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Differential requirement for STAT by gain-of-function and wild-type receptor tyrosine kinase Torso in *Drosophila*

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SUMMARY

Malignant transformation frequently involves aberrant signaling from receptor tyrosine kinases (RTKs). These receptors commonly activate Ras/Raf/MEK/MAPK signaling but when overactivated can also induce the JAK/STAT pathway, originally identified as the signaling cascade downstream of cytokine receptors. Inappropriate activation of STAT has been found in many human cancers. However, the contribution of the JAK/STAT pathway in RTK signaling remains unclear. We have investigated the requirement of the JAK/STAT pathway for signaling by wild-type and mutant forms of the RTK Torso (Tor) using a genetic approach in *Drosophila*. Our results indicate that the JAK/STAT pathway plays little or no role in signaling by wild-type Tor. In contrast, we find that STAT, encoded by *marelle* (*mrl; DStat92E*), is essential for the gain-of-function mutant Tor (Tor^{GOF}) to activate ectopic gene expression. Our findings indicate that the Ras/Raf/MEK/MAPK signaling pathway is sufficient to mediate the normal functions of wild-type RTK, whereas the effects of gain-of-function mutant RTK additionally require STAT activation.

Key words: *Drosophila*, Receptor tyrosine kinase (RTK), Torso, STAT

INTRODUCTION

Overactivation of receptor tyrosine kinases (RTKs), caused by either oversupply of ligands or mutations that result in ligand-independent constitutive activation, has been linked to many cancers and other human diseases (reviewed by Robertson et al., 2000). An important question is why such overactivation, which has been generally presumed to be quantitative in nature, could lead to qualitative changes of cellular properties. It has been proposed that a constitutively activated RTK hyperactivates a canonical downstream signal transduction pathway, such as the Ras-MAPK signaling cassette, and that the qualitative changes in gene expression are determined by the variation in signaling duration and/or intensity (Ghiglione et al., 1999; Greenwood and Struhl, 1997; Marshall, 1995; Sewing et al., 1997; Woods et al., 1997). Alternatively, however, overactivated RTK could signal via alternative pathways that are not essential for or engaged by wild-type RTK under physiological conditions, thus augmenting the signaling capacity of this overactivated RTK.

To investigate how overactivation of an RTK results in aberrant gene expression, we chose to study the Torso (Tor) pathway in the early *Drosophila* embryo. Tor is a fly RTK most homologous to the mammalian PDGF receptor. During *Drosophila* development, Tor specifies cell fates in the terminal regions of the embryo (Duffy and Perrimon, 1994). Tor mRNA is synthesized during oogenesis, deposited into the unfertilized egg, and translated following fertilization. Tor proteins are uniformly distributed on the cell membrane of the early embryo, but are activated at the terminal regions by a ligand that diffuses from the egg poles (Casanova and Struhl, 1993; Sprenger and Nusslein-Volhard, 1992). Previous studies have documented that Tor activates the evolutionarily conserved Ras1/Draf/MEK/MAPK signaling cassette (Duffy and Perrimon, 1994) to induce the expression of target genes such as *tailless* (*tll*) (Pignoni et al., 1990; Pignoni et al., 1992), which is essential for specifying cell fates in the terminal regions (Steingrimsson et al., 1991). The current model is that the *tll* promoter is repressed in the early embryo. The MAPK pathway abrogates *tll* repression, thereby enabling *tll* activation by an unknown ubiquitous transcription factor(s) (Liaw et al., 1995; Paroush et al., 1997).

tll expression at the posterior end is precisely restricted in a domain from 0 to 15% of the egg length (EL) in wild-type embryos (Fig. 1A). The extent of this domain is a sensitive readout of the strength of Tor activation (Hou et al., 1995; Li et al., 1998; Li et al., 1997). Thus, a decrease in Tor signaling, such as caused by *tor* or *Draf* loss-of-function mutations, results in reduction or elimination of *tll* expression in the posterior domain in a manner reflecting the severity of the mutation

(<15%EL; not shown). Conversely, in *tor* gain-of-function (*tor*^{GOF}) mutations associated with an increase in Tor signaling, expansion of the posterior *tll* expression domain towards the middle region of the embryo is observed (>15%EL; Fig. 1C). The signal generated by either wild-type Tor or Tor^{GOF}, as visualized by the *tll* expression readout, can be completely blocked by null mutations in *Draf* (also known as *pole hole*; *phl*) (Ambrosio et al., 1989). Thus, it has been proposed that the major output of Tor signaling is the activation of MAPK.

In a recent genetic screen intended to isolate additional components of RTK signaling, we identified the *Drosophila* STAT, encoded by *marelle* (*mrl; DStat92E*), as an essential mediator of Tor^{GOF} (W. L., unpublished data). This suggests that STAT might be required for signaling by the Tor RTK.

The JAK/STAT pathway was first elucidated by studying the mechanisms of interferon signaling. In the canonical model, STAT is activated by the cytoplasmic tyrosine kinase JAK, which itself is activated by a non-tyrosine kinase receptor in response to ligand binding (Darnell et al., 1994). It is now well established that activation of STAT is associated with many cancers and other human diseases (Sahni et al., 1999; Su et al., 1997), and indeed, activated STAT3 behaves as an oncogene in causing cellular transformation and tumor formation (Bromberg et al., 1999).

JAK and STAT proteins are conserved between flies and humans (Binari and Perrimon, 1994; Hou et al., 1996; Yan et al., 1996). The *hop* and *mrl* genes were isolated in genetic screens for determining the maternal effects of zygotic lethal genes (reviewed by Hou and Perrimon, 1997). Embryos lacking the maternal product of either *hop* or *mrl* exhibit identical morphological defects when their cuticles are examined at the end of embryogenesis. They are missing the fourth and fifth ventral abdominal denticle belts, A4 and A5, respectively (see Fig. 2B). In the early embryo, Hop and Mrl are essential for the correct expression of a number of segmentation genes including *even-skipped* (*eve*) and *runt* that are normally expressed in alternating parasegments, forming seven stripes along the anteroposterior axis (Hou and Perrimon, 1997).

To understand the mechanism by which STAT is involved in RTK signaling, we investigated the requirement of the JAK/STAT pathway downstream of the *Drosophila* RTK Torso (Tor). Our results show that the effects of Tor^{GOF} require not only the Ras/Raf/MEK/MAPK pathway, but also the *Drosophila* STAT protein Mrl. Mutations in *mrl* suppress the ectopically expressed *tll* and embryonic defects caused by Tor^{GOF}. In contrast, Mrl is not essential for the normal *tll* expression patterns controlled by wild-type Tor. These results indicate that the biological effects of wild-type Tor and Tor^{GOF} require distinct downstream signaling components.

MATERIALS AND METHODS

Genetics and examination of embryos

The *tor*^{GOF} alleles used in this study are *tor*^{Y9} and *tor*^{RL3} (Klingler et al., 1988). The *Ras1*$^{\Delta C40B}$ (Hou et al., 1995), *mrl*^{6346} (Hou et al., 1996) and *hop*^{C111} allele (Binari and Perrimon, 1994) used in this study are strong or null alleles. The Sevenmaker (SEM) allele of the *rl* locus is a gain-of-function allele (Brunner et al., 1994). *Draf*^{C110} and *Draf*^{PB26} are weak and intermediate alleles, respectively (Melnick et al., 1993). We used the dominant female sterile (DFS) technique (Chou and Perrimon, 1992) to generate homozygous germline clone (GLC) embryos for null alleles, such as *mrl*^{6346}, *hop*^{C111}, to test genetic interactions. Since *hop* and *Draf*, *mrl* and *Ras1*, are located on the same chromosome arm, respectively, we generated recombinant chromosomes to test the double mutant GLC phenotypes. To generate *mrl* GLC embryos from *tor*^{Y9}/+ females, we crossed *w*; *tor*^{Y9}/CyO; FRT^{82B} [*ovo*^{D1}, *w*$^{+}$]/TM3 males to *y w* hs-Flp/*y w* hs-Flp; +/+; FRT^{82B} *mrl*^{6346}/TM3 females to produce *y w* hs-Flp/*w*; *tor*^{Y9}/+; FRT^{82B} *mrl*^{6346}/FRT^{82B} [*ovo*^{D1}, *w*$^{+}$] females. To remove the maternal *mrl* gene product from the embryos produced by *rl*^{SEM}/+ females, we crossed *y w* hs-Flp; *rl*^{SEM}/CyO; [*ovo*^{D1}, *w*$^{+}$]/+ males to *y w* hs-Flp/*y w* hs-Flp; +/+; FRT^{82B} *mrl*^{6346}/TM3 females and generated *y w* hs-Flp/*y w* hs-Flp; *rl*^{SEM}/+; FRT^{82B} *mrl*^{6346}/FRT^{82B} [*ovo*^{D1}, *w*$^{+}$] females.

Co-immunoprecipitation

To extract embryonic proteins, embryos of 0 to 4 hours after egg-laying were collected and homogenized in Buffer A [10 mM Tris-HCl pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.1% Triton X-100; Protease Inhibitor cocktail (Sigma), and 1 mM PMSF final concentration]. To treat embryos with vanadate, a protein tyrosine phosphatase inhibitor, sodium orthovanadate (Sigma) was added to Buffer A prior to homogenization at 1 mM final concentration. To immunoprecipitate Tor from embryo extracts, we incubated anti-Tor antibody (Cleghon et al., 1996) with wild-type and *tor*^{GOF} embryo extracts (200 μl), respectively, at 4°C overnight at 1:200 dilution. The immunoprecipitates were resolved by 8% SDS-PAGE and blotted with anti-Tor antibody at 1:5000 dilution (Cleghon et al., 1996) to reveal the presence of Tor. The blot was then stripped of antibodies and reprobed with an anti-Mrl antibody (raised by immunizing rat with bacterially expressed Mrl) at 1:500 dilution to detect whether Mrl was bound to Tor in the embryo extracts.

Plasmids and fly transformants

A PCR based mutagenesis was performed on a 5.9 kb *tll* upstream regulatory fragment (Liaw et al., 1995) to introduce nucleotide changes in the two Mrl-binding sites. As a result, site 1 was changed from A**TTC**TGG**GAA**T to ATGCGGCCGCT to create a *Not*I site (underlined), and site 2 from A**TTC**TTC**GAA**AGAC to ATTCTTCGGTACC to create a *Kpn*I site (underlined). A *lacZ* reporter transgene was generated by replacing the wild-type *tll* regulatory region with this mutant 5.9 kb fragment in a *tll-lacZ* fusion gene (Liaw et al., 1995) and used to transform *Drosophila* by P element-mediated transformation.

RESULTS

Mrl mediates the effects of Tor^{GOF} on embryos

To determine whether Mrl plays a role in Tor^{GOF} signaling, we examined the phenotype of embryos derived from female germ cells that carry a *tor*^{GOF} mutation and lack *mrl* activity (see Materials and Methods). Strikingly, these embryos exhibited the characteristic *mrl* mutant phenotype, while the *tor*^{GOF} segmentation phenotype was mostly suppressed (Fig. 1). Consistent with the cuticle phenotype, the domain of *tll* expression in these embryos was nearly wild type (Fig. 1E). This suppression is not allele-specific, as a second *tor*^{GOF} mutant allele was also suppressed by lack of Mrl in embryos (data not shown). Both alleles of *tor*^{GOF} are due to point mutations in the extracellular, ligand-binding domain, presumably causing ligand-independent dimerization of the receptor (Sprenger and Nusslein-Volhard, 1992). These results demonstrate that removal of *mrl* suppresses the effects of *tor*^{GOF} mutation on *tll* expression and larval cuticles, suggesting that Mrl mediates the effects of Tor^{GOF}.

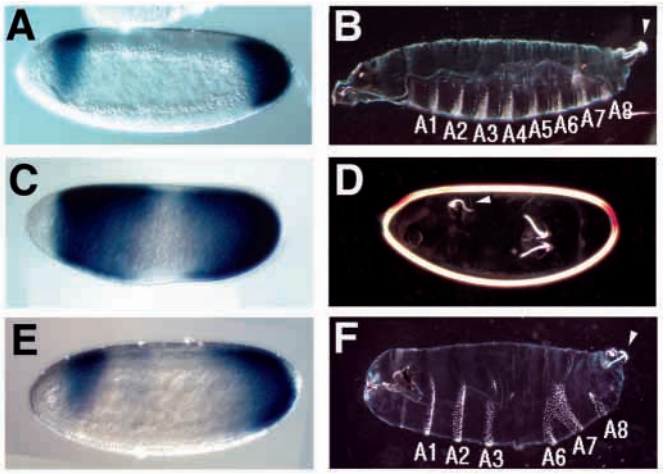

Fig. 1. The *tor*^{GOF} mutant phenotype is suppressed by loss of *mrl* activity. (A,C,E) *tll* mRNA expression patterns. (B,D,F) Larval cuticles. (A) In wild-type embryos, *tll* expression is restricted to the anterior and posterior poles. The posterior *tll* expression domain occupies 15% of egg length (EL). The anterior *tll* expression is controlled by both the Tor and Bicoid pathways (Pignoni et al., 1992) and is not discussed in this paper. (B) A wild-type larva has eight ventral abdominal denticle bands (A1 to A8), and posterior spiracles containing Filzkörper materials (arrowhead). (C) Embryos laid by females heterozygous for *tor*^{Y9}, a gain-of-function allele, show expansion of *tll* expression domains that causes enlargement of the terminal cell fates at the expense of the central cell fates (Klingler et al., 1988). As a consequence, (D) most denticle bands are frequently deleted, and occasionally ectopic Filzkörper material can be observed (arrowhead). (E) Removal of the maternal *mrl* gene product from *tor*^{Y9} embryos resulted in a reduction of the ectopic *tll* expression associated with *tor*^{Y9} to levels similar to those of wild-type embryos (see Materials and Methods). (F) The ventral denticle bands were mostly restored in these embryos and they exhibit phenotypes that are similar to *mrl* embryos derived from homozygous germline clones (GLC embryos). Similar results were obtained when a second gain-of-function *tor* mutation, *tor*^{RL3}, was used (data not shown).

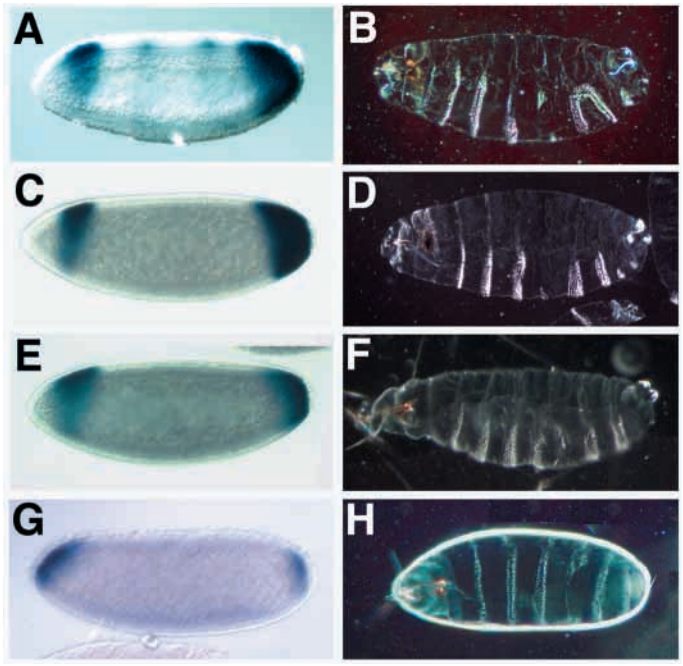

Fig. 2. Mutations in *hop* or *mrl* do not enhance *Draf* or *Ras1* mutant phenotypes. (A) In *mrl* GLC embryos, the size of the posterior *tll* expression domain appears similar to wild type. (B) *hop* or *mrl* GLC embryos have identical phenotypes (*mrl*^{6346} mutant is shown) with a characteristic deletion of A5. (C) The *tll* expression pattern in *hop*^{C111} *Draf*^{C110} GLC embryos is indistinguishable from that in *Draf*^{C110} GLC embryos. (D) These embryos exhibit cuticle phenotypes that resemble those of *hop*^{C111} embryos. (E) The size of the posterior *tll* expression domain in *hop*^{C111} *Draf*^{PB26} double GLC embryos is similar to that of *Draf*^{PB26} GLC embryos. (F) The cuticles of these embryos exhibit defects of both *hop*^{C111} and *Draf*^{PB26} mutants. (G) 19% of the *Ras1*$^{\Delta C40B}$ *mrl*^{6346} double mutant embryos (*n*=18/97) have residual posterior *tll* expression that is similar to *Ras1*$^{\Delta C40B}$ GLC embryos. (H) The cuticle defects of the *Ras1*$^{\Delta C40B}$ *mrl*^{6346} double GLC embryos are a combination of those associated with *Ras1*$^{\Delta C40B}$ and *mrl*^{6346} mutants, respectively, i.e., they show characteristic deletion of A4 and A5 due to the *mrl*^{6346} mutation and defects in posterior structures similar to *Ras1*$^{\Delta C40B}$ and *mrl*^{6346} GLC embryos.

Mrl and Hop are not essential for wild-type Tor signaling

To determine whether mutations in the JAK/STAT pathway show genetic interactions with members of the Ras1/Draf pathway, we generated embryos doubly mutant for various combinations of alleles. We used two *Draf* mutations with reduced activities, *Draf*^{C110} and *Draf*^{PB26}, as well as a null *Ras1* mutation, *Ras1*$^{\Delta C40B}$. Unlike *Draf* null GLC embryos, which exhibit no posterior *tll* and cuticle structures, *Draf*^{C110} GLC embryos have a wild-type cuticle and show a near wild-type *tll* expression (not shown) (see also Melnick et al., 1993). *Draf*^{PB26} GLC embryos have reduced posterior *tll* expression domains to 6-10% EL, and defects in the posterior cuticle structures that include frequent deletions of A8 (not shown) (see also Melnick et al., 1993). While most of the *Ras1*$^{\Delta C40B}$ GLC embryos are identical to *tor* or *Draf* null embryos and exhibit no posterior *tll* expression and cuticle structures, about 20% of these embryos have residual posterior *tll* expression as well as posterior cuticle structures due to a Ras1-independent activation of Draf (see also Hou et al., 1995).

Since the phenotypes associated with *tor*^{GOF} are suppressed by a null *mrl* mutation, we investigated whether Mrl or Hop activities are essential for the expression of *tll* in wild-type embryos. We found that in either *mrl* or *hop* mutant embryos, the posterior domain of *tll* expression, which is invariably reduced in mutations that affect Tor signaling, appears wild type (about 15% EL; Fig. 2A), indicating that the Hop/Mrl pathway is not essential for the wild-type patterns of *tll* expression.

These results, however, do not fully exclude the possibility that Hop and Mrl constitute a branch of the Tor signaling pathway that acts in parallel and redundant to the Ras1-MAPK branch, and that the inability to detect any influence of the JAK/STAT pathway on wild-type *tll* expression could result from a compensatory up-regulation of the Ras1/Draf/MEK/MAPK pathway. We therefore examined the role of the JAK/STAT pathway in a number of sensitized genetic backgrounds wherein the efficiency of Tor signaling had been

compromised. First, we examined *tll* expression and cuticle phenotype in embryos that were doubly mutant for a *hop* null allele and weak alleles of *Draf*. Elimination of *hop* did not increase the severity of the *Draf* mutations in these assays (Fig. 2C-F). Second, we examined the phenotype of embryos doubly mutant for *mrl* and *Ras1*. A fraction (about 20%) of *Ras1* null mutant embryos exhibits residual *tll* expression due to activation of Draf by a Ras1-independent mechanism (Hou et al., 1995; Li et al., 1998; Li et al., 1997). Removal of *mrl* activity did not enhance the *Ras1* phenotypes (Fig. 2G,H). Thus, neither Hop nor Mrl appear to be required for *tll* expression patterns in wild-type embryos, therefore they are unlikely to be integral components of the Tor pathway. This conclusion, however, does not apply to Tor^{GOF} since we find that Mrl activity is required for the full activity of Tor^{GOF}.

Tor^{GOF} is capable of activating Mrl

The above results are consistent with the possibility that Tor^{GOF} causes Mrl activation to exert its biological functions. To test whether Tor^{GOF} can cause Mrl activation, we examined Mrl activity in *Drosophila* Schneider (S2) cells transfected with DNA encoding different Tor molecules. As reported previously (Yan et al., 1996), transfection of Hop into S2 cells increased Mrl DNA-binding activity in these cells (Fig. 3A, lane 4). This increase in DNA binding was specific to Mrl, as addition of an anti-Mrl antibody causes the bound complex to be supershifted (Fig. 3A, lane 6). Interestingly, transfection of Tor or Tor^{GOF} also resulted in activation of endogenous Mrl in S2 cells (Fig. 3A, lane 2 and 3). Based on the intensity of the gel shift bands, Tor and Tor^{GOF} activate Mrl to levels similar to those observed after Hop transfection (Fig. 3A, lane 4). In these transfection experiments, Tor and Tor^{GOF} similarly activated Mrl, presumably because when overexpressed in transfection experiments wild-type Tor can dimerize, mimicking the effect of Tor^{GOF} mutations. These results are consistent with our hypothesis that Tor^{GOF} causes Mrl activation in vivo.

Tor^{GOF} activates Mrl independently of MAPK and JAK and is capable of associating with Mrl

How does Tor^{GOF} RTK activate Mrl? There are at least three possible mechanisms through which STAT activation by RTK can occur. RTK could directly bind and activate STAT proteins (Fu and Zhang, 1993). Alternatively, STAT could be indirectly activated by the RTK, either via JAK or MAPK (Wen et al., 1995). Genetic evidence allows us to rule out the possibilities that Tor^{GOF} activates Mrl via JAK or MAPK. First, we examined whether removal of Hop activity modifies the tor^{GOF} phenotype. Surprisingly, a *hop* null mutation did not suppress tor^{GOF} (Table 1), indicating that unlike Mrl, Hop is not required for ectopic *tll* expression. Second, removal of *mrl* did not suppress $rl^{Sevenmaker}$ (rl^{Sem}) (Table 1), which encodes a GOF mutant form of *Drosophila* MAPK (Brunner et al., 1994), suggesting that Mrl is not essential for the effects of GOF mutation in MAPK. To test for a physical interaction between Mrl and Tor^{GOF}, we immunoprecipitated Tor from wild-type and tor^{GOF} embryos, respectively, with anti-Tor antibody (Cleghon et al., 1996), and examined the presence of Mrl in the immune complexes. As shown in Fig. 3B, we detected a specific band corresponding to Mrl in the immunoprecipitates. The Tor-Mrl association, however, is only observed in the presence of vanadate (a general tyrosine phosphatase

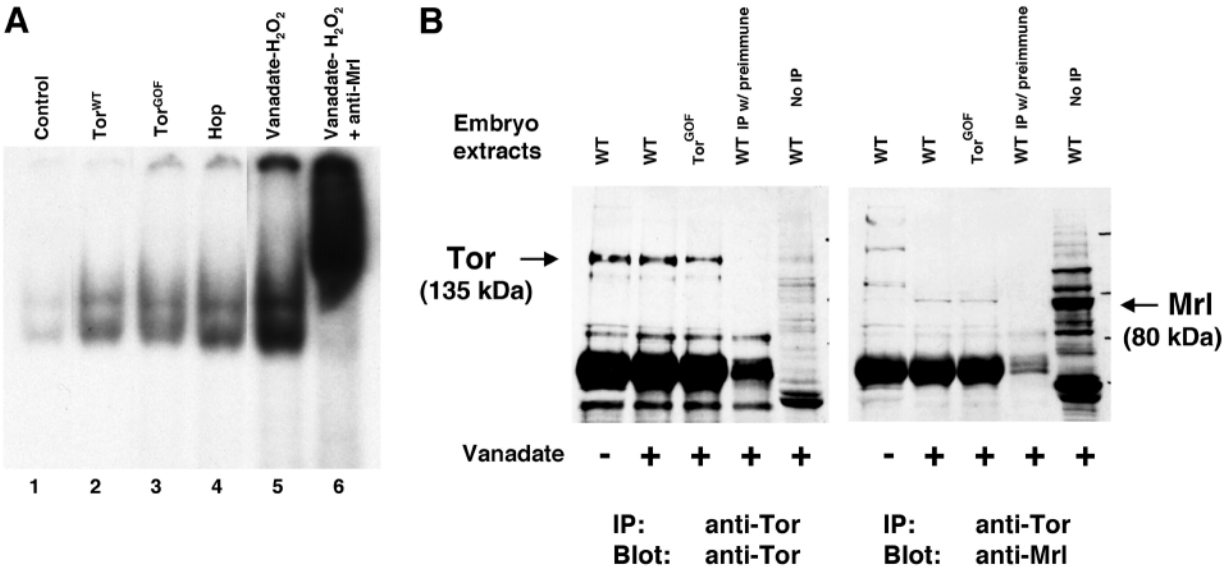

Fig. 3. Tor^{GOF} activates and associates with Mrl. (A) Mrl activity was measured by a gel mobility shift assay in S2 cell extracts using an oligonucleotide (top strand sequence: GGATTTTTCCCGGAAATG. Bottom strand sequence: GACCATTTCCGGGAAAAA) optimal for Mrl binding (Yan et al., 1996). Control (lane 1) shows basal levels of Mrl activity in S2 cells. Transfection of Hop (lane 4; 10 μg) resulted in a significant increase in Mrl DNA-binding activity. Transfection of DNA encoding wild-type Tor (lane 2; 10 μg) or Tor^{GOF} (lane 3; 10 μg) significantly increased the DNA-binding activity of Mrl to levels similar to those observed following Hop transfection. Cells treated with the vanadate-H_2O_2 (100 μM sodium orthovanadate, 1 mM hydrogen peroxide) (see Sweitzer et al., 1995) strongly activate Mrl (lane 5) and result in similar gel-shift bands. Addition of an anti-Mrl antibody caused a supershift of the protein-DNA complex (lane 6), suggesting it is due to Mrl-oligonucleotide association. (B) Tor protein was precipitated with anti-Tor antibody (Cleghon et al., 1996) from wild-type and tor^{GOF} embryo extracts, respectively. Note Mrl (~80 kDa) was co-precipitated with Tor (135 kDa) from both wild-type and tor^{GOF} embryos in the presence of vanadate.

Table 1. *hop* or *mrl* mutations do not suppress the phenotypes associated with tor^{GOF} or rl^{GOF}, respectively

Maternal genotype	Percentage of embryos with <4 denticle belts	Percentage of embryos with ≥4 denticle belts	Total number of embryos scored
tor^{Y9}/+; +/+	94.0 (*n*=376)	6.0 (*n*=24)	400
tor^{Y9}/+; hop^{C111} GLC	91.3 (*n*=94)	8.7 (*n*=9)	103
rl^{SEM}/+; +/+	10.7 (*n*=43)	89.3 (*n*=357)	400
rl^{SEM}/+; mrl^{6346} GLC	15.1 (*n*=13)	84.9 (*n*=73)	86

GOF mutations in *tor* and, to a lesser extent, in *rl* often result in deletions and disruption of the ventral denticle belts in the embryo. Strong alleles are associated with deletions of most or all denticle belts (see Fig. 1D); weaker alleles exhibit partial deletions or disruptions of ventral denticle belts (not shown) (Klingler et al., 1988). We categorized the embryonic phenotypes of GOF mutations into strong (<4 denticle belts) and weak (≥4 denticle belts) classes. Removal of maternal *hop* activity did not cause significant changes in the cuticle phenotypes associated with tor^{Y9}. Similarly, removal of maternal *mrl* did not significantly suppress rl^{SEM}.

inhibitor), suggesting that this interaction takes place only when the cytoplasmic protein phosphorylation status is preserved, or when Tor and/or Mrl have been activated by the presence of vanadate. Altogether, these results are consistent with a direct activation of Mrl by Tor^{GOF}, possibly following recruitment of Mrl to phosphotyrosine residues on the Tor RTK via SH2-phosphotyrosine peptide interaction.

Mrl-binding sites in *tll* promoter are essential only for Tor^{GOG}-induced ectopic *tll* expression

Since Mrl activation is required for ectopic *tll* expression induced by Tor^{GOF}, we examined whether Mrl-binding sites (TTCNNNGAA) were present in the regulatory region of the *tll* gene. A search in the *tll* regulatory region revealed two putative Mrl-binding sites with the consensus TTCNNNGAA located at –2357 (site 1) and –2462 (site 2) upstream of the *tll* transcription start site (Fig. 4B). These two sites were able to bind Mrl, although site 2 showed a much lower affinity (Fig. 4A). Interestingly, the two Mrl sites are located 105 bp apart in the *tll* regulatory region. This configuration is reminiscent of that existing in the *eve* stripe 3 enhancer, where cooperative binding of two Mrl homodimers was demonstrated (Yan et al., 1996). To assess the functional relevance of the two Mrl sites in *tll* expression, transgenes containing the 5.9 kb regulatory fragment upstream of the *tll* transcription start site fused to the *lacZ* gene were introduced into flies. This 5.9 kb fragment had been shown previously to drive *lacZ* expression in a pattern almost identical to that of the endogenous *tll* gene (Fig. 4C) (see also Rudolph et al., 1997). Accordingly, *lacZ* expression is greatly expanded in a tor^{GOF} background (Fig. 4D). A 5.9 kb fragment with the two Mrl binding sites mutated, showed wild-type activity for *lacZ* expression in wild-type embryos (Fig. 4E), suggesting that these Mrl-binding sites are dispensable for *tll* expression under normal Tor signaling. However, in a tor^{GOF} background, the mutant 5.9 kb fragment shows greatly diminished ability to drive *lacZ* expression in an expanded domain compared to the situation when the Mrl binding sites are wild type (compare Fig. 4D and F). These results are consistent with the genetic results that Mrl is required for the full activity of gain-of-function, but not wild-type Tor.

DISCUSSION

A general assumption regarding the pathophysiology inherent to gain-of-function RTKs has been that more activity of the receptor translates into a higher level of activation of the downstream signaling pathway, in our case the Ras1/Draf/MEK/MAPK pathway. The requirement of STAT in RTK signaling has been controversial. Contrary to general expectations that higher MAPK activation accounts for the effects of RTK overactivation, we provide genetic evidence that wild-type and gain-of-function mutant RTKs require distinct downstream signaling components to exert their effects. Signal transduction by Tor^{GOF} requires *Drosophila* STAT (Mrl). In contrast, Mrl is not essential for the Tor RTK to promote

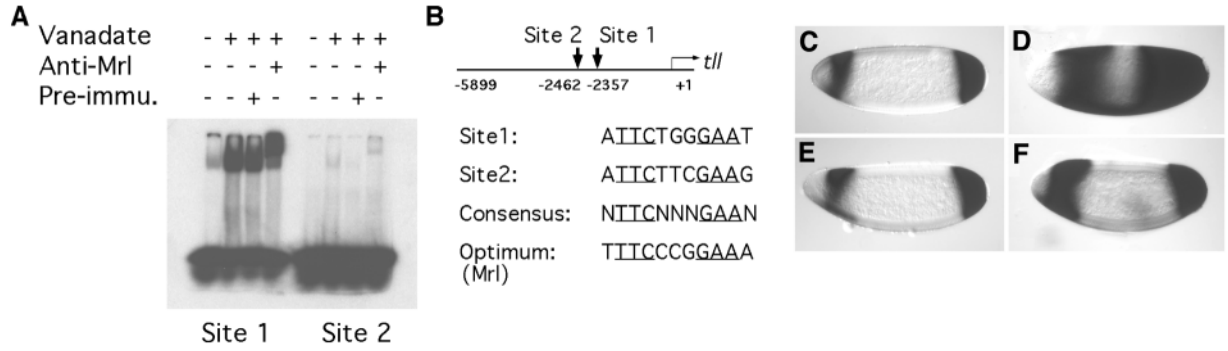

Fig. 4. Mrl-binding sites in the *tll* promoter. (A) The binding of Mrl to sites 1 and 2 was assayed by a gel mobility shift assay using synthetic oligonucleotides corresponding to the two sites and surrounding sequences. The Hop/Mrl pathway was activated by treating S2 cells with vanadate-H_2O_2 (Sweitzer et al., 1995). We find that site 1 binds strongly to Mrl, while the affinity of site 2 is lower. Addition of anti-Mrl antibody produced a supershift for each protein-oligo complex, which is consistent with the binding of Mrl to these sequences. (B) The positions of the two Mrl-binding sites are shown relative to the *tll* transcription start, and their sequences are shown and compared with STAT-binding consensus and optimal Mrl-binding sequences. (C) The 5.9 kb regulatory fragment upstream from the *tll* transcription start site is sufficient to drive *lacZ* expression in a pattern similar to that of endogenous *tll* in wild-type embryos. (D) In tor^{GOF} embryos, this promoter fragment drives *lacZ* expression in expanded domains. (E) A mutant 5.9 kb fragment was generated by disrupting both Mrl-binding sites (see Materials and Methods). In wild-type embryos, the expression pattern of *lacZ* driven by this mutated 5.9 kb fragment was not affected. (F) However, in tor^{GOF} embryos, the expansion of *lacZ* expression pattern was reduced.

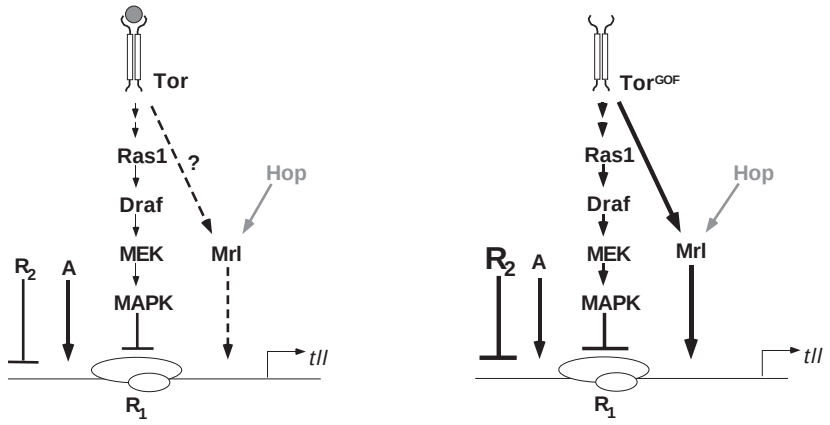

Fig. 5. Differential requirement of STAT for RTK signaling. The RTK Tor induces the expression of its target gene *tll* by derepression via activating the Ras-MAPK signaling pathway. Additional yet unidentified activators (A) and repressors (R_2), which may or may not be controlled by Tor, determine the transcription levels of *tll* in a combinatorial manner. The activators (A) and repressors (R_2) are unevenly distributed in cells along the anteroposterior axis of the embryo. In the central region of the embryo, there are higher levels of the repressors or lower levels of activators than the posterior region (only one possibility is shown). Mrl (STAT) is not essential for *tll* expression under wild-type conditions. However, Tor^{GOF} activates Mrl. Activated Mrl is required to overcome the higher levels of repressors (R_2) in tissues where *tll* is not normally expressed, resulting in developmental abnormalities. Arrow and bar indicate activation and repression, respectively. Dotted lines represent undermined events.

normal *tll* expression patterns in wild-type embryos. Most, if not all, of the deleterious effects (as visualized by expansion of *tll* expression and cuticle phenotype) can be explained by Mrl activation, and not through a higher output of MAPK activity. Thus signaling downstream of wild-type and overactivated RTK differs not only in strength, but also in quality.

In this manuscript we demonstrate that Tor^{GOF} requires Mrl but not Hop for its ability to induce ectopic target gene expression and causing deleterious effects on embryos. In addition, we show that Tor^{GOF} can associate with and cause Mrl activation in embryos and transfected cells. These results are most consistent with a model in which Tor^{GOF} directly phosphorylates Mrl, which in turn binds to the *tll* promoter to exacerbate its expression levels. Activation of STAT by RTKs has previously been suggested following studies in cultured mammalian cells. For example, transfected EGF or PDGF receptors can directly interact with and activate STAT by phosphorylation (Fu and Zhang, 1993; Paukku et al., 2000). Taken together with these studies, our results seem to suggest that the intracellular kinase domain of several RTK proteins may have an intrinsic ability to activate STAT proteins.

To account for the involvement of Mrl in *tll* regulation we propose that a hyperactivated RTK requires a downstream pathway that is not essential for wild-type RTK under normal physiological situations. In wild-type embryos, Tor is activated only in the two terminal regions and defines the spatial limits of *tll* expression domains by relieving the transcriptional repressors bound to the *tll* promoter. Mrl is not an essential factor for *tll* activation in the terminal regions, although it remains to be determined whether Mrl contributes to the activation of *tll* expression redundantly with other yet unidentified factors. In *tor*^{GOF} mutant embryos, Tor^{GOF} is constitutively active in all regions of the embryo and causes ectopic *tll* expression. In this case, Mrl activation is indispensable for the ectopic *tll* expression in the central regions of the embryo. The differential requirement for Mrl in central and terminal regions might be due to the lack of other activators of *tll* and/or the presence of additional repressors in the central region of the embryos. Consistent with this idea, we and others have previously shown that, in the absence of Tor signaling (such as in *tor* mutant embryos), *tll* can be induced by uniformly expressing activated forms of downstream signaling components (such as Ras^{V12} or 14-3-3). The resulting induction of *tll* expression happens preferentially in the terminal regions (Greenwood and Struhl, 1997; Li et al., 1998; Li et al., 1997). Thus *tll* expression could be determined by the balance between repressors and activators that can bind to the *tll* promoter (Fig. 5).

Our findings may explain some of the conflicting observations on the role of STAT in RTK signaling in mammals. For example, thanatophoric dysplasia type II (TD II) dwarfism in humans is caused by mutations that lead to constitutive activation of a human RTK FGF receptor 3 (FGFR3). Similar to Tor^{GOF} activating Mrl, it has recently been shown that an activated mutant FGFR3 specifically activates STAT1 in both human patient tissues and mouse models. The activated STAT1 in this case induces expression of the cell-cycle inhibitor $p21^{WAF1/CIP1}$, resulting in growth inhibition of bone tissues (Sahni et al., 1999; Su et al., 1997). However, STAT1 is not known to be required for bone development. STAT1 knockout mice have perfect bones, although they exhibit defective immune systems (Durbin et al., 1996; Meraz et al., 1996). This might be explained by a redundancy among different STAT proteins. Alternatively, STAT1 may not be required for normal FGFR3 signaling in bone development. The presence of several STAT genes in mammals makes it technically difficult to distinguish between the above two possibilities using the mouse as a genetic model. In contrast, the presence of a single JAK and a single STAT gene in *Drosophila* allows us to examine the relationship between RTK and JAK/STAT signaling, without being limited by gene redundancy. Our observations in *Drosophila* suggest that the TD II syndrome in humans could be explained if STAT1 is not normally required for FGFR3 signaling, but it becomes essential only for the activating mutant FGFR3.

Altered gene expression is commonly found in cancerous growth. The initiation and maintenance of the changes in gene expression often require the activation of multiple signaling molecules. STAT activation is found in many human cancers or transformed cells (Bromberg et al., 1999; Campbell et al., 2001; Catlett-Falcone et al., 1999; Garcia et al., 1997). In light of our finding in *Drosophila*, STAT activation might play essential roles for the activation of genes that are required for malignant growth and other pathological conditions. More importantly, we found that STAT activation is insignificant for the normal patterns of gene expression that are controlled by an RTK. It would be interesting to investigate if it is generally true that STAT activation is an important factor only in aberrant RTK signaling. If so, a broad implication of our results is that STAT rather than Ras, should be viewed as premier target for drug interference in the treatment of human diseases and cancers associated with hyperactivation of receptor tyrosine kinases.

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