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Differential control of cell affinity required for progression and refinement of cell boundary during *Drosophila* leg segmentation

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Abstract

Domain boundary formation in development involves sorting of different types of cells into separate spatial domains. The segment boundary between tarsus 5 (Ta5) and the pretarsus (Pre) of the *Drosophila* leg initially appears at the center of the leg disc and progressively sharpens and expands to its final position, accompanied by down-regulation of the cell recognition molecule Capricious and Tartan and cell displacement from Ta5 to Pre across the boundary. Capricious and Tartan are controlled by transcription factor Bar and Al, and their loss of function leads to reduction of cell affinity to wild type neighbors and cell displacement activities. In addition, although the mutant cells formed Ta5/Pre boundary, its progression and sharpening were compromised. Cells overexpressing Capricious or Tartan became invasive within Ta5 and Pre, sometimes escaping the compartmental restriction of cell movement. Dynamic spatiotemporal regulation of cell affinity mediated by Capricious and Tartan is a key property of refinement of the Ta5/Pre boundary.

Keywords: *Drosophila*; Leg; Segmentation; Cell affinity; Cell adhesion; Capricious; Tartan; LRR protein

Introduction

Cell boundary formation has been thought to involve cell recognition and cell sorting (Moscona and Moscona, 1952; Townes PL, 1955), and is mediated by specific cell-surface receptors (Thiery et al., 1977; Yoshida-Noro et al., 1984). Differential expression of cell adhesion molecules in critical stage of morphogenesis (Hatta et al., 1987), and the reconstruction of differential cell sorting by cloned homophilic cell adhesion molecules in model systems (Miyatani et al., 1989; Nose et al., 1988) suggest that cell adhesion molecules are important mediators of cell sorting. However, the extent to which cell adhesion molecules contribute to domain boundary formation in the developmental context remains to be elucidated (Takeichi et al., 2000).

In tissue development, cell sorting often takes place in the context of the epithelium, in which polarized epithelial cells maintain close contact with each other and form a coherent sheet of cells that constitutes the organ primordium. As typified by the regional specification of neuroepithelium in the development of the vertebrates central nervous system (Lumsden, 2004) and in insect limb formation (Irvine and Rauskolb, 2001), organ primordia are subdivided into several domains that are distinguished by specific gene expression patterns and cell morphologies, and their boundaries often give rise to the morphological borders of mature organs (Mirth and Akam, 2002). Organ primordia grow by cell proliferation, and newly born cells must assume their proper position with respect to the domain boundaries in the field through cell recognition processes.

A well-described case of domain boundaries is the developmental compartment formation seen in the *Drosophila* wing imaginal discs (Garcia-Bellido et al., 1973; Morata and

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Lawrence, 1975; Zecca et al., 1995). Each segment consists of posterior (P) cells, which express the transcription factor Engrailed (En), and anterior (A) cells, which do not. En expression is inherited by all descendents of P cells and instructs the P cells to segregate from A cells, forming a sharp AP compartment boundary (Kornberg et al., 1985). In a similar manner, the homeobox protein Apterous (Ap) specifies the dorsal compartment of the wing primordia (Diaz-Benjumea and Cohen, 1993). In each case, cell lineage restriction by En or Ap is thought to specify a difference in cell affinity and cells of different compartment sort from each other to segregate and form sharp cell boundaries. Early commitment of cell fate is an essential mechanism of compartment boundary formation. However, compartments have been observed in very few parts of the insect epidermis (Bryant and Schneiderman, 1969).

A more general class of epithelial domain boundaries is that formed independently of early cell lineage restriction, such as the rhombomere boundary in the vertebrate hindbrain. Rhombomeres are a series of seven distinct bulges that are transiently visible during the CNS development (Lumsden, 2004; Lumsden and Keynes, 1989). The rhombomere boundary marked by Krox 20 expression is initially diffuse and composed of cells with graded expression of Krox 20, but progressively sharpened (Irving et al., 1996). Cell fate decision between two alternative segment identities and cell sorting contribute to the formation and maintenance of the sharp boundaries (Cooke and Moens, 2002). Transplantation experiments revealed that rhombomere grafts of heterotopic origins were segregated out from the neighbor but homotopic grafts mixed well with neighbors. From these observations, it was suggested that cell sorting is driven by two distinct mechanisms; heterophilic cell repulsion and homophilic cell mixing, each are thought to be mediated by specific cell surface molecules (Guthrie et al., 1993).

The Eph/Ephrin system is known as a mediator of repulsive cell signaling during rhombomere boundary formation (Cooke and Moens, 2002; Cowan and Henkemeyer, 2002). Sek-1 is a member of Eph receptor that is expressed in rhombomere 3 and 5 under control of Krox20 and helps cells to segregate from neighbors during the rhombomere development (Theil et al., 1998). On the other hand, the molecular mechanisms that mediate homophilic cell mixing are unknown.

Capricious (Caps; (Shinza-Kameda et al., 2006; Shishido et al., 1998) and Tartan (Trn) (Chang et al., 1993) are transmembrane proteins with multiple leucine rich repeats (LRRs), which are found in various membrane proteins with signaling receptor or ligand functions (Battye et al., 2001; Buchanan and Gay, 1996; Eldon et al., 1994; Nose et al., 1992; Schneider et al., 1991), or with a specific cell adhesion activity (Eldon et al., 1994; Krantz and Zipursky, 1990; Nose et al., 1992; Schneider et al., 1991). Caps is involved in targeting motoneurons to specific muscles during embryonic neuromuscular junction formation (Shishido et al., 1998) and in targeting photoreceptor axons to optic lobe (Shinza-Kameda et al., 2006). In each case, Caps is expressed on the surface of both cells making contact, suggesting it mediates homophilic cell recognition. This idea is supported by the observation that Caps induces the homophilic aggregation of cultured cells (Shinza-Kameda et al., 2006). In addition, based mainly on gene misexpression studies Caps and Trn have been implicated in cell sorting in the *Drosophila* wing disc (Milan et al., 2005; Milan et al., 2001). However, their requirement for the cell boundary formation was not clear. Here we addressed the roles of Caps and Trn during *Drosophila* leg development and report that Caps and Trn play key roles in homophilic cell affinity that helps the progression and sharpening of a presumptive segment boundary.

Materials and methods

Fly strains

$caps^{GS13628}$ is a homozygous viable strain with a Genes Search element (Toba et al., 1999) inserted upstream of the *caps* promoter. $trn^{28.4}$ carries a small deletion of *trn* gene with a defective placW P-element vector remaining in the promoter region and was used as a null allele of *trn* (Chang et al., 1993). $caps^{GS13628}$, $trn^{GS10885}$, $UAS\text{-}BarH1^{M13}$ and $UAS\text{-}BarH2^{F11}$ (Sato et al., 1999) were used for Gal4-induced misexpression, and $hh\text{-}DsRed\ (hh^{PyR215})$ (Akimoto et al., 2005) was used as a P cell marker. $caps\text{-}lacZ\ (caps^{02937})$ (Shishido et al., 1998), $ptc\text{-}GAL4\ (ptc^{551.9})$ (Flybase ID=FBal0138169), and $Dll\text{-}Gal4\ (Dll^{md23}$; FBal0054121) were obtained from the Bloomington Drosophila Stock Center. $caps\text{-}GAL4\ (caps^{NP3294})$ (Hayashi et al., 2002), *UAS-mCD8::GFP* (FBtp0002652), and *ey-FLP5* (FBti0015983) flies were obtained from the *Drosophila* Genetic Resource Center, Kyoto Institute of Technology.

A signal sequence prediction program (AHIRU) written by Kagayaki Kato was used to select genes encoding potentially secreted proteins. Lines with GS element insertions near those genes were crossed to *Dll-Gal4*, and the progenies that survived beyond the pharate adult stage were examined for leg phenotypes.

Mutagenesis

EMS induced *caps* alleles were isolated by a screen for revertants of the lethality caused by the ectopic expression of $caps^{GS13628}$. $caps^{GS13628}$ males were treated with EMS and crossed to *ptc*-GAL4 females. Among 304,500 progeny, 50 viable revertants were isolated, 17 of them were found to have mutations affecting the Caps coding region. *caps trn* double mutant chromosomes were obtained by P-elements mobilization. Putative recombinant lines were balanced and confirmed for non-complementation with *caps* and *trn* alleles. Molecular integrity of the *caps-trn* locus was assessed by monitoring the presence of a series of STS markers (marker names and positions are indicated above the horizontal line in Fig. 4H). Quantitative PCR (ABI PRISM 7000 Sequence Detection System) with primer sets that amplify 80–150 bp fragments of the locus allowed detection of deletions in genomic DNAs of heterozygous flies. Primer sequences are available upon request. Open reading frame of *caps* was also sequenced to check the presence of $caps^{C28fs}$ point mutation.

$trn^{\Delta 73}\ caps^{GS1}$

This chromosome was isolated from $trn^{28.4}/caps^{C28fs}$ heterozygous males and carries a small deletion of the region containing probe −0.3 k in the *trn* promoter region (Fig. 4H), linked to a new GSv6 insertion into the promoter region of wild type *caps* allele, likely as a result of complex recombination, deletion and P-element transposition.

$Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$

This chromosome was recovered from a recombinant $trn^{GS10885}\ caps^{C28fs}$ chromosome, and carries a deletion uncovering all the STS markers between −3.1 k and +110.9 k, and deletes *trn, CG33262* and *CG11281*. This deficiency was probably produced by simultaneous mobilization of the two P-elements and retains $caps^{C28fs}$.

Estimation of CG33262 and CG11281 mRNA expression

mRNA was extracted from 30 leg discs from late L3 larvae using QuickPrep Micro mRNA Purification Kit (Amersham Biosciences). cDNA was synthesized

by Cloned AMV RT (Invitrogen) using an oligo dT primer. Quantitative PCR was performed with ABI PRISM® 7000 using SYBR® Green PCR master mix, and the data was analyzed by SDS Software (Applied Biosystems). Primer sets of the STS marker CG33262_2 (*CG33262*), CG11281_2 (*CG11281*), +110.9 k (*caps*) (Fig. 4H), and DE-cad (DE-cadherin) were used to detect each transcript. The primer set for +8.6 k was used to estimate the level of background transcription from the intergenic region. Reactions without reverse transcription (−RT) were included as negative control. Serially diluted genomic DNA was included as a positive control template to derive standard curves to estimate mRNA copy numbers. Copy number per cell was calculated on the basis of estimated cell number in mature leg disc (10,000 cells per disc) and the fraction of caps+ cells per leg disc (5%).

Mosaic analysis

Mitotic recombination in the leg disc was induced by the FLP/FRT technique (Xu and Rubin, 1993) using *hs-flp* or *ey*-FLP5, which induces a high frequency of recombination in the leg disc (Tsuji et al., 2000). Flip-out clones were induced by heat-shock in L2 stage, except for experiment described in Fig. 1G (L3). The genotypes used for generating clones were as follows. Wild-type clones: *ey-FLP5/+; FRT2A/ubi-GFP FRT2A*. caps: *ey-FLP5/+; caps^{C28fs} FRT2A/ubi-GFP FRT2A*. trn: *ey-FLP5/+; trn$^{28.4}$ FRT2A/ubi-GFP FRT2A*. *Df(3L)trn$^{\Delta 17}$, caps^{C28fs}*: *ey-FLP5/+; Df(3L)trn$^{\Delta 17}$, caps^{C28fs} FRT2A/ubi-GFP FRT2A*. *trn$^{\Delta 73}$ caps^{GS1}*: *ey-FLP5/+; trn$^{\Delta 73}$ caps^{GS1} FRT2A/ubi-GFP FRT2A*. Large mutant clones in the adult: *y hs-FLP f^{36a}/f^{36a}; Df(3L)trn$^{\Delta 17}$ caps^{C28fs} FRT2A/M(3)65F P{f^{+}13}77A FRT2A*. Bar: *y Df(1)Bar$^{263\text{-}20}$ FRT19A/y arm-lacZ FRT19A; ey-FLP/+*. GFP-expressing clones: *hs-FLP; Ay-Gal4 UAS-mCD8*::*GFP/+*. Caps-expressing clones: *hs-FLP; Ay-Gal4 UAS-mCD8*::*GFP/+; caps^{GS13628}/+*. Trn-expressing clones: *hs-FLP; Ay-Gal4 UAS-mCD8*::*GFP/+; trn^{GS10885}/+*.

Immunostaining

Dissection and antibody staining were performed by standard procedures (Sullivan et al., 2000). Anti-Caps (rabbit, 1:200; Shishido et al., 1998), anti-Trn (rabbit, 1:2000; Chang et al., 1993), anti-Bar (rabbit, 1:20; Higashijima et al., 1992) and anti-Al (rat, 1:100; Campbell et al., 1993) antibodies were used to monitor each genes. The 40-1A anti-beta-Gal and the 1D4 anti-Fas2 were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Rhodamine-labeled Phalloidin (Molecular Probe) was used to label F-actin.

Results

The Ta5/Pre boundary is formed independent of cell lineage restriction

We first describe the segmentation process in the distal tip of the leg (Kojima, 2004). The leg disc is divided into several concentric domains with the distal tip at the center and the proximal domain at the periphery (Fig. 1C). Each domain develops into segmental units called the coxa, trochanter, femur, tibia, tarsal segment (Ta) 1–5, and pretarsus (Pre), in a proximal to distal order (Figs. 1A and B). Specification of the distal leg segments becomes apparent during the second larval instar (L2), when a pair of homeobox genes, *BarH1* and *BarH2* (hereafter collectively called *Bar*), begin expression in the central domain of the leg discs (Campbell, 2005; Higashijima et al., 1992; Kojima et al., 1991) (Fig. 1E). At mid-third instar (L3), Bar expression at the center of the leg disc is reduced and is replaced by the expression of the *aristaless* (*al*), *clawless* (*cll*), and *lim1* homeobox genes under the influence of EGFR signaling, and the boundary between Bar and Al expression becomes sharpened by late L3, due to their mutual repression (Campbell, 2002; Campbell, 2005; Campbell and Tomlinson, 1998; Galindo et al., 2002; Kojima et al., 2005; Pueyo and Couso, 2004; Schneitz et al., 1993; Tsuji et al., 2000). The ring of cells maintaining Bar expression becomes Ta4 and Ta5 (Kojima et al., 2000), and the Al-expressing cells become Pre.

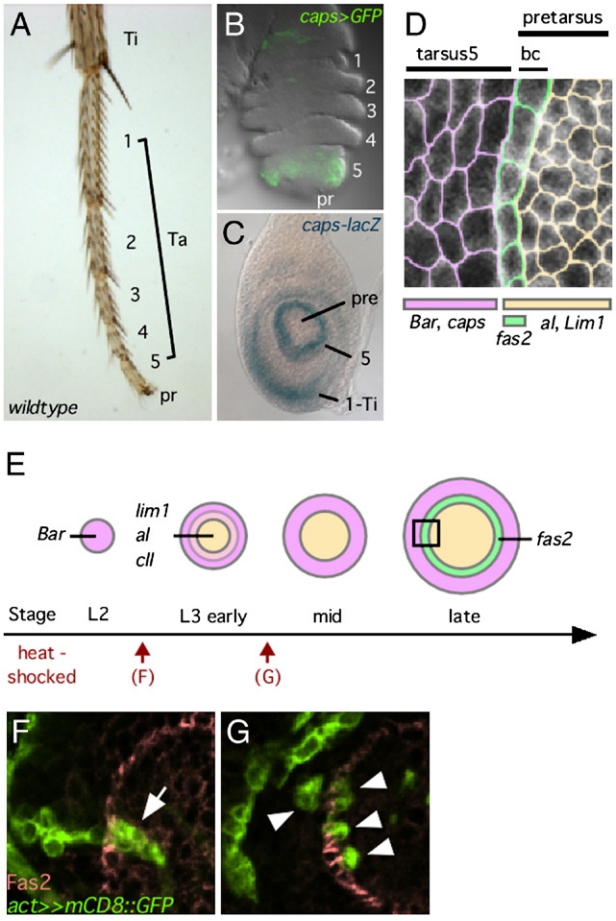

Fig. 1. Development of distal part of the *Drosophila* leg. (A) Distal tip of an adult mesothoracic leg of *Drosophila*. Ti, Tibia; Ta, tarsal segment; pre, pretarsus. (B) A pupal leg of a *caps-GAL4 UAS-mCD8*::*GFP* fly 5 h after puparium formation. (C) *caps-LacZ* expression in the Ta5 and Ta1/Ti domains of a late L3 leg disc. (D) Apical cell boundaries in the Ta5/Pre region at late L3 (boxed in panel E) visualized by F-actin staining. Note that the cells in Pre are more compact than the cells in Ta5; note also the rectangular shape of the border cells that separate them. (E) Schematic diagram of gene expression in the center of a developing leg disc. *Bar* genes are expressed in a broad central domain at L2 (pink). At early to mid L3, Al, Lim1, and Clawless become activated (yellow) and replace the expression of Bar. At late L3, Fas2-expressing border cells (green) appear at the boundary of the Bar- and Al-expressing regions and separate Ta5 and Pre. (F, G) Cell marking experiments. Flip-out recombination was induced to label cells with GFP at L2 (F) or early L3 (G). (F) A 16-cell clone (arrow) crossing the Ta5/Pre boundary marked by Fas2 expression (red). (G) Four-cell clones abutting the Ta5/Pre boundary from the distal side (arrowheads) or proximal side (arrowhead). No clones crossing the boundary were observed (N=20).

The Ta5/Pre boundary is a boundary between cells with different shapes. The Al-expressing Pre cells are densely packed at the apical surface, while the Ta5 cells have a larger apical cell surface (Fig. 1D). The compact apical shape of the Al positive cells was already evident at mid L3 (apical cell surface area=2.75±0.20 μm in mid L3, 2.62±0.33 μm in late L3)

and their number doubled by late L3 (192.8±28.7 cells in mid L3, 393.8±31.1 cell in late L3). The outermost row of Al positive cells took on their characteristic rectangular shape and expressed Fasciclin 2 (Fas2), a cell-surface molecule with homophilic cell adhesion activity (Grenningloh et al., 1991) (Figs. 1D and 2F). The shapes of these "border cells" and the expression of Fas2 are regulated by both Al and Bar (Kojima et al., 2000).

To ask if the Ta5/Pre boundary is permissive to cell movement, we used the flip-out recombination technique to label clones of cells. Clones induced by heat-shock during L2 to early L3 were 16–32 cells in size, and frequently found to cross the Ta5/Pre boundary (Figs. 1E, F). On the other hand, none of the clones induced in mid L3 (about 4 cells in size) crossed the Ta5/Pre boundary ($N=20$, only clones touching the boundary scored, Fig. 1G). The results indicate that the presumptive Ta5/Pre border is permissive to cell movement when it is expanding and sharpening, and becomes refractory when the sharp boundary is established.

Caps and Trn are expressed at the Ta5/Pre region under the control of Bar

To identify molecules that contribute the sharp boundary formation in the leg, we screened for genes involved in leg segmentation using the Gene Search System (GS), a gain-of-function screening system (Toba et al., 1999). We first computationally screened the *Drosophila* genome sequence (Release 3) using a signal peptide prediction algorithm (PSORT) (Bannai et al., 2002) and selected 3192 genes that potentially encoded secreted proteins. Among these, 407 genes that were located close to the insertion sites of GS elements (Sakata et al., 2004; Aigaki et al., unpublished) were tested for a misexpression phenotype. A total of 921 insertion lines were crossed to the *Dll*-Gal4 driver and 110 of them caused visible defects in the adult leg. *caps* was one of genes that caused aberrant cell sorting behavior and was chosen for further analyses.

caps was expressed in two circular domains covering Ta5 and a broad band spanning the tibia and Ta1 in the L3 leg disc (Fig. 1C) (Mirth and Akam, 2002). This specific pattern was maintained after eversion of the pupal leg discs (Fig. 1B). *caps* expression was first detected at early L3 in a pattern overlapping with the Bar proteins (Fig. 2A), and it became excluded from the Al positive cells at mid L3 (Fig. 2B). At this stage, Trn was expressed in Caps-expressing cells (Fig. 2C). By late L3, the internal (distal) limit of the ring of *caps* expression precisely coincided with that of *Bar* in the Ta5 cells, and abutted the expression of Al and Fas2 (Figs. 2D, F). An identical pattern of *trn* expression was observed in Ta5 cells at late L3 (Fig. 2G).

We next investigated the regulation of Caps and Trn expression. In clones lacking both the *BarH1* and *BarH2* functions, the expression of both Caps and Trn was abolished cell-autonomously (Figs. 3A, B). Furthermore, the ectopic expression of *BarH1* (data not shown) or *BarH2* (Figs. 3C, D) allowed Caps and Trn expressions to persist in the Pre region. These results suggest that the *Bar* genes regulate the expression of Caps and Trn in Ta5 and Pre.

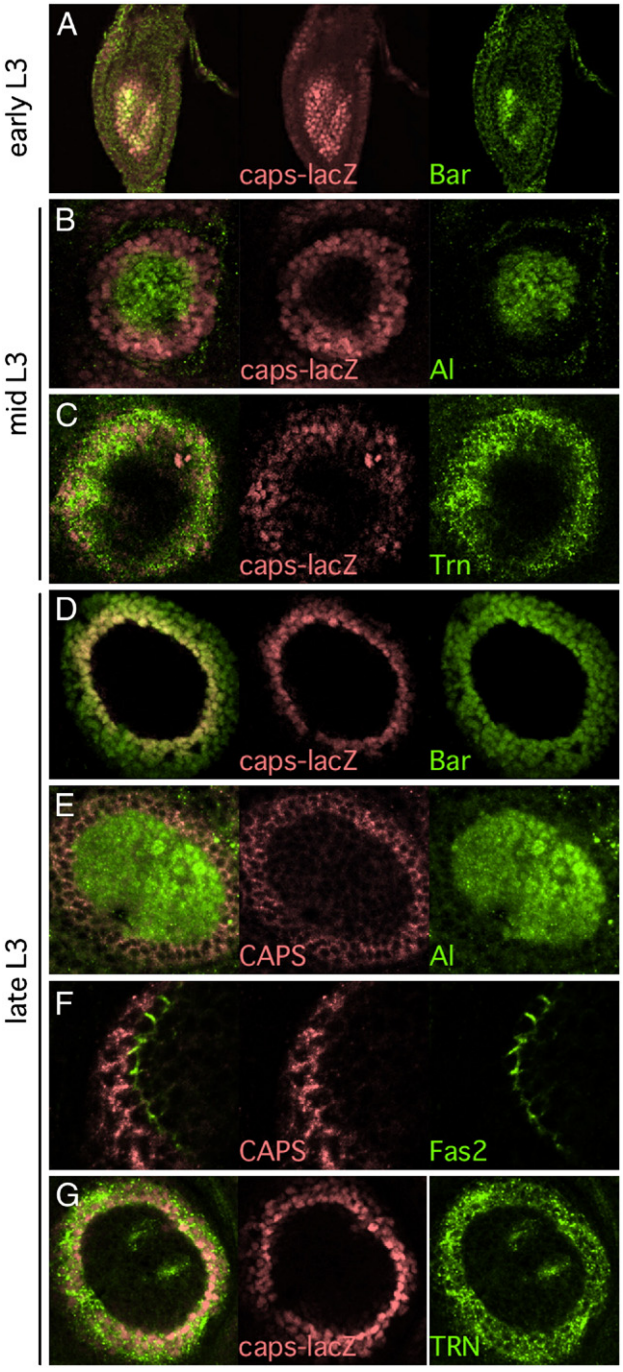

Fig. 2. Expression of Caps and Trn and their relationship with Bar, Al, and Fas2 in the Leg Disc. Caps is shown in red; Bar/Al/Fas2/Trn expressions are shown in green. (A) In the early L3 leg disc, Caps was expressed in the central domain, where the Bar genes were expressed. (B) At mid L3, the Caps and Al expressions became separated. (C) Caps-expressing cells express Trn at mid L3. (D) At late L3, the Bar and *caps-lacZ* expression domains overlapped in the Ta5 domain and formed a sharp boundary at the distal boundary. Bar signals were also detected weakly in the Ta4 domain. (E) The Caps and Al expression domains did not overlap. (F) Fas2 was expressed in the border cells located immediately adjacent to the internal border of the Caps expression. (G) Trn and Caps expression overlapped at late L3. Trn is also expressed in two spots in Pre, which do not correspond to the craw primordia.

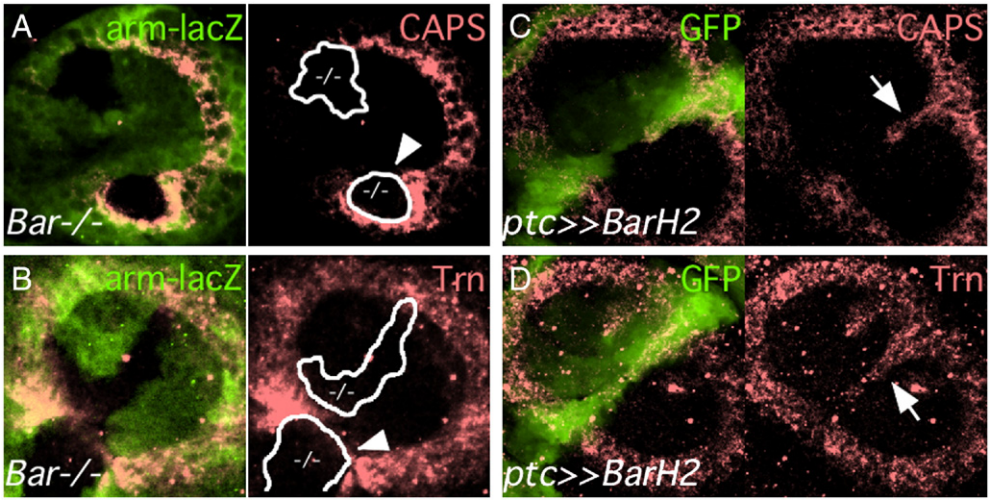

Fig. 3. Cell autonomous regulation of Caps and Trn by Bar. (A, B) Caps and Trn signals (red) were abolished in Bar mutant clones, which can be identified by the lack of GFP marker (green). (C, D) Ectopic expression of Caps and Trn was induced (arrows) in the region where BarH2 was expressed by the *ptc* enhancer (*ptc > BarH2,* green).

Deficiency of Caps and Trn functions impairs sharpening and progression of Ta5/Pre boundary

The progressive down-regulation of Caps and Trn in the presumptive Pre region at the early-to-mid L3 correlated with the establishment of the sharp Ta5/Pre boundary. Previously loss-of-function analysis of Caps and Trn has been performed using a recombinant chromosome carrying the hypomorphic allele $caps^{65.2}$ (Shinza-Kameda, 2006) and a *trn* allele with unknown severity (Milan et al., 2001). Mitotic clones homozygous for this chromosome showed unusually round up shape in wing disc, but did not reveal any obvious phenotype in the adult (Milan et al., 2002; Milan et al., 2001). To study complete loss of function phenotypes, we isolated EMS-induced *caps* alleles that were classified into three categories. The first class (C28fs, I179fs, Q213st, L269fs, N338fs, and Q373st; fs denotes frame shift and st denotes truncation mutations) encoded truncated proteins that are likely to have little or no activity. The second class included point mutations (S103L, L266F) and deletion of the 6th leucine rich repeat (ΔLRR6) that are strong loss-of-function mutations with defects in the extracellular domain. The third class included 8 hypomorphic mutations with a single amino acid alteration (P45S, C58Y, N71I, P72S, L77Q, H118Y, L472P, and T501I). $caps^{C28fs}$ was used as a complete null allele. We also isolated a small deficiency $Df(3L)trn^{\Delta 17}$ in $caps^{C28fs}$ chromosome (Fig. 4H). The deficiency removes the ~100 kb interval between *trn* and *caps*. In addition, we used a chromosome carrying hypomorphic alleles $trn^{\Delta 73}$ $caps^{GS1}$.

Two putative genes *CG33262* and *CG11281* have been annotated in this interval. We conducted quantitative PCR analyses to estimate the level of their mRNA expression in the leg disc (Table 1). Copy number of *caps* mRNA was about 1200 per cell (assuming the expression in 5% of total leg disc cells), and that of *DE-cadherin* was about 190 per cell (assuming ubiquitous expression). On the other hand, the levels of *CG33262* and *CG11281* transcripts were 1.85 and 0.43 per cell, even with the assumption that expression of those genes are limited to 5% of total cells. Those numbers were smaller than the signal of probable sporadic transcripts from the intergenic region between *trn* and *CG33262* detected with the primer set +8.6 k. These data suggest that the transcript levels of *CG33262* and *CG11281* are expressed no more than the level of background transcription in L3 larval leg discs.

To evaluate the requirement of Caps and Trn for Ta5/Pre the boundary formation, we induced homozygous clones of mutant chromosome at L2, before Caps and Trn were down regulated in the presumptive Pre region, and assessed boundary formation phenotype by Fas2 expression. No defect in the position of Fas2 stripe was observed in wild-type, $caps^{C28fs}$ or $trn^{28.4}$ single mutant clones (Figs. 4A–C). Fas2 stripes were also formed in the clones of $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ cells, however, their positions were often shifted centrally (5/15). This boundary shift was most obvious when the position of the Fas2 stripe in a mutant clone in the A compartment was compared to that in adjacent wild-type P-compartment cells (Fig. 4D lower arrow, F arrowhead), suggesting that the distal to proximal progression of presumptive Ta5/Pre boundary is delayed in some $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones. We also found that the normally one-cell-wide stripe of Fas2 expression sometimes expanded to two to three cells (2/15, Fig. 4G). The results suggest that Caps and Trn are dispensable for the formation of Ta5/Pre boundary, but are required for its progression and sharpening. The incomplete penetrance of the phenotype in $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones also suggests that additional genes may be involved in this process.

We also studied the phenotype of $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ in the adult leg by inducing large clones by the minute technique (Fig. 5). Those legs showed defective joint formation at the Ta5/Pre that was consistent with the defective Ta5/Pre boundary formation observed in the leg disc. Ta4/Ta5 boundaries in the mutants were also defective. This phenotype might have been missed in the leg disc, because of the lack of an appropriate marker for this boundary. In addition Pre and claws in the mutants were stunted, as can be expected if the reduction of Pre primordia would reduce the size of presumptive claws.

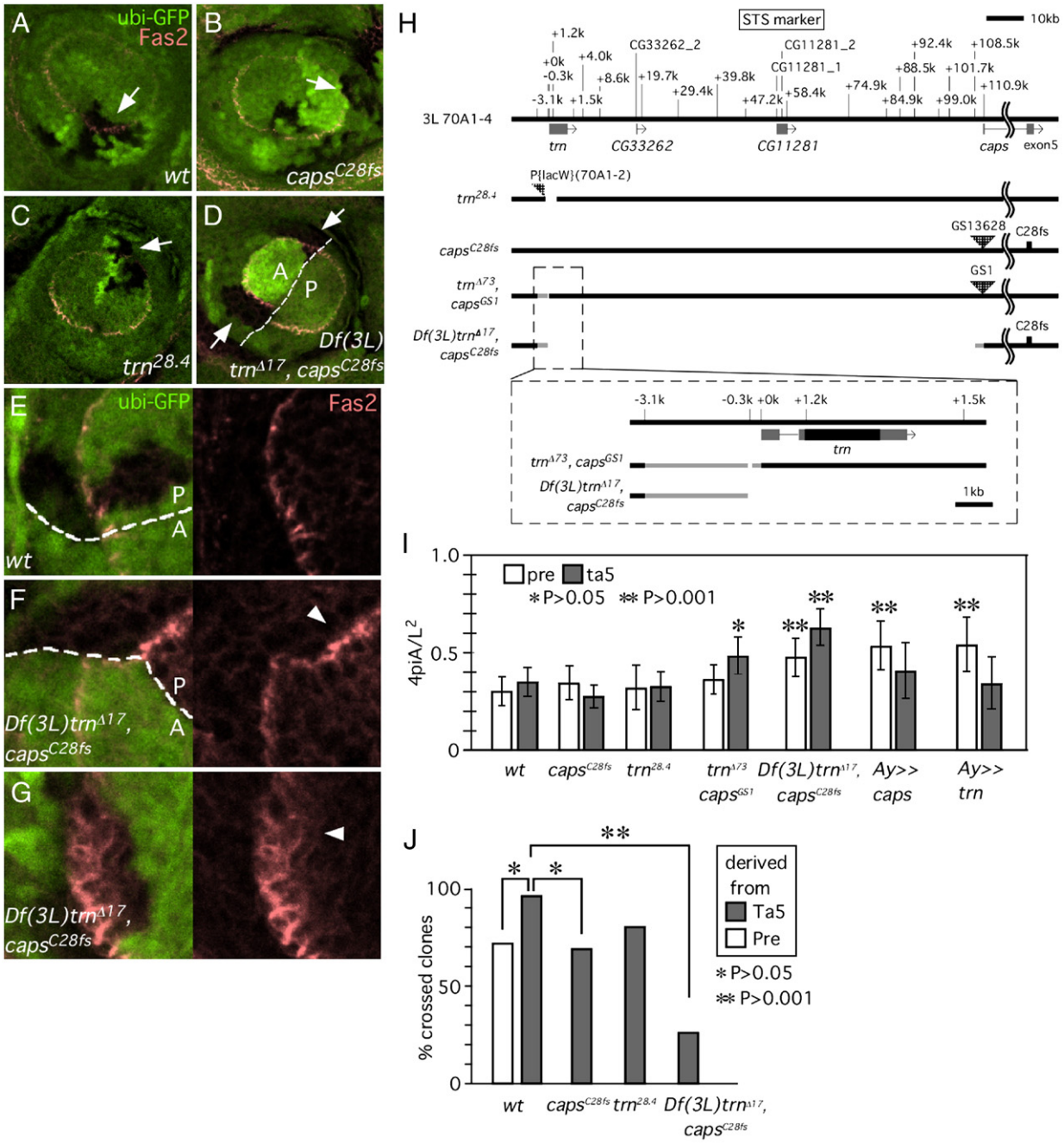

Fig. 4. Caps and Trn control cell affinity and the Ta5/Pre boundary formation in the leg. (A–D) Clone shape assay in the leg disc. Mitotic recombination clones were marked by the lack of two copies of *ubi-GFP*; i.e., they appear as unmarked areas. The Ta5/Pre boundary was visualized with Fas2 (red). (A–C) Wild type, $caps^{C28fs}$, and $trn^{28.4}$ clones (GFP negative cells) showed irregular boundaries (arrows). (D) $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones (GFP negative cells) were more rounded in shape and had smooth boundaries (arrows). Dotted line indicates the A/P boundary. (E) Magnified image of wild type clone crossing the Fas2 stripe. Ta5 is on the left; Pre is on the right. (F–G) A $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clone spanning the Ta5/Pre boundary. Fas2 expression often shifted centrally (F, arrowhead), or expanded (G, arrowhead). (H) Physical map of the *trn* and *caps* region. P-element insertions are shown by triangles. *caps* exon 2–4 are omitted. Breakpoints in mutants are indicated as gray lines. $trn^{28.4}$ retains 5′ end of P{lacW}70A1-2 and deletes the *trn* 5′ ORF sequence (Chang et al., 1993). $caps^{C28fs}$ has a frame-shift mutation at the signal peptide region of the Caps protein. $trn^{\Delta 73}$ $caps^{GS1}$ has a small deletion in *trn* 5′UTR and *caps* hypomorphic mutation caused by an GS vector insertion at −7 bp from *caps* transcriptional *start site*. $Df(3L)trn^{\Delta 17}$ deletes *trn*, *CG33262* and *CG11281*. Positions of STS markers are indicated above the map. The *trn* region is enlarged at the bottom to show the positions of deletion breakpoints. (I) Circularity index (CI: 4piA/L2) of the clones as a measure of "roundness". More than 10 clones were measured in each case. Error bars indicate standard deviation. *=$P<0.05$, **=$P<0.001$. (J) Clone displacement assay. Clones adjacent to the Ta5/Pre boundary were classified into touch or cross category, and their percentage was plotted. The origin of the clones was estimated by the position of their sister clones. Wild-type clones derived from the Ta5 crossed Ta5/Pre boundary more frequently than in the opposite direction, and this frequency was significantly decreased in *caps* or *trn* clones and in $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones. *=$P<0.05$, **=$P<0.001$.

Table 1

Probe	Estimated transcript copy number per cell *			
	Ubiquitous expression in the leg disc[a]		Specific expression in 5% of the leg disc[b]	
	+RT	(−RT)	+RT	(−RT)
CG33262_2	0.09	(ND)	1.85	(ND)
CG11281_2	0.02	(0.01)	0.43	(0.12)
caps	60.50	(0.08)	1209.72	(1.59)
DE-cad	187.20	(0.29)	–	(–)
+8.6 k	0.21	(0.02)	4.15	(0.39)

* Calculation of transcript copy number was based on the assumption that the genes are expressed ubiquitously (a) or in 5% of total leg disc cells (b).

Caps and Trn are involved in cell mixing and proximal to distal cell displacement

To further understand the cellular phenotype of Caps and Trn deficiency, we employed two assays to assess the behavior of mutant cells. In the first assay, the short-range cell mixing activity was assessed by measuring the smoothness of the clone outlines. If labeled cells do mix well with neighbors, then the clones would develop a rough shape. Wild-type clones in both Ta5 and Pre showed a highly complex shape (Fig. 4A). The cell mixing activity was quantified using a Circularity index ($CI=4\pi A/L^2$, A=area, L=perimeter). The CI of the complete circle is 1.0. Wild-type clone in Ta5 and Pre domains both showed a low CI (Fig. 4I), suggesting that cells mixed quite well in the developing leg disc. We found that CI's of clones deficient for *caps* or *trn* were not significantly different compared to wild type clones in both Ta5 and Pre (Figs. 4B, C, I). Clones of $trn^{\Delta 73}$ $caps^{GS1}$ had higher CI in Ta5, suggesting that overlapping activities of Caps and Trn are required to keep Ta5 cells mixed. Further increase of CI was observed in $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones in both Ta5 and Pre.

The second assay determined the extent and orientation of long-range cell displacement across the Ta5/Pre boundary (Fig. 4J). Clones located at the Ta5/Pre boundary were grouped into either the "touch" or "cross" category. By locating the position of their sister clones, it was possible to determine whether the clone of interest stayed near its site of origin, or whether it spread beyond the Ta5/Pre boundary. In the case of wild-type cells, the frequency of clones crossing the boundary from Ta5 to Pre (95.2%) was significantly higher than that of clones crossing in the opposite direction (72.0%; Fig. 4J). This directed displacement of cells from Ta5 to Pre may account at least in part for the expansion of the presumptive Ta5/Pre boundary and increased cell number in the Pre region during the mid-to-late L3 stage. The displacement rate of cells from Ta5 to Pre was reduced in $caps^{C28fs}$ (68.8%) and $trn^{28.4}$ clones (80.0%), and was further reduced in the $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones (asterisks; 26.7%). No statistically significant differences were observed in the Pre to Ta5 displacement rate between wild type, *caps*, *trn*, and $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones (data not shown). Taken together, these results suggest that an overlapping function of Caps and Trn are required to maintain cell affinity in Ta5 and Pre region, and to help the proximal-to-distal cell displacement during the progression of the Ta5/Pre boundary.

Caps and Trn induce invasive cell behavior

To further test the roles of Caps and Trn in cell affinity determination in the leg, we compared the behavior of clones of

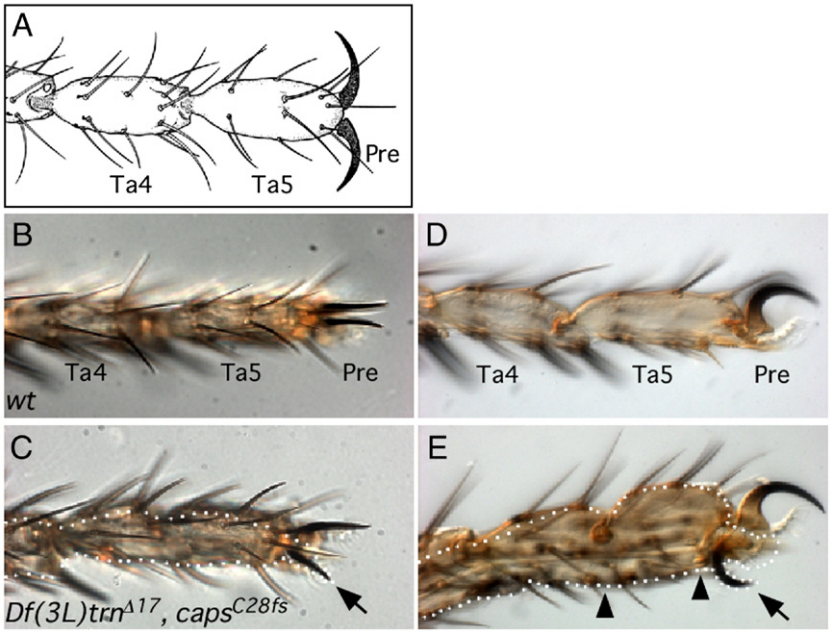

Fig. 5. Adult leg phenotype of *trn–caps* deficiency. (A) Dorsal view of a male fore leg. (B, D) Dorsal (B) and lateral (D) views of wild-type adult legs. (C, E) Dorsal (C) and lateral (E) views of mutant legs having large $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ clones occupying most of P compartment in tarsus and pretarsus (white dots). Pre became smaller as evidence by the small claws (arrows). The joint were lost or reduced at Ta5/Pre and Ta4/Ta5 junctions indicated by the thickening of cuticles characteristics (arrowheads) and the claws rotated abnormally (arrows).

cells over-expressing Caps or Trn. Caps or Trn expressing-cells in the proximal region of the leg disc formed clones with a smooth outline and often rounded up and sorted out from the epithelium, as previously observed in the wing disc (8 clones in each case; Fig. 6B, arrow). We also observed that when Caps and Trn were expressed broadly by the *Dll-Gal4* driver, adult leg formed protuberances and inside vesicles (Fig. 6C, data not shown). On the other hand, when Caps or Trn expression was induced in the future Ta5 regions, the clone outline was rough with low CI values, suggesting that those cells mixed well with their neighbors in this region (Figs. 4I and 6A, B, arrowhead). Clones in Pre region had intermediate CI values (Figs. 4I and 6A, arrow), suggesting that those cells can mix with Pre cells, but less efficiently than with Ta5 cells. This differential cell mixing behavior of Caps and Trn expressing cells correlate with to the down-regulation of Caps and Trn in Pre at the late L3 larvae. Thus the cell mixing behaviors of Caps positive cells may be explained by the homophilic cell recognition activity of Caps (Shinza-Kameda et al., 2006), which would mediate the binding to other cells expressing endogenous Caps in the Ta5/Pre region.

We also used *ptc-GAL4* to overexpress Caps or Trn in a stripe of A cells along the AP compartment boundary (Figs. 6D, F) and followed their movement in the leg disc. In the distal region of the leg disc, Caps-overexpressing cells often invaded the basal side of the P compartment and intermingled with posterior cells labeled with the *hh-DsRed* marker (Fig. 6G). Trn overexpression caused a similar phenotype, but the effect was less severe (Fig. 6H). The cell-invasion phenotype of Caps-expressing cells was most prominent in the central part of the leg disc, where endogenous Caps and Trn are expressed. In extreme cases, the Caps-expressing cells became aligned in a circular pattern like that of the endogenous Caps expression (Fig. 6E). This observation suggests that Caps and Trn confer on cells invasive cell mixing activity that can override the AP compartment restriction. Those animals hatched with malformed legs and were unable to walk.

Discussion

Homophilic cell affinity and mixing by Caps

Segmentation of the distal leg of *Drosophila* is initiated by the activation of EGFR signaling at the center of the disc (Campbell, 2002; Galindo et al., 2002), which leads to the activation of Caps and Trn under control of Al and Bar, and the expansion and sharpening of the Ta5/Pre boundary. Since Caps and Trn are dispensable for Ta5/Pre boundary formation marked with the stripe of Fas2, they are subordinate genes determining cell affinity. The rough borders of wild type clones in Ta5/Pre region suggest that those cells frequently exchange their positions with neighbors. Clones of $Df(3L)trn^{\Delta 17}$ $caps^{GS1}$ sorted from neighboring wild type Ta5 cells, suggesting that Caps and Trn are required to keep $Caps^{+}$ Trn^{+} cells mixed with neighbors. The results of gain-of-function experiments support this idea, as previously described for the analyses in the lateral part of the wing disc (Milan et al., 2002). Although the authors suggested that the Caps extracellular domain acts on a putative

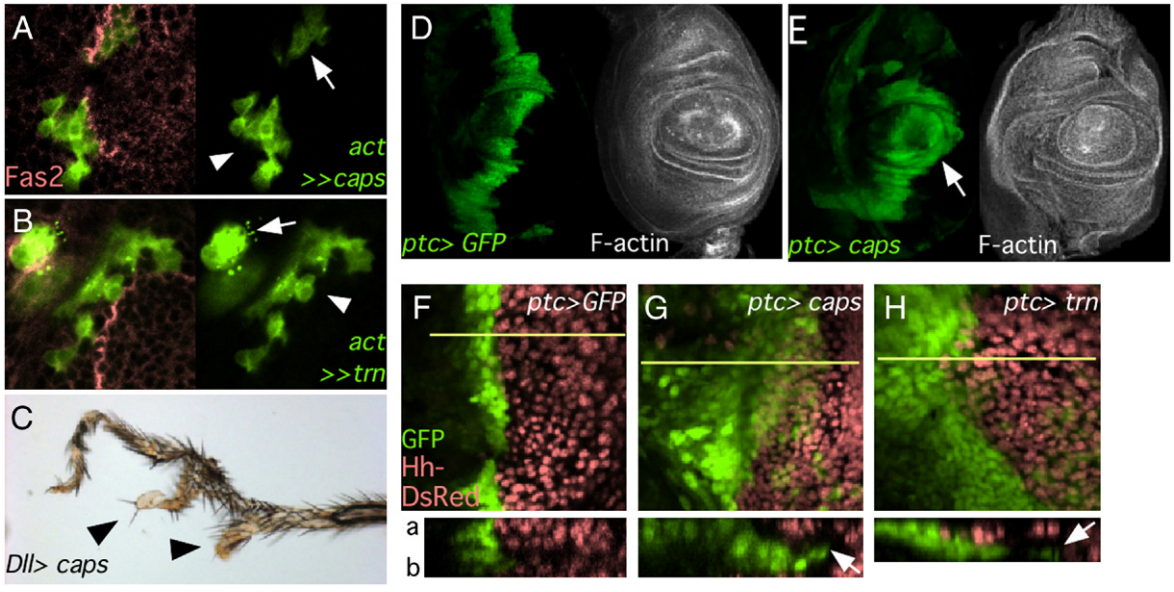

Fig. 6. Phenotypes of Caps/Trn misexpressing cells. (A) Caps-expressing clones. In the Ta5, clones were located in the epithelium and had an irregular outline (arrowhead). The clone in Pre region was also in the epithelium (arrow). (B) Trn-expressing clone in the Ta5 domain (arrowhead) was also located in the epithelium, but clones from the proximal region where Caps and Trn was not expressed were sorted out of the epithelial layer (arrow). (C) An adult foreleg derived from a *Dll>caps* fly. Unusual epidermal protuberances were observed (arrowheads). (D) Low-magnification view of a leg disc with *ptc>GFP*. (E) A leg disc with $ptc>caps^{GS13628}$. Cell invasion induced by Caps was prominent at the center of the leg disc (arrow) and formed a circle in the Ta5/Pre region. (F) Anterior cells abutting the posterior region were marked by GFP in the late L3 leg disc of a *ptc-GAL4/UAS-GFP* (*ptc>GFP*) animal. Posterior cells were marked with the nuclear hh-DsRed marker. Lower panel shows the z section of the region marked with a yellow line. a: apical, b: basal. (G) In the $ptc>caps^{GS13628}$ leg disc, Caps-misexpressing cells invaded into the endogenous Caps domain beyond the A/P compartment boundary from the basal side (arrow). (H) $ptc>trn^{GS10885}$ leg disc. Trn-misexpressing cells also invaded the posterior region (arrow).

receptor (Milan et al., 2002; Milan et al., 2005), recent finding that Caps mediates homophilic cell adhesion in S2 cells (Shinza-Kameda et al., 2006), and the isolation of a point mutation in the Caps intracellular domain (T501I) that abrogates the invasive activity of Caps (this work) suggest that Caps acts as a receptor promoting cell exchange through homophilic cell recognition, an activity termed mixing (Guthrie et al., 1993). The molecular function of Trn is less clear, but the similarity of its structure and activity to Caps suggest that Trn might act redundantly with Caps in homophilic cells recognition and mixing.

Roles of Caps and Trn in domain boundary progression

To understand the role of Caps and Trn in Ta5/Pre segment boundary formation, we must consider both the cell mixing function of Caps and Trn, and the spatio-temporal regulation of their expression (Fig. 7). At early third instar, Caps and Trn are expressed in the region covering the future Ta5 and Pre, and would permit cells to flexibly exchange their positions with those of their neighbors, thereby maintaining a fluid state of cell mixing. This hypothesis is supported by the slightly increased CI of *caps trn* double mutant clones in the future Pre region (Fig. 4I). Through the mid-to-late third instar, Caps and Trn were down regulated in the central domain. Caps- and Trn-dependent cell mixing persists and could shift cells that have lost Caps/Trn expression into the future Pre region. The imbalance of cell mixing activity in the $Caps^{+}$ Trn^{+} and $Caps^{-}$ Trn^{-} domains would allow the observed proximal-to-distal flow of cells across the Bar/Al boundary. Finally, the expression of Fas2 in the row of cells abutting the distal side of the Caps-expressing cells may stabilize the Ta5/Pre boundary.

The Caps/Trn-mediated cell mixing appears to be required for the distal-to-proximal progression of the Ta5/Pre boundary,

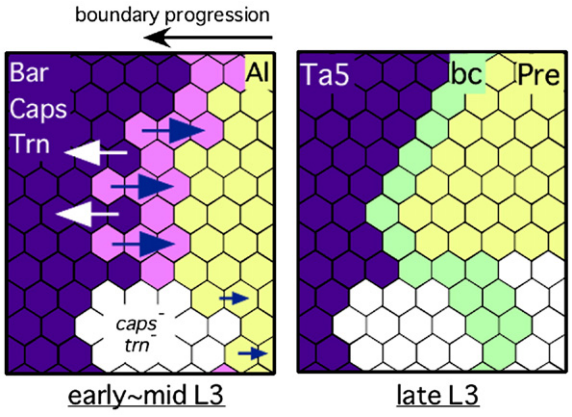

Fig. 7. A model for Ta5/Pre boundary formation in the developing leg disc. Illustrations of models for the segment boundary formation between Ta5 and Pre in the early to mid L3 (left) and late L3 (right) are shown. Bar+ cells: violet. Al+ cells: yellow. Overlapping Bar and Al expression: bright purple. Border cells: green. Gray arrow indicates progression of the Bar/Al boundary accompanied by cell fate change. Blue and white arrows show cell sorting caused by Caps and Trn cell mixing activity. In clones lacking the Caps and Trn functions (white), the cell segregation (small blue arrows) and the progression of the Bar/Al boundary was delayed.

during which Caps and Trn are gradually repressed in a distal-to-proximal direction. The phenotypes of the $Df(3L)trn^{\Delta 17}$ $caps^{C28fs}$ clones shown in Fig. 4 suggest that when the prospective Ta5/Pre boundary hit the clones from the distal side, the sudden loss of Caps and Trn activity prevents or delays the boundary progression. We speculate that this phenotype was caused by the reduction of cell affinity in the mutant clones to mix with both presumptive Ta5 and Pre cells, causing the mutant cells, retaining Ta5 identity, to segregate from both Ta5 and Pre (Fig. 4I). This change in cell affinity might delay the proximal to distal cell flow by the time Al begins expression and determines the Fas2 expression border (Fig. 7). Consistent with these phenotypes, small Pre segment and faulty junction formation were observed in the adult legs with large clones of $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ (Fig. 5). A possibility remains that the two putative genes (*CG33262* and *CG11281*) uncovered by this deficiency may also contribute the observed phenotype. However, the very low, if any, expression of those putative genes in leg disc (Table 1) makes it difficult to assess their roles in leg segmentation. We suggest that cell mixing promoted by Caps and Trn helps the boundary to sharpen and progress by selectively pulling $Caps^{+}$ Trn^{+} cells into the Ta5 region through homophilic cell affinity and by displacing the cells that have turned off Caps and Trn into the future Pre region (Fig. 7).

Roles of Caps and Trn in Ta5/Pre border refinement

The classical model of cell affinity boundary formation proposes that differential expression of cell adhesion molecules creates cell affinity boundaries where domain borders forms (Moscona and Moscona, 1952; Townes PL, 1955). Although the coincidence of Caps and Trn expression border at the Ta5/Pre border fits with this model, we found that the complete removal of those genes still allowed the expression of Fas2 (Fig. 4F), suggesting that cells at the Ta5/Pre border can turn on Fas2 expression and form the unique rectangular shape in the absence of Caps and Trn activities. The Fas2 expression is activated by Al in cells abutting Bar expressing neighbors (Kojima et al., 2000). Fas2 may stabilize the Ta5/Pre border independently of Caps and Trn functions after the border is placed at the proper position by Caps and Trn. Occasionally in $Caps^{-}$ Trn^{-} cells Fas2 expression expanded to 2–3 cells. This blurred border phenotype might indicate that the border refinement by Caps and Trn is essential for Al^{+} cells to limit Fas2 expression in a single row of cells. On the other hand, Bar mutant clones showed strong cell sorting phenotype in Ta5 (Fig. 3A). It is thus likely that Bar controls additional target genes which, together with Caps, Trn and Fas2, carry out the cell affinity boundary formation (Kojima et al., 2000), and to promote the Ta5/Pre boundary progression and sharpening.

In addition to Ta5, *caps* expression has been observed in proximal cells of pupal tarsal leg segments undergoing joint formation (Mirth and Akam, 2002). Here we observed that adult legs carrying clones of $Df(3L)trn^{\Delta 17}$, $caps^{C28fs}$ are defective in Ta5/Pre and Ta4/Ta5 joint formation. This might reflect that proper boundary formation is prerequisite for leg segmentation, or independent late roles of Caps and Trn in leg joint formation.

Further analyses of joint cell differentiation processes will be required to clarify those issues.

Our finding that Caps and Trn promote cell boundary progression and sharpening is somewhat different from the previously proposed model that these molecules mediate cell sorting at the dorsal–ventral compartment boundary of the wing disc (Milan et al., 2001, 2005). Milan et al. assessed the cell-sorting activity of Caps or Trn by using a rescue assay in which they sought to complement a defect in the DV compartment boundary formation in *ap* mutants. Although apparently sharp DV boundaries were created by the expression of Caps and Trn driven by the *ap-Gal4* driver in *ap* mutants, it is not clear whether the Caps and Trn activities revealed in this assay reflect their endogenous function in cell segregation. This is because Caps and Trn are not normally expressed in D cells at the late third instar, when the wing discs were examined in the assay; the *ap-Gal4* expression forced the Caps and Trn expression in D cells at this stage, even in *ap* mutants. Therefore, the segregation of cells at the DV boundary may simply reflect the cell-sorting effect of ectopic overexpression of Caps and Trn. In addition, *caps trn* double mutant clones did not alter wing patterning except for cell round up phenotypes that were observed in both D and V compartment (Milan et al., 2002). We therefore suggest that Caps and Trn may act primarily to help cells to locate appropriate place according to their positional identity by maintaining homophilic cell affinity. Their roles in the progression of Ta5/Pre boundary uncovered in this work and in medio-lateral cell positioning in the wing disc (Milan et al., 2002) can be understood in this context.

A large number of LRR proteins are encoded in the genome of higher metazoans. It would be interesting to investigate whether cell mixing and cell displacement also play active roles in other cases of cell boundary progression by helping cells to create and maintain sharp boundaries.

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