* drives expression in mesectoderm at gastrulation and continues until early stage 10. The delay in expression of genes driven by *twist-GAL4* (as compared to the wild-type onset of *twist* expression) occurs because Gal4 must be expressed and then translated before it can activate transcription from the UAS construct (*12*) [A. Brand, A. Manoukian, N. Perrimon, in Drosophila melanogaster*: Practical Uses in Cell and Molecular Biology*, L. Goldstein and E. Fyrberg, Eds. (Academic Press, San Diego, CA, 1994), p. 643–654]. This delay is 30 to 45 min in the early embryo.
17. Embryos carrying *twist-GAL4;UAS-twist* constructs show patterning defects ventrally as the amount of *twist* increases. Maintained *twist* expression in the mesectoderm leads to a disruption of the central nervous system as midline cells are transformed toward mesodermal fates (detectable by myosin and D-MEF2 expression). This in turn causes a disturbance in the normal development of the ventral ectoderm as the two halves of the developing nervous system are apparently pushed apart by these cells. The result of this is a disruption in the normal patterning of the ventral muscles, which depends on ventral ectoderm. Genes that are activated by Twist, such as *D-MEF2*, were activated in these experiments in a way that reflected the pattern of ectopic *twist* expression.
18. Increasing the amount of *twist* was accomplished by establishing two additional stocks: one that consisted of *yw UAS-twist; UAS-twist* and one that consisted of *yw twist-GAL4; twist-GAL4*. Thus, embryos could be obtained with the following Gal4;UAS configuration: one copy of *twist-GAL4;UAS-twist*; two copies of *twist-GAL4*; one copy of *UAS-twist*; two copies of *twist-GAL4*; and two copies of *UAS-twist*. Expression of *twist* could be increased further by performing the experiments at 29°C rather than at 25°C, as the Gal4 system is temperature-sensitive and shows increased activity at higher temperatures (*12, 32*).

19. Expression of the founder cell marker gene, *vestigial*, can be detected extending dorsally into the ectopic muscles forming in the region of the heart (M. K. Baylies and M. Bate, unpublished results).
20. The temperature sensitivity of the *twist* allelic combination $twist^{v50}/twist^{ry50}$ has been described (*29*) [M. Leptin, J. Casal, B. Grunewald, R. Reuter, *Development 1992 Supplement* 23 (1992)]. At 18°C, embryos carrying this allelic combination survived until adulthood, whereas at 29°C, these embryos died during embryogenesis. We have sequenced each allele (K. Lewis and M. K. Baylies, unpublished results), and the changes are as follows: for $twist^{v50}$, Pro^{22} (Ccc) to Ala^{22} (Gcc); for $twist^{ry50}$, Gln^{251} (cAg) to Leu^{251} (cTg).
21. Reduction of Twist activity does not necessarily lead to the ectopic induction of visceral or cardiac mesodermal markers. Thus, whereas larger amounts of Twist inhibit visceral and cardiac mesoderm formation, reduced amounts of Twist may not in themselves be sufficient to initiate the formation of visceral or cardiac muscle. We suggest that there are additional factors that must be present if visceral or cardiac mesoderm is to form (*27, 33*).
22. The *daughterless-GAL4* line [A. Wodarz, U. Hinz, M. Engelbert, E. Knust, *Cell* **82**, 67 (1995)] expresses Gal4 ubiquitously, starting at cellularization and continuing throughout the remainder of embryogenesis. Ectopic Twist expression under the control of *daughterless GAL4* prevents ectodermal differentiation and eliminates the ectodermally derived cuticle. In situ hybridizations were performed with a *Distal-less (Dll)* cDNA fragment (*32*). *Dll* is expressed solely in ectoderm and in ectodermally derived tissues [S. M. Cohen, *Nature* **343**, 173 (1990)]. To assay for the epidermis and nervous system, we made cuticle preps of, and stained with horseradish peroxidase antibody, respectively, the experimental embryos (*32*). In addition, no cell death, as assayed by acridine orange incorporation [K. White *et al.*, *Science* **264**, 677 (1994)], was found in early embryos, which excludes the possibility that ectopic Twist causes ectodermal cell death.
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24. Intrinsic and extrinsic influences upon the mesoderm (*33*) include the following: *even-skipped/fushi tarazu* (M. Bate, E. Rushton, M. K. Baylies, in preparation), *wingless* (*32*), and *decapentaplegic* (*27*) [M. Frasch, *Nature* **374**, 464 (1995)].
25. Homologs of *twist* include the following: Xtwist [N. D. Hopwood, A. Pluck, J. B. Gurdon, *Cell* **59**, 893 (1989)]; Mtwist [C. Wolf *et al.*, *Dev. Biol.* **143**, 363 (1991)]; Meso-1 [M. A. Blanar *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5870 (1995)]; and bHLH-EC40 [E. E. Quertermous, H. Hidai, M. A. Blanar, T. Quertermous, *ibid.* **91**, 7066 (1994)].
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Structural Evidence for Functional Domains in the Rat Hippocampus

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The hippocampus has two major outputs: multisynaptic pathways to the cerebral cortex and a massive descending projection directly to the lateral septal part of the basal ganglia. Here it is shown that the descending output is organized in such a way that different hippocampal regions map in an orderly way onto hypothalamic systems mediating the expression of different classes of goal-oriented behavior. This mapping is characterized by a unidirectional hippocampo-lateral septal projection and then by bidirectional lateral septo-hypothalamic projections, all topographically organized. The connectional evidence predicts that information processing in different regions of the hippocampus selectively influences the expression of different classes of behavior.

Multimodal information processing in the hippocampus (Ammon's horn) is important for memory formation (*1*), and the results of this processing are sent to two major forebrain locations (*2*). One projection reaches broad parts of the cerebral cortex via the parahippocampus and apparently influences the long-term storage of memories. The other projection is topographically organized and descends through the precommissural fornix to the lateral septal nucleus (LS). It was shown several years ago (*3*) that the better known postcommissural fornix projection to the mammillary body arises not in the hippocampus proper but rather in the adjacent subicular complex of the parahippocampus. The present work was designed to clarify the possible functional significance of the descending output of the hippocampus, which remains enigmatic, by reexamining the structural organization of input and output pathways of the LS.

First, we carefully examined the spatial distribution of several neurotransmitter-related mRNAs. LS neurons are known to express the neuropeptides enkephalin (*4*) and somatostatin (*5*), as well as glutamic acid decarboxylase (GAD) (*6*), the enzyme responsible for synthesizing the neurotransmitter γ-aminobutyric acid (GABA). Serial section in situ hybridization analysis through the rostrocaudal extent of the LS, with adjacent sections labeled for the expression of enkephalin and somatostatin mRNA, reveals that somatostatin hybridization is concentrated dorsocaudally, whereas enkephalin hybridization is concentrated rostroventrally (Fig. 1A), although the two cell types are not segregated completely. In contrast, GAD is expressed abundantly throughout the LS, including a distinct ventromedial part with very little enkephalin or somatostatin mRNA expression. This evidence suggested that the LS may be divided into rostral (LSr), caudal (LSc), and ventral (LSv) parts, respectively.

To test this hypothesis, we examined neural inputs and outputs of the three parts with anterograde and retrograde tracing methods. First, we made 22 simultaneous injections of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHAL) and the retrograde tracer fluorogold (FG) into various parts of the LS and confirmed the results with 13 single PHAL and 4 single FG injections (in 39 animals in all). Second, we reexamined a collection of more than 100 anterograde autoradiographic experiments that were done with ^{3}H–amino acid injections throughout the hippocampal formation and used in previous work (*3, 7, 8*).

The double injections were used to plot LS connections with the hypothalamus. PHAL injections in the LSc labeled a dense pathway to and through the far lateral hypothalamus that terminates densely in the lateral supramammillary nucleus (Fig. 1, B and D), and accompanying FG injections retrogradely labeled neurons in the same two hypothalamic regions. Thus, the LSc establishes bidirectional connections with the far lateral hypothalamus and lateral supramammillary nucleus. In contrast, PHAL injections in the LSr labeled major inputs to the medial preoptic nucleus (lateral part), anterior hypothalamic nucleus and adjacent perifornical region of the lateral hypothalamic area, ventrolateral ventromedial nucleus and adjacent tuberal nucleus, and posterior hypothalamic nucleus (Fig.

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