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Distinct functions of the leucine-rich repeat transmembrane proteins Capricious and Tartan in the *Drosophila* tracheal morphogenesis

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Abstract

A key step in organogenesis of the *Drosophila* tracheal system is the integration of isolated tracheal metameres into a connected tubular network. The interaction of tracheal cells with surrounding mesodermal cells is crucial in this process. In particular, single mesodermal cells called bridge-cells are essential for the guided outgrowth of dorsal trunk branches to direct formation of the main airway, the dorsal trunk. Here, we present evidence that the two leucine-rich repeat transmembrane proteins Capricious and Tartan contribute differently to the formation of branch interconnections during tracheal development. Capricious is specifically localized on the surface of bridge-cells and facilitates the outgrowing dorsal trunk cells of adjacent metameres toward each other. We show that Capricious requires both extracellular and intracellular domains during tracheal branch outgrowth. In contrast, Tartan is expressed broadly in mesodermal cells and exerts its role in tracheal branch outgrowth through its extracellular domain. We propose that Capricious contributes to the instructive role of bridge-cells whereas Tartan provides permissive substrate for the migrating tracheal cells during the network formation.

Keywords: *Drosophila*; *capricious*; *tartan*; Bridge-cell; Tracheal development

Introduction

Cell motility and tissue movements are crucial in many biological processes including organ development of multicellular animals. The migrating cells recognize external cues and substrates in order to move in an organized pattern along distinct paths. Cell surface proteins are central in receiving and transferring the external signals provided by long-range acting chemoattractants and/or short-range cell-to-cell communication.

The *Drosophila* tracheal system is a particularly traceable in vivo model for studying molecular and cellular mechanisms of directed cell movements. The development of the tracheal system initiates with differentiation of ectodermal cells in ten isolated clusters at each side of the embryo. Cell movements, which are strictly coordinated along or across the surrounding tissues, lead to stereotypical branch outgrowth from these clusters. Finally, fusion of branches from adjacent cell clusters results in interconnection of separated units into a three-dimensional network of tubes (Manning and Krasnow, 1993; Samakovlis et al., 1996a,b).

The initial outgrowth of tracheal cells from the clusters is triggered by *branchless* (*bnl*), a gene encoding a *Drosophila* FGF homolog (dFGF/Bnl). Bnl is expressed dynamically in small groups of cells surrounding the tracheal primordia and acts as a chemoattractant that guides outgrowth of primary tracheal branches (Sutherland et al., 1996; Metzger and Krasnow, 1999). In addition to the Bnl-signaling, a cellular guidance mechanism is essential for normal outgrowth of dorsal trunk branches. This guidance requires single mesodermal cells called bridge-cells,

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which are positioned posteriorly next to the tracheal cell clusters and serve as guidance posts for the outgrowing dorsal trunk branches (Wolf and Schuh, 2000). Initially, filopodia-like extensions from anterior and posterior dorsal trunk cells get in touch with the bridge-cells, slide along the bridge-cell surface and contact the opposite extensions (Wolf et al., 2002). The dorsal trunk fusion process then starts with deposition of the epidermal adhesion protein DEcadherin (DEcad) at the contact point between the two fusion cells (Tanaka-Matakatsu et al., 1996). Localized at the adherens junctions, DEcad interacts with α and β-catenin (*armadillo*) that subsequently bind the actin cytoskeleton. A cytoskeleton-associated plakin Short Stop (*shot*), which interacts with both actin and microtubules, is required for DEcad accumulation at the fusion site (Lee and Kolodziej, 2002). Finally, each of fusion cells forms an intracellular tube and the two lumens fuse and become continuous.

The molecular mechanisms underlying the initial cell-to-cell contacts between the extending dorsal trunk cells and the guiding bridge-cells are not yet known. The transcription factor Hunchback (Hb) was shown to play a key role in bridge-cell differentiation, which in turn is necessary for dorsal trunk fusion (Wolf and Schuh, 2000). In addition, a recent study showed that the transcription factors Extradenticle (Exd) and Homothorax (Hth) are also expressed in the bridge-cells (Merabet et al., 2005). However, neither of these transcription factors nor Bnl may be directly involved in the recognition of bridge-cells by tracheal extensions. Thus, it has been speculated that this process is mediated by extracellular matrix and adhesion molecules that are expressed on the surfaces of tracheal and bridge cells (Wolf et al., 2002).

Here, we show that a cell adhesion molecule Capricious (Caps), which represents a transmembrane protein containing the leucine-rich repeat (LRR) motifs (Shishido et al., 1998), is specifically expressed in the bridge-cells and is involved in the formation of dorsal trunk branches. Analysis of both loss- and gain-of-function mutations indicates that Caps function is important for the bridge-cells to guide efficiently the extending dorsal trunk cells along their surface. In contrast to Caps, another cell adhesion protein Trn, which is highly related to Caps and also consists of LRR motifs (Milan et al., 2001), is expressed broadly in the muscle founder cells. Loss- and gain-of-function phenotypes of *trn* mutants suggest that Trn may provide a permissive substrate for the migrating dorsal and lateral trunk cells. Interestingly, protein domain swap experiments demonstrate that the extracellular domains of both proteins are largely functionally equivalent while their intracellular domains are distinct during tracheal development. Whereas the intracellular domain of Trn is dispensable for its function, the intracellular domain of Caps is essential and contributes to the function of bridge-cells during tracheal pathfinding.

Materials and methods

Screening of P-element strains

2460 lethal *P*-element *Drosophila* strains from the Szeged Stock Center (Deak et al., 1997) were screened as described previously (Samakovlis et al., 1996a) for expression in metamerically repeated cells. The *P*-insertion sites of such strains were localized by plasmid rescue and sequenced to identify the responsible genes (Hemphälä et al., 2003).

Fly stocks

The following mutant alleles and fly strains were used: $caps^{l(3)02937}$ (Carrera et al., 1998), $caps^{l(3)05121}$ (Abrell and Jäckle, 2001), $caps^{65.2}$, *caps-lacZ*, (Shishido et al., 1998), $trn^{28.4}$, $trn^{\Delta 2.9}caps^{65.2}$ double mutant (Milan et al., 2001) and *trn-lacZ* (Chang et al., 1993). The tracheal cells were labeled by staining cytoplasmic β-Gal in the *lacZ* enhancer trap line *1-eve-1* (Perrimon et al., 1991). To drive *GAL4* expression in mesodermal cells, we used *twi-GAL4* (Greig and Akam, 1993). For misexpression studies *UAS-caps* (Shishido et al., 1998), $UAS\text{-}caps^{Ed}$, $UAS\text{-}caps^{Id}$ (Taniguchi et al., 2000), *UAS-trn* (Milan et al., 2001), $UAS\text{-}trn^{Ed}$ (Milan et al., 2002), or *UAS-lacZ* were used. All fly strains were bred at 25°C.

Transgene expression constructs

$UAS\text{-}caps^{Ed}trn^{Id}$ and $UAS\text{-}trn^{Ed}caps^{Id}$ were generated by introducing $caps^{Ed}trn^{Id}$ and $trn^{Ed}caps^{Id}$ hybrid constructs into pUAST (Brand and Perrimon, 1993). $Caps^{Ed}Trn^{Id}$ consists of extracellular domain of Caps (aa 1–470) and intracellular domain of Trn (aa 469–737) while $Trn^{Ed}Caps^{Id}$ contains extracellular domain of Trn (aa 1–492) and intracellular domain (aa 487–532) of Caps. In order to generate $UAS\text{-}caps^{\Delta RH}$, $UAS\text{-}caps^{\Delta Y}$ and $UAS\text{-}caps^{\Delta PDZ}$ strains, RH (aa 478–479), Y (aa 507) and VTEL (aa 537–540) in Caps intracellular domain were replaced with glycine or stop codon through site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene) and inserted into pUAST vector. The transgene constructs were verified by sequencing and used for germline transformation. Finally, in vivo expression was confirmed by in situ hybridization and antibody staining of transgene embryos. Upon ectopic expression, the transgene constructs reveal membrane associated expressions of modified Caps or Trn protein that are indistinguishable from wild-type Caps and Trn localization, respectively (see Supplement Material Fig. 1). Ectopic expression of $Caps^{\Delta PDZ}$ could not be confirmed with the given anti-Caps antibody. However, the correct expression and localization of $Caps^{\Delta PDZ}$ cannot be ruled out because the anti-Caps antibody was produced against a protein sequence (AAGGYPYIAGNSRMI-PVTEL) that contains the PDZ sequence and thus may fail to detect Caps lacking the PDZ sequence (Shishido et al., 1998). We speculate that $Caps^{\Delta PDZ}$ is normally expressed since we observe an ectopic phenotype after $Caps^{\Delta PDZ}$ expression (Fig. 6B). For further analysis, only homozygous viable P-element insertion lines were used. Also, two independent P-element insertion lines of each transgene construct were examined to exclude phenotypes caused by the P-element integration sites.

Statistical analysis

For the analysis of tracheal phenotypes, at least three independent antibody stainings were performed. The interruptions in dorsal and lateral trunks were counted at least two independent times. The total number of interruptions were divided by total number of possible connections (18 in each embryo) and converted to the percentage of total connections. The rescued interruption rates were calculated by dividing the interruption rates by the interruption rate of $trn^{28.4}$ mutant embryos. N1 and N2 describe two separate countings of embryos or two independent P-element insertion lines of a single transgene construct. The average of N1 and N2 and the respective standard deviation (depicted by ± and error bar in the charts) are presented throughout this study.

Immunostaining and in situ hybridization

Immunostainings and RNA in situ hybridizations with whole-mount embryos were performed as previously described (Goldstein and Freyberg, 1994). Primary antibodies used were the following: monoclonal antibody 2A12 (DSHB, Iowa) to visualize tracheal lumen, anti-Caps (kindly provided by S.M. Cohen; Milan et al., 2001), anti-Trn (kindly provided by A. Laughon; Chang et al., 1993), anti-β-Galactosidase (Promega), anti-Hb (Wolf et al., 2002), anti-

digoxigenin-AP, anti-fluorescein-AP, anti-digoxigenin-POD and anti-fluorescein-POD (Roche). Secondary antibodies used were the following: biotinylated goat anti-mouse IgM (Jackson), biotinylated horse anti-mouse IgG, biotinylated goat anti-rabbit IgG, AP coupled goat anti-rabbit IgG (Vector Laboratories), goat anti-mouse IgG-Cy3, goat anti-rabbit IgG-Cy3, goat anti-mouse IgG-Cy2, goat anti-rabbit IgG-Cy2, goat anti-guinea pig-Cy3 (Molecular Probes). The antisense RNA probes were generated from cDNAs of *caps* (Shishido et al., 1998), *trn* (EST GH10871), *hb* (EST LD34229) and *btl* (Glazer and Shilo, 1991). The ESTs were ordered from ResGen. Vectastain® ABC elite kit or ABC-AP kit (Vector Laboratories) and/or Tyramide Signal Amplification (NEN) were used to enhance signal detection.

DNA sequence alignments

The *Anopheles gambiae* gene *agCP14407* was identified as a Caps homologous gene by using the blastp program at NCBI (Altschul et al., 1990). The alignment using the lalign program (Myers and Miller, 1989) was used to align Caps and agCP14407. The REP search program (Andrade et al., 2000) was used to search for repeats in agCP14407. In addition, the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to detect putative transmembrane domains.

Results

Capricious is specifically expressed in the bridge-cell

In order to identify genes that are distinctively expressed in the bridge-cells, we screened 2460 lethal *P*-element containing *Drosophila* strains from the Szeged Stock Center (Deak et al., 1997) for reporter gene expression in the bridge-cells (see Materials and methods). Embryos from one of the *P*-element lines showed β-Galactosidase (β-Gal) expression in metamerically repeated cells at the presumptive bridge-cell positions (Fig. 1A). Sequencing of genomic DNA flanking the *P*-element revealed a gene called *capricious* (*caps*), which encodes a transmembrane protein with 14 leucine-rich repeat (LRR) motifs in the extracellular domain (Shishido et al., 1998). These LRR motifs are frequently found in cell adhesion molecules and may mediate cell-to-cell interactions (Kobe and Deisenhofer, 1994). Previous work showed that *caps* affects the target specificity of muscle 12 motorneurons during innervation in larva (Shishido et al., 1998) and it mediates layer-specific photoreceptor targeting in the visual system (Shinza-Kameda et al., 2006). Caps has been also shown to contribute to the affinity boundary formation between the dorsal and the ventral compartments during wing development (Milan et al., 2001). It was speculated that Caps exerts these functions by interacting with cell surface molecules of neighboring cells (Shishido et al., 1998; Milan et al., 2001).

In situ hybridization with *caps* antisense probe revealed that endogenous *caps* is expressed in single cells in each metamere as it was observed in *caps-lacZ* embryos (data not shown; see Fig. 1A). Further analysis of *caps* expression in embryos harboring a specific tracheal marker (*1-eve-1*; see Materials and methods) revealed *caps* expression specifically in single cells that are positioned at the posterior lateral side of each tracheal metamere (Fig. 1B). This distinct expression disappears during stage 13 when dorsal trunk cells fuse with each other (data not shown). Earlier work demonstrated that nuclear *lacZ* expression in *caps-lacZ* enhancer trap line E2-3-27 coincides with the expression pattern of endogenous *caps* (Shishido et al., 1998). Thus, *caps-lacZ* (E2-3-27) embryos were double stained with anti-Hb antibody to label the bridge-cells and with anti-β-Gal antibody to determine *caps* expression to verify that the metamerically repeated cells expressing *caps* are indeed the bridge-cells (Figs. 1C–E). During stage 11, Hb is expressed not only in the bridge-cells but also in the more ventrally positioned anchor-cells (Fig. 1C; Wolf and Schuh, 2000). Colocalization of Hb and β-Gal is found in the bridge-cells but not in the anchor-cells, demonstrating that Caps is expressed selectively in the bridge-cells (Figs. 1D, E).

Caps contributes to the formation of dorsal trunk

Lack of bridge-cell function disrupts formation of a continuous dorsal trunk (Wolf and Schuh, 2000). In order to determine whether *caps* is required for the bridge-cell function, the tracheal system development of various *caps* mutant alleles ($caps^{l(3)02937}$, $caps^{l(3)05121}$ and $caps^{65.2}$) was investigated. Embryos of these *caps* alleles reveal a discontinuous dorsal trunk formation (compare Figs. 1F with G). We noticed that the frequency of interruptions vary among the embryos. To analyze the tracheal phenotype more accurately, we counted interruptions in the dorsal and the lateral trunk and represented them as percentage of all possible fusions. All three *caps* mutant alleles revealed similar rate of dorsal and lateral trunk interruptions and the interruption rate in $caps^{65.2}$ embryos is shown representatively (Fig. 1H). We observed only 0.2% and 0.4% interruptions in the dorsal trunks of wild-type embryos at stage 14 and during later stages, respectively, while we detected 20.4% and 12.4% dorsal trunk interruptions in $caps^{65.2}$ mutants at the corresponding stages. The significant variation in the severity of dorsal trunk phenotypes implies that the extension of dorsal trunk cells towards the corresponding targets may be stalled rather than be completely inhibited when Caps is lacking. In contrast, lateral trunk breaks were not significantly increased in *caps* mutants (5.8%) compared to wild-type embryos (4.1%) during stage 14 to 16.

Previous study revealed that Hb expression is essential for the viability of bridge-cells (Wolf and Schuh, 2000). In mutant embryos lacking *hb*, the bridge-cells undergo apoptosis and the dorsal trunk cells cannot be guided efficiently. Consequently, discontinuous dorsal trunks are formed. As *caps* mutant embryos revealed similarly disrupted dorsal trunks, we asked whether this phenotype is also result of apoptotic bridge-cells. Hb is expressed in the bridge-cells of *caps* mutant embryos from stage 10 to 13 as found in wild-type embryos indicating that the viability of bridge-cells is not affected in *caps* mutants (Fig. 1I). Furthermore, it was recently shown that *bnl* is specifically expressed in the bridge-cells (Merabet et al., 2005). Thus, we analyzed *bnl* expression in *caps* mutant embryos and found that *bnl* is normally expressed in the bridge-cells of those embryos (Figs. 1J–L). In summary, the interruptions in the dorsal trunk despite the viable bridge-cells and the normal *bnl* expression in *caps* mutant embryos suggest that *caps* function is important for the bridge-cells to properly guide the dorsal trunk cells during tracheal morphogenesis.

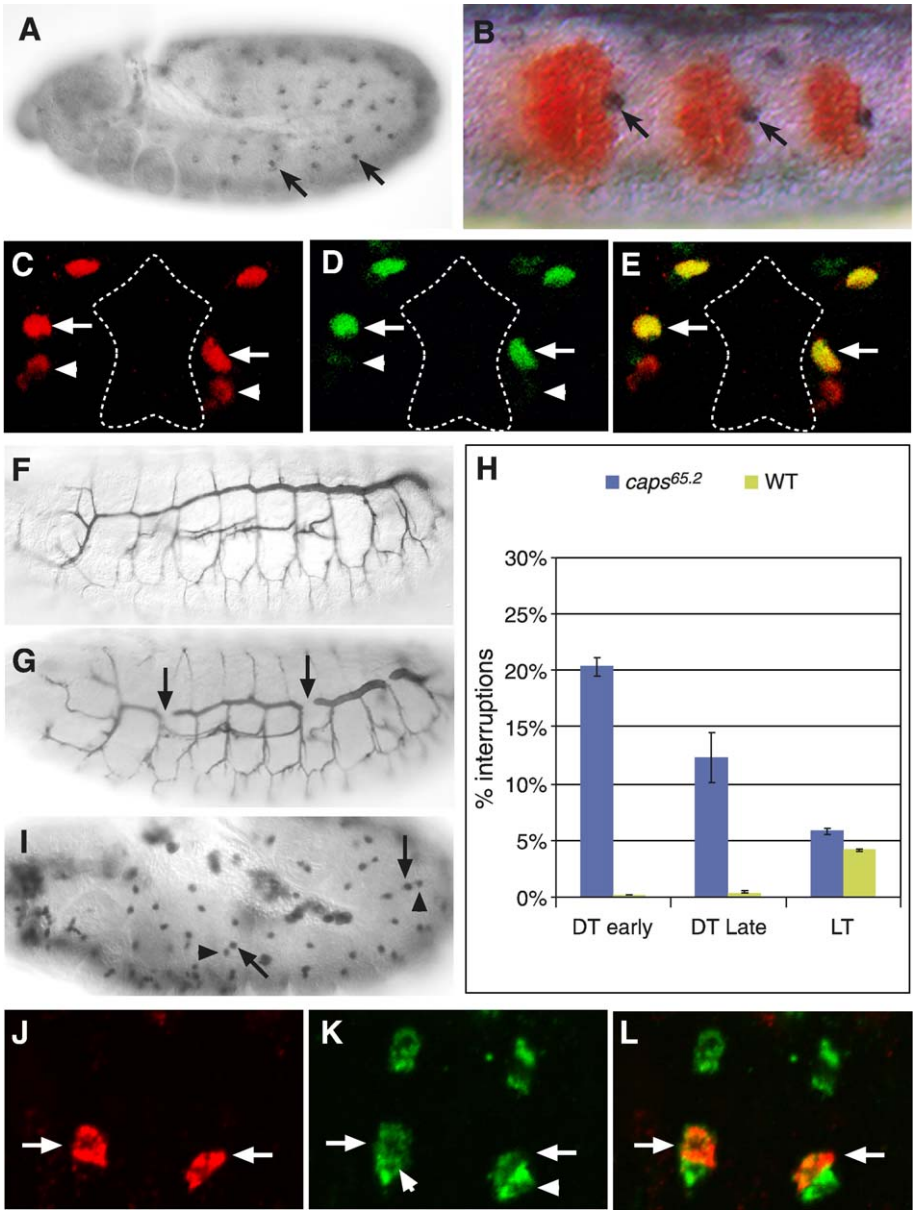

Fig. 1. Selective expression of *caps* in the bridge-cell is important for dorsal trunk formation. (A) *caps-lac*Z embryos were stained with anti-β-Gal antibody. A stage 10 embryo shows specific expression of β-Gal in metamerically repeated cells (arrows) on the lateral side of the embryo. (B) *1-eve-1* enhancer trap embryos were stained with anti-β-Gal antibody (red) to visualize the tracheal cells. The embryos were also hybridized in situ with *caps* antisense RNA (blue). A stage 10 embryo reveals that *caps* expressing cells are directly adjacent to the posterior lateral side of each tracheal metamere (arrows). (C–E) *caps-lac*Z embryos were stained with anti-β-Gal (green) and anti-Hb (red) antibodies. The white dotted lines indicate the presumptive positions of the tracheal metameres during stage 12. C. A stage 12 embryo shows Hb expression (red) in the bridge-cells (arrows) and the more ventral anchor-cells (arrowheads). (D) The same embryo shows β-Gal expression (green), which reveals Caps expression in the bridge-cells (arrows) but not in the anchor-cells (arrowheads). (E) The overlay of green and red channels reveals colocalization of β-Gal (Caps) and Hb selectively in the bridge-cells (arrows). (F, G) Wild-type (F) or $caps^{65.2}$ embryos (G) were stained with the 2A12 antibody to visualize tracheal lumen. (F) A wild-type embryo at stage 14 shows a continuous dorsal trunk. (G) In contrast, a $caps^{65.2}$ embryo at stage 14 reveals gaps in the dorsal trunk at several positions (arrows). Other tracheal branches appear to be as in wild-type embryos. (H) Interruptions of fusion points in $caps^{65.2}$ and wild-type embryos were counted for the dorsal trunks at early and late stages and for the lateral trunks. Early stage is defined as stage 14. Late stage is defined as later than stage 14. For all figures, the following abbreviations are used: DT early, dorsal trunk in early stage embryos; DT late, dorsal trunk in late stage embryos; LT, lateral trunk; N, total number of fusion points counted. Homozygous $caps^{65.2}$ mutant embryos: DT early = 20.4% ± 0.8% (N1 = 918, N2 = 702), DT late = 12.4% ± 2.2% (N1 = 1008, N2 = 990), LT = 5.8% ± 0.4% (N1 = 378, N2 = 396). Wild-type (OreR) embryos: DT early = 0.2% ± 0% (N1 = 558, N2 = 540), DT late = 0.4% ± 0.1% (N1 = 594, N2 = 576), LT = 4.1% ± 0.1% (N1 = 594, N2 = 612). Note: Homozygous mutant embryos of $caps^{l(3)02937}$ and $caps^{l(3)05121}$ reveal tracheal interruptions as found for $caps^{65.2}$. (I) $caps^{65.2}$ mutant embryos were stained with anti-Hb antibody to label the bridge-cells. A stage 12 $caps^{65.2}$ embryo reveals Hb expression in the bridge-cells (arrows) as well as in the anchor-cells (arrowheads), similarly as in the wild-type embryos. (J–L) $caps^{65.2}$ embryos were hybridized in situ with *bnl* antisense RNA (red), *hb* antisense RNA (green) and *lac*Z antisense RNA (not shown) to identify the homozygous *caps* mutant embryos. (J) A homozygous $caps^{65.2}$ mutant embryo at stage 12 reveals *bnl* expression in the bridge-cells (arrows). (K) Normal *hb* expression is detected in the bridge-cells (arrows) as well as in the anchor-cells (arrowheads). (L) The overlay reveals coexpression of *bnl* and *hb* in the bridge-cells (arrows).

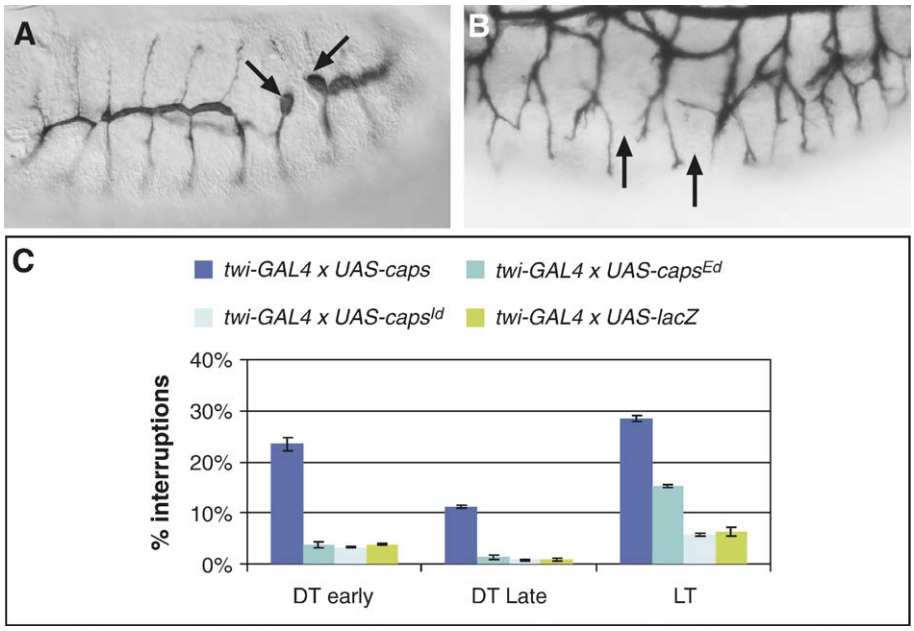

Fig. 2. Misexpression of *caps* in mesodermal cells results in disrupted dorsal trunk and lateral trunk formation. (A, B) Embryos carrying *UAS-caps* and *twi-GAL4* were stained with the 2A12 antibody to visualize the tracheal lumen. (A) A whole-mount embryo at stage 14 reveals dorsal trunk interruptions at several fusion points. Some of the truncated dorsal trunk branches appear in abnormal directions (arrows) instead of fusing with their adjacent targets. (B) A whole-mount embryo at stage 15 also shows a discontinuous lateral trunk (arrows indicate lateral trunk interruptions). (C) Interruptions of fusion points were counted for the dorsal trunks at early and late stages and for the lateral trunks. Embryos bearing *twi-GAL4* and *UAS-caps*: DT early = 23.5% ± 1.3% (N1 = 594, N2 = 576), DT late = 11.2% ± 0.4% (N1 = 576, N2 = 612), LT = 28.4% ± 0.6% (N1 = 594, N2 = 504). Embryos bearing *twi-GAL4* and *UAS-caps^{Ed}*: DT early = 3.7% ± 0.6% (N1 = 486, N2 = 414), DT late = 1.4% ± 0.5% (N1 = 468, N2 = 486), LT = 15.2% ± 0.3% (N1 = 396, N2 = 522). Embryos bearing *twi-GAL4* and *UAS-caps^{Id}*: DT early = 3.2% ± 0.2% (N1 = 504, N2 = 450), DT late = 0.7% ± 0.2% (N1 = 504, N2 = 450), LT = 5.8% ± 0.3% (N1 = 468, N2 = 468). Embryos bearing *twi-GAL4* and *UAS-lacZ*: DT early = 3.8% ± 0.2% (N1 = 576, N2 = 414), DT late = 0.8% ± 0.2% (N1 = 414, N2 = 486), LT = 6.4% ± 0.8% (N1 = 486, N2 = 432).

Next, we used the UAS-GAL4 system (Brand and Perrimon, 1993) to investigate whether ectopic *caps* expression in mesodermal cells would interfere with the normal dorsal trunk branch outgrowth. Similarly as in *caps* mutant embryos, the dorsal trunk is interrupted at several positions in embryos expressing ectopic *caps* through *twi-GAL4* (Fig. 2A; compare with Fig. 1G). Occasionally, we observe some dorsal trunk branches in abnormal directions rather than in the normal anterior–posterior direction (arrows in Fig. 2A). Interestingly, ectopic Caps expression affects the outgrowth of not only the dorsal trunk cells but also the lateral trunk cells. When Caps is overexpressed in mesodermal cells surrounding the lateral trunk, a discontinuous lateral trunk is formed (Fig. 2B). Significantly lower interruption rates in the embryos carrying *twi-Gal4* and *UAS-lacZ* demonstrate that the phenotype of ectopic *caps* expression is not an intrinsic effect of the *UAS-GAL4* system (Fig. 2C). Once again, we observe fewer breaks in the dorsal trunk at late stages than at early stages (11.2% compared to 23.5%). Previous studies of Caps in boundary formation during wing disc morphogenesis revealed that Caps requires only its extracellular domain (Ed) for its function (Milan et al., 2002). Thus, we asked whether the intracellular domain (Id) and/or the Ed of Caps is sufficient to mediate Caps function during tracheal development as well. We therefore expressed two deletion constructs lacking either the Id or the Ed of Caps in mesodermal cells and analyzed their effects on the tracheal development. When either the Id or the Ed of Caps is lacking, normal development of the tracheal system was observed. For example, while the misexpression of complete Caps in the mesodermal cells resulted in 23.5% interruptions in the dorsal trunk of early stage embryos, only 3.7% or 3.2% interruptions were observed in the embryos misexpressing either the Ed or the Id of Caps, respectively (Fig. 2C). These observations indicate that ectopic Caps interferes with normal migration of tracheal cells so that the dorsal and the lateral trunk branches fail to interconnect properly. Furthermore, both Id and Ed are required for ectopic Caps function during tracheal morphogenesis.

Mesodermal Tartan expression is essential for normal formation of dorsal and lateral trunk

Caps shares high sequence homology with another cell adhesion molecule named Tartan (Trn). It was demonstrated that both proteins share redundant function in the formation of affinity boundary between dorsal and ventral compartments during wing imaginal disc development (Milan et al., 2001). To test a possible role of *trn* during tracheal formation, we analyzed branch interconnections in *trn* mutant embryos. Similar to *caps*, *trn* mutants reveal discontinuous dorsal trunks (Fig. 3A). Furthermore, *trn* and *caps* mutants show a comparable rate of interruptions in the dorsal trunk in early as well as late stages. For example, *caps* mutants contain 20.4% interruptions in the dorsal trunks at early stages, while *trn* mutants reveal 20.5% interruptions (Fig. 3B). However, whereas *caps* mutants form normal lateral trunks, *trn* embryos reveal 21.7% disconnections

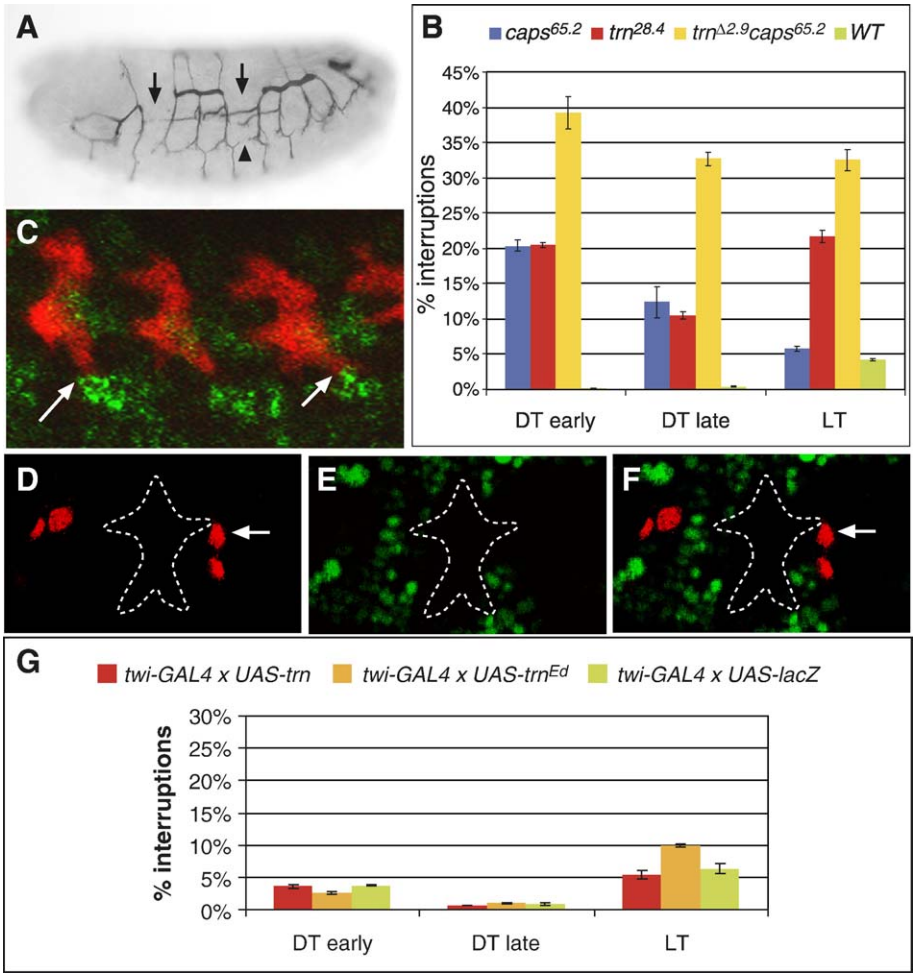

Fig. 3. Mesodermal *tartan* expression contributes to normal formation of the tracheal system. (A) A stage 14 $trn^{28.4}$ mutant embryo stained with the 2A12 antibody reveals gaps in the dorsal trunk (arrows) and the lateral trunk (arrowhead). (B) Interruptions of tracheal fusion points were counted in $caps^{65.2}$, $trn^{28.4}$, $trn^{\Delta 2.9}caps^{65.2}$ double mutant and wild-type embryos. Homozygous $caps^{65.2}$ mutant embryos: see Fig. 1H. Homozygous $trn^{28.4}$ mutant embryos: DT early = 20.5% ± 0.3% (N1 = 774, N2 = 810), DT late = 10.5% ± 0.5% (N1 = 720, N2 = 1476), LT = 21.7% ± 0.9% (N1 = 612, N2 = 684). $trn^{\Delta 2.9}caps^{65.2}$ double mutant embryos: DT early = 39.2% ± 2.3% (N1 = 540, N2 = 522), DT late = 32.7% ± 0.9% (N1 = 342, N2 = 306), LT = 32.5% ± 1.5% (N1 = 360, N2 = 378). Wild-type embryos: see Fig. 1H. C. *1-eve-1* embryos were hybridized in situ with *trn* antisense RNA (green) to label *trn* expression and then stained with anti-β-Gal antibody (red) to mark the tracheal cells. A whole-mount embryo at stage 12 shows that *trn* is expressed in mesodermal cells adjacent to the tracheal metameres. Some lateral trunk cells appear to be in direct contact with *trn* expressing cells (arrows). (D–F) *trn-lacZ* embryos were stained fluorescently with anti-β-Gal (green) and anti-Hb (red) antibodies. The white dotted lines indicate the presumptive positions of tracheal metameres during stage 12. (D) A whole-mount embryo at stage 12 shows Hb expression in the bridge-cells (arrows). (E) The same embryo reveals β-Gal expression in a large number of mesodermal cells (muscle founder cells). (F) No colocalization of β-Gal and Hb is detectable in the bridge-cells (arrow) and other mesodermal cells, indicating lack of Hb and Trn coexpression in mesodermal cells. (G) Interruptions of tracheal fusion points in embryos misexpressing *trn*, trn^{Ed} or *lacZ* were counted. Embryos bearing *twi-GAL4* and *UAS-trn*: DT early = 3.6% ± 0.4% (N1 = 522, N2 = 450), DT late = 0.7% ± 0% (N1 = 450, N2 = 468), LT = 5.3% ± 0.6% (N1 = 450, N2 = 468). Embryos carrying *twi-GAL4* and *UAS-*trn^{Ed}: DT early = 2.5% ± 0.2% (N1 = 378, N2 = 468), DT late = 1.0% ± 0.1% (N1 = 450, N2 = 468), LT = 9.9% ± 0.2% (N1 = 378, N2 = 396). Embryos bearing *twi-GAL4* and *UAS-lacZ*: see Fig. 2C.

in lateral trunks (Figs. 3A, B). Since both *caps* and *trn* mutants show discontinuous dorsal trunks, we asked whether the functions of *caps* and *trn* are synergistic or additive. The interruption rates in dorsal and lateral trunks of $trn^{\Delta 2.9}caps^{65.2}$ double mutant embryos are close to the sum of interruption rates in *trn* or *caps* single mutant embryos (Fig. 3B) and suggest that *caps* and *trn* function additively. Since both *caps* and *trn* mutants demonstrate interrupted dorsal trunks, we asked whether *trn* is also expressed in the bridge-cells. In contrast to *caps*, *trn* is expressed in metamerically repeated clusters of founder cells located laterally between the tracheal metameres during early tracheal development (Artero et al., 2003, Fig. 3C). The immediate juxtaposition between the tracheal extensions and the muscle founder cells expressing *trn* suggests a direct contact between these cells (Fig. 3C). In order to determine whether the bridge-cells are included in these clusters of cells, *trn-lacZ* embryos, which reveal β-Gal expression in *trn* patterns (data not shown; Chang et al., 1993), were double labeled with anti-Hb and anti-β-Gal antibodies. Interestingly, our results indicate that *trn* is not expressed in the bridge-cells, suggesting that *trn* contributes to dorsal trunk formation through a different mechanism than *caps* (Figs. 3D–F). Overexpression of *trn* in mesodermal cells further demonstrates that *trn* functions distinctively from *caps*. Whereas embryos ectopically expressing *caps* reveal more than 20% interruptions in dorsal and lateral trunks (Fig. 2C), embryos overexpressing *trn* or Trn

extracellular domain (Trn^{Ed}) only show interruption rates which are comparable to that found in control embryos overexpressing *lac*Z (Fig. 3G).

Thus, although function of *caps* and *trn* overlaps during wing development, their roles differ during tracheal development. First, we observe a similar dorsal trunk phenotype in *caps* and *trn* mutants, but we detect lateral trunk breaks only in *trn* mutant embryos. Second, *caps* and *trn* are expressed in different patterns: *caps* is visible distinctively in the bridge-cells whereas *trn* is detectable in founder cells but not in the bridge-cells. Finally, ectopic expression of *caps* in mesodermal cells interferes with dorsal trunk formation. In contrast, *trn* overexpression does not affect tracheal development. These results suggest that restricted expression of Caps contributes to the bridge-cell function while the broad mesodermal expression of Trn supports the outgrowth of tracheal branches.

The extracellular domains of Caps and Trn provide similar function during tracheal morphogenesis

Since mesodermal overexpression of *trn* did not influence normal function of *trn*, we investigated whether it can compensate for the tracheal defects in *trn* mutants. As expected, the interruption rates in dorsal trunk could be effectively rescued (Fig. 4). If Trn acts as a cell adhesion molecule whose major function is to make a direct contact with other cell adhesion molecules on the surface of dorsal trunk cells, then its Ed might be sufficient for the normal role during tracheal morphogenesis. Our observation that the ectopic mesodermal Trn expression rescues the *trn* tracheal phenotype prompted us to analyze whether the Ed including the transmembrane region of Trn would be sufficient to rescue *trn* tracheal dorsal trunk defects. Indeed, overexpression of Trn^{Ed} in mesodermal cells efficiently rescues the dorsal trunk breaks of *trn* mutants (Fig. 4). This result implies that Trn Ed is sufficient for the proper function of Trn during dorsal trunk development.

Alignment of Caps and Trn amino acid sequences revealed that most amino acid residues comprising the extracellular domains and the transmembrane domains in Caps and Trn are either similar or identical. However, the intracellular domains are distinct and lack any considerable similarity. Since the Trn Ed could rescue the tracheal phenotype in *trn* mutants and is similar to Caps Ed, we asked whether Caps Ed would also be able to rescue the tracheal defect in *trn* mutants. Thus, we examined Caps, $Caps^{Id}$ and $Caps^{Ed}$ for their abilities to rescue *trn* tracheal phenotype by overexpressing the corresponding constructs in *trn* mutant embryos. We found that 47.5% of the *trn* dorsal trunk defects could be rescued by expressing the $Caps^{Ed}$ construct, compared to 92.4% rescue by complete *trn* (Fig. 4). This result implies that Caps extracellular domain is competent to substitute partially for Trn function during dorsal trunk formation. In contrast, when Caps or $Caps^{Id}$ was overexpressed in *trn* mutant embryos, the defects in dorsal trunk formation could not be rescued but instead, became even more severe (Fig. 4). These results infer that the extracellular

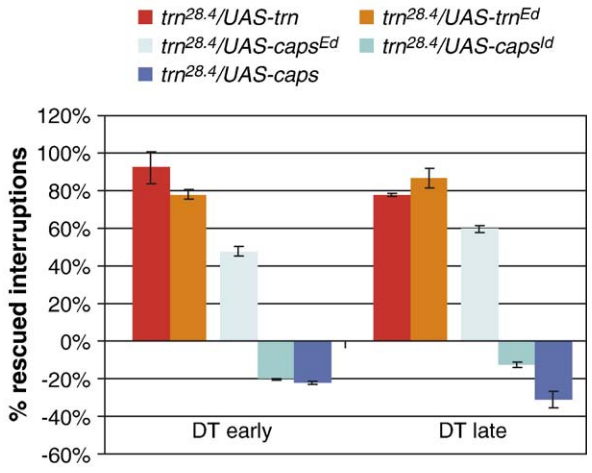

Fig. 4. The extracellular domains of Caps and Trn reveal similar function during tracheal morphogenesis. Interruptions of tracheal branch fusion in $trn^{28.4}$ embryos carrying various *caps* and *trn* constructs were counted and compared to the interruptions in $trn^{28.4}$ mutant embryos (20.5%) and presented in percentage of rescued interruptions. $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-trn*: DT early = 92.4% ± 8.4% (N1 = 756, N2 = 1152), DT late = 77.6% ± 0.9% (N1 = 702, N2 = 954). $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-*trn^{Ed}: DT early = 78.1% ± 2.6% (N1 = 432, N2 = 414), DT late = 86.6% ± 5.2% (N1 = 396, N2 = 504). $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-*$caps^{Ed}$: DT early = 47.5% ± 2.6% (N1 = 558, N2 = 666), DT late = 59.9% ± 1.9% (N1 = 936, N2 = 576). $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-*$caps^{Id}$: DT early = −20.4% ± 0.7% (N1 = 522, N2 = 558), DT late = −12.7% ± 1.5% (N1 = 522, N2 = 504). $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-caps*: DT early = −22.4% ± 0.9% (N1 = 576, N2 = 558), DT late = −31.1% ± 4.7% (N1 = 576, N2 = 576).

domain of either Caps or Trn can mediate *trn* function during dorsal trunk development.

The intracellular domain specifies caps function

Loss- and gain-of-function experiments clearly demonstrate that Caps and Trn play different roles during tracheal development and it seems unlikely that the extracellular domains of both proteins provide such differences. In order to gain more insight into what dictates the functional specificity of Caps and Trn, two different hybrid proteins consisting of either the Ed (aa 1–470) of Caps and the Id (aa 469–737) of Trn ($Caps^{Ed}Trn^{Id}$) or the Ed (aa 1–492) of Trn and the Id (aa 487–532) of Caps ($Trn^{Ed}Caps^{Id}$) were constructed (Fig. 5A). We investigated whether these hybrid proteins could rescue the tracheal defects of *trn* mutants. Overexpression of $Caps^{Ed}Trn^{Id}$ in *trn* mutant embryos rescued the tracheal defects. In contrast, $Trn^{Ed}Caps^{Id}$ failed to rescue the dorsal trunk interruptions and instead enhanced the tracheal phenotype of *trn* mutants, i.e., such embryos revealed 11.2% more dorsal trunk interruptions than *trn* mutants (Fig. 5B). Even though the $Trn^{Ed}Caps^{Id}$ hybrid protein contains the complete extracellular domain of Trn, it not only fails to rescue *trn* deficiencies in tracheal development, but rather aggravates the dorsal trunk fusion defect. These data demonstrate that the $Trn^{Ed}Caps^{Id}$ hybrid protein functions similar to Caps, indicating that the Id of Caps most likely mediates its functional specificity.

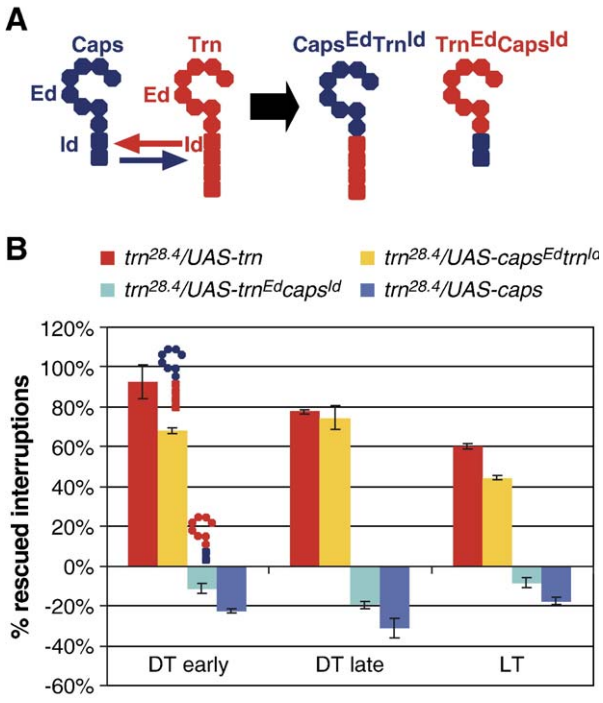

Fig. 5. The intracellular domain of Caps mediates functional specificity. (A) Generation of hybrid proteins is schematically shown. The Id of Trn is fused to the Ed of Caps to generate $Caps^{Ed}Trn^{Id}$ hybrid protein. Similarly, $Trn^{Ed}Caps^{Id}$ hybrid protein is generated by attaching the Id of Caps to the Ed of Trn. Ed, extracellular domain; Id, intracellular domain. (B) Interruptions of tracheal branch fusion in $trn^{28.4}$ embryos carrying *trn*, *caps* and the hybrid constructs were counted and presented in percentage of rescued interruptions. $trn^{28.4}$ embryos bearing *twi-GAL4* and *UAS-trn*: see Fig. 4. $trn^{28.4}$ embryos bearing *twi-GAL4* and $UAS\text{-}caps^{Ed}trn^{Id}$: DT early = 67.9% ± 1.2% (N1 = 252, N2 = 594), DT late = 74.5% ± 6% (N1 = 450, N2 = 450), LT = 44.3% ± 1.1% (N1 = 342, N2 = 612). $trn^{28.4}$ embryos bearing *twi-GAL4* and $UAS\text{-}trn^{Ed}caps^{Id}$: DT early = −11.2% ± 2.5% (N1 = 594, N2 = 450), DT late = −19.2% ± 1.8% (N1 = 486, N2 = 468), LT = −8.6% ± 2.4%(N1 = 138, N2 = 117). For $trn^{28.4}$ embryos carrying *twi-GAL4* and *UAS-caps*: DT early and DT late (see Fig. 4). LT = −17.6% ± 1.6% (N1 = 504, N2 = 486).

Caps intracellular domain contains functionally essential motif

Although Caps and Trn are both cell adhesion molecules with largely functionally equivalent extracellular domains during tracheal formation, they operate through divergent mechanisms. Our results demonstrate that Trn does not require its intracellular domain for its role during outgrowth of dorsal trunk cells whereas Caps needs its intracellular domain for proper function. To analyze the intracellular domain of Caps in more detail and to identify conserved protein domains, we searched for homologous protein sequences (see Materials and methods). We identified a putative *Anopheles gambiae* protein agCP14407, which shows 40% sequence identity to Caps. Structure and sequence analysis predicts that agCP14407 contains 14 LRR motifs, a putative transmembrane domain and a signal peptide at the amino-terminus as found for Caps. The high similarity in sequence and structure suggests that agCP14407 (*Aga*Caps) is the *Anopheles* homolog of the *Drosophila* Caps. Within the intracellular domains of Caps and *Aga*Caps, three domains are predominantly conserved (Fig. 6A): an RHR motif immediately after the transmembrane domain, a putative tyrosine phosphorylation site and finally a putative PDZ-binding motif. Interestingly, even though the last four amino acids in *Aga*Caps and Caps are not identical, both match one of the consensus C-terminal peptide sequences recognized by PDZ-proteins: X-S/T-X-L, where X is an unspecified amino acid (Sheng and Sala, 2001).

In order to analyze potential functions of the conserved motifs, point mutations leading to amino acid substitutions were introduced into *caps* cDNA sequence through site-directed mutagenesis (see Materials and methods). RH in RHR motif and Y were substituted with glycine whereas a stop codon was introduced before VTEL (Fig. 6A). The function of these altered proteins was analyzed in vivo by ectopic mesodermal expression in embryos. Misexpression of either $UAS\text{-}caps^{\Delta Y}$ or $UAS\text{-}caps^{\Delta PDZ}$ using the *twi-GAL4* driver reveals tracheal defects (Fig. 6B), and suggest Caps-like functions of these mutagenized proteins. In contrast, both ectopic expressions of $Caps^{\Delta RH}$ or β-Gal as control barely show any defects in tracheal development (Fig. 6B). These results suggest that the RHR motif is essential for Caps activity during the formation of tracheal network.

Discussion

Caps expression in the bridge-cells contributes to the normal dorsal trunk development

Single mesodermal cells called bridge-cells are required to provide local guidance for the outgrowing dorsal trunk branches (Wolf and Schuh, 2000). Here, we present evidence that Caps contributes to the bridge-cell's function. Caps is specifically expressed in the bridge-cells. When *caps* is lacking, the bridge-cells may not bind the dorsal trunk cells effectively and thus fail to provide sufficient guidance. Consequently, discontinuous dorsal trunks are formed. As only dorsal trunk cells require bridge-cell's guidance, other tracheal branches are not affected by lack of *caps* activity.

What is the function of Caps in the bridge-cells? Caps might be involved in directly mediating communication between the bridge-cells and the tracheal cells, as it represents a transmembrane protein with 14 leucine-rich repeats (LRR) in the extracellular domain (Shishido et al., 1998). The crystal structure of a ribonuclease inhibitor containing 15 LRRs revealed that the LRRs are arranged consecutively and parallel to a common axis so that the conformation resembles a horseshoe (Kobe and Deisenhofer, 1995a), which is ideal for mediating cell-to-cell interactions (Kobe and Deisenhofer, 1995b). Possibly, the extracellular domain of Caps assumes the horseshoe-like conformation as well and binds molecules localized on the surface of dorsal trunk cells. Thus, Caps may provide an important link between the guiding bridge-cells and the outgrowing tracheal cells.

When *caps* is ectopically expressed in mesodermal cells, discontinuous dorsal as well as lateral trunks are formed. Moreover, some dorsal trunk cells grow dorsally or ventrally

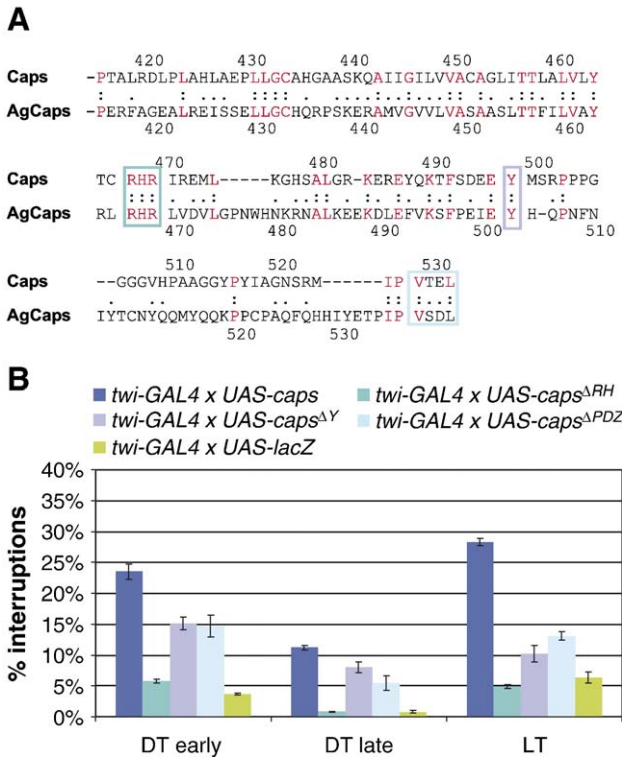

Fig. 6. The RHR motif in the intracellular domain is essential for Caps function. (A) Sequence alignment of the intracellular domains of *Drosophila* Caps and *Anopheles* Caps (AgCaps) using the lalign program (Myers and Miller, 1989). Single letter code was used for the amino acid residues. Red letters and (:) represent identical residues between Caps and AgCaps; (.) describes conserved residues. The boxed residues represent three different conserved motifs: RHR motif (violet), putative tyrosine phosphorylation site (blue) and putative PDZ binding motif (turquoise). (B) Interruptions of tracheal branch fusion in embryos that misexpress *caps* or mutagenized *caps* constructs. Embryos bearing *twi-GAL4* and *UAS-caps*: see Fig. 2C. Embryos bearing *twi-GAL4* and *UAS-caps*$^{\Delta RH}$: DT early = 5.8% ± 0.3% (N1 = 486, N2 = 450), DT late = 0.9% ± 0.1% (N1 = 504, N2 = 432), LT = 4.9% ± 0.3% (N1 = 450, N2 = 486). Embryos bearing *twi-GAL4* and *UAS-caps*$^{\Delta Y}$: DT early = 15.2% ± 1.0% (N1 = 900, N2 = 540), DT late = 8.0% ± 0.9% (N1 = 900, N2 = 486), LT = 10.3% ± 1.3% (N1 = 900, N2 = 450). Embryos bearing *twi-GAL4* and *UAS-caps*$^{\Delta PDZ}$: DT early = 14.7% ± 1.8% (N1 = 486, N2 = 432); DT late = 5.5% ± 1.2% (N1 = 432, N2 = 504), LT = 13.1% ± 0.7% (N1 = 414, N2 = 468). Embryos bearing *twi-GAL4* and *UAS-lacZ*: see Fig. 2C.

rather than anteriorly towards their adjacent targets. The outgrowing tracheal cells may adhere to ectopic Caps on the surface of mesodermal cells, which normally do not express Caps, rather than binding endogenous Caps on the bridge-cells. As a result, these tracheal cells may become disoriented and extend in abnormal directions. This ability of ectopic Caps to stall normal development of dorsal trunk branches suggests that selective expression of Caps in the bridge-cells is important for the specific local guidance for the outgrowing dorsal trunk cells. However, more severe dorsal trunk defects were observed in *hb* mutant embryos, which lack the bridge-cells (Wolf and Schuh, 2000). This observation suggests that additional components besides Caps are involved in the bridge-cell's guidance. Such components may include the chemoattractant Bnl, which is also expressed in the bridge-cells (Merabet et al., 2005).

Caps may function as a cell adhesion molecule and a receptor

Previous results demonstrated that Caps plays an essential role during pathfinding of motorneurons, layer-specific targeting in the visual system and boundary formation in wing imaginal discs (Shishido et al., 1998; Shinza-Kameda et al., 2006; Milan et al., 2001). Normally, Caps is expressed in a subset of CNS neurons including aCC, RP2, RP5 and U motorneurons (Shishido et al., 1998). When Caps is over-expressed in all neurons, the axons of muscle 12 motorneurons (MNs) become misrouted. Similar results are obtained when Caps^{Ed} lacking the intracellular domain is misexpressed in neurons. However, when Caps^{Id} lacking the extracellular domain is misexpressed, no defects can be observed in pathfinding of muscle 12 MNs. These results clearly indicate that a neural expression of Caps^{Ed} is sufficient to misroute muscle 12 MNs (Taniguchi et al., 2000). During boundary formation of the wing discs, cells incorrectly specified for their position undergo apoptosis because they fail to express Caps (Milan et al., 2002). Again, exclusive expression of Caps^{Ed} is sufficient to prevent apoptosis of misspecified cells. During these developmental events, Caps is thought to function as a cell adhesion molecule providing specific affinity between different cells and thus, may require only its membrane-anchored extracellular domain. In contrast, muscularly expressed Caps requires extracellular and intracellular domain to establish aberrant synapses of muscle 12 MNs (Taniguchi et al., 2000). Similarly, only embryos misexpressing complete Caps reveal interruptions in dorsal and lateral trunks. These results suggest that Caps requires its intracellular domain both for its function during the establishment of motorneuron synapses and during tracheal morphogenesis. This hypothesis was further strengthened by investigations using Caps and Trn hybrid proteins in ectopic expression assays. Misexpression of Trn^{Ed}Caps^{Id} in mesodermal cells results in discontinuous tracheal branches. Moreover, misexpression in *trn* mutants aggravates the tracheal defects. These observations suggest that the Trn^{Ed}Caps^{Id} hybrid protein functions similar to Caps even though it contains only the intracellular domain of Caps. Thus, it is the intracellular domain that determines specificity of Caps function during tracheal morphogenesis.

Previously, it was postulated that Caps might interact with receptors on specific motorneurons via its extracellular domain and transmit the signal into muscles via its intracellular domain (Taniguchi et al., 2000). Likewise, Caps may function as a signal transmitter between the tracheal cells and the bridge-cells. Interestingly, proteins containing LRRs are predicted to undergo conformational changes upon binding the ligand or other proteins. These changes do not involve the usual movement of separate domains relative to each other, but rather an elastic alteration of the entire structure (Kobe and Deisenhofer, 1995b). Such conformational changes may subsequently induce Caps to interact with other intracellular proteins involved in signaling pathways or regulation of cytoskeletal structures. It appears that depending on the cell type, Caps may act as a cell adhesion molecule or as a receptor that relays signals from the outside to the inside of cells.

Sequence alignment of Caps from *Drosophila melanogaster* and *Anopheles gambiae* reveals evolutionary conservation of three putative motifs within the intracellular domain: a putative tyrosine phosphorylation site, a predicted PDZ binding motif and a conserved RHR motif. Site-directed mutagenesis of these putative functional motifs and in vivo analysis shows that only the RHR motif is essential for Caps function during tracheal formation. This RHR motif is not yet recognized as a functional motif by protein data bases (Gasteiger et al., 2003). Therefore, no information regarding other proteins containing such a motif or prediction about its putative function is known. Located immediately after the transmembrane domain, the RHR motif may contribute to conformational changes, which enable Caps to transmit extracellular signals to small membrane-associated proteins that bind components of signaling pathways or cytoskeleton.

Caps and Trn act dissimilarly during tracheal development

During the *Drosophila* wing development, Caps and Trn share redundant function and contribute evenly to the formation of affinity boundary between the dorsal and ventral compartments (Milan et al., 2001). This result is rather expected as only the extracellular domain of Caps or Trn is required during the establishment of boundary in wing discs and the extracellular domains of Caps and Trn are 65% identical. Similarly, Trn requires only its extracellular domain to mediate its function during the establishment of tracheal network. Our results demonstrate that the extracellular domain of either Trn or Caps can rescue the *trn* tracheal phenotype. Thus, the extracellular domains of both Caps and Trn provide the mesodermal cells with a substrate that mediates a normal tracheal branch progression in *trn* mutants.

On the other hand, Trn and Caps are expressed in divergent patterns and contribute differently to the formation of continuous tracheal branches. Whereas *caps* is expressed selectively in the bridge-cells, *trn* is detected in broad subsets of mesodermal cells excluding the bridge-cells. Furthermore, ectopic expression of Caps in mesodermal cells disrupts formation of normal tracheal interconnections while ectopic expression of Trn in mesodermal cells does not affect tracheal development. Rather, it rescues the tracheal defects of *trn* mutant embryos. Finally, although Caps requires both its extracellular and intracellular domain for proper function during the formation of tracheal branches, Trn needs only its extracellular domain.

Based on these observations, we propose the following model for Caps and Trn functions during tracheal development: Caps is important for the bridge-cell, which provides instructive cues for the extending dorsal trunk cells, while Trn contributes to permissive matrix function of mesodermal cells for normal tracheal branch outgrowth (Fig. 7). Localized at the surface of bridge-cells, Caps may bind to cell surface molecules on tracheal cells and allow the tracheal cells to extend along the bridge-cells so that they can find the correct targets. In the absence of *caps*, the bridge-cells cannot mediate their local guidance as effectively and consequently, the dorsal trunk cells fail to interconnect to their targets. On the other hand, when *caps* is expressed in additional mesodermal cells besides the bridge-cells, then the nearby tracheal cells can also adhere to these mesodermal cells through Caps and extend in unspecified directions. Thus, disconnected tracheal branches are formed (Fig. 7A). Previous studies indicate that specific interactions between cell surface

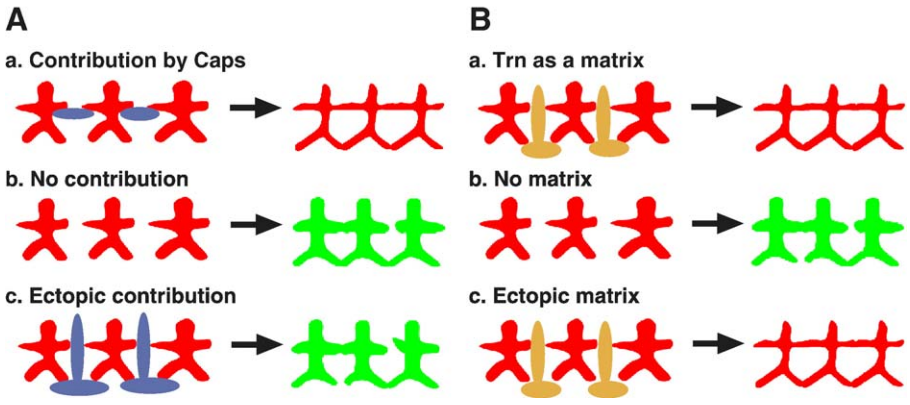

Fig. 7. Model of Caps and Trn function during tracheal system development. (A) Schematic diagram of Caps function during tracheal formation. (a) WT: wild-type expression of Caps (blue) in the bridge-cells interconnects juxtaposing dorsal trunk cells (red). Caps may mediate direct interaction between the bridge-cells and tracheal cells and facilitate tracheal branches towards their proper targets. As a result, a continuous dorsal trunk is formed. (b) Lack of Caps: when Caps is lacking in the bridge-cells, guidance function cannot be mediated properly and dorsal trunk cells fail to find their tracheal targets efficiently. Consequently, a discontinuous dorsal trunk (green) is generated. (c) Ectopic Caps: when mesodermal cells (blue) besides the bridge-cells misexpress *caps*, the outgrowing dorsal trunk cells may bind ectopic Caps and become detached and/or extend in abnormal directions. Thus, an interrupted dorsal trunk is formed. (B) A schematic diagram of Trn function during tracheal formation. (a) WT: wild-type expression of Trn (orange) in mesodermal cells located between dorsal trunk and lateral trunk cells. The tracheal cells may use the surrounding mesodermal cells as a matrix to determine their positions and migrate towards their targets. Trn expressed on the surface of mesodermal cells may interact with cell surface molecules on tracheal cells and thereby mediate a normal outgrowth of tracheal cells. (b) Lack of Trn: when *trn* is lacking in mesodermal cells, the matrix function cannot be mediated properly and tracheal cells fail to find their targets efficiently within a specified period of time. Consequently, discontinuous tracheal branches (green) are generated. (c) Ectopic Trn: overexpression of Trn in mesodermal cells (orange) mimics closely the endogenous Trn expression in wild-type embryos. As a matrix molecule, ectopic Trn may allow the outgrowing tracheal cells to navigate normally through mesodermal cells and connect properly to their targets. Note: The diagram is an oversimplification since only about 20% of the dorsal trunk interconnections are affected in *caps* and *trn* mutant embryos.

proteins on the tracheal cells and the surrounding mesodermal cells are crucial for migration of tracheal cells (Franch-Marro and Casanova, 2000). The mesodermal cells may serve as a matrix enabling (and facilitating) the tracheal cells to recognize the correct path and to migrate efficiently. These cell-to-cell interactions might involve transmembrane proteins such as Trn. Localized at the surface of mesodermal cells, Trn may interact directly with other molecules on the tracheal cells and thereby support the tracheal cells to extend across the mesodermal sheet. When *trn* is absent, the navigation of outgrowing tracheal cells is partially hampered and they cannot migrate along their paths as efficiently. Consequently, they fail to connect to their targets. Overexpression of Trn in mesodermal cells mimics the wild-type expression of Trn and does not affect the progression of tracheal cells (Fig. 7B).

Our results suggest that migrating tracheal branches require an adhesive substrate provided by the broad expression of Trn in the immediate proximity. The major airway, the dorsal trunk, relies additionally on instructive guidance by the extending bridge-cells expressing Caps, which may bind to surface molecule(s) on the progressing tracheal cells. The intracellular domain of Caps might induce signaling leading to cytoskeletal changes that generate a “pulling” force of the bridge-cells on the migrating tracheal cells. Identification of extracellular binding partners on tracheal cells and intracellular interaction partners of Caps may elucidate molecular mechanisms underlying transmission of external cues in the bridge-cells that induce intracellular events leading to cellular guidance.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.04.462.

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