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A Genetic Link between Morphogenesis and Cell Division during Formation of the Ventral Furrow in *Drosophila*

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Summary

Stages in development with rapid transitions between mitosis and morphogenesis may require specific mechanisms to coordinate cell shape change. Here we describe a novel mitotic inhibitor that acts during *Drosophila* gastrulation to counteract String/Cdc25, specifically in the cells that invaginate to form the mesoderm. We have identified two genes, *frühstart* and *tribbles*, that are required for this ventral inhibition. *tribbles* encodes a kinase-related protein whose RNA, however, is also present outside of the ventral region. Effective inhibition of mitosis in the cells of the ventral furrow depends on the transcription factor Snail that triggers the ventral cell shape changes. When overexpressed in a microinjection assay, Tribbles directly inhibits mitosis. We propose that Frühstart and Tribbles form a link between the morphogenetic movements and mitotic control.

Introduction

Morphogenetic processes and cell division both rely on the reorganization of the cytoskeleton. Dual use of the cytoskeleton can potentially cause an interference between these two processes especially during stages when development progresses rapidly. *Drosophila* provides a suitable model system to detect such interference as its early development alternates rapidly between mitotic programs and morphogenetic movements (Foe, 1989; reviewed in Foe et al., 1993). Following fertilization the embryo undergoes a series of rapid nuclear replications with cell cycle lengths of 8 to 15 min and no intervening cytokinesis. After 13 such divisions, the cell cycle pauses in G2 of interphase 14 and cellularization, a morphogenetic program with a length of about 50 min, starts to enclose the nuclei in newly emerging cell membranes. During gastrulation the newly formed cells then change their shape, and morphogenetic movements rearrange their positions to one another (reviewed in Costa et al., 1993 and Leptin 1995). One of the most prominent of these morphogenetic movements is the formation of the ventral furrow, which brings the mesoderm anlage into the interior of the embryo. However, it is also during gastrulation that cell division resumes. During this time mitosis occurs in an asynchronous manner separated in at least 25 domains (Foe, 1989). This asynchrony allows morphogenesis and cell division to occur simultaneously in different regions of the embryo and sequentially in specific primordia. For example, the ventral-most cells first form the ventral furrow and only after this invagination is completed, do they go into mitosis.

Entry into mitosis is positively controlled by String, the homolog of Cdc25, which is necessary and sufficient for mitosis during gastrulation (Edgar and O'Farrell, 1989, 1990). The expression pattern of *string* RNA closely matches the mitotic pattern. During the cleavage stage *string* RNA is uniformly distributed, but then degraded at the pause in mitosis and the transition to cellularization. *string* RNA subsequently reappears at the beginning of gastrulation in a pattern preceding the mitotic domains. In all domains except one, mitosis starts a few minutes after *string* RNA is expressed. Mitotic domain 10, which comprises most of the mesoderm anlage, behaves differently, in that the gap between *string* RNA expression and entry into mitosis is much longer.

Here we describe a novel mitotic inhibitor that counteracts String specifically in the cells involved in ventral furrow formation during gastrulation. We have identified two zygotic genes required for this ventral inhibition, *tribbles* (*trbl*) and *frühstart* (*frs*), and show that *trbl* encodes a protein with partial homology to serine-threonine protein kinases. We also show that Trbl itself can directly inhibit mitosis when overexpressed in a microinjection assay. Ventral inhibition depends on the program of cell shape changes that are induced by the nuclear protein Snail, although the transcription of *trbl* is not regulated by it. We propose that Frs and Trbl form a link between morphogenetic movements and mitotic control.

Results

Ventral Cells Contain a Mitotic Inhibitor Counteracting *string*

Although the transcription pattern of *string* generally prefigures the pattern of mitotic domains in the early gastrula, there is one striking exception. *string* RNA is first expressed on the ventral side of the embryo, in a region destined to form mesoderm (Figures 1A and 1B). The cells in this region, however, divide only as the tenth mitotic domain, after they have formed a ventral furrow and completed their invagination into the interior (Figure 1C). The delay in their mitosis suggests that ventral cells contain a factor lengthening the gap between appearance of *string* RNA and entry into mitosis. This delay involves a subtle titration of *string* activity, since it can be shortened by addition of two more copies of the *string* region raising the copy number of *string* to four (see Experimental Procedures). Under these conditions the ventral cells divide at about the same time as the cells of domains 1 to 3 (Figure 1D), which matches the *string* RNA pattern more closely than it does in wild-type embryos. Only the mitosis in domain 10 is shifted in these experiments, the order of the other mitotic domains is not changed.

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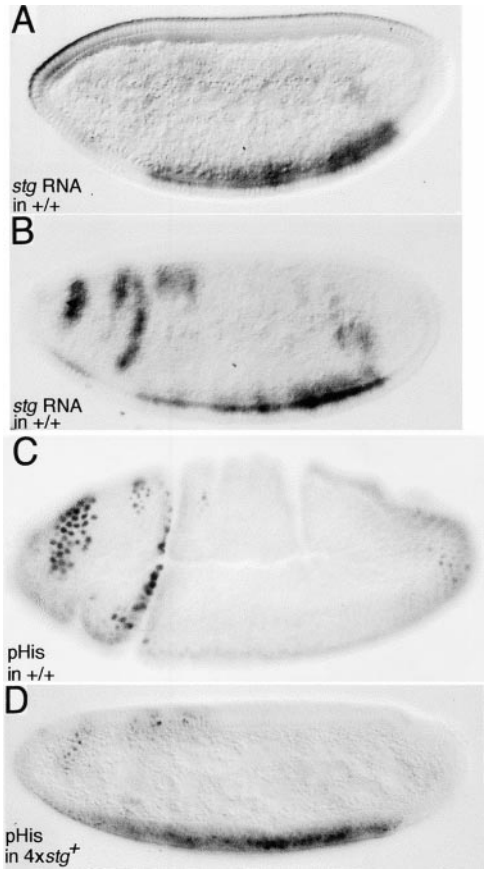

Figure 1. Delayed Mitosis in the Ventral Furrow Region

(A and B) In situ hybridization for *string* RNA in wild-type embryos. The embryo in (B) is slightly older than the embryo in (A). (C and D) Mitotic nuclei are visualized by staining for phospho-histon3 (pHis). The cells of domain 10 do not yet divide in (C). (D) Embryo with four copies of *string* (overlay picture). The ventral cells (domain 10) divide at the same time as domain 1 to 3 (see Experimental Procedures).

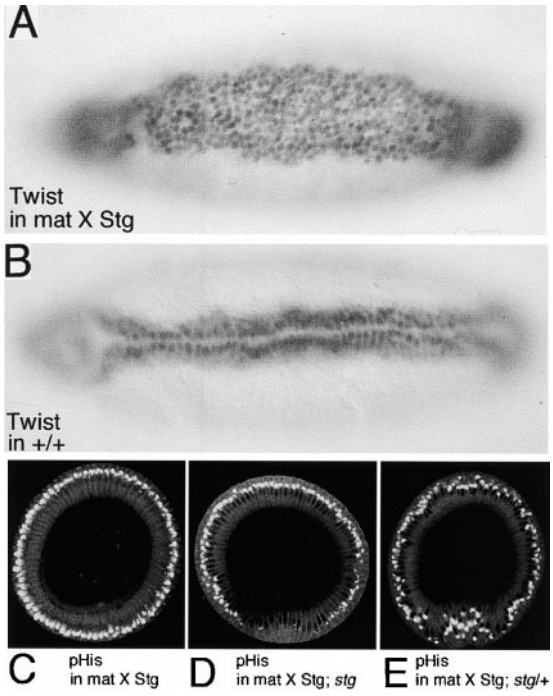

Figure 2. Ventral Cells Are Less Sensitive to *string*

(A and B) Embryos stained for Twist, marking the ventral cells. (A) *string* overexpression driven by four copies of maternal Gal4 (mat x Stg). In wild-type embryos (B) the ventral cells form a furrow, in (A) the ventral cells remain on the surface of the embryo. (C–E) Optical cross sections of embryos stained for Neurotactin (cell outline) and phospho-histon3 (mitotic nuclei), in which *string* RNA is uniformly expressed. *string* expression is driven by four (C) or two (D and E) copies of maternal Gal4. The zygotic genotypes are +/+ or *string*/+ in (C and E) and *string*/*string* in (D). In (D) the ventral cells changing their shape do not enter mitosis at the same time as the other cells. In (E) the cells contain endogenous and exogenous *string* RNA.

To examine more stringently whether the factor counteracting *string* is specific for ventral cells, we expressed exogeneous *string* RNA at the same level in all cells of the embryo, using a UAS-String transgene driven by a maternally provided Gal4 in embryos otherwise homozygous for a *string* deletion. Four copies of maternally provided Gal4 produced high levels of *string* activity, indicated by the uniform entry of all cells into mitosis immediately at the beginning of gastrulation (Figures 2A–2C). In these embryos ventral furrow formation is inhibited, confirming the observations by Edgar and O'Farrell (1990). Using females with three or two Gal4 insertions, expression of *string* was gradually lowered. This shifted the onset of mitosis to a time when the first mitoses normally occur in wild-type embryos. Under these conditions differences in the behavior of the cells become apparent. In spite of the uniform *string* expression, the ventral cells undergoing cell shape changes to form the ventral furrow enter mitosis later than the other cells (Figure 2D). This special behavior of the ventral cells is not observed in *string* heterozygous embryos which have endogeneous as well as exogeneous *string* RNA (Figure 2E). We conclude from these experiments that ventral cells contain a dosage-sensitive factor, the ventral inhibitor, that counteracts *string* activity and that the delay of mitosis in domain 10 of wild-type embryos is due to this factor.

Two Zygotic Genes Are Required for the Ventral Inhibitor

In order to identify components that constitute the ventral inhibitor, we performed a genome-wide screen (Müller et al., 1999) for loci that are required for a delayed mitosis in domain 10. By screening 99% of the genome, we identified two novel loci, *frühstart* and *tribbles*. In embryos deficient for either of these genes, cells in the ventral domain are the first to enter mitosis, such that their pattern of *string* RNA expression and the mitotic pattern match each other (Figures 3A–3D). The order of the other mitotic domains is not altered, suggesting that *frs* and *trbl* act specifically in the ventral cells. The double mutant *frs trbl* shows the same phenotype as the single mutants (Figures 3D and 3H), suggesting that *frs* and *trbl* are nonredundant genes in a common process.

As a consequence of the early mitosis, the mesodermal precursors remain on the surface and do not form a proper ventral furrow (Figures 3E–3H). This defect is

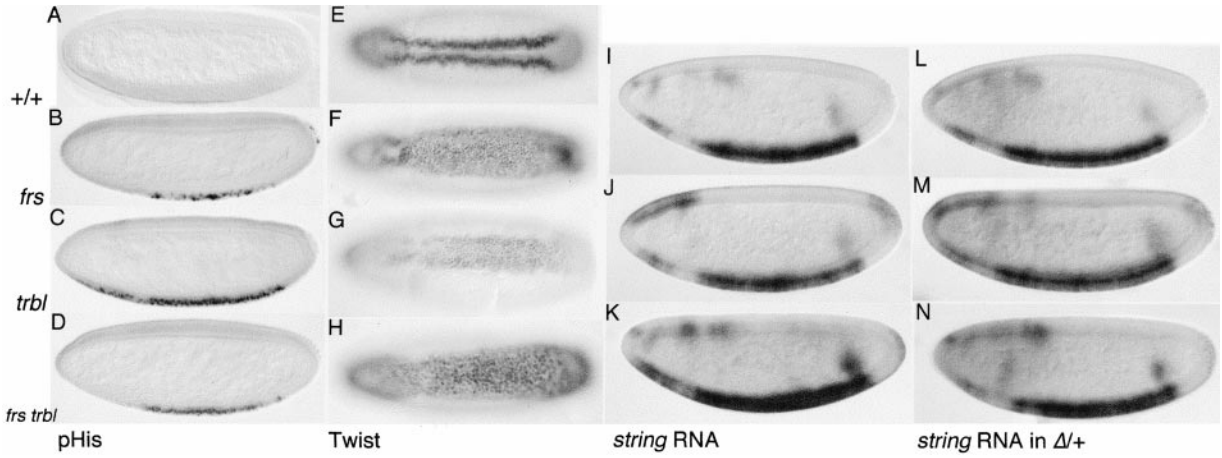

Figure 3. Phenotype of *frs* and *trbl*

(A–D) Mitotic nuclei detected by phospho-histon3 staining (pHis). (E–H) Ventral cells are marked by staining for Twist. (I–N) in situ hybridization for *string* RNA with double staining for the reporter gene (anterior expression) on the balancer chromosome not marking the homozygous embryos (I–K). The genotypes are as follows: (A and E) +/+, (B, F, and I) *frs*, (C, G, and J) *trbl*, (D, H, and K) *frs trbl*, (L–N) corresponding heterozygous or wild-type siblings of (I–K). *frs* is Df(3L)XG10, *trbl*, EP(3)3519, and *frs trbl* double mutant is Df(3L)BK10 Df(3L)rdgC-co. Embryos in (A–H) were from crosses with compound-3 females.

similar to that observed in embryos in which all cells have been forced into mitosis by increased *string* dosage or *string* overexpression (Figures 1D and 2A; Edgar and O'Farrell, 1990). Although other zygotically active genes are known to affect formation of the ventral furrow, *frs* and *trbl* are unique in that their defects solely depend on the premature mitosis. In double mutant *frs string* and *trbl string* embryos, no mitosis takes place during gastrulation, and the ventral furrow forms as in wild-type (data not shown). The premature mitosis in *frs* or *trbl* embryos is not caused by overexpression of *string* RNA in the ventral region, as *string* RNA is present in comparable amounts in mutant embryos (Figures 3I–3K) and with a similar pattern as in their heterozygous siblings (Figures 3L–3N) or wild-type embryos. Since *String* is the rate-limiting factor for entry into mitosis during gastrulation, this observation suggests that *frs* and *trbl* counteract *string* via a posttranscriptional mechanism.

trbl Encodes a Protein with an Unusual Protein Kinase Domain

To determine the biochemical nature of the ventral inhibitor, we isolated the *trbl* gene. Using deficiency chromosomes, we mapped the gene to the chromosome bands 77C-D, but could not identify a point mutation with the ventral furrow phenotype in several collections of lethal mutations mapping to this region. We did, however, identify two semi-viable lines (EP(3)3519 and EP(3)1119) whose P insertions cause the same gastrulation phenotype as deficiency chromosomes. In both lines the transposon inserted into the 5′ untranslated region of a single transcription unit in the 77C region (Seher and Leptin, 2000; BDGP; data not shown). To show that the absence of this transcription unit indeed causes the gastrulation defect, we induced revertants of EP(3)3519. Precise excision of the transposon (141 out of 150 lines) reverts the mutant to full viability and normal gastrulation. Imprecise excision (9 of the 150 revertants) does not restore the mutant phenotype. These results, coupled with the rescue experiment using UAS-trbl that is presented below, indicate that the identified transcript encodes the wild-type *trbl* product.

The sequence of the *trbl* transcript contains an open reading frame encoding a protein of 488 amino acid residues with homology to serine-threonine protein kinase domains. The most closely related proteins in budding yeast (*S. cerevisiae*) are Snf1 and Hsl1 and in fission yeast (*S. pombe*), the SNF1-like protein kinase and Nim1 (Figure 4). Furthermore, protein kinases with a similar degree of identity are present in many organisms. For many of these related proteins, however, the physiological function is not known. Comparison of the primary structure with the consensus sequence of protein kinases shows severe deviations from the kinase consensus sequence (Hanks and Hunter, 1995). Three of the invariant residues are changed in Trbl: a KR change in the ATP binding site in subdomain II, an NR change in the catalytic loop within subdomain VIB, and a DS exchange in subdomain VII. In addition, the highly conserved histidine of the catalytic loop is substituted by a leucine. These severe deviations from the consensus make it unlikely that Trbl is a functional protein kinase. A second striking feature of the primary sequence is the abundance of serine residues in the N-terminal region of Trbl. Twenty-two of the first 49 residues are serines, which includes a piece with nine consecutive serines.

Expression of *trbl* Is Not Restricted to the Ventral Furrow

trbl transcript is absent from early cleavage embryos and accumulates to high levels at the beginning of cycle 14. During cellularization, expression levels decline, but transcripts are present throughout gastrulation, and in fact persist until late embryogenesis (Figures 5A–5C and data not shown). At the beginning of gastrulation, the RNA distribution is not uniform. Highest levels are seen

```
consensus                                          g G   g v
     NIM1  ~~~~~~~~~~ ~~~~~~~MVK RHKNTIGVWR LGKTLGTGST SCVRLAKHAK
     SNF1  HHHHGHGGSN STLNNPKSSL ADGAHIGNYQ IVKTLGEGSF GKVKLAYHTT
     trbl  VKLIQQRYLI SAQPSHISAA AAAKTPASYR HLVDLTASNL RCVDIF.... 150
                                       /->     I          <-/

consensus       a K                        E         h
     NIM1  TGDLAAIKII PI...RYAS. ......IGME ILMMRLLRHP NILRLYDVWT
     SNF1  TGQKVALKII NK...KVLAK SDMQGRIERE ISYLRLLRHP HIIKLYDVIK
     trbl  TGEQFLCRIV NEPLHKVQRA YFQLQQHDEE LRRSTIYGHP LIRPVHDIIP 200
           /->    II            <-//->    III   <-//->        IV

consensus
     NIM1  .DHQHMYLAL EYVPD..... .......GEL FHYIRKHGPL SEREAAHYLS
     SNF1  .SKDEIIMVI EYA.G..... .......NEL FDYIVQRDKM SEQEARRFFQ
     trbl  LTKDRTYILI APVPQERDST GGVTGVYENL HTYIRHAKRL CETEARAIFH 250
           <-//->     V                           <-//->      VIA

consensus           h      hrD k   N            k  Df  g
     NIM1  QILDAVAHCH RFRFRHRDLK LENI.LIKVN EQQIKIADF. GMATVEPNDS
     SNF1  QIISAVEYCH RHKIVHRDLK PENL.LLD.E HLNVKIADF. GLSNIMTDGN
     trbl  QICQTVQVCH RNGIILRDLK LKRFYFIDEA RTKLQYESLE GSMILDGEDD 300
                <-//->   VIB         <-//->      VII     <-/

consensus       g       pE              D     g          p
     NIM1  CLENYCGSLH YLAPEIV.SH KPYRGAPADV WSCGVILYSL LSNKLPFGGQ
     SNF1  FLKTSCGSPN YAAPEVI.SG KLYAGPEVDV WSCGVILYVM LCRRLPFDDE
     trbl  TLSDKIGCPL YTAPELLCPQ QTYKGKPADM WSLGVILYTM LVGQYPFYEK 350
           /->   VIII     <-//->    IX                    <-//

consensus                                          R
     NIM1  NTDVIYNKIR HGAYDLPSSI SSAAQDLLHR MLDVNPSTRI TIPEFFSHPF
     SNF1  SIPVLFKNIS NGVYTLPKFL SPGAAGLIKR MLIVNPLNRI SIHEIMQDDW
     trbl  ANCNLITVIR HGNVQIPLTL SKSVRWLLLS LLRKDYTERM TASHIFLTPW 400
           ->       X       <-//->      XI                  <-
```

Figure 4. Sequence Analysis of the Trbl Kinase Domain

Comparision of the kinase domains of NIM1 (SWISS-PROT P07334), SNF1 (SWISS-PROT P06782), and Trbl (GenBank AF204688) with the protein kinase consensus (Hanks and Hunter, 1995). The invariant residues of the consensus are indicated by captial letters in bold, highly conserved residues, by the lowercase letters. The Roman numerals describe the kinase subdomains. The underlined residues of Trbl are deviations from the consensus. The lysine of the catalytic center in subdomain *VI*B is mutated to an arginine in the TrblK266R allele. Within the kinase domain Trbl and NIM1 share 30% identical residues, Trbl and SNF1, 29%.

in ventral cells; the extent of this expression, however, is wider than the ventral furrow itself and thus wider than the domain in which *trbl*-dependent mitotic delays are observed (Figures 5D–5F). To test the significance of this expression pattern, we expressed *trbl* RNA uniformly with a UAS-trbl driven by a maternal Gal4. When supplied in this manner, exogeneous *trbl* RNA can restore the mitotic delay and the ventral furrow defect observed in homozygous mutant deficiency embryos. This confirms that *trbl* is the only gene in the 77CD region that is required for the mitotic delay in domain 10 (Figure 6A). However, ectopic expression does not affect the timing and order of other mitotic domains in the embryo (Figure 6B). Thus *trbl* RNA expression itself is not sufficient to account for the delay in mitosis in the ventral domain. We therefore suggest that an additional factor may control activation of Trbl protein in the ventral cells.

The Ventral Inhibitor Depends on Mesodermal Programming

In embryos mutant for either *snail* or *twist*, no ventral furrow forms and cells are shifted to more lateral fates

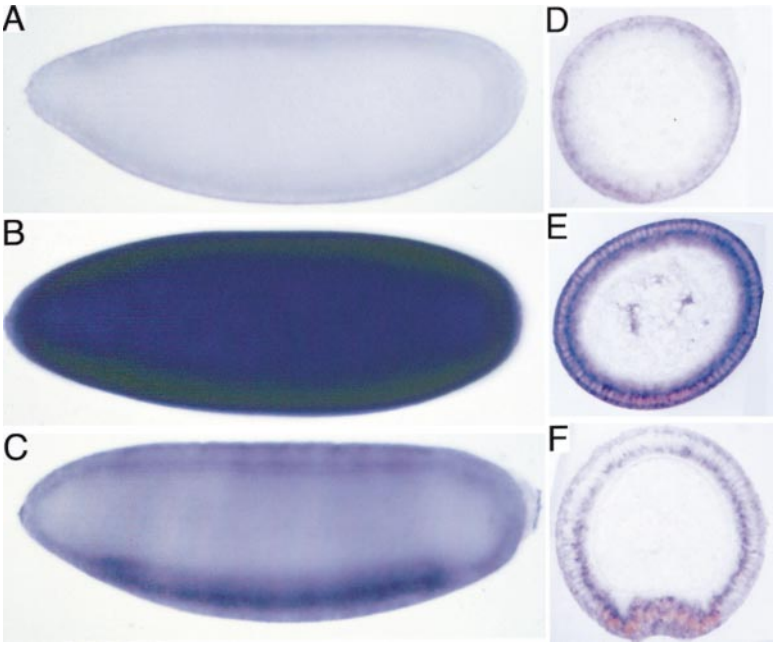

Figure 5. Expression of *trbl* RNA

(A–F) In situ hybridization for *trbl* RNA, (A and D) cleavage stage, (B and E) early cellularization, (C and F) early gastrulation. (D–F) Cross sections stained in addition for Twist (red stain) to mark the ventral cells.

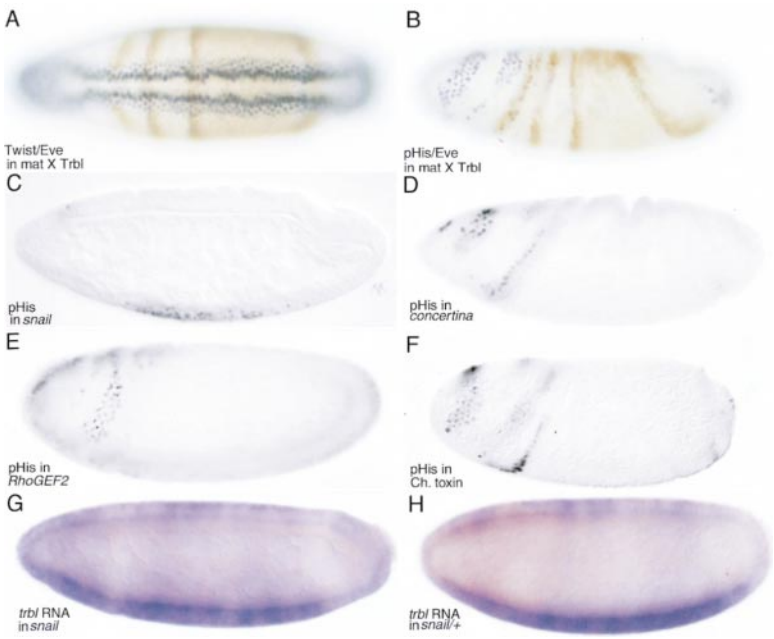

Figure 6. Ventral Inhibition Depends on *snail*

(A and B) Embryos homozygous for a *trbl* deficiency, in which *trbl* is uniformly expressed driven by a maternal Gal4, stained for Even-skipped (Eve, stripped pattern) and (A) Twist and (B) phospho-histon3. Homozygous embryos were recognized by the *knirps* phenotype (stripes 4–6 are absent) of the Even-skipped expression pattern. Such embryos are rescued for the ventral furrow defect (A), but show normal mitotic pattern elsewhere (B). (C–F) Mitotic staining by phospho-histon3 antibody with the following genotypes: (C) *snail*, 3x*twist*$^{+}$ embryo from the cross compound-2 females $\times$ *snail*, (D) from *concertina* female, and (E) from female with *DRhoGEF2* germline. (F) Wild-type embryo injected with cholera toxin. (D)–(F) are overlay pictures. (G–H) In situ hybridization for *trbl*, in a (G) *snail* embryo and in a *snail*/+ embryo, marked by the reporter gene (anterior expression, red stain) of the balancer chromosome.

(Leptin and Grunewald, 1990). *string* RNA is not present in domain 10 and mitotic patterns in the ventral region of these mutant embryos are difficult to evaluate (Arora and Nüsslein-Volhard, 1992; data not shown). We have found that *string* RNA is restored to wild-type levels in the prospective domain 10 of *snail* mutants carrying three copies of wild-type *twist* (see Experimental Procedures; data not shown). In such mutants, the ventral cells are the first ones to divide (Figure 6C), indicating that *snail* is required for the function of the ventral inhibitor. One possibility would be that the persistence of *trbl* expression in the ventral region requires mesodermal determination and thus wild-type *snail* activity. However, *snail* mutants show a normal pattern of *trbl* expression and maintain *trbl* expression in the ventral domain (Figures 6G and 6H). Similarly, in *twist* homozygous mutants and in embryos homozygous for deficiencies for *frs* the expression of *trbl* is not changed (data not shown). Because *snail* embryos do not show a ventral mitotic inhibition, even though their *trbl* expression is normal, we conclude that some aspect of mesodermal determination mediated by *snail* is required for Trbl activation.

One possible mechanism would be that the cell shape changes triggered by mesodermal programming might in some way activate *trbl*. Although no downstream genes are known that totally block these cell shape changes, two pathways have been identified that show less severe effects than *snail* itself. The Fog/Concertina pathway coordinates apical constriction of cells in the ventral furrow (Costa et al., 1994), and RhoGEF2 is required for their apical flattening (Barrett et al., 1997; Häcker and Perrimon, 1997). We examined the mitotic pattern in embryos mutant for each pathway. In both cases, the mitotic pattern showed the same delay as in wild-type, indicating that, despite the abnormal cell shape changes in these mutants, the ventral inhibitor is still activated (Figures 6D and 6E). To test whether we can inhibit mitosis by ectopic cell shape changes outside the ventral region, we induced apical flattening of all cells by activation of the Gα protein Concertina with cholera toxin (Morize et al., 1998). Embryos injected with cholera toxin display the characteristic *concertina* gain-of-function phenotype, whereas the mitotic domains in the anterior region are present in the normal extent and pattern (Figure 6F). Thus, the apical flattening, which is the first step in the series of shape changes that leads to the ventral furrow, is not sufficient to activate the ventral inhibitor. However, given the established redundancy in the cell shape change pathways that are involved in ventral furrow formation, our results do not rule out that mesodermal cell shape changes do indeed ultimately account for Trbl activation. Alternatively, the function of Snail may be more direct, if for example Snail was found to regulate the transcription of *frs*, or a presently unknown component of the ventral inhibitor (see Discussion).

Overexpression of *trbl* Inhibits Mitosis

To directly investigate the activity of *trbl* on mitosis, we used the rapid cell cycle of cleavage stage embryos as an assay. *trbl* is not yet expressed during this stage as it appears only at the beginning of cellularization when the rapid nuclear divisions stop. We microinjected large amounts of synthetic *trbl* mRNA into the posterior half of the embryos, followed the subsequent nuclear divisions, and fixed the embryos before reaching gastrulation. In addition, the progression of the cell cycle after injection was recorded in embryos that express a GFP-Histon fusion protein which labels interphase and mitotic chromosomes (data not shown). The nuclei at the injection site do not participate in the last mitosis (13th division) resulting in larger nuclei and lower density at the injection site (Figure 7, 17 out of 29 embryos scored). As a negative control for this assay, we injected mRNA of the unrelated serine-threonine protein kinase Pelle. This did not inhibit mitosis (34 embryos scored). We have shown above that presence of *trbl* alone is not sufficient to inhibit mitosis and that it requires an additional trigger

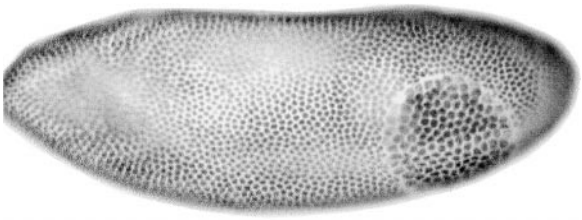

Figure 7. Overexpression of *trbl* RNA

Injected embyro fixed and overstained with phospho-histon3 antibody, so that also interphase nuclei become labeled. *trbl* mRNA was injected during cleavage stage into the posterior half of the embyro. In one experiment for each for the different mRNA, the number of embryos with a mitotic defect was determined by visual observation: *trbl*, 17 of 29, *trbl*K266R, 20 of 41, and *pelle*, 0 of 34.

in the ventral cells. It is possible that we are overriding this requirement by providing *trbl* in excess in the microinjection assay.

The deviations from the consensus of protein kinases make it unlikely that Trbl is a functional protein kinase. To test this possibility further, we generated an allele of Trbl (K266R) mutated at a crucial lysine of the catalytic center thought to govern the specificity of kinases for their targets (see Figure 4). We changed this lysine to an arginine, a change which should maintain the configuration of the active site but alter its specificity. A lysine is present in all serine-threonine protein kinases, whereas an arginine is characteristic of conventional tyrosine kinases (Hanks and Hunter, 1995). When injected into embryos, this allele also induced a premature pause in the cell cycle (20 out of 41 embryos scored), confirming that Trbl does not function as a serine-threonine protein kinase.

Discussion

During gastrulation in *Drosophila*, the main trigger for mitosis is *string* expression. *string* is transcribed in a spatial pattern controlled by the anterior–posterior and dorsoventral patterning systems (Edgar et al., 1994). Expression of *string* RNA in a given domain precedes mitosis by a few minutes. By analyzing the exception to this rule, domain 10 on the ventral side at the embryo, we uncovered an additional mode of regulation that is not based on transcription. Although *string* is expressed in these cells, they do not divide until they are internalized. This delay depends on the activity of the *frs* and *trbl* genes and is restricted to the ventral furrow, even though *trbl* RNA is also present outside of this domain and the *trbl* mutation can be rescued by uniform exogeneous expression. This suggests that *trbl* activity is triggered by an input which is present only in the ventral furrow region.

Given its early strong expression at the onset of cycle 14, it is possible that *trbl* has a function earlier in development as well. For example, *trbl* may function redundantly with the degradation of maternal *string* RNA to induce the transition from rapid nuclear divisions to the cellularization program. We only rarely see mitotic defects in *frs* and *trbl* embryos before gastrulation (unpublished observations), which indicates that in most cases reduction of maternal *string* RNA is sufficient to ensure a pause in mitosis during cellularization. On the other hand, exogeneous *string* expression driven by a maternal Gal4 driver did not result in an additional round of mitosis during cellularization (unpublished observations), suggesting that a factor other than *string* degradation may contribute to the mitotic block observed in early cycle 14 embryos. Given its expression pattern and activity in the injection assay, that other factor might be Trbl.

The mitotic behavior of the cells in the ventral furrow reflects a delicate balance between activating and inhibiting activities. Even a 2-fold increase of the *string* activity can override the ventral inhibition. A similar dosage sensitivity is observed in ventral cells when *string* is overexpressed in embryos that have no endogeneous *string*. On the other hand, an increase in the inhibitor blocked the cell cycle, e.g., in the injection experiment, where a gain-of-function activity of *trbl* is introduced. The inhibition is sharply restricted to an area at the injection site, visualizing the dosage dependence of the system. The pathways and targets of these activities are not known in detail, but the well-characterized mechanisms of cell cycle regulation in yeast may provide insight into where this balance might be taking place at a molecular level.

In fission yeast (*S. pombe*) as in *Drosophila*, mitosis is positively activated by the action of Cdc25, the homolog of *Drosophila* String. The Cdc25 protein achieves this activation by hydrolyzing phospho-tyrosine 15 of Cdc2, thus activating Cdc2 (Russel and Nurse, 1986; Millar and Russel, 1992). A negative pathway acts on this very same residue through the protein Wee1, which phosphorylates tyrosine 15 thus inhibiting Cdc2 and mitosis (Russel and Nurse, 1987a; Featherstone and Russel, 1991; Parker et al., 1991). Wee1 is itself negatively regulated by the protein kinase Nim1 (Russel and Nurse, 1987b; Coleman et al., 1993; Parker et al., 1993; Wu and Russel, 1993). We propose that the Wee1 pathway may also be present in *Drosophila* and that this pathway constitutes the ventral mitotic inhibitor revealed in our experiments. A homolog of Wee1 is known to be expressed in the early *Drosophila* embryo, but its physiological function has yet to be characterized in detail (Campbell et al., 1995). Intriguingly, Trbl is closely related to Nim1. However, there are two important differences between Trbl and Nim1. First, whereas Nim1 activates mitosis by inhibiting the negative regulator Wee1, Trbl activity inhibits mitosis. Second, unlike the kinase Nim1, Trbl is a nonfunctional kinase. It is therefore possible that a true functional homolog of Nim1 exists in *Drosophila* and that the closely related Trbl protein competes or interferes with the action of this functional kinase on Wee1. In this way Trbl activity would lead to the activation of Wee1 and thus inhibition of Cdc2 and mitosis.

It is not known how Nim1 is activated in yeast cells. During *Drosophila* gastrulation, however, we know that *trbl* activity depends on Snail, the trigger for ventral furrow formation. We propose two models for how Trbl might be activated in the ventral furrow region. In the first model, Snail and Twist would induce transcription of an activator of Trbl, predicting an expression pattern that is restricted to the ventral furrow. This activator

might be *frs* or a further component of the ventral inhibitor. We have not isolated *frs* yet, but none of the candidate genes in the *frs* region that we have looked at so far display expression restricted to the ventral region. It is also unlikely that there exists another gene with a similar mutant phenotype, as we identified only *frs* and *trbl* in our genome-wide screen. We therefore favor a second model.

In the second model, ventral inhibition is an integral part of the morphogenetic program. Frs and Trbl would be activated by a component involved in the cell shape changes, and the expression of *frs*, as it is the case for *trbl*, would not necessarily be restricted to the ventral region. This second model seems to be more attractive given the known cytoskeletal interaction of the Nim1-related kinase Hsl1 of *S. cerevisisae*. Although it is a larger protein with additional domains, Hsl1 shares high homology with Nim1 and Trbl within the kinase domain. The kinase activity of Hsl1 depends on septins that are localized at the bud neck. Hsl1 activates Cdc2 via its effect on Swe1, the homolog of Wee1, thus forming a link between the cytoskeleton and cell cycle regulators (Barral et al., 1999). Although the situation in flies is slightly different, Trbl might be activated by changes in the cytoskeleton and transmit the signal to D-Wee1 and Cdc2. In this case the signal would ultimately be inhibitory.

We have characterized the novel function of two genes that connect a morphogenetic process with the cell cycle. The presence of such a delicate regulatory system suggests that morphogenesis and cell proliferation are not simply proceeding side by side, but that direct coordination between these two processes occurs, at least during rapid developmental transitions. Future research will show whether similar mechanisms and homologs of *frs* and *trbl* coordinate morphogenetic movements and cell proliferation in other higher organisms as well.

Experimental Procedures

Genetic Experiments

If not otherwise indicated, genetic material and fly stains are described by Lindsley and Zimm, 1992, in the Flybase (http://flybase.bio.indiana.edu/), and by the Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org). Commonly used procedures and protocols were applied, if not otherwise indicated (Ashburner, 1989; Robert, 1998). C(2) and C(3) are compound chromosomes that segregate the left and right chromosome arms. Following mutations and alleles were used: *snail*^{IIG}, *concertina*^{RC10}, *DRhoGEF2*$^{1.1}$, *string*^{AR2}, *string*^{7M53}, *twist*^{ID96}, *snail*^{IIG} *twist*^{Df560}. The germline clones of *DRhoGEF2* were induced by FRT/Flipase and selected by *ovo*$^{D1\text{-}2R}$.

frühstart (*frs*) maps to 71CD. Several deficiencies restrict *frs* to a region that contains about 14 transcription units (data not shown; BDGP). Deficiencies were isolated by X-ray-induced excision of the w^+ insertion in the line B204, which maps to 71DE (Rose et al., 1997). If not otherwise stated, the *frs* mutation was Df(3L)XG10. The gastrulation phenotype is fully penetrant in a cross with C(3) females. The lethal point mutations that map to this region do not show the gastrulation phenotype of the deficiencies.

tribbles (*trbl*) is defined by two P element insertions, EP(3)3519 and EP(3)1119 (Rørth et al., 1998). The gastrulation phenotype is fully penetrant in a cross with C(3) females. Homozygous flies are semi-viable. Their ventral furrow defect and early ventral mitosis are similar to the phenotype of the deficiencies Df(3L)rdgC-co and Df(3L)ri79c. Revertants of EP(3)3519 were induced by X-ray resulting in four semi-viable and four lethal lines. In addition the P element in EP(3)3519 was mobilized with transposase. Out of 150 white revertants, 8 were semi-viable and one was lethal. All of the semi-viable and lethal revertants display the same gastrulation phenotype as the deficiencies. Five of the 141 viable revertants were tested for the gastrulation phenotype. Embryos from these five lines gastrulate normally.

For the genome-wide screen involving aneuploid embryos (Müller et al., 1999) fixed embyros were stained with p-histon3 antibody. In most crosses the deficiency embryos could be recognized by secondary staining (e.g., for Even-skipped) that uncovered a linked patterning gene.

snail 3x*twist*$^+$ embryos: One quarter of the embryos from the cross C(2) females × *snail* have one 2L with the *snail* mutation and three copies of 2R including *twist*$^+$. The restoration of *string* expression in domain 10 is due to the third copy of *twist*$^+$, because restoration is not observed in the cross C(2) females × *snail twist*.

4x*string* embryos: Embryos with three or four copies of *string* contained the two wild-type copies and in addition two copies of a duplication of the *string* region on the Y chromosome. These duplications are cytologically visible and comprise several chromosome bands. The embryos were obtained by crossing males carrying a duplication to females with a C(3) chromosome. The transpositions Tp(3;Y)J55 (cytology: 98A; 100B) and Tp(3;Y)B219 (94C; 100A) contain the *string* locus (99A), Tp(3;Y)B204 (93B; 98) has the breakpoint proximal to *string*. From the cross with Tp(3;Y)B204, half of the embryos are 3x*string*, the other half are 1x*string*, and 8% (8 out of 114) showed an early ventral mitosis. 1/8 of the embryos from the crosses with Tp(3;Y)B219 and T(3;Y)J55 are 4x*string*, and 1/4 are 3x*string*. 23% (38 of 160, B219) and 19% (40 of 208, J55) of such egglays show an early mitosis in the ventral region.

string was uniformly expressed with the following cross at 18°C: mat67; *string*/TM3 × UAS-String; *string*/TM3. For the crosses with three or four copies of maternal Gal4, an insertion on the third chromosome (mat15) was added to the stock, as a recombinant with *string* or in trans to mat15 *string*. The UAS-String transgene is described by Johnston and Edgar (1998). mat67 and mat15 are maternally expressed Gal4 transgenes (St Johnston, Cambridge, UK), *string* was the allele *string*^{AR2}, which is a deletion of the *string* locus (Edgar et al., 1994). The TM3 balancer contained an hb-lacZ reporter gene. Overexpression of *string* in a wild-type background was done with the cross: mat67; mat15 × UAS-String.

Exogeneous *trbl* was expressed with the following cross at 25°C: mat67; Df(3L)ri79c/TM3 × UAS-trbl; Df(3L)ri79c/TM3. Df(3L)ri79c is a deficiency of *trbl* and also *knirps*. The TM3 balancer is marked with an hb-lacZ reporter gene.

Histology

Fixations and the protein and RNA in situ detection were according to general protocols (Tautz and Pfeifle, 1989; Robert, 1998; Müller et al., 1999). The following antibodies were used: anti-Twist (S. Roth, Cologne, FRG), anti-Even-skipped (J. Reinitz, New York, USA), anti-β-galactosidase (Boehringer Mannheim, FRG), anti-phospho-histon3 (Upstate Biotech., USA), and anti-Neurotactin (monoclonal BP106). For proteins the staining was by coupled peroxidase (Vectastain, Vector Lab., Burlingame, USA), for RNA, by coupled alkaline phosphatase (Boehringer Mannheim, FRG). The detection of RNA was by probes labeled with Digoxigenin (Boehringer Mannheim, FRG). Detection by immunofluorescence was with the coupled dyes Alexa-488 and Alexa-546 (Molecular Probes, USA). Double detections of RNA and protein were performed sequentially, first RNA, then the antigen. The stained specimens were embedded in Durcupan (Fluka, Switzerland) or Aquapolymount (Polysciences, USA) and documented with a Zeiss Axioplan microcope with Nomarski or bright-field optics on a EPY64T film (Kodak, USA). For overlays the first picture was taken with Nomarski optics, the second one at a different focal plane with bright-field optics. After digitalization the pictures were merged with the computer program Photoshop (Adobe, USA). Histological sections were 5 to 10 μm thick. The sections (approximately 35% to 65% egg length) for fluorescence microscopy were cut manually in aquapolymount with a 26G3/8 cannula. Photographs of them were taken with a Zeiss LSM 510 confocal microscope.

Molecular Genetics

The EcoRI/XhoI fragment of EST GH18646 (BDGP, obtained from Research Genetics, Huntsville, USA) containing the *trbl* cDNA was cloned into pUAST and pCS2 (R. Rupp, Tübingen, FRG) to result in pUAS-trbl and pCS-trbl, respectively. The *trbl*K266R allele was obtained by a two-step PCR protocol (Higuchi et al., 1988), ligated first into pKSBluescript and then into pCS2. w^+ flies were transformed with pUAS-trbl. mRNA was synthesized in vitro with SP6 polymerase (Boehringer Mannheim, FRG) and linearized plasmids pCS-trbl, pCS-trblK266R, and pNB-pelle as templates (Großhans et al., 1999).

Microinjection of Embryos

Microinjection was performed as described (Großhans et al., 1999). RNA was injected into the posterior half of the embryos at a concentration of 1 mg/ml. Cholera toxin (Sigma, USA) was injected as described by Morize et al. (1998). The fly strain carrying a histon2A-GFP fusion protein is described in Clarkson and Saint (1999).

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