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Trachealess (Trh) regulates all tracheal genes during Drosophila embryogenesis

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Abstract

The Drosophila trachea is a branched tubular epithelia that transports oxygen and other gases. *trachealess* (*trh*), which encodes a bHLH-PAS transcription factor, is among the first genes to be expressed in the cells that will form the trachea. In the absence of *trh*, tracheal cells fail to invaginate to form tubes and remain on the embryo surface. Expression of many tracheal-specific genes depends on *trh*, but all of the known targets have relatively minor phenotypes compared to loss of *trh*, suggesting that there are additional targets. To identify uncharacterized transcriptional targets of Trh and to further understand the role of Trh in embryonic tracheal formation, we performed an in situ hybridization screen using a library of ~100 tracheal-expressed genes identified by the Berkeley Drosophila Genome Project (BDGP). Surprisingly, expression of every tracheal gene we tested was dependent on Trh, suggesting a major role for Trh in activation and maintenance of tracheal gene expression. A re-examination of the interdependence of the known early-expressed transcription factors, including *trh*, *ventral veinless* (*vvl*) and *knirps*/*knirps-related* (*kni*/*knrl*), suggests a new model for how gene expression is controlled in the trachea, with *trh* regulating expression of *vvl* and *kni*, but not vice versa. A pilot screen for the targets of Vvl and Kni/Knrl revealed that Vvl and Kni have only minor roles compared to Trh. Finally, genome-wide microarray experiments identified additional Trh targets and revealed that a variety of biological processes are affected by the loss of *trh*.

Keywords

Drosophila; *knirps*; *kni*; trachea; *trachealess*; *trh*; *ventral veinless*; *vvl*

Introduction

Tubular organs are required in multicellular organisms to transport essential substances into and out of the body and from one part of the body to another. The Drosophila trachea is a branched network of tubular epithelia that transports oxygen and other gases, and is an excellent model for discovering the cellular and molecular events underlying epithelial tube formation. The trachea develops from twenty placodes, or plates, of polarized ectodermal cells, ten on each side of the embryo from the second thoracic segment (T2) to the eighth abdominal segment (A8). Through a series of coordinated cell shape changes, tracheal cells

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invaginate to form small, internalized epithelial sacs, undergoing their final round of mitotic division during this process. Once the tracheal cells are internalized, small groups of cells in distinct positions within each tracheal sac begin to migrate in stereotypical patterns to form the primary branches. Dorsally-positioned branches migrate anteriorly and posteriorly and fuse to the equivalent branches in adjacent segments to create a continuous dorsal trunk that runs along the anterior-posterior axis of the embryo. The remaining branches either extend to target organs or fuse with either adjacent or contra-lateral tracheal branches to ultimately form a contiguous tubular network (Manning and Krasnow, 1993).

Much is known about specification of the Drosophila trachea. The earliest events are controlled by global patterning genes, including the dorsal-ventral, homeotic, and segment polarity genes (Kerman et al., 2006). Patterning genes both limit the number of segments in which trachea form and determine the anterior-posterior and dorsal-ventral location of the primordia within each segment. *spalt* (*sal*), which encodes a zinc finger transcription factor initially expressed in broad domains in both anterior and posterior regions of the embryo, limits tracheal formation to the ten segments from T2 through A8 (Kühnlein and Schuh, 1996). The dorsal limit on trachea formation is set by the dorsal patterning gene *decapentaplegic* (*dpp*) (Isaac and Andrew, 1996; Wilk et al., 1996). The segment polarity gene *wingless* (*wg*) limits formation of tracheal primordia to the cells between its stripes of expression within each segment (de Celis et al., 1995; Wilk et al., 1996), whereas STAT92E, the single Drosophila STAT, which also functions as a segment polarity gene in early development, is essential for tracheal formation (Brown et al., 2001; Li et al., 2003; Sotillos et al., 2010).

Three known transcription factors are specifically expressed early in the cells that will develop as trachea: *trachealess* (*trh*), *ventral veinless* (*vvl*) and *knirps*/*knirps-related* (*kni*/*knrl*). *trh* encodes a basic helix-loop-helix (bHLH)-PAS transcription factor, which is essential for trachea formation; in the absence of *trh* function, the precursor cells fail to undergo any of the morphogenetic events of tube formation and remain at their site of origin (Isaac and Andrew, 1996; Wilk et al., 1996). Interestingly, its mammalian orthologue NPAS-3 is required for lung development and homeostasis (Zhou et al., 2009). *vvl* encodes a POU-domain containing transcription factor also required for tracheal development; in the absence of *vvl*, tracheal cells invaginate but fail to migrate appropriately (Anderson et al., 1995; de Celis et al., 1995). *knirps* (*kni*) and *knirps-related* (*knrl*) are two closely linked homologous genes that encode zinc finger proteins (Chen et al., 1998). Both genes are expressed throughout the early tracheal primordia, but their expression becomes restricted to specific branches at later stages. Loss of *kni* and *knrl* disrupts migration of a subset of tracheal branches within each segment (Chen et al., 1998).

Previous studies have revealed that the initial expression of *vvl* and *trh* is independent of each other, and that early tracheal expression of *kni* is also independent of *trh* (Boube et al., 2000). Indeed, more recent findings reveal that the initial expression of both *trh* and *vvl* is directly and independently activated by STAT92E (Sotillos et al., 2010). *vvl* has been reported to be required for the maintenance of *trh* expression, suggesting that although the initial expression of early tracheal transcription factor genes is independent of the others, these genes subsequently become dependent on each other for their continued expression (Zelzer and Shilo, 2000).

The early-expressed tracheal transcription factors not only regulate each other but also regulate key downstream targets required to form trachea. For example, Trh and Vvl work together to control *breathless* (*btl*), which encodes an FGF receptor essential for tracheal migration (Anderson et al., 1996; Klämbt et al., 1992; Ohshiro and Saigo, 1997; Wilk et al., 1996), and *rhomboid* (*rho*), which encodes a transmembrane protease that processes the

EGF ligand Spitz and is required for complete internalization of tracheal precursors (Lee et al., 2001; Llimargas and Casanova, 1999; Nishimura et al., 2007). Trh also independently regulates *tracheal defective* (*tdf*) and *pebble* (*peb*, also known as *hindsight* [*hnt*]), which encode two putative transcription factors (Boube et al., 2000; Eulenberg and Schuh, 1997; Yip et al., 1997), and Vvl is thought to independently regulate *thick veins* (*tkv*), a Dpp receptor, which is required in the subset of tracheal cells that migrate to form specific dorsal-ventral branches (Llimargas and Casanova, 1997). Kni/Knrl are expressed in the entire trachea initially, then become restricted to specific branches where they are required to repress expression of *sal*, their only known tracheal target gene (Chen et al., 1998). Altogether, the studies of gene regulation in the trachea suggest a model wherein the early-expressed transcription factors control both unique and overlapping sets of targets genes to drive tracheal formation (Boube et al., 2000).

Since the loss of known targets of Trh results in phenotypes that are relatively mild compared to loss of *trh*, additional downstream targets of this key regulator must exist. To find such targets, we performed an in situ hybridization screen and carried out a genome-wide microarray analysis. To our surprise, we discovered that expression of nearly every gene expressed in the trachea requires *trh*. A re-examination of the regulatory relationships among the early expressed tracheal transcription factors leads to a new model for how gene expression is controlled in this tissue.

Materials and methods

Fly strains

For the in situ hybridization screen, two EMS-induced *trh* alleles, trh^{1} (trh^{5D55}) and trh^{2} (trh^{7J83}), and the trh^{8} protein-null P-element excision allele were used. For the microarray experiments, the trh^{8} allele was used. vvl^{6A3} (a kind gift from Dr. Jordi Casanova) and *Df(3L)ri-79c* (a deficiency deleting both *kni* and *knrl*; Bloomington stock center) were used for the pilot screen for Vvl and Kni/Knrl downstream genes. *Df(3L)BSC247*, *Df(3L)BSC362* (deficiencies deleting *trh*) and *Df(3L)Exel6109* (a deficiency deleting *vvl*) were obtained from the Bloomington stock center.

Whole-mount In situ hybridization

The *trh* mutant alleles were balanced either over TM3 carrying a *ftz-lacZ* transgene marker or over TM3 carrying a *twi-GFP* transgene marker. cDNA clones for the tracheal-expressed genes were obtained from the laboratories of Allan Spradling and Philip Beachy. In situ hybridization with gene specific probes was performed as previously described (Lehmann and Tautz, 1994) either in combination with a *lacZ* or *GFP* probe to distinguish the homozygous mutant embryos from their heterozygous siblings or by pre-sorting GFP-negative trh^{2} homozygous embryos using a COPAS embryo sorter (Union Biometrica). vvl^{6A3} and *Df(3L)ri79C* lines were balanced over TM3, *twi-GFP* balancer and homozygous embryos were pre-sorted before in situ hybridization. Images were obtained using an Axiophot compound microscope (Carl Zeiss, Inc) configured with a digital camera (Coolpix 4500; Nikon).

Double Fluorescent In Situ Hybridization and Antibody Labeling

Fluorescent in situ hybridization with mRNA probes and antibody staining with α-Trh was performed as described (Knirr et al., 1999). Images were obtained under identical settings for each probe using a confocal LSM 510 Meta microscope (Carl Zeiss, Inc.).

Immunohistochemistry

Embryo fixation and staining were performed as described (Reuter et al., 1990). The primary antibodies used were rat α-Trh (Ward et al., 1998), rat α-Vvl (a gift from S. Certel; 1:50), guinea pig α-Kni (a gift from S. Small; 1:20), mouse α-βgal (Promega; 1:500 for fluorescence; 1:10,000 for HRP), rabbit α-GFP (Molecular Probes; 1:40,000), and mouse α-Tgo (Developmental Studies Hybridoma Bank; 1:2). For HRP staining, Biotin-labeled secondary antibodies were used at 1:500 dilution (Molecular Probes). HRP images were taken with an Axiophot microscope (Carl Zeiss, Inc.) and Coolpix 4500 camera (Nikon). For fluorescent staining, Alexa-488- or Alexa-568-labeled secondary antibodies were used at a 1:500 dilution (Molecular Probes). Confocal images were obtained using a LSM 510 Meta confocal microscope (Carl Zeiss, Inc.).

Microarray experiments to identify Trh downstream genes

Three independent samples of stage 11-16 trh^{8} homozygous mutant embryos and three of wild type (Oregon R) embryos were isolated using a COPAS Select embryo sorter (Union Biometrica). Total RNA was isolated using Trizol (Invitrogen) and cleaned up with the RNeasy kit (Qiagen). Total RNA (100ng) was labeled according to standard Affymetrix protocols and hybridized to the Drosophila genome 2.0 chip. After scanning, intensity values were normalized by RMA (Irizarry et al., 2003a; Irizarry et al., 2003b) and statistical analysis was performed using Spotfire software (TIBCO). Trh target genes were identified based on a 1.4 fold change in gene expression, with a p-value ≤0.05. This fold change was selected because many known confirmed Trh target genes fall into the range of a 1.4 fold or more change.

Microarray accession nos

All microarray data have been deposited in Gene Expression Omnibus (GEO), accession no. GSE28780.

Real-time quantitative PCR (RT-qPCR)

Total RNA from three independent samples each of wild-type and trh^{8} homozygous mutant embryos (stage 11-16) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with oligo(dT20V) primers for the two-step RT-qPCR assays. Gene-specific primers were used to amplify the corresponding cDNA by RT-qPCR on a CFX96 Real-Time PCR Detection System (BioRad) using the SYBR Green Supermix (BioRad). Primer sets for each gene were designed against the complete nucleotide sequence, as deposited on FlyBase, using PerlPrimer. The specificity of each primer set and molecular weight of the amplicon were monitored by dissociation curve analysis and verified by running the PCR products against size standards on a 1.5% agarose gel. A sample volume of 20 μl was used for all assays, which contained a 1× final concentration of SYBR Green mix, 200nM gene specific primers, and 4μl of diluted template. All samples were run in triplicate. Genes for the RT-qPCR were chosen based on their fold change in the microarray analysis (−72.8 to −1.4 fold for downregulated genes and 1.4 to 10.5 fold change for upregulated genes) and the Gene Ontology (GO) cluster into which they have been categorized.

Results

trh autoregulates in the trachea but not in other tissues

trh encodes three alternatively spliced transcripts, designated RA-RC (BDGP; Fig.1A). *trh-RA* and *trh-RB* transcripts share all exons except a small additional exon (exon 6) found in the *RA* form. The *trh-RC* transcript includes all but the first exon of *trh-RB* but also contains several unique 5′ protein coding exons. All three splice forms include the bHLH, PAS-A and

PAS-B domains (Fig.1A). As previously reported, *trh* mRNA is expressed in the tube-forming cells of the salivary duct, trachea and filzkörper, and in a subset of cells in the central nervous system (CNS) (Isaac and Andrew, 1996; Wilk et al., 1996). In situ hybridization with all of the splice form-specific probes (exon 6 of RA form, exon 1 for RA/RB-specific form, and exon 2 of RC form) recapitulated the previously published *trh* mRNA expression pattern (Isaac and Andrew, 1996), suggesting that all three splice forms are expressed similarly (Fig.S1).

Before initiating our screens for transcriptional targets of Trh, we first characterized the three *trh* alleles with the most severe and consistent tracheal invagination defects, two EMS-induced alleles, trh^{1} and trh^{2}, and a P-element excision allele, trh^{8} (Isaac and Andrew, 1996; Jürgens et al., 1984; Wilk et al., 1996). For the EMS alleles, we generated and sequenced PCR fragments spanning the entire *trh* coding region from homozygous mutant embryos. We discovered several nucleotide substitutions. Most are silent mutations that do not affect the amino acid sequence, but one of the substitutions (ATC to AAC) changed an Ile to Asn in the PAS-A domain in both trh^{1} and trh^{2} (Fig.1A). This residue is conserved in all Drosophila species and in some insects, including the silkworm (Fig.1B). A conserved Val or Leu residue is found in the Trh orthologs in other insects and crustaceans, and the orthologous mouse and human NPAS3 proteins have a conserved Met in this position (Fig. 1B). Previous studies showed that the PAS domain of Trh confers target gene specificity (Zelzer et al., 1997), and our molecular characterization of *trh* mutants further confirms that the PAS domain is critical to Trh function. The trh^{8} allele is a $rosy^{-}$ excision derivative of the trh^{3} enhancer trap line (also known as trh^{10512}) in which a 14.5 kb PZ element had inserted right after nucleotide 14 of the 5′ UTR of the *trh-RA* and *trh-RB* transcripts (Isaac and Andrew, 1996). Genomic PCR and sequence analysis of trh^{8} revealed an 8949 bp internal deletion of the PZ element (2740-11687 nt), leaving the flanking genomic region intact (Fig.1A).

Next, we examined *trh* mRNA and protein expression in the mutant alleles. *trh* is reported to regulate its own expression in the trachea, but not in the salivary duct (Haberman et al., 2003; Wilk et al., 1996). Indeed, although *trh* mRNA was detected in the early trachea in all three *trh* alleles, the number of *trh*-expressing cells decreased over time and *trh* mRNA was not detected after early st.14 (Fig.1C). Failure of the tracheal cells to express *trh* mRNA is not due to cell death; we and others did not detect any significant increase of apoptosis in the tracheal cells of these mutants (Zelzer and Shilo, 2000). Moreover, expression of three pro-apoptic genes, *hid*, *reaper* and *grim*, was unaffected by loss of *trh* (data not shown). Finally, tracheal cells persist through at least embryonic stage 15 in *trh* mutants based on the expression patterns of a *lacZ* reporter gene driven by two independent *vvl* enhancer fragments; β-gal protein expression (which persists longer than *lacZ* RNA or the endogenous *vvl* RNA/protein) from these enhancers is maintained in the uninvaginated tracheal cells of *trh* mutant embryos through stage 15 (Sotillos et al., 2010). Expression of *trh* mRNA in the salivary duct and filzkörper was at approximately wild-type levels with all three alleles, suggesting that *trh* does not autoregulate in either tissue (Haberman et al., 2003). Interestingly, *trh* expression in the CNS was unaltered in trh^{1} and trh^{2}, but was not detected in trh^{8} mutant embryos (Fig.1C). In trh^{1} and trh^{2}, α-Trh staining showed a very similar pattern to that of the mRNA; protein was detected in tracheal cells until early st.14 and absent at later stages (Fig.1D). These data suggest that the trh^{1} and trh^{2} alleles make non-functional Trh protein that fails to maintain its own expression. In the trh^{8} mutants, no protein expression was detected at any stage, revealing that trh^{8} is protein null (Fig.1D). The strong α-Trh signals detected in the secretory portion of the salivary glands at st.13 and later are nonspecific; the same staining was also detected in embryos homozygous for a deficiency that deletes *trh* and several flanking genes (Fig.1D; data not shown).

Trh maintains the expression of all tracheal genes

To identify additional transcriptional targets of Trh required for tracheal tube formation, we performed an in situ hybridization analysis with cDNA clones for ~100 genes expressed in the trachea based on findings from the Berkeley Drosophila Genome Project (BDGP; http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl). We examined the expression of each gene in both wild type and *trh* mutant embryos. Homozygous *trh* embryos were unambiguously distinguished from their heterozygous siblings by including probes specific for a marker on the balancer chromosome (*lacZ* or *GFP*) in the in situ hybridization mix. For the genes whose expression overlapped significantly with the balancer chromosome marker, we presorted the *trh* null homozygous embryos from their non-mutant siblings, which carried a GFP marker on the balancer chromosome, using a COPAS embryo sorter. The genes, cDNAs, and the *trh* allele(s) used for the in situ screen are included in Table 1.

Surprisingly, we found that tracheal expression of all the genes we examined was completely absent in *trh* mutants at late stage 12 and later, suggesting that *trh* may be required for maintaining expression of all tracheal genes. The affected genes include those expressed in the entire trachea (Fig.2A-E) as well as genes expressed in only a subset of branches or in limited tracheal cell types (Fig.2F-H). The absence of tracheal gene expression is not due to a loss of tracheal cells since we have shown that the cells are present and express *trh* mRNA (and Trh protein) until embryonic stage 14 in the *trh* mutants (Fig. 1C, D). Some of these genes were also expressed in other tissues, but only their tracheal expression was affected by *trh* loss (Fig.2D, H). The absence of tracheal gene expression in the trh^{1} and trh^{2} mutants confirms that the Trh proteins made in the EMS-induced mutants are non-functional.

We then asked if *trh* is also required for the initial expression of tracheal genes. Out of the 96 genes we have examined, 38 genes begin to be expressed at embryonic stages 10 and 11 (referred to as early-expressed genes hereafter). We found that expression of 20 of the 38 early-expressed genes absolutely required *trh* at all stages. Previous studies had suggested that Trh is not required for the initial expression of all tracheal genes; specifically, the early expression of *tkv* was shown to not change in *trh* mutants (Boube et al., 2000). To carefully examine the expression of *tkv* and other early-expressed genes, we performed fluorescent in situ hybridizations with *tkv* and three other early-expressed tracheal genes (*ft*, *CG13333* and *CG8312*). Indeed, their mRNA expression was detected in tracheal cells at stage 10/11, albeit at somewhat reduced levels (Fig.2I, J, L, M and data not shown). Thus, although *trh* is required for the expression of all tracheal genes by late stage 12, the initial expression of a small subset of early expressed genes occurs independently of *trh*.

Trh regulates the expression of Vvl and Kni, but not vice versa

Our unexpected finding that Trh is required to maintain expression of all tracheal genes led us to more carefully investigate the interdependence of the early-expressed tracheal transcription factors. *trh* and *vvl* are among the earliest-expressed tracheal genes (Anderson et al., 1995; Isaac and Andrew, 1996; Wilk et al., 1996) and both genes are directly regulated by STAT92E, which functions in early embryonic patterning (Sotillos et al., 2010). Two other early expressed tracheal transcription factor genes include *kni*, which also functions earlier as a zygotic gap gene, and *knrl*, which has a redundant role with *kni* in the trachea (Chen et al., 1998; Rothe et al., 1989). The initial expression of *trh*, *vvl* and *kni* is reported to be unaffected by the loss of expression of the other two genes (Boube et al., 2000). We hypothesized that these early-expressed transcription factors might become interdependent at later stages, which could explain why the expression all tracheal genes is gone in *trh* mutants by embryonic stage 12. Thus, we examined both the early and late expression of *trh*, *vvl*, and *kni* in embryos mutant for the other genes. First, we examined *trh*

mRNA and protein expression in *vvl* and *kni* mutants, using the null allele vvl^{6A3} and a deficiency that deletes both *kni* and *knrl*, *Df(3L)ri-79C*. Consistent with previous findings (Zelzer and Shilo, 2000), the initial expression of *trh* mRNA was unaffected by the absence of *vvl* or of *kni* (Fig.3B, C). Because of the role of *kni* as a zygotic gap gene, *kni* deficient embryos develop with two relatively normal anterior metameres, one relatively normal posterior metamere and several fused metameres in between; nonetheless, *trh* mRNA was expressed to WT levels in all tracheal cells in these embryos (Fig.3C). Similarly, Trh protein levels appeared normal in both *vvl* and *kni* deficient trachea at early stages (Fig.3E, F). Previous studies reported that although the initial expression of Trh is not affected in *vvl* null mutants, the levels of Trh are reduced over time and become almost undetectable by stage 15 (Zelzer and Shilo, 2000), suggesting a requirement for Vvl in the regulation of Trh. We find that, although the number of *trh*-positive cells in *vvl* null mutants or *vvl* deficiency embryos (*Df(3L)Exel6109*) may be fewer than in wild type, *trh* mRNA and protein expression levels in these cells are comparable to wild-type levels throughout embryogenesis (Fig.3H, K; data not shown). Thus, Vvl plays only a minor role, if any, in maintaining *trh* expression. Similarly, *trh* mRNA and protein levels are comparable to WT at all stages in *kni* deficient embryos, indicating that *kni* is not required for *trh* expression (Fig.3I, L).

The initial early expression of Vvl was not significantly affected in either the *trh* or *kni* mutant backgrounds, consistent with previous findings (Boube et al., 2000; Fig.4B, C). Expression of Vvl was reduced in later stage *trh* mutants and was completely gone in all tracheal cells by embryonic stage 15 (Fig.4E). In contrast, loss of *kni* did not affect Vvl expression at any stage (Fig.4C, F).

Previous studies indicated that the early expression of *kni* is independent of *trh*, suggesting that *kni* may be one of the primary tracheal genes (Boube et al., 2000). Inconsistent with these findings, we observed that the tracheal expression of *kni* was completely absent in *trh* mutants at all stages (Fig.4H, H'). In wild-type embryos, Kni is expressed in a subset of tracheal cells (Fig.4G, G'). Double staining with Trh and Kni antibodies revealed that whereas Trh protein is easily detected in the tracheal cells in trh^{2} mutants at early stages, the Trh-positive cells do not express Kni (Fig.4H, H'). By stage 15, neither Trh nor Kni tracheal expression can be detected (Fig. 4K, K'). In contrast, Kni is expressed to wild-type levels in the tracheal cells of *vvl* mutants at all stages (Fig.4I, I', L, L').

Taken together, this analysis reveals that expression of both *trh* and *vvl* is initiated independently of other tracheal genes and that maintenance of their expression absolutely requires Trh. As observed with most tracheal genes analyzed in our in situ analysis, *kni* requires Trh to both initiate and maintain its tracheal expression. A small number of tracheal genes behave like *trh* and *vvl*, where initial expression is independent of other tracheal genes. Nonetheless, every gene expressed in the late trachea required *trh*, regardless of whether initial expression was Trh-dependent or not.

Interestingly, both *trh* and *vvl* null embryos appear to have fewer tracheal primordial cells, based on the early expression of Trh, Vvl and/or Tango (Fig. 2I-M; data not shown). This observation suggests that activation of both Trh and Vvl by the upstream patterning genes (JAK/STAT signaling in the absence of Sal, Wg and Dpp signaling) may be required for committing cells to a tracheal lineage. This idea is consistent with the finding that ectopic expression of *trh* can induce formation of additional trachea, but only in cells that also express *vvl* (Boube et al., 2000; Wilk et al., 1996; Zelzer and Shilo, 2000).

Vvl regulates only a subset of tracheal genes whereas Kni does not affect expression of any of the tested genes

Our findings that *vvl* is not required for *trh* expression led us to ask how many tracheal genes require Vvl. A pilot in situ analysis revealed that only six out of 21 tested tracheal genes were affected by loss of *vvl*. Three genes, *mipp1*, *CG4726* and *CG3777*, were completely absent at all stages (Table 2). Expression of *serrano* (*sano*), which encodes an apically-enriched cytoplasmic protein that controls tracheal tube length (Chung et al., 2009), was significantly reduced at early stages and disappeared completely at later stages (Table 2; Fig.5A). Levels of *CG33006* and *CG32499* were strongly reduced at all stages (Table 2; Fig. 5B, D). The remaining 16 genes were expressed to wild-type levels in the *vvl* mutants (Table 2; Fig.5C, E, F). Thus, we estimate that Vvl contributes to the expression of approximately 25 - 30% of all tracheal genes. We also performed fluorescent in situ with *tkv* probe in *vvl* mutants to examine it more carefully since *tkv* was the only known gene whose early expression is regulated by Vvl but not by Trh (Boube et al., 2000; Llimargas and Casanova, 1997). Inconsistent with the previous studies, however, levels of *tkv* mRNAs in the *vvl* mutant tracheal cells were as high as levels in *trh* mutants (Fig.2J, K), suggesting that the initial expression of *tkv* is not dependent on *trh* or *vvl*.

Only a single target of Kni has ever been identified; Kni represses *sal* expression in dorsal tracheal cells (Chen et al., 1998). The observation that *kni* does not affect the expression of any other known downstream gene suggests that it might act in parallel in early tracheal development (Boube et al., 2000). A pilot in situ screen of 32 tracheal genes, including all 21 of the genes we tested in *vvl* mutants, revealed that not a single tested gene was affected by loss of *kni* (Table 2). This finding suggests that Kni has a very minor role in this tissue compared to Trh or even to Vvl. Consistent with our failure to find additional targets of Kni, genetic studies support a model wherein *sal* may well be the only transcriptional target of Kni in this tissue (Ribeiro et al., 2004).

Microarray experiments identify additional novel Trh downstream genes

Our in situ hybridization screen identified about 100 tracheal-expressed genes regulated by Trh, but many Trh targets will have been missed since the BDGP expression datasets are incomplete and cover only about 1/3 of the genome. To find additional genes regulated by Trh, we performed microarray analysis comparing RNA from wild type and *trh* mutant embryos. 983 genes had expression levels reduced by 1.4-fold or greater; a number chosen because many of the Trh target genes identified in our in situ screen were included in this set (35 out of the 98 known genes; Fig.6A and Table S1). The expression patterns for about 1/3 of the target genes identified by microarrays are available in the BDGP dataset (356 genes), with 26.1% of them being expressed in the trachea (93 genes) (Table S1). In addition to tracheal-expressed targets of Trh, the microarray analysis also identified many novel targets expressed in the other tissues where Trh is normally expressed, such as the filzkörper, central nervous system (CNS), salivary duct and early salivary gland, suggesting that they are regulated by Trh in those tissues (19.9%; 71 genes; Table S1). To validate the microarray data, we chose 11 novel target genes (one gene without a known expression pattern and ten genes with known expression patterns in the trachea, filzkörper or salivary duct), and performed in situ hybridization in both wild type and *trh* mutant embryos. All eleven genes were affected by loss of *trh* (Fig. 6B and Table S1). *Spn43Aa*, which encodes a serine protease inhibitor, is expressed in several tissues including the trachea, and its tracheal expression requires *trh* at all stages (Fig. 6B). *Osi19*, the gene with greatest reduction in expression levels in *trh* mutants relative to WT, is specifically expressed in the late trachea, and as observed with all of the tracheal genes we have examined, this expression is gone in *trh* mutants (Fig. 6B). *CG12009*, which encodes a tracheal-specific

chitin-binding protein, and *CG13640*, a novel filzkörper-specific gene, also absolutely require *trh* for their tracheal expression (Fig. 6B).

We also performed real-time quantitative PCR (RT-qPCR) with a dozen downregulated Trh target genes to validate microarray data. The set included four genes with known expression in the salivary duct, CNS or epidermis, and eight genes without any known expression patterns (Table S1). Their fold change ranges from −72.8 to −1.4 (Table S1). All but one of the genes showed reduced levels of expression in *trh* mutants compared to WT and seven showed significant reduction by this analysis, including *Glucose dehydrogenase* (*Gld*), which is expressed in the salivary duct and other tissues, *Synapse protein 25* (*Snap25*), which is expressed in the CNS midline, and several novel genes such as *CG14934*, whose expression pattern is not known (Fig. 6C and Table S1). Four other genes, including *Glutamate receptor I* (*Glu-RI*) and *CG31955*, also showed reduced expression, but not to statistically significant levels (Fig. 6C and Table S1).

The microarray experiments not only identified downregulated genes but also a number of genes whose expression went up in *trh* mutants. RT-qPCR with ten of these genes with expression levels increased by 1.4 to 10.5 fold, revealed that genes with four fold or greater change by microarray analysis also showed significant increases by this assay, suggesting that a four fold change is a reasonable cut-off for upregulated genes (Fig. 6D and Table S2). The 26 genes with four-fold or greater increases in expression levels in *trh* mutants include *Rieske iron-sulfur protein* (*RFeSP*), which encodes a Ubiquinol-cytochrome-c reductase, *GstD4* and *GstD7*, both of which encode Glutathione S transferases, and *Cyp12a4*, which encodes a protein with putative electron carrier activity that also has been shown to be upregulated in response to insecticides (Fig.6D and Table S2; King-Jones et al., 2006). Although we do not yet understand the roles of all the genes whose expression is affected by loss of *trh*, our validation of the microarray data by in situ hybridization and RT-qPCR suggest that the genes identified by this method are indeed regulated by Trh.

To ask which biological processes are affected by Trh, gene ontology (GO) clustering was performed using DAVID, a program that identifies enriched biological clusters in a given dataset relative to their representation in the entire genome (Dennis et al., 2003; Hosack et al., 2003; Huang da et al., 2009). This analysis revealed that a range of biological processes are affected by the absence of *trh*, which is not surprising since all tracheal-expressed genes are downregulated in *trh* mutants. The affected biological processes include “glucose metabolic process”, “proteolysis”, “chitin metabolic process” and “nucleotide catabolic process”, with an enrichment of the associated GO terms by at least twofold compared to the Drosophila genome (Table 3). Genes with function linked to the tricarboxylic acid cycle (TCA)/aerobic respiration were highly enriched, which makes sense since the trachea is a respiratory organ (Table 3). Many genes involved in synapse transmission were also enriched, perhaps revealing a role for Trh in the CNS, where it is also expressed (Table 3).

Discussion

To identify downstream targets of Trh, we performed two large-scale screens, including an in situ hybridization screen of approximately 100 tracheal-expressed genes and a genome-wide microarray analysis. Surprisingly, our in situ screen revealed that *trh* is required for maintaining expression of all tested tracheal-expressed genes, suggesting that *trh* is required for tracheal cell identity, a role similar to that of the *C. elegans* pharyngeal regulator *pha-4* (Gaudet et al., 2004) and the mouse pancreatic regulator Pdx1 (Jonsson et al., 1994; Svensson et al., 2007). A re-examination of the regulatory hierarchy among the early-expressed transcription factors and the pilot in situ screen for Vvl and Kni targets during tracheal formation suggest a new model wherein Trh plays a far greater role than these other

transcription factors in regulating the expression of tracheal genes. Indeed, loss of *vvl*, the gene previously thought to function in parallel with Trh (Boube et al., 2000), affects expression of only an estimated 25-30% of tracheal genes, whereas loss of *kni*/*knrl* affects expression of only a single known tracheal gene. A role for Trh in tracheal cell identity and in regulating all tracheal genes is consistent with both loss-of-function and overexpression phenotypes, wherein all of the morphological events of trachea formation completely fail to occur in *trh* null embryos and formation of extra tracheal segments can be induced by global expression of a *trh* cDNA (Isaac and Andrew, 1996; Wilk et al., 1996).

Direct versus indirect regulation by Trh

The genes affected by loss of *trh* have variable expression patterns both in terms of when and where they are expressed in the trachea (Fig.2). Presumably, many of the earliest-expressed genes could be direct targets of Trh, whereas the genes expressed only at late stages could include both direct and indirect targets since *trh* continues to be expressed in all tracheal cells through the end of larval life. The tracheal genes whose expression begins only late in embryogenesis or whose expression is limited to only a subset of tracheal cells must also be regulated by other tracheal-expressed transcription factors. Indeed, many transcription factors were identified as Trh targets in our screen. Examples include Xbp1 and CrebA, two bZip transcription factors that regulate secretory function in multiple secretory organs (Abrams and Andrew, 2005; Fox et al., 2010; Lee et al., 2005; Shaffer et al., 2004), No Ocelli (Noc), a zinc finger transcription factor controlling morphogenesis of specific tracheal branches (Dorfman et al., 2002), Molting defective (Mld), another Zinc finger transcription factor involved in ecdysone biosynthesis (Neubueser et al., 2005), and Limpet (Lmpt), a zinc finger protein of unknown function (Table 1). Our microarray experiments also identified a number of completely uncharacterized putative transcription factors downregulated in *trh* mutants (Table S1). A cascade of transcriptional regulation could explain how so many genes are affected by the loss of only a single transcription factor and could explain the range in temporal expression observed with the different tracheal genes. The genes downstream of Trh also have a range of spatial expression patterns; some genes are expressed in the entire trachea, some are expressed in only specific branches, and others are expressed in only specific cell types, such as in the fusion cells (Fig. 2). The differential expression of genes within the trachea suggests that many Trh targets also require input from branch-specific or cell-type specific pathways, many of which have already been described, including FGF, Wg, Dpp and Notch signaling (Chiang et al., 1995; Chihara and Hayashi, 2000; Franch-Marro and Casanova, 2002; Ikeya and Hayashi, 1999; Llimargas, 1999, 2000; Steneberg et al., 1999; Vincent et al., 1997). Nonetheless, our finding that Trh is required for the expression all tracheal genes, regardless of when and where they are expressed, suggests a role for Trh in both specifying and maintaining the tracheal cell fate.

Although many of the early-expressed (stage 10/11) tracheal genes are completely dependent on *trh* at all stages, some tracheal genes, including four genes we have examined by fluorescence in situ analysis (*tkv*, *ft*, *CG13333* and *CG8312*), are expressed in *trh* mutant tracheal cells at stage 10/11, suggesting that another factor may be required for regulating the initial expression of a subset of tracheal genes. It is unlikely, however, that *trh* and *vvl* independently activate expression of these early genes, because all of the four genes whose early expression is either unaffected or only slightly downregulated by *trh* were still expressed in *trh vvl* double mutant trachea at early stages (data not shown). Another possibility is that expression of a few early-expressed tracheal genes could be activated by the same factors that initiate *trh* and *vvl* expression, such as STAT92E (Fig. 7). A third possible explanation is based on the expression pattern of many of these early genes. Three of the four genes, including *tkv*, *ft* and *CG13333*, are not exclusively expressed in the

tracheal cells. Instead, they are expressed in a broad ectodermal domain that overlaps the early trachea (data not shown). Gene expression in this broader domain may not necessarily be regulated by tracheal-specific transcription factors.

A new transcriptional hierarchy of tracheal formation

trh, *vvl* and *kni*/*knrl* are among the earliest-expressed transcription factors in tracheal formation and their initial expression has been reported to be independent of one another (Boube et al., 2000; Zelzer and Shilo, 2000). However, our re-examination of the expression of each of the early-expressed transcription factors in the mutant background of the other two genes revealed that not all of the reported changes in gene expression are correct. Consistent with previous findings, the early expression of *trh* and *vvl* are independent of each other; *vvl* is dispensable for the maintenance of *trh*, however, whereas *trh* is absolutely required for the maintenance of *vvl*. Also, *kni* tracheal expression is completely dependent on *trh*, suggesting that *kni* is just one of many transcriptional targets of *trh*, rather than being among the earliest-expressed transcription factors whose activation is directly dependent on the early patterning genes (Fig. 7).

Consistent with a new transcriptional hierarchy, expression of only a subset of tracheal genes depends on *vvl*. Our pilot in situ screen suggests that *vvl* is required for the expression of approximately 25-30% of tracheal genes and that Vvl plays a relatively minor role in the transcriptional regulation of tracheal genes compared to Trh. Indeed, *vvl* mutants have milder tracheal defects than *trh* mutants; whereas no morphogenetic events occur in *trh* mutants, *vvl* mutant tracheal cells do invaginate and form rudimentary branches (Fig. 3). Identification of the full transcriptional targets of *vvl* will lead to further understanding of the function of *vvl* during tracheal formation. Consistent with our finding that *kni* is just one of many *trh* targets, *kni* itself had only one previously known tracheal target, *sal*, which is repressed by Kni in dorsal tracheal cells (Chen et al., 1998). We failed to identify any additional transcriptional targets of *kni* in our pilot in situ screen.

Microarray experiments identify novel Trh targets

Our microarray experiments using mRNA from stage 11-16 embryos not only identified a number of novel Trh targets, but also confirmed many of the Trh target genes identified in our in situ screen. 35 out of the 98 genes we examined in the in situ screen were reduced by 1.4 fold or more in *trh* mutants compared to WT. There could be several reasons why all of the in situ targets were not included in the microarray targets: Many Trh downstream genes showed less than 1.4 fold change in the microarray analysis. An example is *kkv*, which encodes chitin synthase that regulates tracheal tube diameter (Tonning et al., 2006); *kkv* was down only 1.36 fold in the *trh* mutants. A similarly mild change in expression levels was observed with many Trh downstream genes, including many genes that have only transient tracheal expression and genes that are expressed in multiple other tissues, where expression is unaffected by loss of *trh*. The change in mRNA levels in the trachea relative to the whole embryos that were used in the microarray experiments resulted in total averaged mRNA changes that are likely too small to be detected in the microarray. We also found a number of genes that showed more than 1.4 fold change, but had p-values greater than 0.05. Although the three sets of our microarray samples showed more than 98% correlation, the occasional outliers made the fold change non-significant for some known Trh targets. In summary, although the whole-genome approach was successful in revealing new Trh target genes, the sensitivity limitations of this type of study will result in many target genes being missed. Nonetheless, microarray experiments identified almost 1,000 transcriptional targets of Trh, many of which are novel targets and a subset of which we have confirmed by either in situ hybridization or RT-qPCR. GO clustering revealed that Trh downstream genes are involved in various steps in numerous biological processes, which is consistent with our

findings that Trh functions very early during tracheogenesis and controls all of the genes required to form of this organ. Microarray experiments also identified a number of targets expressed in the other tissues such as CNS providing potential insight into which aspects of neuronal development are under the control of Trh.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

trh encodes three splice forms that are expressed in the same spatial and temporal patterns.

trh autoregulates in the trachea, but not in the other tissues.

trh regulates expression of all tracheal genes based on in situ analysis of ~100 genes.

ventral veinless (*vvl*) regulates only 25-30% of tracheal genes.

knirps (*kni*) regulates only a single tracheal gene.

Contrary to published findings, Trh maintains *vvl* and activates *kni*, but neither *kni* nor *vvl* affect *trh*.

Microarray studies reveal that Trh regulates a broad range of biological processes.

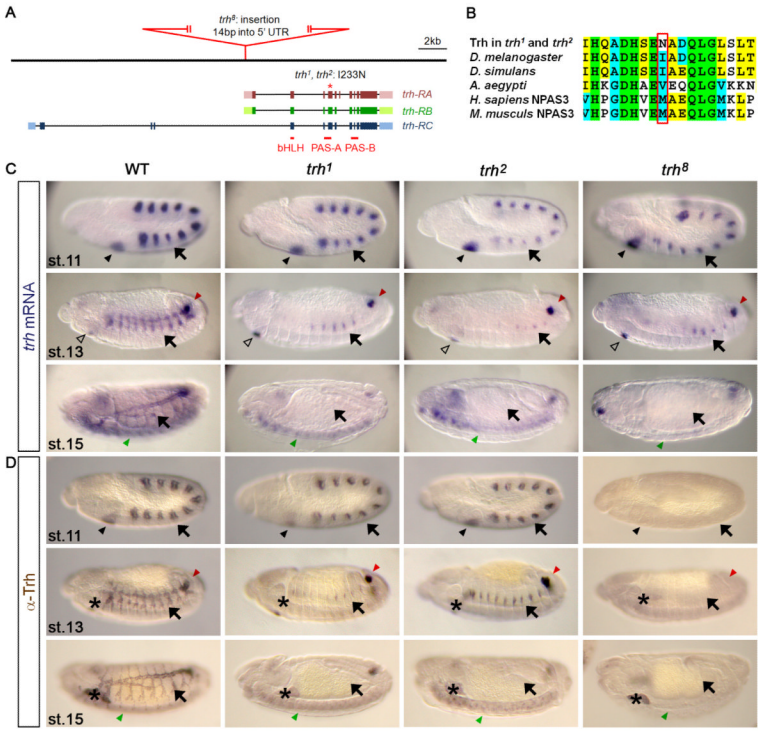

Fig.1.

Trh autoregulates in the trachea but not in other tissues. (A) *trh* has three known alternative transcripts (*RA*, *RB* and *RC*), which encode nearly identical proteins that share the bHLH domain and two PAS domains (FlyBase). trh^1 and trh^2 are EMS-induced mutations. trh^8 is an excision allele derived from a P element inserted 14 bp into the 5′ UTR of the *trh-RA* and *trh-RB* isoforms. (B) The amino acid sequences of the C-terminal region of PAS-A domain are aligned. The Ile (I) to Asn (N) mutation in trh^1 and trh^2 is conserved in Trh orthologues from other species (either the same or a similar residue is always found in this position). The amino acids completely conserved in all species are in green, the identical residues are in yellow, the similar residues are in cyan, and the different residues are in white. (C) A comparison of *trh* mRNA levels in wild type and *trh* mutant alleles reveals a loss of *trh* expression in the trachea (arrows) at later stages but no changes in the levels of expression in the salivary duct, filzkörper or CNS in *trh* mutants. (D) Trh protein levels are similar to *trh* mRNA levels in WT, trh^1 and trh^2 mutants, whereas no Trh protein is detected in trh^8 mutants. Arrows indicate the trachea, black arrowheads indicate the early salivary gland, open arrowheads indicate the salivary duct, red arrowheads indicate the filzkörper, and green arrowheads indicate the CNS. The asterisk indicates cross-reactivity of the Trh antiserum with the secretory portion of the salivary gland, which does not express *trh* in late embryos.

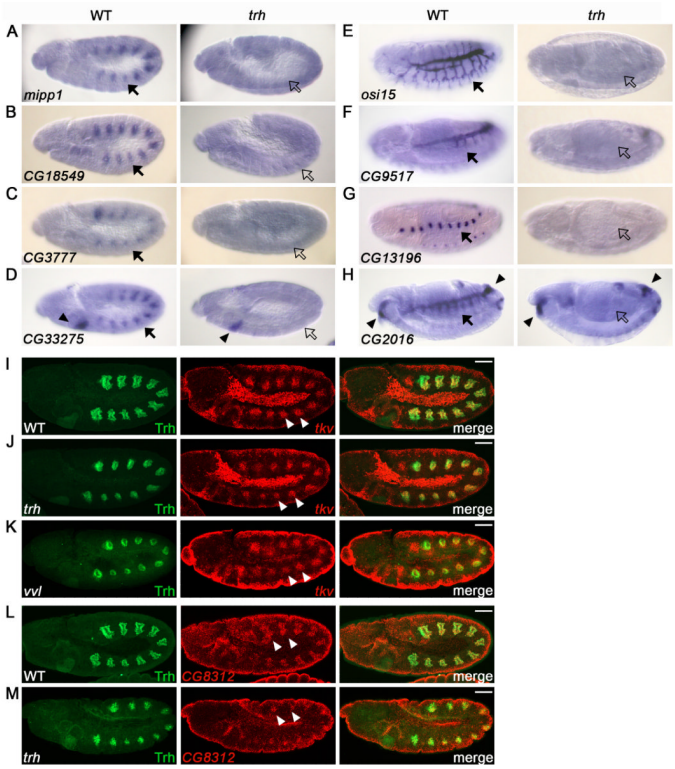

Fig.2.

trh regulates all tested tracheal genes. (A-H) Shown are in situs of several tracheal-expressed genes in wild-type and *trh* mutant embryos. Embryos in A-D are stage 11 and those in E-H are stage 15. At later stages, some genes are expressed in the entire trachea (E), whereas other genes are expressed in a subset of tracheal branches (F, H) or in specific tracheal cell-types (G). Regardless of the expression pattern of each gene, the tracheal expression in wild type (black arrows) is completely absent in the *trh* mutants (open arrows). Note that only tracheal expression is dependent on *trh*; expression in the other tissues, such as the salivary gland (D) or head/tail region (H), is not affected by the absence of *trh* (arrowheads in D, H). (I-K) Fluorescent in situ hybridization of *tkv* (red) with α-Trh staining (green) was done with wild type (I), trh^{2} (J) and vvl^{6A3} (K) mutants. *tkv* mRNA expression is still present although slightly reduced in the dorsal tracheal cells at stage 11 in both *trh* and *vvl* mutant embryos. (J, K) Fluorescent in situ hybridization reveals reduced expression of the early-expressed tracheal gene *CG8312* in *trh* mutants (M) compared to wild type (L). White arrowheads in I-M indicate *tkv* or *CG8312* mRNA expression in WT or *trh* mutant embryos. Scale bars: 50μm.

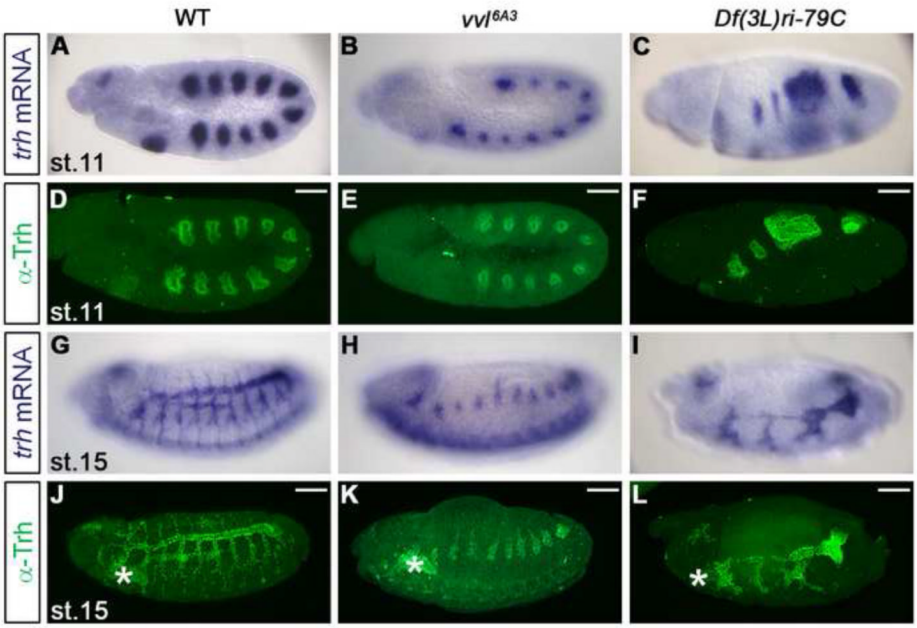

Fig.3.

trh expression is largely unaffected by loss of *vvl* or *kni*. *trh* mRNA (A-C, G-I) and Trh protein (D-F, J-L) are expressed in the trachea of both *vvl* mutants and embryos deficiency for both *kni* and the related *knrl* gene (*Df(3L)ri-79C*) at both early (A-F) and late (G-L) stages. Asterisks in J-L indicate nonspecific background staining with the α-Trh antisera in the late embryonic salivary gland at late stages. Scale bars: 50μm.

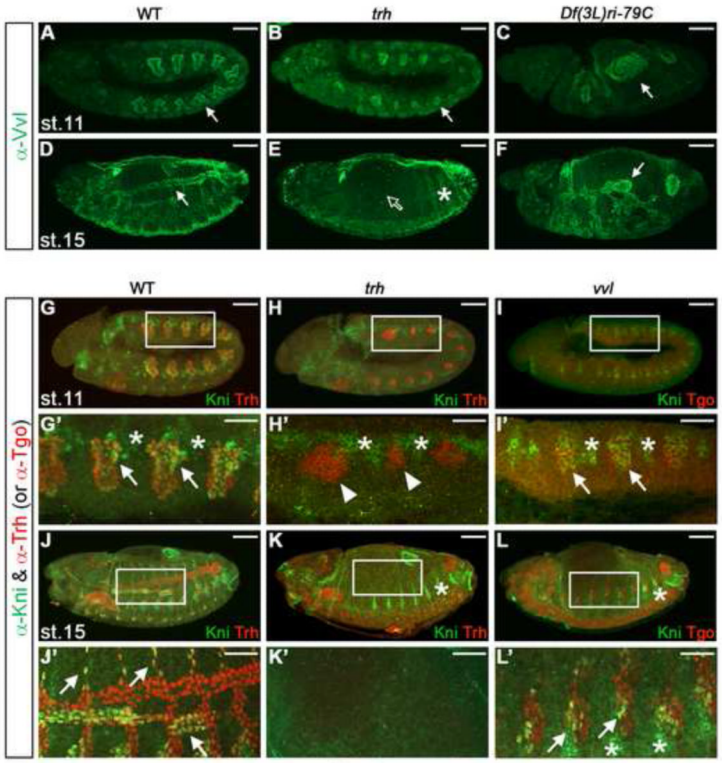

Fig. 4.

Trh is required for the expression/maintenance of Vvl and Kni. (A-F) α-Vvl staining (green) in wild type, *trh*^{2} and *Df(3L)ri-79C* embryos. Wild type expression of Vvl is shown in A and D (arrows). The early expression of Vvl is relatively normal in early *trh* mutants (B, closed arrow), but subsequently disappears (E, open arrow). Asterisk indicates non-tracheal Vvl expression. Vvl protein is expressed to WT levels in *Df(3L)ri-79C* embryos at all embryonic stages (C, F, arrows). (G-L) Kni (green) expression requires *trh* but not *vvl*. Higher magnifications of the boxed region of G-L are shown in G'-L'. α-Trh or α-Tgo (red) signals mark the tracheal cells. Arrows indicate the Kni- and Trh (or Tgo)- doubly positive tracheal cells. Kni expression is completely absent in the *trh* mutant tracheal cells at all stages (H, K). Trh-positive cells in H' do not express Kni in the *trh*^{2} mutants (arrowheads). In contrast, Kni is normally expressed in the *vvl* mutants (I, L). Asktersisks in G'-I', K, L and L' are non-tracheal cells expressing Kni. Embryos in A-C, G-I, and G'-I' are stage11, and those in D-F, J-L, and J'-L' are stage15. Scale bars: 50μm in A-L; 20μm in G'-L'.

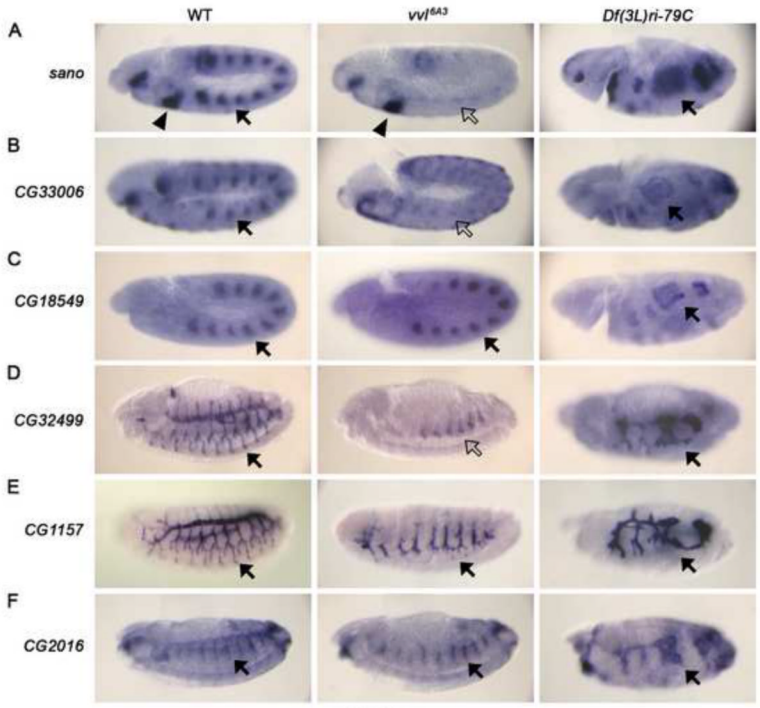

Fig. 5.

Vvl regulates a subset of tracheal genes whereas Kni does not affect expression of any of the tracheal genes tested. (A-F) Shown are examples of *in situ* hybridizations with tracheal-expressed genes in *vvl* and *Df(3L)ri-79C*. Embryos in A-C are stage 11 and those in D-F are stage 15. (A, B) Compared to wild-type tracheal expression levels (black arrows), *sano* and *CG33006* levels are significantly reduced in vvl^{6A3} mutants (open arrows). Note that salivary gland expression of *sano* is not affected by *vvl* loss (arrowheads in A). (C) *CG18549* is expressed to wild-type levels in *vvl* mutants and in *Df(3L)ri-79C* embryos. (D) *CG32499* is expressed in *vvl* mutants (open arrow), but in fewer cells compared to wild type (black arrow). (E, F). Two additional examples of tracheal genes (*CG1157* and *CG2016*) whose expression is unaffected by loss of *vvl* or *kni* are shown.

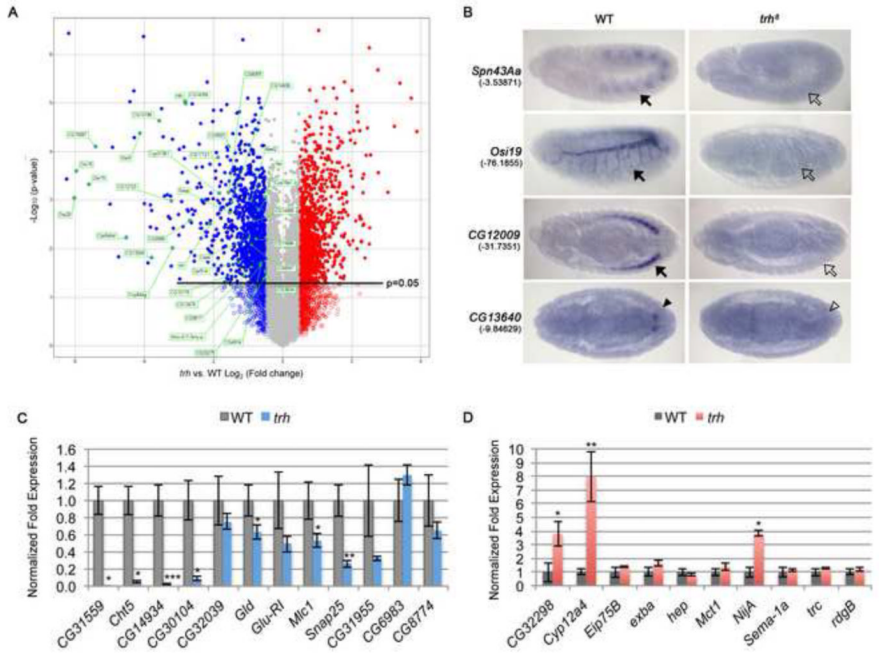

Fig. 6.

Microarray experiments identify additional genes regulated by Trh. (A) Volcano plot showing changes in expression levels and statistical significance of Trh target genes. Transcripts elevated 1.4 fold or more in *trh* mutants are labeled red. Transcripts reduced 1.4 fold or more are labeled blue. Trh downstream genes identified in the in situ screen are marked in green. Closed circles indicate significance with a p-value ≤0.05. Open circles indicate non-statistically significant changes. (B) Examples of the expression patterns of novel Trh targets identified by microarray in both wild type and *trh* mutant embryos. Tracheal-specific genes (*Spn43Aa*, *Osi19*, and *CG12009*, black arrows) as well as those expressed in other Trh-expressing tissues, such as the filzkörper (*CG13640*, black arrowhead), require *trh* for expression. Open arrows and arrowheads indicate the absence of expression in the *trh* mutant trachea and filzkörper, respectively. (C, D) RT-qPCR with downregulated genes (C) and upregulated genes (D). The fold changes were normalized to a housekeeping gene (*actin5c*) and the relative levels of normalized expression were shown compared to WT (1.0). Error bars represent standard error of the mean. *, $p<0.05$; **, $p<10^{-3}$; ***, $p<10^{-6}$.

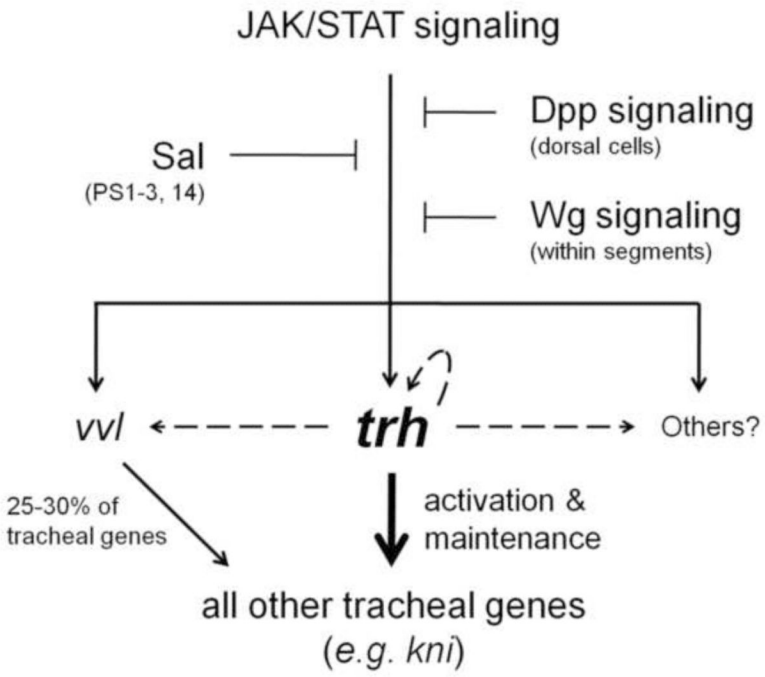

Fig. 7.

A model for gene regulation during tracheal development. The global patterning genes including Dpp, Wg and Sal specify the region where tracheal primordia form. Stat92E directly and independently regulates the expression of *trh* and *vvl*, the two earliest-expressed tracheal transcription factors. Trh is required for expression of all tracheal genes whereas Vvl is required for the expression of only 25-30% of tracheal genes. Trh also maintains its own expression and is required for the maintenance of *vvl* and the few other tracheal genes whose initial expression is independent of Trh (dotted lines).

Table 1

Gene names, cDNAs and the *trh* mutant alleles used in the in situ screen

Early-expressed genes, expression is dependent on Trh at all stages

gene name	cDNA used	*trh* allele(s) used
Mipp1	GM09242	1
CG33167/CG33275	GM01778	1
heph	LD03185	1
dp	SD02173	1
CG17218	RE65321	1
vari	RH61449	1
Rab23	RH23273	1
CG4726	RE01809	8
CG32276	RE27904	1
jbug	GH03013	1, 8
CG18507	RE25285	8
trh	RH17284	2
Gtp-bp	LD25651	1
Xbp1	GH09250	8
CG8965	GH01721	1
noc	LD28078	8
kkv	LD43683	1
CG18549	RE35942	1
CG3777	RH61716	1, 2
sano	RE56731	1, 2

Early-expressed genes, late expression is dependent on Trh, early expression is downregulated in *trh* mutants

gene name	cDNA used	*trh* allele(s) used
CG13333	LD15563	1*, 8
tkv	GH25238	2*, 8
CG8312	GH04568	1*, 8
ft	LD19710	1*, 8

Early-expressed genes, late expression is dependent on Trh, early expression is not clearly affected by *trh* loss

gene name	cDNA used	*trh* allele(s) used
pyd	LP05923	1, 8
mfas	GH11519	1

Early-expressed genes, late expression is dependent on Trh, early expression is not clearly affected by *trh* loss

gene name	cDNA used	*trh* allele(s) used
sala	RE40068	1
CG32499	RE51076	8
CG33006/CG4844	LD10058	1
tgo	LD32037	2, 8
ogre	HL01248	1
CG11069	LD11139	1
CG4115	RH61375	1
CG3624	GH11432	1
CG12581	GM02933	1
Tsf2	LD22449	1
CrebA	RE64328	1
mld	SD03914	8

Late-expressed tracheal genes (st12-16), all are dependent on Trh

gene name	cDNA used	*trh* allele(s) used	gene name	cDNA used	*trh* allele(s) used
CG32473	GM08240	1	*CG13196*	RE44287	8
CG9990	LD15982	1	*CG13049*	RE26528	1
CG4914	LP11612	1	*Lmpt*	RE65447	1
PFE, rdo	GH07373	1	*Cpr64Ad*	RE69226	8
Loco	GH08607	1	*CG12206*	RE48393	1
Osi15	RE74918	1	*Cyp301a1*	RE71294	1
CG3153	RE36503	2	*CG10175*	RE02195	1
CG2016	RE32803	1	*CG14636*	RE60714	1
CG4324	RE53026	1	*Cyp18a1*	RE70470	8
ImpE1	RE39082	1	*CG6055*	RE43931	8
CG16786	RE48511	1	*RpS12*	LD04944	8
CG5873	RE26705	1	*CG13676*	RE01745	8
CG14866	RE26319	8	*Ptp4E*	GH15539	8
CG17121	RH48101	8	*emp*	GH06663	8
CG2663	RE73641	8	*CG1440*	LD46760	8
CG32473	RE62048	8	*CG30101*	LD27203	8
Ast	RE16553	8	*Knk*	RE24065	8
CG9517	RE28171	8	*CG9503*	RE09982	8
CG1299	RH04813	8	*obst-A*	LD43683	8
Cyp313b1	RE08823	2	*Best2*	RE18408	8
Cpr51A	RE08808	1, 8	*Amy-p*	GH10266	8
CG15786/CG42749	RE24790	8	*CG14356*	RE49262	1
CG12723	RE28286	8	*Osi9*	RE71995	1
Dnd	RE02160	1	*serp*	RE22242	1

Late-expressed tracheal genes (st12-16), all are dependent on Trh

gene name	cDNA used	*trh* allele(s) used	gene name	cDNA used	*trh* allele(s) used
Path	RH24992	2	*CG11686*	RH19248	2
m2	RE15854	8	*CG6847*	RH14406	1
CG15887	RE53127	8	*Gasp*	LD05259	1
Ccp84Ag	RE04513	8	*Osi20*	RE13893	1
Osi18	RE07882	8	*CG5590*	GH01709	1

* fluorescent in situ

Table 2

Tracheal genes regulated by Vvl and Kni

Gene name	Regulated by Vvl?	Regulated by Kni?
Mipp1	Yes, disappears	No
Tsf2	No	No
dnd	No	No
CG32499	Yes, significantly reduced	No
CG4726	Yes, disappears	No
CG18507	No	No
trh	No	No
CG13333	No	No
noc	No	No
CG33006/CG4844/CG18431	Yes, significantly reduced	No
tgo	No	No
ogre	No	No
CG18549	No	No
CG3777	Yes, disappears	No
CG1157	No	No
CG2016	No	No
CG32473	No	No
CG12723	No	No
Ccp84Ag	No	No
CG12206	No	No
sano	Yes, reduced early and disappears later	No
kkv	N.T.	No
CG3624	N.T.	No
CG33167/CG33275	N.T.	No
dp	N.T.	No
mfas	N.T.	No
vari	N.T.	No
CG4115	N.T.	No
CG31121	N.T.	No
CG10479	N.T.	No
CG11069	N.T.	No
CG8965	N.T.	No

N.T., not tested

Table 3

Clustering analysis of Gene Ontology (GO) terms for genes downregulated in *trh* mutants

Annotation Cluster*	GO term	Fold enrichment	P-value
1 (6.62)	glucose metabolic process	4.8	7.9×10^{-8}
	monosaccharide metabolic process	3.7	2.8×10^{-7}
	hexose metabolic process	3.8	6.1×10^{-7}
	e.g. *Gapdh1*, *Gapdh2*, *Gld*[a], *Pglym78*, *Tpi*		
2 (5.81)	proteolysis	1.8	5.4×10^{-8}
	peptidase activity	1.7	4.8×10^{-6}
	peptidase activity, acting on L-amino acid peptides	1.7	1.4×10^{-5}
	e.g. *Dip-B*, *stv*, *CG4386*[a], *CG8774*[a], *CG9372*[*]		
3 (4.15)	hexose catabolic process	4.5	3.9×10^{-5}
	glucose catabolic process	4.5	3.9×10^{-5}
	cellular carbohydrate catabolic process	4.2	4.3×10^{-5}
	monosaccharide catabolic process	4.4	4.9×10^{-5}
	glycolysis	4.7	1.9×10^{-4}
	alcohol catabolic process	3.8	2.1×10^{-4}
	- all genes are included in Cluster 1		
4 (4.04)	serine-type endopeptidase inhibitor activity	3.4	5.1×10^{-5}
	peptidase inhibitor activity	3.0	8.3×10^{-5}
	enzyme inhibitor activity	2.7	9.1×10^{-5}
	endopeptidase inhibitor activity	2.9	1.8×10^{-4}
	e.g. *Dbi*, *Spn43Aa*[*], *Spn5*, *Timp*, *CG14298*		
5 (3.37)	aminoglycan metabolic process	2.6	4.6×10^{-5}
	pattern binding	2.4	2.3×10^{-4}
	polysaccharide binding	2.4	2.3×10^{-4}
	chitin metabolic process	2.6	5.3×10^{-4}
	ChtBD2	2.6	5.9×10^{-4}
	Chitin binding protein, peritrophin-A	2.4	1.5×10^{-3}
	chitin binding	2.3	2.3×10^{-3}
	e.g. *obst-B*[*], *Cht5*[a], *CG10287*, *CG12009*[*], *CG17905*		
6 (3)	contractile fiber	6.0	2.0×10^{-4}
	contractile fiber part	5.8	7.8×10^{-4}
	sarcomere	6.0	2.2×10^{-3}
	myofibril	5.6	3.0×10^{-3}
	e.g. *Mlc1*[a], *Mf*, *Prm*, *Tm2*, *up*		
7 (2.71)	nucleobase, nucleoside and nucleotide catabolic process	7.0	1.1×10^{-3}
	nucleobase, nucleoside, nucleotide and nucleic acid catabolic process	7.0	1.1×10^{-3}

Annotation Cluster*	GO term	Fold enrichment	P-value
	nitrogen compound catabolic process	5.8	2.8×10^{-3}
	nucleotide catabolic process	6.8	4.6×10^{-3}
	e.g. *b*, *Gyc-89Db*, *CG4827* ,*CG6330*, *CG30104*[a]		
8 (2.71)	Aamy	6.5	1.4×10^{-3}
	Glycosyl hydrolase, family 13, subfamily, catalytic region	6.0	2.0×10^{-3}
	Glycosyl hydrolase, family 13, catalytic region	5.6	2.9×10^{-3}
	e.g. *Amy-d*, *LvpD*, *LvpH*, *CG14934*[a], *CG30359*		
9 (2.53)	serine-type peptidase activity	1.8	4.8×10^{-4}
	serine hydrolase activity	1.8	5.4×10^{-4}
	Tryp_SPc	1.8	2.2×10^{-3}
	Peptidase S1A, chymotrypsin	1.7	5.2×10^{-3}
	serine-type endopeptidase activity	1.6	7.3×10^{-3}
	Peptidase S1 and S6, chymotrypsin/Hap	1.6	8.0×10^{-3}
	Peptidase S1/S6, chymotrypsin/Hap, active site	1.7	1.1×10^{-2}
	- all genes are included in Cluster 2		
10 (2.37)	acetyl-CoA metabolic process	4.2	1.0×10^{-3}
	tricarboxylic acid cycle	4.1	2.7×10^{-3}
	acetyl-CoA catabolic process	4.1	2.7×10^{-3}
	coenzyme catabolic process	4.0	3.3×10^{-3}
	aerobic respiration	3.8	3.9×10^{-3}
	cofactor catabolic process	3.8	3.9×10^{-3}
	Citrate cycle (TCA cycle)	2.2	6.9×10^{-2}
	e.g. *l(1)G0255*, *Nc73EF*, *CG6439*, *CG7920*, *CG10622*		
11 (2.12)	transmission of nerve impulse	1.9	4.1×10^{-3}
	synaptic transmission	1.9	5.7×10^{-3}
	cell-cell signaling	1.7	1.9×10^{-2}
	e.g. *CanB*, *Nmdar1*, *SerT*, *Snap25*[a], *svp*		
12 (2.05)	Intrinsic to membrane	1.2	3.6×10^{-3}
	Integral to membrane	1.2	4.2×10^{-3}
	transmembrane	1.2	4.8×10^{-2}
	e.g. *btl*, *Glu-RI*[a], *mthl8*, *slif*, *CG10960*		

*The enrichment score is shown in parentheses.

[a]validation by in situ

[b]validation by RT-qPCR