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Regulation of Invasive Cell Behavior by Taiman, a *Drosophila* Protein Related to AIB1, a Steroid Receptor Coactivator Amplified in Breast Cancer

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Summary

Steroid hormones are key regulators of numerous physiological and developmental processes, including metastasis of breast and ovarian cancer. Here we report the identification of a *Drosophila* gene, named *taiman*, which encodes a steroid hormone receptor coactivator related to AIB1. Mutations in *tai* caused defects in the migration of specific follicle cells, the border cells, in the *Drosophila* ovary. Mutant cells exhibited abnormal accumulation of E-cadherin, β-catenin, and focal adhesion kinase. TAI protein colocalized with the ecdysone receptor in vivo and augmented transcriptional activation by the ecdysone receptor in cultured cells. The finding of this type of coactivator required for cell motility suggests a novel role for steroid hormones, in stimulating invasive cell behavior, independent of effects on proliferation.

Introduction

Steroid hormones are key regulators of reproductive biology and have potent effects on both the development and physiology of cells of the reproductive organs. In addition, steroid hormones play a key role in the development and progression of breast, ovarian, and prostate cancers (Schneider and Birkhauser, 1995). In fact, antiestrogens, such as tamoxifen, are effective therapeutic agents for these types of cancers (Cuzick, 1996; Green and Furr, 1999; Jordan, 1999).

Steroid hormones exert their effects by binding to nuclear receptors, which activate transcription in a ligand-dependent manner. Transcriptional activation also requires ligand-dependent association with coactivator proteins such as AIB1, a steroid receptor coactivator of the p160 class (Leo and Chen, 2000) that is amplified in breast and ovarian cancer (Anzick et al., 1997).

A key feature of cancer progression is increased motility and invasive behavior of the tumor cells. In addition to its central role in metastasis, cell motility is a fascinating feature of normal embryonic development. However, the molecular mechanisms that control when and how a stationary epithelial cell acquires invasive properties are not understood in detail. It is clear though that changes in gene expression, cell adhesion, and cytoskeletal organization are likely to be important.

We have been studying a small group of follicle cells in the *Drosophila* ovary, the border cells, as a model system for a forward genetic approach to the study of cell motility (reviewed in Montell, 1999). The border cells originate within an epithelium of approximately 1100 follicle cells, which surround a cluster of 16 germline cells to form an egg chamber (King, 1970). Early in oogenesis, a pair of specialized follicle cells, called polar cells, differentiates at each end of the egg chamber (Ruohola et al., 1991; Margolis and Spradling, 1995; Tworoger et al., 1999). The anterior polar cells recruit an additional four to eight cells, and this cluster then detaches from the follicle cell epithelium and invades the neighboring group of fifteen nurse cells.

A number of genes are known to be required to convert the border cells from stationary, epithelial cells to invasive, migratory cells. The first gene found to be required for border cell migration was *slow border cells* (*slbo*), which encodes a basic region/leucine zipper transcription factor (Montell et al., 1992) related to the mammalian CCAAT enhancer binding protein (C/EBP) family. It has been proposed that expression of C/EBP is one factor controlling the timing of border cell migration during oogenesis.

Several genes have been identified whose expression is regulated, either directly or indirectly, by *slbo* (reviewed in Montell, 1999). One of these is *breathless* (*btl*), which encodes a *Drosophila* FGF receptor homolog that is known to be required for directional guidance of migrating tracheal cells during embryonic development. A second target for *slbo* is the gene *shotgun* (*shg*), which encodes *Drosophila* E-cadherin (Niewiadomska et al., 1999), a homophilic cell–cell adhesion molecule. E-cadherin expression is required both in the nurse cells and in the border cells for normal border cell migration (Oda et al., 1997; Niewiadomska et al., 1999). This finding was somewhat paradoxical since E-cadherin is generally believed to promote the formation of stable adherens junctions and epithelial morphology, rather than the transient cell–cell adhesions required for cells to migrate (Birchmeier et al., 1991; Behrens, 1993; Shiozaki et al., 1996; Guilford, 1999). Nonetheless it is clear that E-cadherin-mediated cell adhesion is essential to border cell migration (Niewiadomska et al., 1999).

In a genetic screen for mutations that cause border cell migration defects in mosaic clones, we identified a number of mutations on chromosome 2R that cause defects in border cell number or position (Liu and Montell, 1999). Here we report identification of a novel locus, *taiman* (*tai*, pronounced ti-maan'), from a screen for mutations on chromosome 2L that caused border cell defects in mosaic clones.

Results

tai, a New Locus Required for Border Cell Migration

We carried out a screen for mutations on the left arm of the second chromosome (2L) that cause border cell

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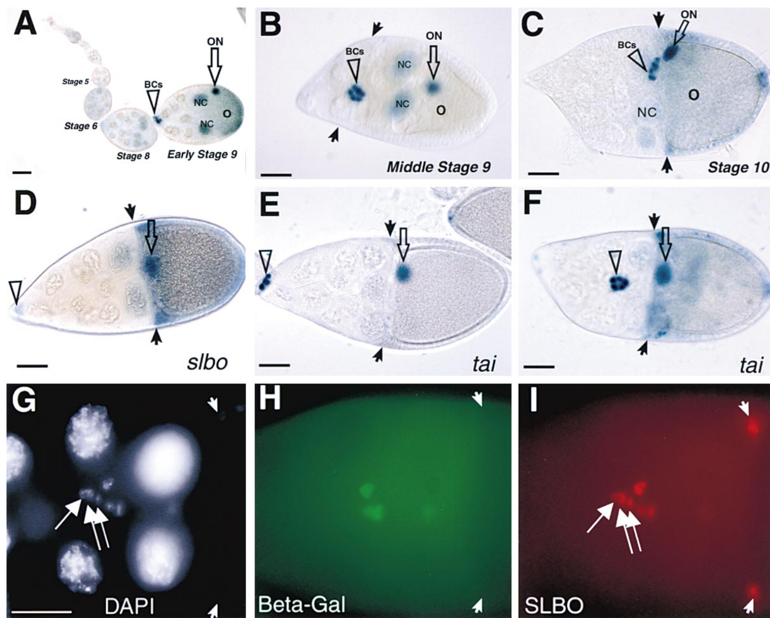


Figure 1. The *tai* Mutant Phenotype

(A–C) Nomarski optics images of wild-type egg chambers stained for β-galactosidase activity to reveal the expression pattern of enhancer trap PZ6356, which is expressed at highest levels in border cells (BCs, open arrowheads) and the oocyte nucleus (ON, open arrows). Lower levels of expression can be seen in other follicle cells and in the nurse cells (NC) closest to the oocyte (O). The small arrows indicate the normal extent of migration of the border cells at the stage shown. (A) Early stages of oogenesis up to stage 9. (B) A mid stage 9 egg chamber. The border cells have migrated partway towards the oocyte. (C) A stage 10 egg chamber. Border cell migration is complete. (D) A stage 10 *slbo* mutant egg chamber. Migration has failed and β-galactosidase expression from the PZ6356 enhancer trap is markedly reduced in the border cells. (E) An egg chamber with a *tai* mutant clone. The border cells have failed to migrate and are located at the anterior tip of the egg chamber. They still express normal levels of β-galactosidase from the PZ6356 enhancer trap insertion, which is on the same chromosome arm as the *tai* mutation, in this experiment. (F) A second egg chamber with a *tai* mosaic clone. In this case, partial migration has occurred. (G–I) Fluorescence micrographs of a border cell cluster. (G) DAPI staining of a border cell cluster undergoing partial migration through the nurse cells. (H) The same egg chamber as in (G), stained with anti-β galactosidase antibody. In this experiment, β-galactosidase expressing cells are wild type for *tai* and β-galactosidase negative cells are mutant for *tai* (see Experimental Procedures for details). (I) The same egg chamber as in (G) and (H), stained with an antibody against the SLBO protein. Note that both wild-type and *tai* mutant border cells (arrows) express SLBO. In addition, the wild-type cells are located at the front of the cluster and the mutant cells are at the back. This is typical of clusters composed of a mixture of wild-type and mutant cells. Scale bars = 50 μm

migration defects in mosaic clones (see Experimental Procedures for details). Border cell position was monitored using β-galactosidase expression from an enhancer trap line, PZ6356 (Tinker et al., 1998) (Figures 1A–1F). In wild-type egg chambers, border cells originate from the anterior follicle cell epithelium (Figure 1A) and migrate during stage 9 in between the nurse cells (Figure 1B). Migration is complete by stage 10, at which point the border cells are invariably found adjacent to the oocyte nucleus (Figure 1C). In *slbo* mutants, migration is prevented and the border cells are usually located at the extreme anterior end of the nurse cell cluster (Montell et al., 1992, Figure 1D), and PZ6356 expression in the border cells is significantly reduced (R. Tinker and D. J. M., unpublished data and Figure 1D).

Of 2885 mutant lines screened for migration defects, one mutant named *taiman*^{61G1} (*tai*^{61G1}, meaning "too slow") was selected for further study. In egg chambers containing *tai*^{61G1} mosaic clones, PZ6356 expression was unaffected (Figures 1E and 1F). In some egg chambers containing *tai*^{61G1} clones, migration was completely inhibited, and border cells remained at the anterior tip of the egg chamber (Figure 1E). In other egg chambers, the border cells migrated partway (Figure 1F). Border cell clusters that underwent partial migration were typically composed of a mixture of heterozygous and homozygous mutant cells.

Border cells mutant for *tai* expressed wild-type levels of the SLBO protein, indicating that the *tai* mutant phenotype was not due to reduced expression of SLBO. For example, in a border cell cluster composed of a mixture of wild-type cells and cells homozygous mutant for *tai*, SLBO protein was expressed similarly in all of the cells (Figures 1G–1I).

Mislocalization of Adhesion Molecules in *tai* Mutant Border Cells

A second protein that is known to be required for border cell migration is *Drosophila* E-cadherin. To determine whether the *tai* migration defect might be due to reduction in *D*E-cadherin expression, egg chambers containing *tai* mutant clones were stained with antibodies against *D*E-cadherin. In all wild-type stages examined, *D*E-cadherin accumulated in the central, nonmigratory polar cells, as well as in the junctions between individual border cells (Niewiadomska et al., 1999, Figures 2A–2C). *D*E-cadherin colocalized with cortical F-actin in those locations (Figures 2A′, 2B′, and 2C′). Prior to migration, when the border cells were still part of the follicular epithelium, *D*E-cadherin also accumulated at the junctions between border cells and nurse cells (Figures 2A and 2A′). However, once the border cells left the follicular epithelium and invaded the neighboring germline cell cluster, much less *D*E-cadherin staining was evident at the junctions between the nurse cells and border cells (Figures 2B and 2B′) relative to the level between border cells or in the polar cells. When migration was complete, *D*E-cadherin accumulated again in the junctions between the border cells and the oocyte (Figures 2C and 2C′).

In *tai* mutant clusters, *D*E-cadherin staining was abnormally elevated at the border cell/nurse cell junctions (Figures 2E and 2E′). In contrast, in *slbo* mutants, *D*E-cadherin expression fails to rise at the time of migration

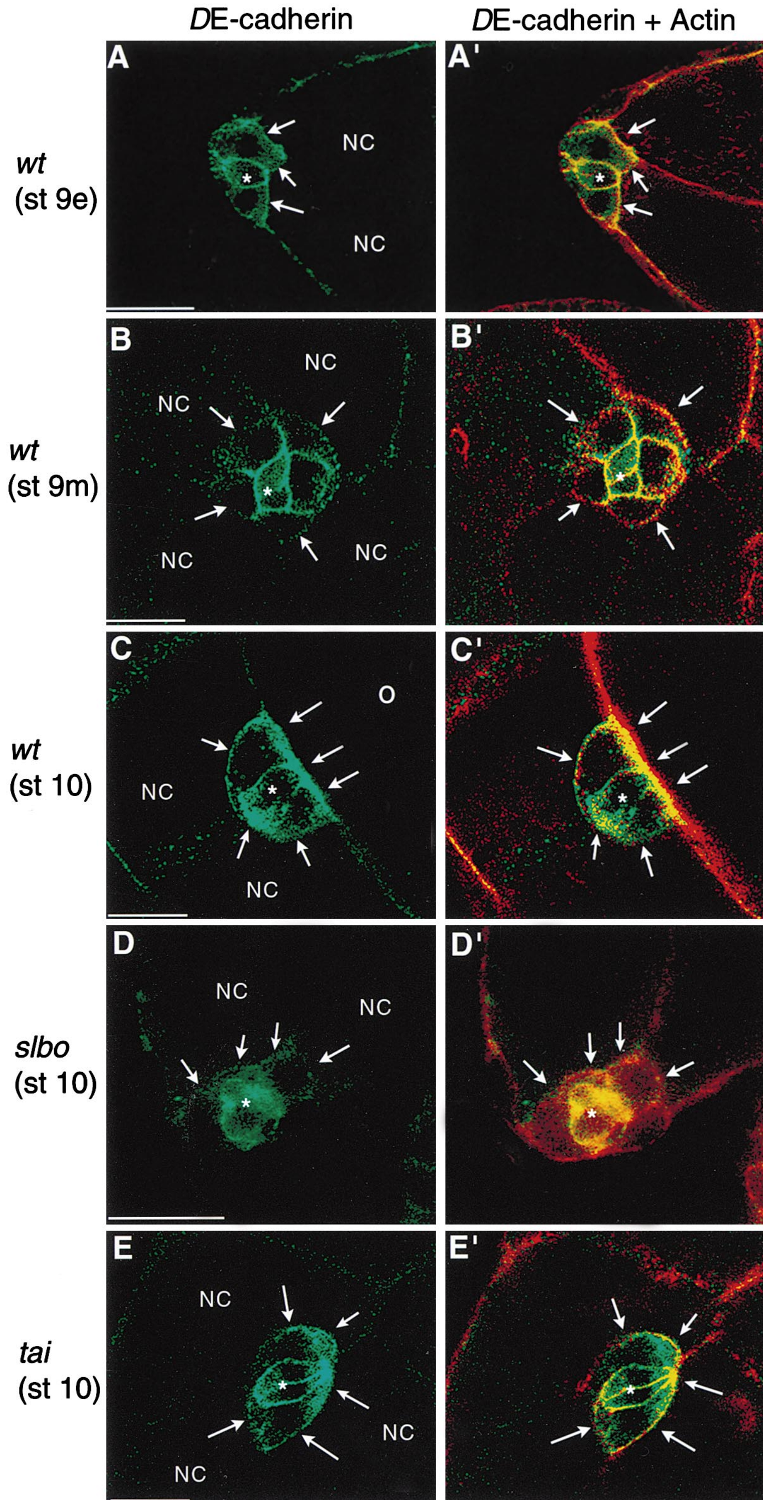


Figure 2. Alterations in *D*E-Cadherin Accumulation during Border Cell Migration

Fluorescence confocal micrographs of border cell clusters stained for *D*E-cadherin (green) and filamentous actin (red). Colocalization appears yellow. A and A′ show a wild-type border cell cluster, with central polar cells (*), in early stage 9, prior to border cell migration. Note the coaccumulation (yellow) of *D*E-cadherin and actin at the junctions between border cells and nurse cells (NC) (arrows), in addition to the coaccumulation at the junctions in between border cells. Actin is also highly enriched at the junctions in between nurse cells (NC). B and B′ show a cluster of wild-type border cells amidst the nurse cells during their migration. Note that there is still coaccumulation of *D*E-cadherin and actin at the junctions between border cells; however, only punctate expression of *D*E-cadherin is apparent at the junctions between border cells and nurse cells (NC) (arrows). C and C′ depict border cells after the completion of migration. *D*E-cadherin again accumulates to high levels between the border cells and the neighboring germ cells (arrows). D and D′ show a cluster of border cells from a *slbo* mutant egg chamber. The central polar cells (*) express normal levels of *D*E-cadherin; however, the outer cells only have a basal level of expression of *D*E-cadherin. E and E′ show a *tai* mutant cluster amidst the nurse cells. Note the accumulation of *D*E-cadherin at the junctions between the border cells and germline cells (arrows), which is as high as the accumulation at junctions between border cells. Scale bars = 15 μm.

(Niewiadomska et al., 1999) and *D*E-cadherin immunoreactivity is only detected at high levels within the polar cells (Figures 2D and 2D′). Armadillo (ARM) (Peifer and Wieschaus, 1990) colocalized with *D*E-cadherin in wild-type and mutant border cells (not shown). The abnormal accumulation of *D*E-cadherin and ARM in *tai* mutants did not appear to result from increased transcription of *D*E-cadherin because overexpression of *D*E-cadherin in border cells did not cause a migration defect nor specific accumulation of cadherin staining at the border cell/nurse cell junctions (not shown). Nor did the abnormal accumulation of *D*E-cadherin and ARM appear to be simply a consequence of the migration failure. In addition to *slbo*, we have examined DE-cadherin and ARM

expression in border cells that fail to migrate due to mutations in the *jing* locus (Liu and Montell, 2001), and no defect in either expression or localization of adhesion complexes was observed. Nor were defects in DE-cadherin or ARM expression or localization found in border cells that fail to migrate due to expression of dominant-negative Rac (Murphy and Montell, 1996; E. Fields and D. J. M., unpublished observations).

The accumulation of DE-cadherin at the border cell/nurse cell boundary suggested that the role of *tai* in border cell migration might be to stimulate turnover of adhesion complexes during migration in order to allow forward movement. One protein believed to play a role in turnover of adhesion complexes is FAK (Ilic et al., 1995). *Drosophila* FAK (DFAK) (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999) is highly enriched in the border cells during their migration (Fox et al., 1999), but not in the polar cells (Figures 3A–3D).

To determine whether DFAK expression or localization was affected by mutations that disrupt border cell migration, we stained wild-type and *slbo* mutant egg chambers and compared the staining to that of egg chambers containing *tai* mosaic clones. DFAK expression was significantly reduced in *slbo* mutant border cells (Figures 3E and 3F). Furthermore, the level of reduction correlated with the degree of inhibition of migration. That is, in some *slbo* egg chambers, border cell migration fails completely and the cells remain at the anterior tip. In such egg chambers, DFAK expression was undetectable (Figure 3E). In a minority of *slbo* mutant chambers, the cells migrate a little. In these egg chambers, DFAK expression was reduced compared to wild type, but was detectable (Figure 3F). In *tai* mutant border cells, DFAK expression was present; however, its distribution was altered relative to wild type (Figure 3G). Rather than being evenly distributed throughout the cytoplasm, DFAK appeared to accumulate at the would-be leading edge of the cluster. Some border cell clusters that were mutant for *tai* exhibited partial migration and in these clusters, the abnormal distribution of DFAK was only slightly affected such that little DFAK accumulation could be detected at the most posterior position within the cluster (Figure 3H). Thus, the severity of the migration defect in *tai* mutants correlated with the severity of the defect in DFAK localization.

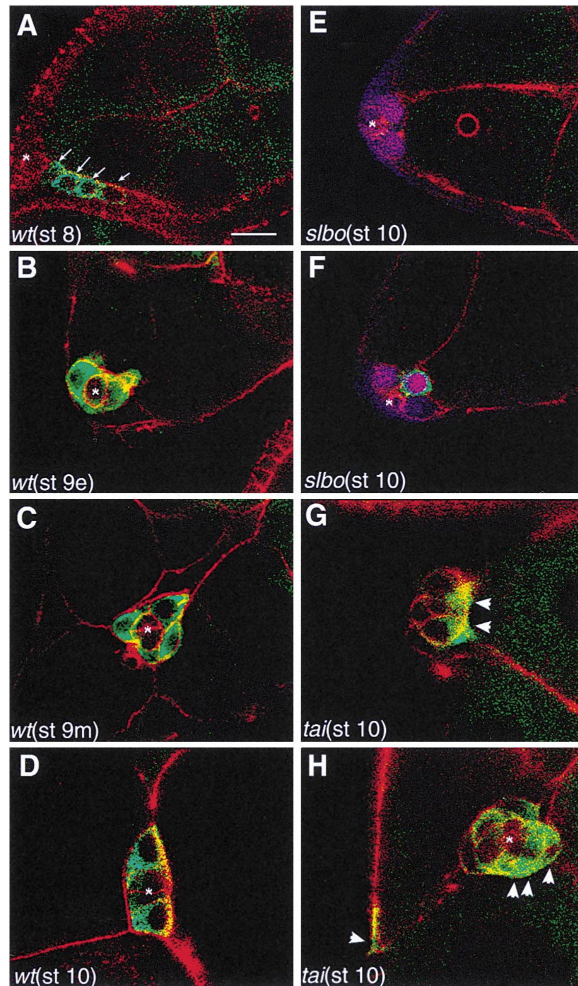


Figure 3. Expression of DFAK in Wild-Type and Mutant Egg Chambers

Confocal micrographs of border cell clusters stained for DFAK (green) and filamentous actin (red). (A–D) Wild-type. (A) Stage 8 prior to border cell migration. DFAK accumulates in a few anterior follicle cells (arrows). (B) Early stage 9, as the border cells initiate migration. (C) Mid stage 9 during migration. (D) Stage 10 when migration is complete. Note the specific accumulation of DFAK in the border cells, but not in the central polar cells (*), and the distribution of DFAK in the border cells is throughout the entire cytoplasm. (E and F) Stage 10 egg chambers of $slbo^{1310}/slbo^{1310}$. Border cells, identified by anti-β-galactosidase staining (purple), remain at the tip of the egg chambers and expressed no detectable DFAK (E), or little DFAK (F). (G and H) Stage 10 egg chambers in which all border cells were homozygous mutant for *tai*. Border cells accumulated normal levels but an altered distribution of DFAK. DFAK appeared to accumulate abnormally at the leading edge of the border cell cluster (arrow heads). Scale bar: 10 μm.

Molecular Characterization of the *tai* Locus

Eight P element alleles of *tai* were identified (see Experimental Procedures), and their insertion points, which spanned more than 60 kb, are shown in Figure 4A. The protein sequence predicted from cDNA analysis and RT-PCR is shown in Figure 4B.

The *tai* locus encoded a protein with amino acid sequence similarity to steroid hormone receptor coactivator proteins of the p160 family (SRCs). SRCs bind to steroid hormone receptor (SHR) complexes in a ligand-dependent manner and potentiate hormone-induced transcriptional activation (Leo and Chen, 2000). The most related protein was AIB1, a steroid hormone receptor coactivator that is amplified in breast and ovarian cancer (Anzick et al., 1997). SRCs typically contain a basic helix-loop-helix (bHLH) domain near the N terminus, a PAS domain, LXXLL motifs, which are responsible for binding to the hormone bound receptor (McInerney et al., 1998), and one or more polyglutamine stretches that mediate transcriptional activation. The predicted TAI protein contained all of the domains featured in the p160 class of SRCs (Figures 4B and 4C), and the top 19 BLAST scores were from members of this family. The highest level of amino acid sequence identity was found in the bHLH domain, which was 48% identical and 71% similar between AIB1 and TAI.

The entire coding sequence was cloned into a P element transformation vector and expressed in transgenic

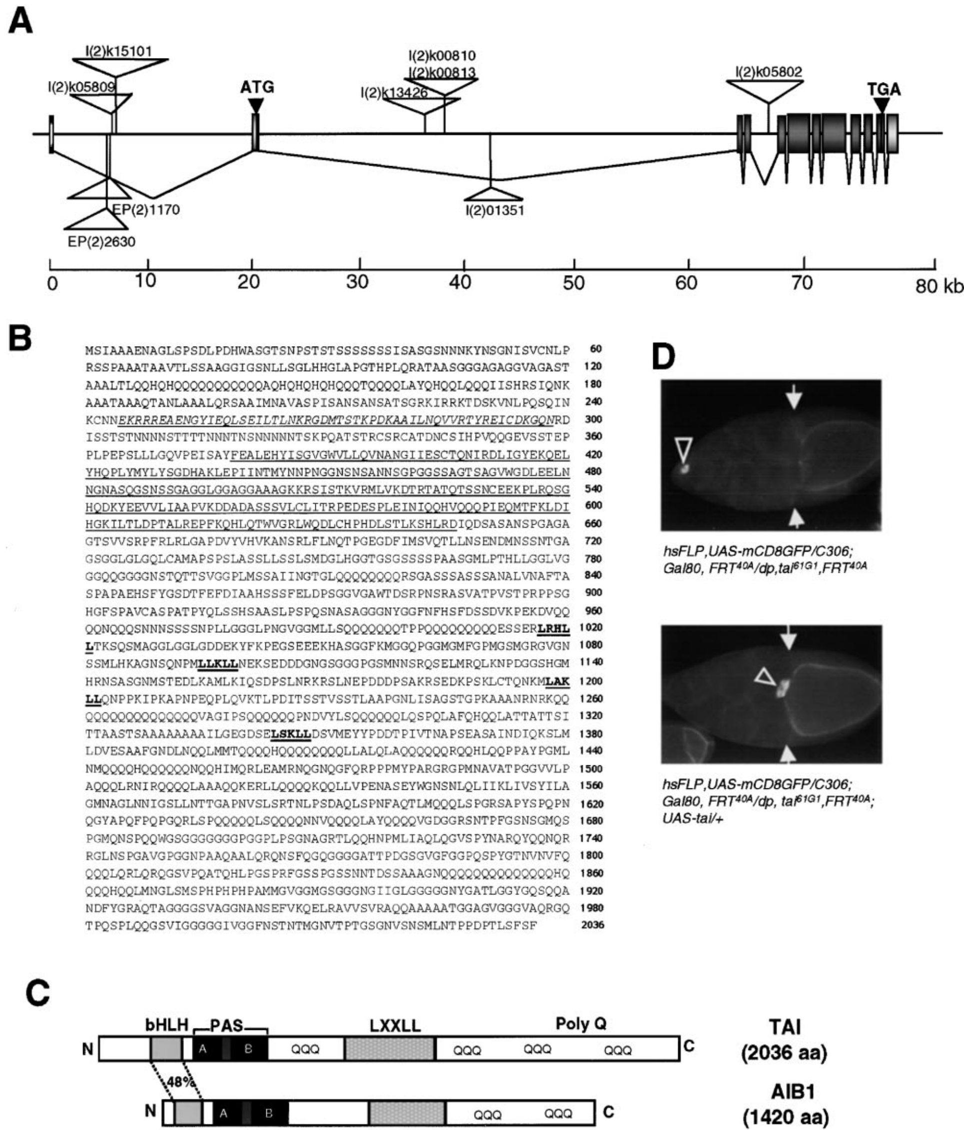


Figure 4. Genomic Organization, Sequence, Homology, and Domain Organization of TAI

(A) The splicing pattern of the major TAI RT-PCR product is indicated, as are the sites of insertion of the eight *tai* P element alleles. The predicted coding sequence is shaded. (B) Deduced amino acid sequence of the major TAI RT-PCR product. The bHLH domain is shown in italics and underlined. The PAS domain is underlined. The LXXLL motifs are shown in bold and underlined. The GenBank accession number for this sequence is AY008258. (C) Schematic diagram of the TAI protein domains compared to AIB1. The percent amino acid identity within the bHLH domain is shown for the human coactivator AIB1, which is also known as SRC3, TRAM-1, RAC3, and ACTR (see for example Suen et al., 1998). (D) Rescue of border cell migration defects in egg chambers expressing the TAI transgene under the control of a border cell enhancer (see Experimental Procedures for details). In this experiment, GFP is expressed only in homozygous mutant cells. In the absence of the transgene, migration was delayed or prevented. In the presence of the transgene, migration was normal.

flies (see Experimental Procedures for details). We employed a system for generating mosaic clones, known as MARCM, in which only homozygous mutant cells express GFP and are therefore easy to identify unambiguously (Lee and Luo, 1999). In the presence of the transgene, but not in its absence, migration was rescued in all *tai* mutant clusters examined (n $>$ 15) (Figure 4D).

We raised antibodies against two different regions of the predicted protein, the PASB domain and the LXXLL domain (see Experimental Procedures). Both antibodies stained follicle cells in the *Drosophila* ovary (Figure 5). Nuclear staining was observed in all of the follicle cells, including the border cells; however, much less protein was detected in the nurse cells. Widespread expression was also detected in embryos (not shown). The nuclei of *tai* homozygous mutant cells did not react with the antibody against TAI (Figures 5D″ and 5E″), though the nuclei were present, as detected by DAPI staining (Figures 5D and 5E). TAI expression was unchanged in *slbo* mutant egg chambers (Figures 5C and 5C′), indicating that *tai* is not a downstream target of *slbo*.

Coexpression and Function of TAI and the Ecdysone Receptor

The similarity of TAI to steroid hormone receptor coactivators suggested that TAI might interact with one or

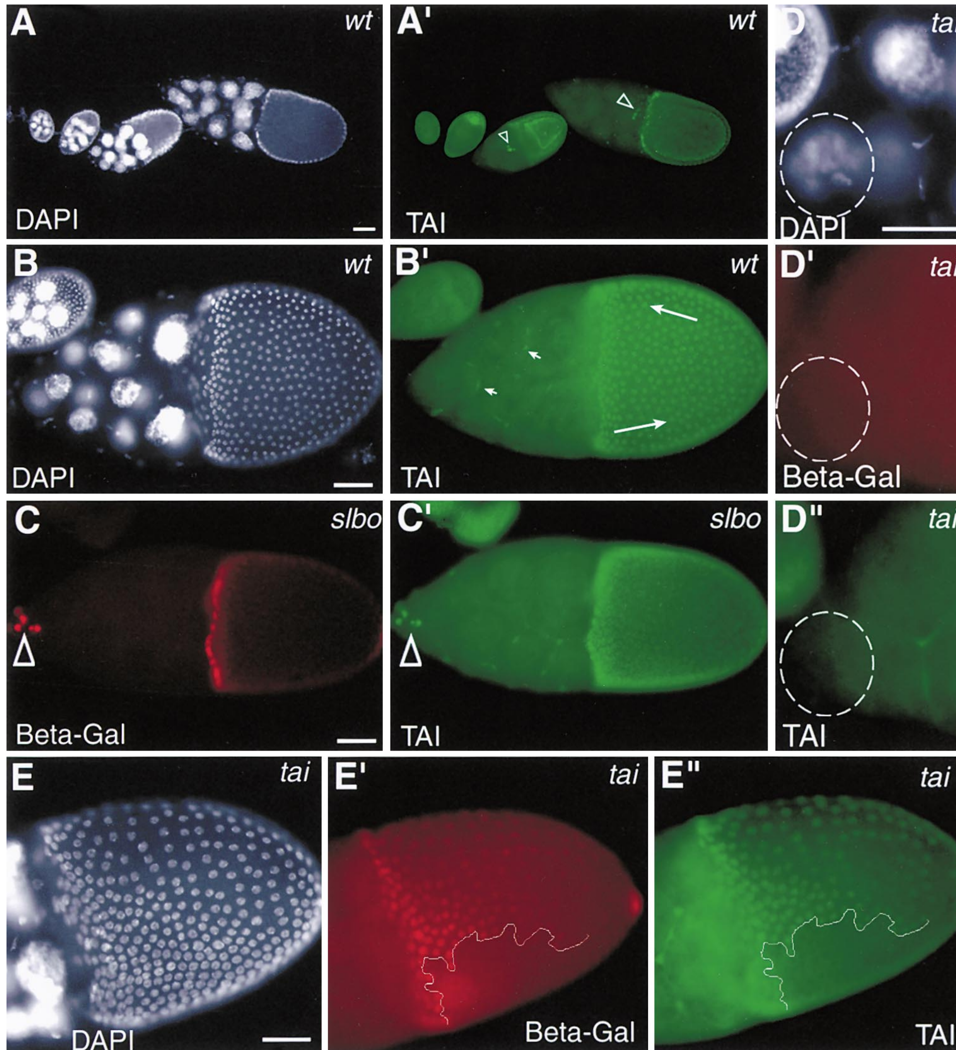


Figure 5. Expression of TAI Protein in *Drosophila* Egg Chambers

(A, B, D, and E) DAPI staining reveals the large nurse cell nuclei as well as the smaller follicle cell nuclei. (A′, B′, C′, D″, and E″) Anti-TAI antibody staining. Follicle cells are brightly stained whereas nurse cells are less brightly stained. (A and A′) Wild-type egg chambers stained for DAPI and TAI showing nuclear localization of TAI protein in all follicle cells including border cells (open arrowheads in A′). (B and B′) Higher magnification of a stage 10 egg chamber focused on the surface to show that nurse cell associated follicle cells (short arrows) and columnar follicle cells (long arrows) are also stained. The border cells are out of the plane of focus. (C) A stage 10 *slbo* mutant egg chamber stained with anti-β-galactosidase antibody to reveal border cell (open arrowheads) and centripetal follicle cell nuclei. (C′) The same egg chamber as in (C) stained with anti-TAI antibody to show that *slbo* mutant border cells express normal levels of TAI (open arrowheads). (D) DAPI staining of a cluster of *tai* mutant border cells that has failed to migrate (circle). (D′) Absence of β-galactosidase staining of the same egg chamber indicates that the border cell cluster is homozygous mutant for *tai*. (D″) Absence of staining of the same cluster with anti-TAI antibodies demonstrates autonomy of the migration defect. (E) DAPI staining of an egg chamber containing *tai* mosaic clones to visualize all nuclei. (E′) Anti-β-galactosidase staining of the same egg chamber. Absence of staining indicates cells that are homozygous mutant for *tai*. (E″) Absence of TAI staining colocalizes with absence of β-gal. Scale bars = 50 μm.

more steroid hormone receptors. The only known steroid hormone in *Drosophila* is ecdysone, and the ovary is a major site of ecdysone synthesis, which peaks at stage 9 (Riddiford, 1993). The functional ecdysone receptor is a heterodimer composed of Ultraspiracle (USP), which is the fly retinoid X receptor (RXR) homolog, and EcR (Yao et al., 1993). To determine whether the ecdysone receptor complex would be a good candidate for interaction with TAI, we examined expression of ecdysone receptor subunits in egg chambers using antibodies against USP, EcR-A, and EcR-B. EcR-A and EcR-B are distinct isoforms of the EcR subunit, which are generated by alternative splicing (Talbot et al., 1993). USP, EcR-A, and EcR-B colocalized with TAI protein in migrating border cells (Figure 6). While USP and EcR-A were expressed generally, in both follicle cells and nurse cells (Figures 6A′ and 6C′), EcR-B (Figure 6B′) and TAI (Figures 6A″, 6B″, and 6C″) were specifically enriched in border cells during the period of their migration.

These observations raised the possibility that the timing of border cell migration might be controlled by ecdysone. To test whether border cell migration was respon-

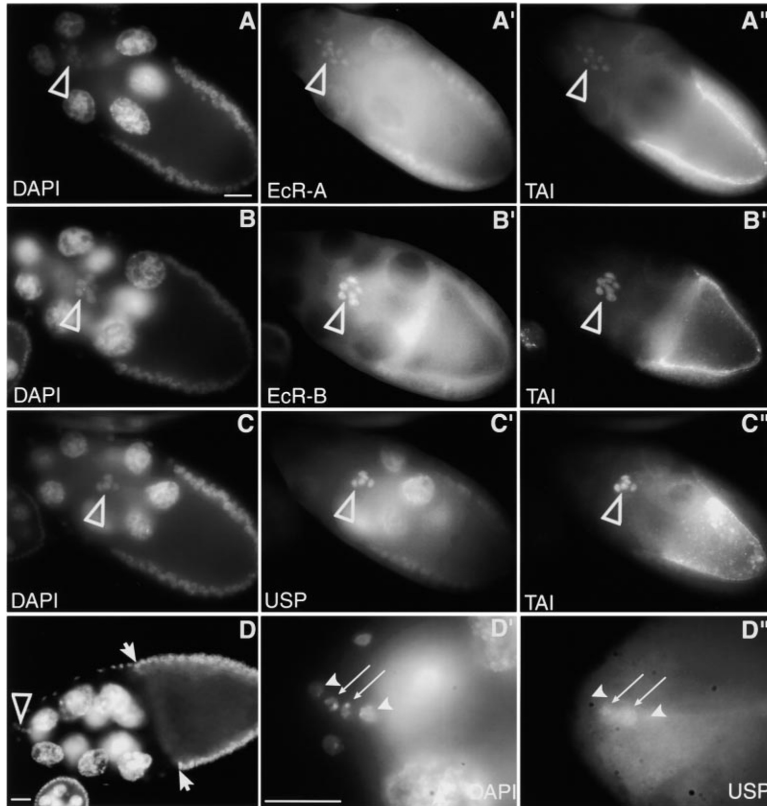


Figure 6. Colocalization of TAI, USP, and EcR in Border Cells and Inhibition of Border Cell Migration by *usp* Mutation

Immunofluorescence micrographs of egg chambers stained with DAPI (A–D and D′), with antibodies against ecdysone receptor subunits (A′–C′, and D″), and with anti-TAI antibodies (A″–C″). (A)–(C″) show wild-type egg chambers while (D), (D′), and (D″) show an egg chamber containing *usp* mosaic clones. EcR-A is expressed in all cells of the egg chamber including border cells (A′, open arrowheads). Anti-EcR-B antibody staining is specifically enriched in border cells (B′), as is TAI (A″, B″, and C″). The USP protein (C′) is expressed in all of the cells but appears to be expressed at highest levels in border cells and the nurse cells that are closest to the oocyte. A stage 10 egg chamber with homozygous *usp* mutant border cells is shown stained with DAPI in (D) and (D′). Mutant border cells are at the anterior tip (open arrowheads), whereas normally they should have arrived at the border between the nurse cells and oocyte (arrows in [D]). (D′) A high magnification view of the DAPI stained border cell cluster shown in (D). (D″) The same egg chamber shown in (D) and (D′) stained with antibodies against the USP protein. Two cells in the cluster that lack USP protein (arrowheads). Two cells in the middle of the cluster, most likely the nonmigratory polar cells, still express USP (long arrows). Scale bars = 25 μm.

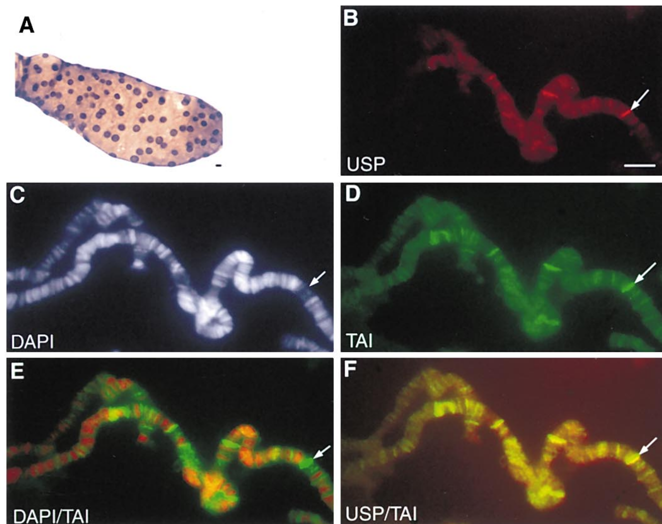
sive to hormone, we analyzed the effects of injecting hormone into female flies. We did not expect that increasing the hormone concentration alone would be sufficient to cause precocious border cell migration because expression of the *slbo* gene and its targets are independently required for migration. Therefore, we first expressed *slbo* precociously using transgenic flies carrying a heat-inducible *slbo* transgene, followed by injection of hormone. Border cell migration was assayed in stage 8 egg chambers dissected from flies treated with heat shock and hormone, and compared to control flies treated with heat shock and ethanol, or with hormone in the absence of heat shock (see Experimental Procedures for details). Precocious border cell migration was observed in 20% of egg chambers that were treated with both heat shock and hormone but not in controls (data not shown). The observed effects were consistent with a role for ecdysone in regulating the timing of border cell migration.

If the rising ecdysone level at stage 9 were required to stimulate border cell migration, then reducing the ecdysone level should cause a delay in border cell migration. The *ecdysoneless* mutant *ecd[1]* is temperature sensitive for production of ecdysone. Females homozygous for *ecd[1]* are sterile when held at the nonpermissive temperature for 5 days, and egg chambers in these flies arrest development at stage 8 and subsequently degenerate. We found that border cells failed to develop in these arrested egg chambers (not shown). However, when *ecd[1]* mutants were held at the nonpermissive temperature for 2 days, some stage 10 egg chambers developed, in which border cells differentiated and expressed SLBO protein. Greater than 50% of these egg chambers exhibited delayed border cell migration (not shown).

Since the effects on border cell migration of increasing or decreasing ecdysone levels could have been indirect, we tested whether there was a cell autonomous requirement for the ecdysone receptor in border cells. The EcR locus is proximal to available FRT insertion sites, preventing mosaic analysis. Therefore, we carried out the analysis using mutations in *usp*. Border cells that were homozygous mutant for a null allele of *usp* exhibited inhibition of border cell migration, but no obvious defects in other follicle cells (Figures 6D, 6D′, and 6D″).

To assess whether TAI and the ecdysone receptor were likely to associate in a complex in vivo, TAI expression was examined in third instar larvae. Both antibodies against TAI reacted specifically with the salivary gland nuclei (Figure 7A), as well as other larval tissues (not shown). Polytene chromosome spreads were stained with antibodies against TAI and USP proteins in a double labeling experiment. As shown in Figure 7D, anti-TAI antibody labeled specific loci on the polytene chromosomes. Moreover, USP and TAI proteins colocalized precisely (Figure 7). Since previous experiments have shown that USP and EcR colocalize as a complex on polytene chromosomes (Yao et al., 1993), these results indicated that TAI colocalized with the functional ecdysone receptor complex at specific target sites.

We also determined whether expression of TAI could enhance ecdysone receptor dependent transcriptional activation in EcR-293 mammalian cells (see Experimental Procedures for details). These cells respond to hormone, either ecdysone or an analog known as ponaster-



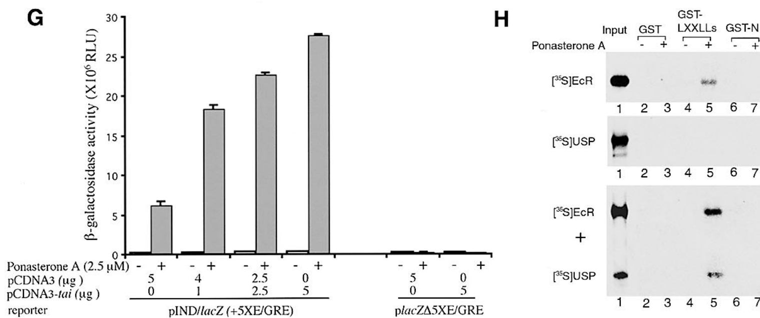


Figure 7. In Vivo and In Vitro Interactions between TAI, EcR, and USP

(A) Staining of a salivary gland with anti-TAI antibodies. TAI is expressed in the polyploid nuclei. (B) Staining of a segment of a polytene chromosome from a late third instar larval salivary gland for USP. (C) DAPI staining of the same chromosome segment. (D) Staining of the same segment with anti-TAI antibodies and a fluorescein conjugated secondary antibody. (E) Overlay image of DAPI and anti-TAI staining. No colocalization is apparent. (F) Overlay of TAI and USP. Overlap appears as yellow. Scale bars = 10 μm. (G) TAI-dependent increases in hormone-regulated transcription by the ecdysone receptor in culture cells. EcR-293 cells were transiently transfected with increasing amounts of pCDNA3-*tai*, decreasing amounts of pCDNA (empty vector), and reporter pIND/*lacZ* or p*lacZ*-Δ5XE/GRE in the absence or presence of 2.5 μM of ponasterone A. β-galactosidase activity is expressed in relative light units (RLU). Data shown represent the mean of three determinations from one of four independent experiments. Error bars indicate the standard errors of the means. (H) GST pulldown assays for hormone-dependent interactions between TAI, EcR, and USP. In vitro translated, [^{35}S]methionine-labled EcRB1 alone (top panel), [^{35}S]methionine-labled USP alone (middle panel), or a mixture of [^{35}S]methionine-labled EcRB1 and [^{35}S]methionine-labled USP (bottom panel) were incubated with GST (lanes 2 and 3), GST fused to the LXXLL domain of TAI (amino acids 1028–1035) (lanes 4 and 5), and GST fused to the N terminus of TAI (amino acids 259–382) (lanes 6 and 7) in the absence (minus sign: lanes 2, 4, and 6) or presence (plus sign: lanes 3, 5, and 7) of 5 μM of ponasterone A. In lane 1, 50% of input protein is shown.

one, with a substantial increase in transcriptional activation of genes placed under the control of a *cis*-acting sequence known as an E/GRE (see Experimental Procedures for details). We compared transcriptional activation in cells expressing varying amounts of TAI in transient transfection assays. As shown in Figure 7G, TAI expression increased transcriptional activation up to 5-fold, in a dose-dependent manner, specifically in the presence of hormone.

Furthermore, a GST-fusion protein containing the region of TAI protein containing the LXXLL motifs predicted to interact with EcR (residues 1028 to 1235 of TAI) associated with in vitro translated EcR in a ligand-dependent manner. The same fusion protein did not associate detectably with USP alone (Figure 7). However, in the presence of EcR and ligand, the TAI-GST fusion protein was able to coprecipitate USP (Figure 7). Taken together, these results suggest that TAI is a bona fide ecdysone receptor coactivator.

Discussion

The *tai* Gene Encodes a *Drosophila* Steroid Hormone Receptor Coactivator

The p160 class of steroid hormone receptor coactivators has been thought to be missing from invertebrates (Freedman, 1999). TAI appears to be a coactivator of this class based not only on amino acid sequence similarity and overall domain structure, but based also on its in vivo colocalization with EcR, its direct, ligand-dependent binding to EcR, and its ability to potentiate hormone-dependent transcription in cultured cells.

The homology of TAI to SRC proteins suggested that TAI might interact with a steroid hormone receptor. Although there are more than 20 genes in *Drosophila* that code for proteins related to nuclear hormone receptors, ecdysone is the only known steroid hormone. Since SRC proteins require the presence of a ligand in order to interact with receptors, the ecdysone receptor seemed like the best candidate partner for TAI. The colocalization of TAI protein with the ecdysone receptor complex at specific chromosomal loci in third instar larva, the direct and ligand-dependent binding of TAI to EcR in vitro, and the ability of TAI to potentiate the ecdysone response in cell culture lend substantial support to this proposal.

The ligand-dependent interaction of TAI with the ecdysone receptor suggests that ecdysone regulates border cell migration. The strongest evidence in support of this is that border cells lacking USP were unable to migrate. Consistent with this observation, Oro et al. (1992) reported that numerous unfertilized eggs were

produced from females lacking *usp* function. Moreover *usp* was required specifically in somatic cells for production of a fertilizable egg (Oro et al., 1992). Although they did not specifically examine border cell migration, defects in border cell migration are known to lead to the production of unfertilized eggs (Montell et al., 1992). We have been unable to test whether EcR loss of function mutations affect border cell migration. This is because the EcR locus, at 42A, is proximal to available FRT insertions, making it impossible to make FLP-mediated mosaic clones. The frequency of X-ray induced mitotic clones is too low to be useful, and marking such clones is problematic. A temperature-sensitive allele of EcR exists and flies at the nonpermissive temperature exhibit a variety of defects in oogenesis, including arrest prior to border cell migration. Even though it was not possible to assess the effect of EcR mutations specifically in the border cells, the observations that hormone injections can lead to precocious border cell migration and that reduced ecdysone levels can lead to delayed migration provide additional support for the hormonal control of migration.

The rise in ecdysone after eclosion, specifically in females, occurs in response to adequate nutrition (Schwartz et al., 1985). In the absence of a rich diet, yolk protein synthesis is inhibited and oogenesis does not progress. Yolk protein synthesis can be restored in the absence of a rich diet by applying ecdysone or juvenile hormone (JH) to cultured ovaries. Recent studies indicate that functional ecdysone receptors are required in the germline for progression of oogenesis through vitellogenesis, the stages during which yolk is taken up by the oocyte (Buszczak et al., 1999; Carney and Bender, 2000). In summary, then, adequate nutrition appears to lead to elevated hormone levels, which in turn stimulate yolk protein synthesis and uptake, and progression of oogenesis beyond stage 8. Together with the results reported here, these findings suggest that a rising ecdysone titer coordinates a variety of events that occur in early vitellogenic egg chambers, including border cell migration.

Steroid Hormones in Cell Motility and Cancer

Our studies suggest a role for steroid hormones in cell motility that is independent of any role in cell proliferation or cell fate determination. If *tai* function were required for follicle cell proliferation, it would not be possible to generate homozygous mutant clones since the homozygous mutant cells would fail to proliferate. We found many homozygous mutant *tai* clones in the follicular epithelium, some of which were quite large; therefore, there did not appear to be a requirement for *tai* function in follicle cell proliferation. In addition, *tai* mutant border cells clearly differentiated from the neighboring follicle cells, based on their morphology, and they continued to express all of the border cell markers we tested. Therefore, there did not appear to be any detectable change of cell fate or differentiation state in these cells. Rather, they appear to have a relatively specific defect in their ability to migrate through the neighboring nurse cell cluster.

These findings may have significance for steroid hormone-dependent human cancers since hormones are known to promote progression of breast, ovarian, and prostate cancers, which includes acquisition of highly invasive characteristics (Cuzick, 1996; Green and Furr, 1999). The prevailing view is that the hormones act to stimulate proliferation of the cancer cells, leading to an increased likelihood of mutation and appearance of an invasive phenotype. However, the data presented in this paper suggest that steroid hormones can also stimulate invasive behavior independently of any discernible effect on proliferation. Thus, steroid hormones, like many peptide growth factors, may possess both mitogenic and motogenic properties. This notion is supported by studies that show effects of an anti-estrogen on metastasis of prostate cancer cells in the rat. Raloxifene, an anti-estrogen, was found to inhibit metastasis of PAIII adenocarcinoma cells to the lymph nodes and lungs, in vivo, without effects on growth of the primary tumor, or proliferation of the PAIII cells in vitro (Neubauer et al., 1995). The treatment also extended the survival of the animals.

The *tai* Gene and Its Interactions Define a *slbo*-Independent Pathway Required for Border Cell Migration

A number of genes that have been described, *slbo*, *jing*, *breathless*, cadherin, and PZ6356 define a *slbo*-dependent pathway required for border cell migration (reviewed in Montell, 1999). Experiments reported here indicate that expression of DFAK also depends upon the *slbo* pathway. In contrast, *tai* function appears to be independent of *slbo,* based on the lack of effect of *slbo* mutations on *tai* expression and the lack of effect of *tai* mutations on *slbo* expression. In addition, overexpression of *tai* failed to rescue even mild *slbo* migration defects and overexpression of *slbo* failed to rescue *tai* migration defects (Bai and Montell, unpublished). Mutations in either *slbo* or *tai* affect cadherin and DFAK. Whereas *slbo* function is required for expression of these two proteins, *tai* function is required for proper subcellular localization of both proteins.

Turnover of Adhesion Complexes in Cell Migration

The finding that *D*E-cadherin is required both in the border cells and in the nurse cells for normal border cell migration was surprising since the prevailing view has been that E-cadherin promotes formation of stable cell–cell adhesion belts and inhibits motility (see for example, Takeichi, 1993). However, there are numerous exceptions to the general correlation of decreased E-cadherin expression with increasing motility. One particularly interesting exception is the human ovarian epithelium (Ong et al., 2000). Normal cells within the human ovarian surface epithelium express relatively low levels of E-cadherin. However, carcinomas derived from this epithelium express high levels of E-cadherin, and overexpression of E-cadherin in T antigen transformed ovarian surface epithelium cells causes them to become invasive and to form distant metastases in nude mice. Thus, in both human and *Drosophila* ovaries, E-cadherin seems to promote rather than inhibit motility.

Why then do some cells respond to increased cadherin expression by increasing invasiveness whereas other cells respond by decreasing invasiveness? We

propose that the difference is that some cell types are capable of rapidly turning over E-cadherin-containing adhesion complexes whereas other cells are not. If the complexes can be turned over efficiently, the cells behave like wild-type border cells and become invasive. If the complexes cannot be turned over efficiently, the cells behave as *tai* mutant border cells, accumulate stable cell–cell adhesion complexes and do not migrate. It will be important to identify the critical downstream targets of TAI because one or more of these may be a protein important for turnover of adhesion complexes.

Steroid hormones may stimulate formation and turnover of cadherin-containing cell adhesion complexes in human breast cancer as well. In support of this, DePasquale has observed that MCF7 human breast cancer cells respond to β-estradiol treatment by extending motile lamellipodia, which make small, transient, E-cadherin-containing contacts with underlying cells (DePasquale, 1999). This behavior is prevented by treatment of the cells with anti-estrogens. Taken together with the proven effectiveness of anti-estrogens in preventing and reversing metastasis of hormone-dependent cancers, these findings suggest that steroid hormones may stimulate invasive cell behavior by facilitating rapid turnover of E-cadherin containing cell adhesion complexes. This could be one mechanism by which amplification of AIB1 contributes to the progression of breast and ovarian cancer.

Experimental Procedures

Drosophila Genetics

The mosaic screen for mutations that caused border cell migration defects was performed much as described for 2R (Liu and Montell, 1999) with the exception of the chromosomal location of FRT. One allele of *tai*, tai^{61G1}, was recovered from a screen of 2885 mutant lines. To map the *tai* locus, the original tai^{61G1} mutant was crossed to the deficiency kit for 2L (Bloomington Stock Center) and scored for lethality. Meiotic recombination mapping was performed by crossing the original tai^{61G1} line to FRT^{40A}. About 150 recombinants were scored for *dp*, *PZ6356*, and border cell migration defects in mosaic clones. All recombinants with border cell migration defects failed to complement *l(2)01351*, a P element within *Df(2L)30A-C*. Precise excision of this P element can reverse the lethal phenotype of the original *l(2)0135*1 line. Other P element alleles of the *tai* locus *l(2)k15101*, *l(2)k00810*, *l(2)k00813*, *l(2)k13426*, *l(2)k08502* were obtained from Szeged while *l(2)k05809*, *EP(2)2603*, *EP(2)1170* were obtained from BDGP. *l(2)01351*, FRT^{40A}/*CyO* was obtained from Norbert Perrimon (Chou and Perrimon, 1996). Border cell migration defects were observed in l(2)01351 mosaic clones, and tai^{61G1}/l(2)k05809 escapers exhibited border cell migration defects and reduced fertility.

To negatively mark mosaic clones in the border cells, *dp,* tai^{61G1}*,* FRT^{40A}/*CyO* flies were crossed to *dp, PZ6356,* FRT^{40A}/*dp, PZ6356,* FRT^{40A}. To positively mark clones in the border cells, the technique known as MARCM was employed as described (Lee and Luo, 1999). The alleles of *slbo* used were $slbo^{e7b}$, $slbo^{1310}$, $slbo^{ry8}$ (Montell et al., 1992).

The ecd^1 allele of *ecdysoneless* was obtained from the Bloomington Stock Center. After 2 days or 5 days at 29°C, ovaries were dissected and stained with anti-SLBO antibodies (Montell et al., 1992). No staining was observed in 5-day ovaries, which combined egg chambers were arrested at stage 8, and degenerating. 2-day ovaries contained stage 10 egg chambers, 50% of which (30/59) exhibited delayed border cell migration. No egg chamber from wild-type females kept at 29°C or from ecd^1 females kept at 20°C exhibited delayed border cell migration.

Hormone Treatment of Ovaries

Flies containing a *hs-slbo* transgene were heat-shocked at 37°C for 1 hr. After 5 hr at 25°C, either 50 nl of 20-hydroxyecdysone (Sigma, 2 ng/nl in Ringer's solution) or 50 nl of ponasterone A (10 ng/nl in ethanol) was injected as described. Ringer's alone or ethanol alone served as controls. *Hs-slbo* flies that had not been heat treated were also injected. Migration was assayed 15 hr later in stage 8 egg chambers, when border cell migration is never observed in wild type. Premature border cell migration was observed in 5 out of 22 egg chambers treated with heat shock and hormone, but not in controls. As expected, most egg chambers treated with hormone were also undergoing degeneration at stage 8.

Molecular Identification of *tai* and Germline Transformation

The sequence flanking l(2)01351 was used in a BLAST search of the entire *Drosophila* genome sequence and matched P1 clone DS06958, a 108,924 base pair genomic sequence, which had been completed by BDGP. Two ESTs, GH06208 and LP10313, were found to correspond to portions of a coding sequence predicted by Genescan. Complete sequencing of these ESTs indicated that GH06208 and LP10313 spanned the translation start site and the predicted 3′ end of a transcript, respectively. To obtain a full-length cDNA of *taiman*, RT-PCR was carried out using an RNA PCR Core kit (PERKIN ELMER) and total RNA.

To construct a *tai* minigene, a BglII to EcoRI fragment of EST clone GH6208 was combined with an EcoRI fragment from P1 clone DS06958 and EST clone LP0313 in pUAST (Brand and Perrimon, 1993). For germline transformation, the pUAST-*tai* DNA was coinjected into w^{1118} embryos with pπ25.7WC helper plasmid according to standard procedures. Several independent transformant lines were established.

Polyclonal Antibodies

Two fragments of the *tai* cDNA (amino acids 1028–1235 and 477–687) were cloned into pGEX-5X-3 (Pharmacia) and used to produce GST fusion proteins, which were used to immunize rabbits (antibody JBL). Sera were diluted 1:2000 to carry out tissue immunostaining. No staining was observed in Df(2L)30A-C homozygous embryos. Preimmune sera failed to stain embryos, egg chambers, or third instar laval salivary glands.

Immunohistochemistry and Immunofluorescence

Ovary dissections were performed in Grace's medium plus 10% calf serum. For β-galactosidase staining, whole ovaries were fixed in PBS containing 0.1% glutaraldehyde for 5 min, rinsed, and stained as described (Montell, 1999). Immunofluorescent localization of proteins was carried out essentially as described. For double or triple labeling, stained egg chambers were incubated with rhodamine-phalloidin (Molecular Probes) at 1:200 for 25 min followed by DAPI at a final concentration of 0.5 μg/ml and incubation for another 5 min. After three washes, egg chambers were mounted in VECTASHIELD® (Vector Laboratories). Salivary glands from third instar larvae were dissected in PBS and fixed in PBS containing 3.7% formaldehyde for 30 min. Staining was detected using the Elite ABC kit (Vector Laboratories). Polytene chromosome staining was carried out as described in Andrew and Scott (1994). Primary antibodies used were: mouse anti-Armadillo (N2 7A1, 1:200) (Developmental Studies Hybridoma Bank); rat anti-DE-cadherin (DCAD2, 1:20) (Uemura et al., 1996); rabbit anti-DFAK (1:1000) (Palmer et al., 1999); mouse anti-EcR-A (15G1a, 1:50), mouse anti-EcR-B1 (AD4.4, 1:50) (Talbot et al., 1993); mouse anti-USP (AB11, 1:50) (Christianson et al., 1992); mouse anti-β-galatosidase (1:500) (Promega); and rabbit anti-β-galatosidase (1:2000) (Cappel). The secondary antibodies used were: horse fluorescein anti-mouse (Vector Laboratories); Cy™5-conjugated donkey anti-rabbit; fluorescein (FITC) conjugated donkey anti-rabbit; fluorescein (FITC) conjugated donkey anti-rat; rhodamine red™-X-conjugated donkey anti-mouse; rhodamine red™-X-conjugated donkey anti-rabbit (Jackson ImmunoResearch).

Cell Culture and Transient Transfections

EcR-293 cells (Invitrogen) were maintained in complete medium containing DEME (GibcoBRL), 10% fetal bovine serum (Gibco BRL) and 400 μg/ml Zeocin™ (Invitrogen). Transient transfections were

performed using the Calcium Phosphate Transfection method (Chen and Okayama, 1987). Transfected cells were re-fed with fresh medium containing either vehicle (ethanol) alone or vehicle plus 2.5 μM Ponasterone A (Invitrogen) and were harvested 20 hr after treatment. Transfections using β-galactosidase as the reporter were assayed using the Galacto-Light Plus™ kit (Tropix). Transfections using luciferase as the reporter were assayed with the Enhanced Luciferase Assay Kit (PHARMINGEN).

GST Pulldown Assays

^{35}S-methionine-labeled EcR and USP were generated in a coupled transcription–translation system, TNT (Promega), using CMX-EcR (T7) and CMX-uspK (T7) constructs (Tsai et al., 1999), respectively, as templates. Glutathione S-transferase (GST) fusion proteins, GST-LXXLLs (amino acids 1028–1235) and GST-N (amino acids 259–382), were expressed in BL21 cells. In vitro GST-pulldown assays were performed as described in Tsai et al. (1999). Where indicated, 5 μM Ponasterone A was added.

Supplemental Experimental Procedures

The Experimental Procedures given here have been abridged due to space constraints. A full-length version of the Experimental Procedures is available online (http://www.cell.com/cgi/content/full/103/7/1047/DC1).

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GenBank Accession Number

The sequence of *taiman* has been deposited in GenBank under the accession number AY008258.