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Ras Pathway Specificity Is Determined by the Integration of Multiple Signal-Activated and Tissue-Restricted Transcription Factors

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Summary

Ras signaling elicits diverse outputs, yet how Ras specificity is generated remains incompletely understood. We demonstrate that Wingless (Wg) and Decapentaplegic (Dpp) confer competence for receptor tyrosine kinase–mediated induction of a subset of *Drosophila* muscle and cardiac progenitors by acting both upstream of and in parallel to Ras. In addition to regulating the expression of proximal Ras pathway components, Wg and Dpp coordinate the direct effects of three signal-activated (dTCF, Mad, and Pointed—functioning in the Wg, Dpp, and Ras/MAPK pathways, respectively) and two tissue-restricted (Twist and Tinman) transcription factors on a progenitor identity gene enhancer. The integration of Pointed with the combinatorial effects of dTCF, Mad, Twist, and Tinman determines inductive Ras signaling specificity in muscle and heart development.

Introduction

A defining feature of metazoan development is the transition from an initially undifferentiated field of cells to one in which cells have unique identities. Intercellular signaling plays a major role in the progressive determination of cell fates, yet a relatively small number of signal transduction pathways is responsible for specifying a broad range of cell types. Although various combinations of signals can alter the effects of an individual pathway, the precise mechanisms by which distinct signaling outputs are generated in different developmental contexts remain to be elucidated. Three key issues relating to this problem must be addressed: first, how cells acquire the competence to respond to inductive signals; second, how multiple signaling inputs are integrated by the targeted cells; and third, how common signals elicit cell type–specific responses.

Developmental competence, signal integration, and response specificity are particularly relevant to signaling by the receptor tyrosine kinases (RTKs). While many RTKs function in the specification of diverse cell types, they can all act through a common pathway involving the activation of the Ras/mitogen activated protein kinase (MAPK) cascade (van der Geer et al., 1994). Ras signaling also plays a role in numerous other developmental processes, including those regulating cellular differentiation, migration, proliferation, and survival (Downward, 1998). It is thus critical to understand how RTK activation can lead to one response in a given cell, but a different response in another. In only a few cases has the basis of this specificity been defined (reviewed by Rommel and Hafen, 1998; Tan and Kim, 1999). For instance, RTKs are sometimes coupled to non-Ras-dependent pathways instead of, or in addition to, the Ras pathway (Clandinin et al., 1998). In other cases, the activities of additional signal transduction pathways or tissue-restricted transcription factors appear to act cooperatively with RTK/Ras/MAPK signaling to mediate response specificity (Maloof and Kenyon, 1998; Tan et al., 1998).

We have been studying cell fate specification in the somatic mesoderm of the *Drosophila* embryo as a model for dissecting the molecular basis of combinatorial signaling involving RTKs. The somatic musculature and the cells that compose the heart develop from specialized cells called progenitors (Carmena et al., 1995). Each progenitor divides asymmetrically to produce two founder cells (Ruiz Gomez and Bate, 1997; Carmena et al., 1998b) which possess the information that specifies individual muscle fate and which seed the formation of multinucleate myofibers (Rushton et al., 1995). We have focused our attention on a small subset of somatic mesodermal cells that express the transcription factor Even skipped (Eve). Eve is expressed in the progenitors and founders of the dorsal muscle fiber DA1 and of a pair of heart accessory cells, the Eve pericardial cells or EPCs (Buff et al., 1998; Carmena et al., 1998a, 1998b). Since *eve* is the earliest known marker for these cells (Carmena et al., 1998a) and is required for their formation (Su et al., 1999), we refer to it here as a progenitor identity gene.

Previous genetic experiments have defined multiple intercellular signaling events that govern the progressive determination of the Eve progenitors (Carmena et al., 1998a). Signaling from both the Wnt family member Wingless (Wg) and the TGFβ family member Decapentaplegic (Dpp) prepatterns the mesoderm and renders cells competent to respond to Ras/MAPK activation. Localized Ras activation within the competence domain determined by the intersection of Wg and Dpp expression occurs through the action of two RTKs, the *Drosophila* epidermal growth factor receptor (DER) and the Heartless (Htl) fibroblast growth factor receptor. This RTK signaling induces two distinct equivalence groups, each of which

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expresses Eve. Lateral inhibition mediated by Notch then selects a single progenitor from each equivalence group.

In the present study, we have explored how the prepattern genes *wg* and *dpp* establish competence for mesodermal cells both to activate and to respond to the Ras/MAPK cascade, how multiple intercellular signals are integrated to establish Eve progenitor fates, and how muscle- and cardiac-specific responses to Ras signaling are generated. We show that Wg provides competence for the generation of the Ras/MAPK inductive signal by regulating the expression of key proximal components of the DER and Htl RTK pathways. Wg and Dpp then create competence for a specific response to the inductive signal both through their own respective downstream transcriptional effectors, dTCF (also called Pangolin; Brunner et al., 1997; van de Wetering et al., 1997) and Mothers against dpp (Mad; Sekelsky et al., 1995), and through their regulation of the mesoderm-specific transcription factors Tinman (Tin; Azpiazu and Frasch, 1993; Bodmer, 1993) and Twist (Twi; Baylies and Bate, 1996). We further demonstrate that specificity of the Ras/MAPK response is achieved though the integration of these signal-activated and tissue-restricted transcription factors, along with the Ras/MAPK-activated Ets domain transcription factor PointedP2 (Pnt), at a single transcriptional enhancer. Our results provide a direct link between the initial axis patterning processes in the early embryo and the subsequent combinatorial signaling events that lead to the progressive determination of muscle and cardiac progenitors.

Results

The Mesodermal Eve Lineage

The Eve progenitors in each mesodermal hemisegment arise during embryonic stage 11 in a dorsal region demarcated by the intersecting domains of Wg and Dpp expression (Carmena et al., 1998a). The cells exposed to both Wg and Dpp are competent to respond to localized Ras signaling, which induces the initial expression of Eve in two clusters of equipotent cells. In each of these equivalence groups, activity of the Notch pathway leads to the rapid refinement of Eve expression to a single muscle or cardiac progenitor. The two Eve equivalence groups arise sequentially. Cluster C2, from which progenitor P2 derives, forms first (Figures 1A and 1B). P2 divides asymmetrically, with one daughter maintaining Eve expression and becoming the founder of the two EPCs ($F2_{EPC}$), and the other losing Eve expression and becoming the founder of muscle DO2 ($F2_{DO2}$; A. C., unpublished data). The second Eve-expressing cluster, C15, forms slightly later and produces the progenitor P15, which in turn divides to yield the founder of the Eve-expressing muscle, DA1, and an Eve-negative cell of as-yet-undetermined identity. Activation of the Ras/MAPK pathway in C15 depends on both the DER and Htl RTKs, but only Htl signaling is required for C2 formation (Buff et al., 1998; Carmena et al., 1998a; Michelson et al., 1998a, 1998b).

Wg Is Required for RTK/Ras Pathway Activation in the Specification of Muscle and Heart Progenitors

The progressive determination of Eve mesodermal progenitors requires that Wg prepattern the mesoderm, rendering cells competent to respond to inductive RTK/Ras signaling (Carmena et al., 1998a). To further investigate the basis of this competence, we considered whether the Ras pathway is active in the absence of Wg signaling by monitoring the expression of the activated, diphosphorylated form of MAPK (Gabay et al., 1997) in *wg* mutant embryos. As previously described, diphospho-MAPK is expressed in progenitor P2 in early stage 11 wild-type embryos (Figure 1B) (Carmena et al., 1998a). Not only is this progenitor missing from *wg* mutant embryos, but activation of MAPK in the C2 equivalence group, which is dependent on Htl, fails to occur (Figure 1C). Similarly, Wg is essential both for P15 formation and for the DER- and Htl-dependent activation of MAPK in the equivalence group from which this progenitor is derived (data not shown) (Carmena et al., 1998a).

We next determined at what level in the RTK/Ras pathway Wg is required for MAPK activation. In *wg* mutant embryos, there is loss of the P2-specific expression of Htl, its specific downstream signaling component, Heartbroken (Hbr, also known as Dof and Stumps; Michelson et al., 1998a; Vincent et al., 1998; Imam et al., 1999), and Rhomboid (Rho), a protein involved in the presentation of the DER ligand Spitz (Bang and Kintner, 2000; Figures 1E, 1I, and 1M). Conversely, constitutive Wg signaling, achieved by ectopic expression of Wg or an activated form of the downstream Wg pathway component Armadillo (Arm; Pai et al., 1997), induces Htl, Hbr, and Rho expression in more dorsal mesodermal cells than the single P2 progenitor found at a comparable developmental stage (Figures 1F, 1J, and 1N and data not shown). This effect is less prominent for Rho than for Htl and Hbr, which may reflect different threshold responses to Wg. Alternatively, the effect on Htl and Hbr may be more pronounced because ectopic Wg signaling prolongs their earlier expression in the entire C2 cluster; Rho, in contrast, is normally expressed in P2 but not in C2, possibly making it more refractory to a prepattern factor such as Wg (data not shown) (Buff et al., 1998; Michelson et al., 1998b). Expanded expression of these RTK pathway components is associated with increased MAPK activation and Eve expression (Figure 1P). However, these effects of Wg hyperactivation are transient, with a normal number of Eve progenitors eventually segregating (Lawrence et al., 1995; Carmena et al., 1998a; see below). Moreover, activated Arm is able to fully rescue Htl, Hbr, Rho, diphospho-MAPK, and Eve expression in *wg* mutant embryos (Figures 1G, 1K, 1O, and 1Q). Htl, Hbr, and Rho expression, as well as MAPK activation, are also Dpp dependent (data not shown). In summary, Wg and Dpp regulate the production of several key proximal components of the DER and Htl signal transduction pathways.

Given the involvement of Wg in the expression of Htl, Hbr, and Rho, we reasoned that a constitutively activated form of Ras1 might bypass the requirement of Wg for MAPK activation. Constitutively activated Ras1, when targeted to the mesoderm of wild-type embryos, leads to an overproduction of Eve progenitors (Gisselbrecht et al., 1996; Buff et al., 1998; Carmena et al., 1998a), as well as to the expected hyperactivation of MAPK in these cells (Figure 1R). In the absence of Wg signaling, diphospho-MAPK expression is restored by activated Ras1. However, despite this recovery of MAPK activa-

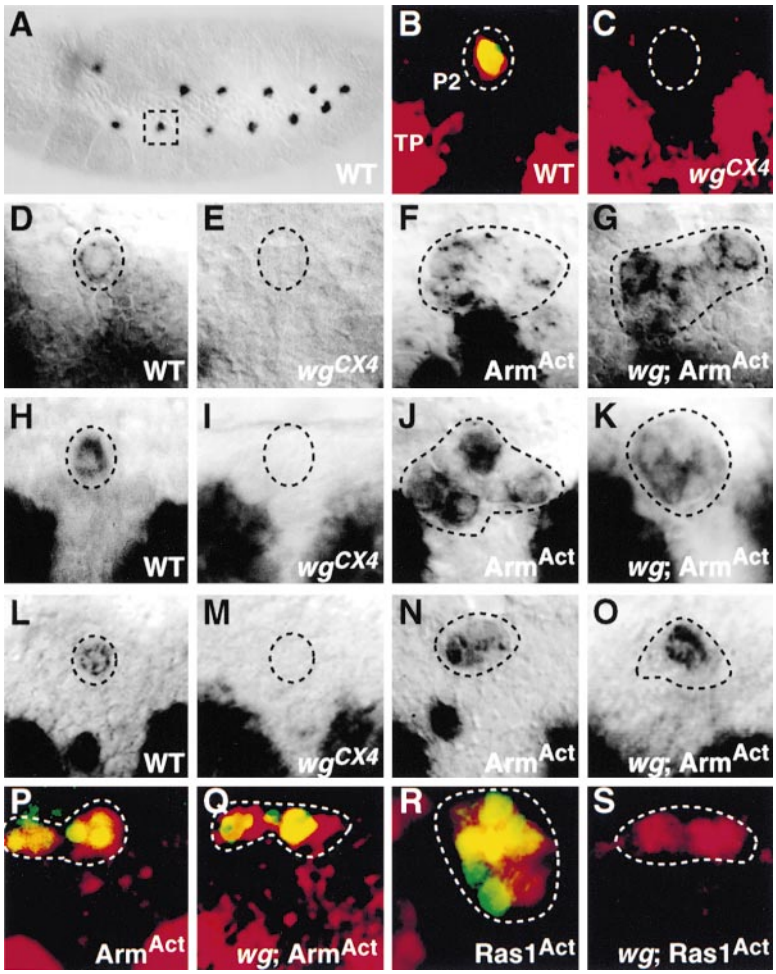

Figure 1. Wg Facilitates RTK/Ras Signaling through Its Effects on Proximal Components of the Htl and DER Pathways

(A) In early stage 11 wild-type (WT) embryos, Eve expression in C2 resolves into one P2 progenitor per hemisegment. The box indicates the portion of a hemisegment that is shown in the remaining panels.

(B) Eve (green) and diphospho-MAPK (red) are coexpressed in P2.

(C) In a *wg* mutant, both Eve and diphospho-MAPK expression is lost.

Expression of Htl protein (D–G), Hbr protein (H–K), *rho* RNA (L–O), and Eve (green) and diphospho-MAPK (red; P–S) in the indicated genetic backgrounds. Like Eve and activated MAPK, Htl, Hbr, and *rho* are present in wild-type P2 and this expression is Wg dependent. Ectopic activated Arm (F, J, N, and P) causes more cells to express Htl, Hbr, *rho*, Eve, and diphospho-MAPK, an effect that occurs at early stage 11 but is transient (data not shown; see Figure 3K). Activated Arm also fully rescues these markers in a *wg* mutant (G, K, O, and Q).

(R) Constitutive Ras1 hyperactivates MAPK (red) and induces extra Eve progenitors (green).

(S) While activated Ras1 restores MAPK activation in a *wg* mutant, Eve expression is not rescued.

tion, constitutive Ras1 does not rescue Eve progenitor formation in a *wg* mutant background (Figure 1S) (Carmena et al., 1998a). This is in marked contrast to the ability of activated Arm to fully rescue RTK signaling and Eve progenitor specification in a *wg* mutant (Figure 1Q). These results suggest that, in addition to enabling activation of Ras/MAPK signaling as a result of the induction of Htl, Hbr, and Rho expression, Wg signaling must contribute other factors that are essential for the specification of mesodermal Eve progenitors.

The Ets Domain Transcription Factors Pnt and Yan Are Involved in Eve Progenitor Formation

Given the importance of Ras/MAPK signaling in Eve progenitor determination (Gisselbrecht et al., 1996; Buff et al., 1998; Carmena et al., 1998a; Michelson et al., 1998b), we determined whether Pnt, an Ets domain transcriptional activator that functions downstream of MAPK (Klämbt, 1993; O'Neill et al., 1994), is also involved in this process. In *pnt* mutant embryos, there is a severe reduction in the number of both Eve progenitors, although this loss is more pronounced for the P15 lineage (Figures 2C, 2D, and 2I). Since mesoderm migration is normal in *pnt* embryos (data not shown), Pnt must only be required for the progenitor specification function of Htl (Michelson et al., 1998b). Consistent with this conclusion, an activated form of Pnt induces extra Eve progenitors (Figure 2H).

In embryos mutant for *yan*, which encodes a MAPK-regulated Ets-domain transcriptional repressor (Lai and Rubin, 1992; O'Neill et al., 1994; Rebay and Rubin, 1995), there is an increased number of Eve progenitors and their differentiated derivatives (Figures 2E and 2F). Conversely, a constitutively activated form of Yan inhibits Eve progenitor formation (Figure 2G). Thus, two MAPK-regulated transcription factors are involved in the development of Eve progenitors.

Characterization of an *eve* Enhancer that Functions Specifically in Muscle and Heart Progenitors

One mechanism that would ensure the convergence of the multiple regulatory inputs required for the formation of P2 and P15 is integration by a transcriptional enhancer. Since Eve expression is the feature that uniquely identifies these progenitors, we investigated whether *eve* itself is a direct target for regulation by both signal-activated and tissue-specific transcription factors.

Regulatory sequences responsible for mesodermal *eve* expression are located approximately 6 kb downstream of the transcription start site (Fujioka et al., 1999; Sackerson et al., 1999). We generated deletions of this region and defined a 312 bp minimal enhancer that we

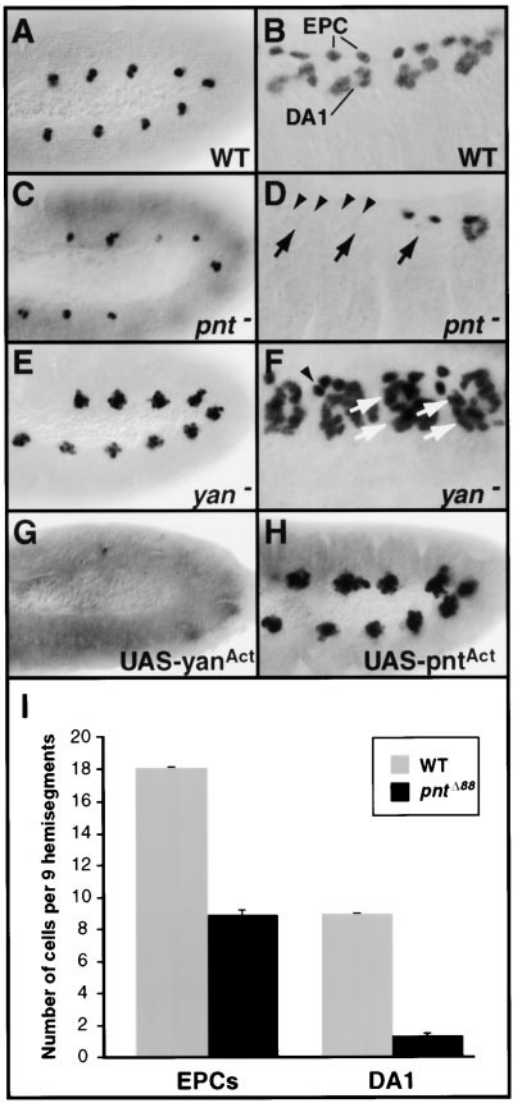

Figure 2. The Ets Domain Transcription Factors Pnt and Yan Are Involved in Eve Progenitor Specification

Embryos were stained for Eve expression at either stage 11 (A, C, E, G, and H) or stages 14–15 (B, D, and F).

(A) Two Eve progenitors are present in each hemisegment at mid stage 11.

(B) By stage 14, the individual EPCs and multinucleate DA1 muscle fiber can be distinguished.

(C) In *pnt*$^{\Delta 88}$ embryos, Eve progenitor formation is variably reduced.

(D) Both the EPCs (arrowheads) and DA1 (arrows) are affected by loss of *pnt* function.

(E and F) In *yan* mutants, there is an increased number of Eve progenitors and their derivatives (arrows, duplicated DA1; arrowhead, extra EPC).

(G and H) Constitutively active forms of *pnt* and *yan* have the opposite effects of their respective loss-of-function mutations.

(I) Quantitation of DA1 and EPC formation in wild-type and *pnt* mutant embryos. Failure of both cell types to develop in *pnt* embryos is highly significant ($P < 0.001$), although there is a greater effect on DA1. Cells were scored in 9 hemisegments on each side of an embryo; wild type, $n = 117$ embryo sides for EPCs, $n = 96$ for DA1; *pnt*$^{\Delta 88}$, $n = 128$ embryo sides for both cell types.

term the *eve* *M*uscle and *H*eart *E*nhancer (MHE; Figures 3A and 3B). When fused to a nuclear-*lacZ* reporter gene, the MHE drives expression in a mesodermal pattern identical to that of the endogenous *eve* gene (Figures 3C–3J). Reporter expression initiates at early stage 11, coincident with the onset of Eve expression in the equivalence group C2 (Figure 3D and data not shown). Following formation of P2, MHE activity is observed in P15 and in the P2 daughters, $F2_{EPC}$ and $F2_{DO2}$ (Figure 3E), then in the EPCs and the F15 daughters of P15 (Figures 3F–3H), and finally in muscle fiber DA1 (Figure 3I). Colocalization of MHE-driven β-galactosidase expression with Runt, which marks the $F2_{DO2}$ founder and muscle DO2 (Figures 3H and 3J; A. C., unpublished data), established that the reporter gene expression present in Eve-negative sibling cells is a result of β-galactosidase perdurance. Of note, the MHE mimics endogenous Eve expression despite its lack of a consensus binding site for the transcription factor Zfh-1 that had previously been proposed to play a role in mesodermal *eve* regulation (Su et al., 1999).

Strikingly, the MHE is only active in a single nucleus of the mature DA1 and DO2 muscles (Figure 3I,J). We infer that these are the original nuclei of the $F15_{DA1}$ and $F2_{DO2}$ founders based on prior reporter expression in those cells (Figure 3E-H). Similar results were obtained when DNA flanking the MHE by several hundred base pairs on either side (+4.96 to +7.36 kb; see Figure 3A), including the previously described Zfh-1 site (Su et al., 1999), was included in the reporter construct (Figure 3J), or when the MHE was placed 3′ to a reporter gene fused to the endogenous *eve* promoter (data not shown; Sackerson et al., 1999). Thus, additional sequences are required for *eve* expression in non-founder myofiber nuclei. Of critical importance to the present study, the MHE fully recapitulates mesodermal Eve expression during the signal-dependent induction of progenitor and founder cells.

The *eve* MHE Is Responsive to Wg, Dpp, and Ras Signaling

Genetic manipulation of the Wg, Dpp, and RTK/Ras signaling pathways causes predictable alterations of endogenous mesodermal Eve expression (Carmena et al., 1998a). We next determined whether the isolated MHE responds appropriately to these signals. In all genetic backgrounds, reporter gene expression corresponded precisely to that of endogenous *eve* (Figures 3K–3P and data not shown). For example, constitutively activated Arm transiently increased the expression of both genes (data not shown). However, as previously reported with respect to Eve (Lawrence et al., 1995; Carmena et al., 1998a) and as described above for Htl, Hbr, Rho, and diphospho-MAPK expression, Wg hyperactivation did not have a stable effect on MHE function (Figure 3K). In contrast, both endogenous *eve* and the MHE-driven reporter were induced throughout the initial competence domain by constitutively activated Pnt (Figure 3L), and expression of both markers extended laterally in the presence of activated Arm plus Pnt (Figure 3M). Ectopic Dpp led to both endogenous Eve and MHE-driven reporter expression in the ventral mesoderm (Figures 3N and 3O), while coexpression of Dpp and activated Ras1 induced expression of both genes in a dorsal–ventral stripe (Fig-

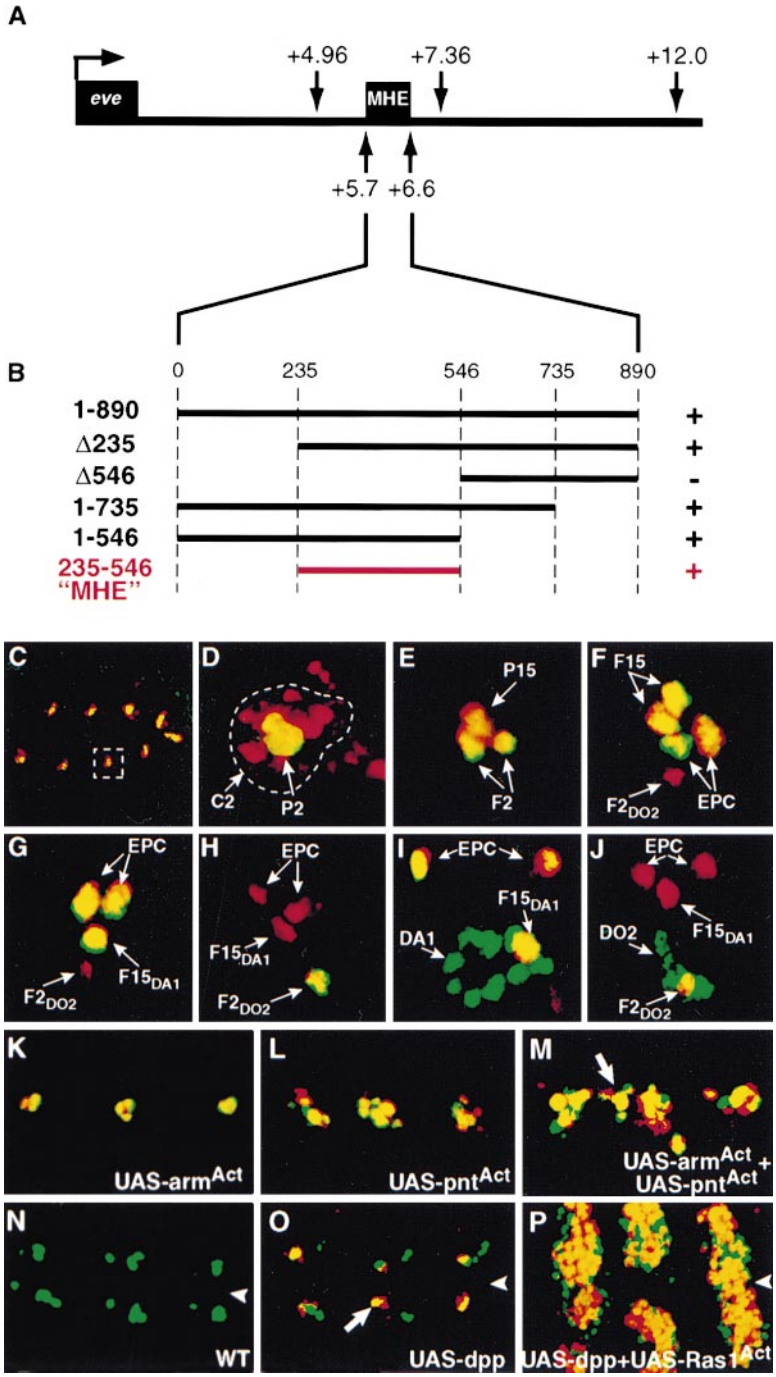

Figure 3. An *eve* Mesodermal Enhancer Recapitulates All Aspects of *eve* Expression during the Specification of Muscle and Cardiac Progenitor and Founder Cells

(A) The *eve* muscle and heart enhancer (MHE) is located 3′ to the coding region.

(B) Sequences tested for MHE activity in transgenic flies. A "+" indicates mesoderm-specific expression.

(C–P) Confocal analysis of reporter gene expression using antibodies to β-galactosidase (red) and either endogenous Eve (C–G, I, and K–P) or Runt (H and J) (green). (C) Reporter expression colocalizes with endogenous Eve at stage 11. The box indicates the portion of one hemisegment enlarged in the following panels. (D) Colocalization of Eve and β-galactosidase in the P2 progenitor, along with fading Eve expression and β-galactosidase perdurance in the former cluster C2 cells (dotted circle). Ectopic β-galactosidase occurs nonspecifically in various transgenic lines and varies considerably among segments and embryos (red, outside dotted circle). (E and F) Coexpression of Eve and the MHE reporter is observed in P15 and the F2 founders, and even later in the EPCs and the sibling F15s. β-galactosidase perdures in $F2_{DO2}$ after Eve expression ceases. (G) Slightly later, only the EPCs and $F15_{DA1}$ retain Eve. (H) $F2_{DO2}$ coexpresses Runt (green) and β-galactosidase (red). (I and J) At later stages, only a single nucleus of each of the DA1 and DO2 muscles expresses β-galactosidase. (K) Activated Arm has no stable effect on Eve progenitors or MHE-dependent expression at late stage 11, while activated Pnt (L) increases expression of both genes. (M) Activated forms of Arm plus Pnt cause a lateral expansion of Eve and MHE-expressing cells (arrow). (N) Stage 11 embryo showing Eve staining in the central nervous system (arrowhead, ventral midline). (O) Ectopic Dpp activates both the endogenous and transgenic MHEs in ventral mesodermal cells. (P) Ectopic Dpp and activated Ras1 generate β-galactosidase-positive Eve progenitors along the entire dorsoventral axis of the embryonic mesoderm.

ure 3P). These results demonstrate that the isolated MHE is responsive to all of the known signals that are essential for the specification of Eve progenitors.

The *eve* MHE Contains Functional Binding Sites for Both Signal-Activated and Tissue-Restricted Transcription Factors

Given that the MHE recapitulates early mesodermal Eve expression, we determined whether this enhancer contains binding sites for candidate signal-dependent and mesoderm-specific transcription factors. We focused on two mesoderm-specific factors, Tin and Twi, as well as the nuclear factors that act downstream of Wg (dTCF), Dpp (Mad) and Ras (Pnt, Yan). A computer-based search of the MHE sequence suggested the presence of potential binding sites for each of these transcription factors. Gel-shift assays confirmed that these putative sites actually bind the relevant factors (Figure 4 and data not shown). This analysis established the existence of one binding site for dTCF, six for Mad, two for Twist, and four each for Tin and Pnt. Since Yan bound to each of the Pnt sites, we refer to these as Ets sites.

To ascertain whether these in vitro binding sites have in vivo functional significance, we mutated the sites,

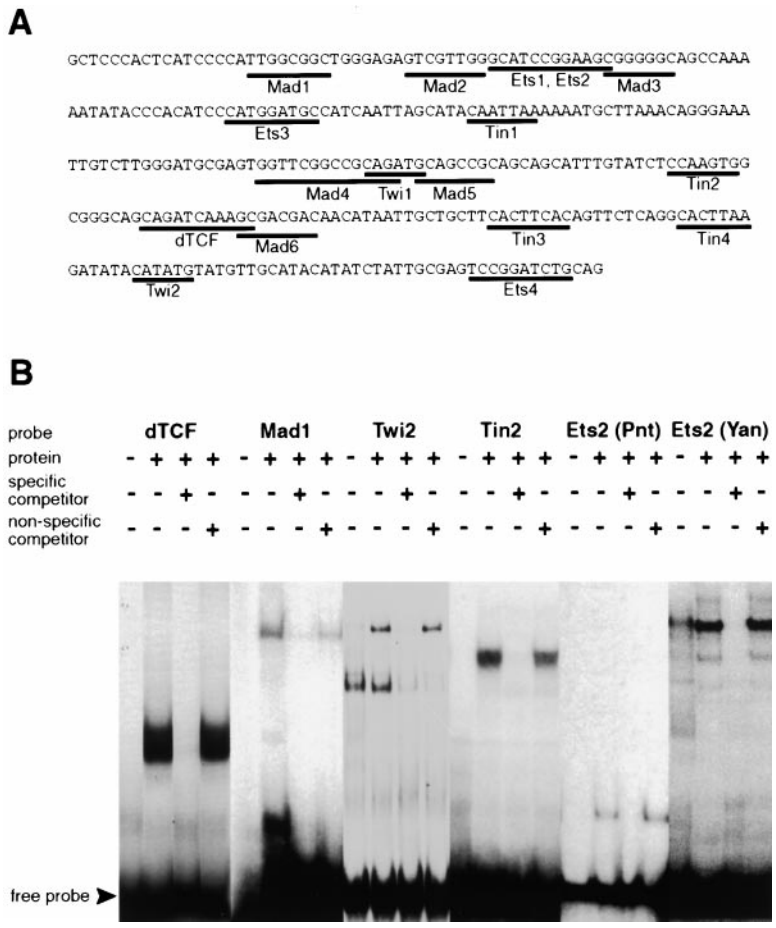

Figure 4. The MHE Contains Binding Sites for Multiple Signal-Activated and Tissue-Restricted Transcription Factors

(A) The 312 bp MHE sequence showing binding sites for dTCF, Mad, Twi, Tin, and the Ets domain factors Pnt and Yan, as identified by gel-shift assays.

(B) Representative gel shifts. Each set of four lanes shows a specific MHE oligonucleotide probe assayed for binding by the corresponding protein. Specific and nonspecific competitor DNAs were present at approximately 150× concentrations. All four Ets sites bind both Pnt and Yan.

both singly and in combination, within the context of the entire MHE. All mutagenesis was by base substitution so as not to affect the spacing between other potential cis-regulatory elements. We tested the ability of the mutated MHEs to drive reporter gene expression in transgenic embryos and compared this expression to that of endogenous Eve (Figure 5 and data not shown). Of the six Mad sites, only Mad4, 5, and 6 were critical for MHE function when inactivated singly or in combination (Figure 5B and data not shown). Mutation of the single dTCF site or of individual binding sites for Twi, Tin, or the Ets factors also led to loss of reporter gene expression in some, but not all, Eve-expressing cells, with some mutant sites associated with a more severe loss than others (Figures 5C–5F and data not shown). Of note, both the EPC and DA1 lineages were affected equally by all of

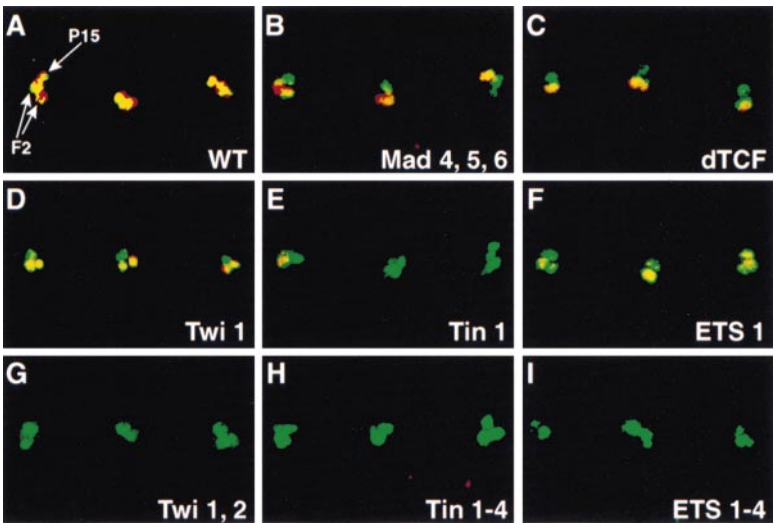

Figure 5. The Identified MHE Binding Sites Are Functional In Vivo

Embryos bearing mutated MHEs driving a nuclear-*lacZ* reporter gene were stained with antibodies to Eve (green) and to β-galactosidase (red). Three representative hemisegments of late stage 11 embryos are shown for each construct. (A) With a wild-type MHE, three cells per hemisegment (P15 and two F2s) express both Eve and β-galactosidase. Mutation of the Mad4, 5, and 6 (B) or the dTCF (C) sites leads to a partial loss of reporter expression, as does mutation of single sites for Twi (D), Tin (E), or the Ets factors (F). Reporter expression is competely lost when all of the sites capable of binding Twi (G) or Tin (H) or the Ets proteins (I) are mutated. Failure to express the reporter construct occurs equally in both the P2 and P15 lineages.

the mutations. In addition, the activity level in those Eve-expressing cells that did maintain reporter gene expression was on average lower than that seen with the wild-type MHE (as assayed by fluorescence intensity of the anti-β-galactosidase signal; data not shown). In contrast to the single site mutants, mutation of the two Twi, all four Tin, or all four Ets sites completely eliminated MHE activity (Figures 5G–5I). We conclude that binding sites for two tissue-specific and three signal-responsive transcription factors are required for full activity of the MHE in both the muscle and the heart lineages.

The finding that the three Wg-dependent factors, dTCF, Twi, and Tin, directly regulate *eve* could explain why activated Ras was incapable of bypassing Wg in the induction of Eve progenitors (Figure 1S). We therefore attempted to rescue Eve expression in *wg* mutant embryos by ectopically expressing Twi and Tin together with activated Ras. However, Eve progenitors were not recovered by this manipulation, perhaps due to the direct requirement of dTCF for *eve* MHE activity. While activated Arm could supply the missing downstream Wg transcription factor in this rescue experiment, Arm alone was capable of fully rescuing not only the Eve progenitors but also all of the Wg-dependent factors that regulate the MHE, including Twi, Tin, and the RTK/Ras pathway components (Figure 1 and data not shown). Thus, we were unable to further evaluate the combined effects of the MHE transcription factors in the absence of Wg signaling. Nevertheless, our rescue and enhancer mutagenesis data strongly support the involvement of Wg as a mesodermal competence determinant both upstream of the Ras pathway and directly (via dTCF) as well as indirectly (via Twi and Tin) in the transcriptional response to inductive RTK signaling.

The Transcription Factors Regulating Mesodermal Eve Expression Act Synergistically at the MHE

Since mutation of any single transcription factor binding site in the MHE caused only a partial loss of enhancer activity, we considered whether different sites might function together synergistically. To test this possibility, binding site mutations for two different activators were combined. Simultaneous mutation of the dTCF and Twi1 sites led to reporter gene expression in approximately 5-fold fewer cells than would be expected from the additive independent effects of each mutation (6% $\pm$ 3% observed vs. 31% predicted, $P < 0.001$; Figure 6A). A similar, though slightly less robust, synergy was observed when the dTCF and Ets3 mutations were combined (20% $\pm$ 3% observed vs. 34% predicted for an additive effect, $P < 0.002$; Figure 6A).

We also assessed whether ectopic coexpression of individual transcription factors or upstream signals would lead to cooperative effects on endogenous Eve expression. As previously reported, ectopic Wg had no effect on Eve expression at late stage 11 (Figure 6C), activated Ras1 induced extra Eve progenitors (Figure 6D), and ectopic Wg plus activated Ras1 caused a lateral expansion of the progenitor clusters (Figure 6E; Carmena et al., 1998a). When Twi was expressed using a *twi*-Gal4 driver, a few Eve-positive cells developed at ectopic positions (Figure 6F). The magnitude of this effect was increased by coexpression of Wg and Twi (Figure 6G), and even more so by coexpression of Twi with activated Ras1. The latter effect strikingly resembles that of Wg plus activated Ras1 (compare Figures 6E and 6H). With the simultaneous ectopic expression of Wg, Twi, and activated Ras1, Eve progenitors formed an almost continuous anteroposterior stripe confined to the dorsal mesoderm (Figure 6I). These results demonstrate a synergistic induction of Eve progenitors by various combinations of Wg, Twi, and activated Ras1 that parallels the synergistic loss of MHE activity seen by mutating the dTCF, Twi, and Ets binding sites. Taken together, these loss- and gain-of-function findings suggest that dTCF, Twi, and Pnt cooperate at the MHE to synergistically regulate Eve transcription and, by extension, to induce the specification of Eve progenitor fates.

Discussion

The convergence of multiple intercellular signals in the determination of cell fates is a recurring theme in animal development. However, the mechanisms by which disparate inputs are integrated to generate specific, coordinated responses remain poorly understood. We have demonstrated that Wg and Dpp coordinate a series of signal-activated (dTCF and Mad) and mesoderm-specific (Twi and Tin) transcription factors in a temporal and spatial pattern that facilitates cooperation with the Ras transcriptional effector Pnt. The synergistic integration of these five transcription factors by a single enhancer generates a specific developmental response to Ras/MAPK signaling. Moreover, Wg and Dpp exert proximal effects in this signaling network by enabling Ras/MAPK activation through the regulated localized expression of upstream components of the RTK signal transduction machinery. A model governing the acquisition of developmental competence, signal integration and response specificity in this system is summarized in Figure 7 and discussed in more detail below.

A Two-Tiered Molecular Basis for Developmental Competence

Since their initial recognition by Stern during genetic investigations of *Drosophila* thoracic bristle formation (Stern, 1954), developmental prepatterns have been found as critical early steps in the progressive determination of various cell types. Our prior studies established that Wg and Dpp prepattern the dorsal mesoderm of the *Drosophila* embryo. Moreover, we proposed that Wg and Dpp function as competence factors for the induction of Eve progenitor fates by the Ras pathway (Carmena et al., 1998a). The present results strongly support this hypothesis and provide evidence that competence in this case results from dual functions of Wg and Dpp in enabling RTK/Ras activation and in facilitating the downstream response to RTK/Ras signaling.

First, Wg is essential for MAPK activation due to its requirement for the expression of Htl, Hbr, and Rho, proximal components of the EGF and FGF pathways. In the absence of Wg, neither FGF nor EGF receptor activation can occur in dorsal mesodermal cells. Consistent with this, gain of Wg function causes both increased

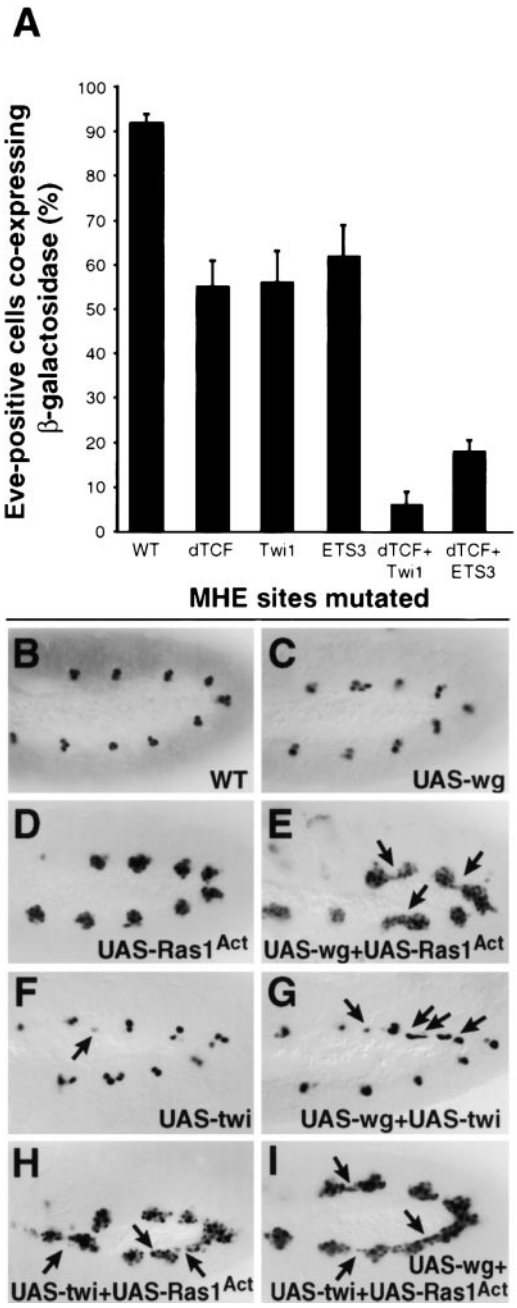

Figure 6. Synergistic Transcription Factor Interactions at the MHE

(A) The percentage of Eve-expressing cells that also express β-galactosidase from single- or double-mutant MHEs were quantitated in stage 11 embryos from at least four independent transgenic lines of each genotype (for wild type, n = 325 cells in 18 embryos; dTCF, n = 459 cells in 29 embryos; Twi1, n = 356 cells in 22 embryos; Ets3, n = 181 cells in 11 embryos; dTCF+Twi1, n = 445 cells in 30 embryos; and dTCF+Ets3, n = 382 cells in 25 embryos).

(B–I) Eve staining in stage 11 wild-type embryos or embryos in which *twi*-Gal4 was used to drive expression of Twi and/or the indicated signaling proteins. As previously described (Carmena et al., 1998a), ectopic Wg has no stable effect on Eve progenitor formation (C), activated Ras1 induces extra Eve cells within a hemisegment (D), and Wg synergizes with Ras1 to induce additional Eve cells between hemisegments (arrows in E). Ectopic Twi leads to occasional extra Eve progenitors (arrow in F), an effect that is substantially increased when combined with ectopic Wg (arrows in G). (H) Coexpression of Twi and activated Ras1 causes a lateral expansion of progenitor clusters similar to that seen with Wg and activated Ras1 (compare with E). (I) Coexpression of Wg, Twi, and activated Ras1 dramatically increases the induction of Eve progenitors within the dorsal Dpp domain.

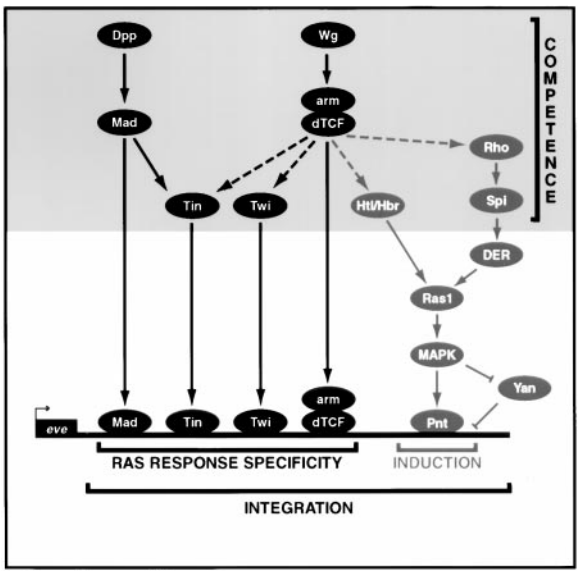

Figure 7. A Model for Developmental Competence, Signal Integration, and RTK/Ras Response Specificity in the Determination of Mesodermal Eve Progenitors

Wg and Dpp provide competence (gray box) through the regulation of tissue-specific transcription factors (Tin and Twi), signal-responsive transcription factors (Mad and dTCF), and proximal components of the RTK/Ras pathways (Htl, Hbr, and Rho). The Ras signaling cascade leads to activation of the inductive transcription factor, Pnt, and inactivation of the Yan repressor. While a direct role for Mad in regulating Tin expression has been demonstrated (solid arrow; Xu et al., 1998), Wg regulation of Tin, Twi, Htl, Hbr, and Rho may be either direct or indirect (dashed arrows). Additional effects of Dpp on the proximal RTK factors (M. S. H., unpublished data) are not illustrated. The five transcriptional activators assemble at and are integrated by the MHE, where they function synergistically to promote *eve* expression. Specificity of the response to inductive RTK/Ras signaling derives from the combinatorial effects of the tissue-restricted and signal-activated transcription factors that converge at the MHE. In the absence of inductive signaling, Yan would repress *eve* by binding to the Ets sites. Since *eve* is a muscle and heart identity gene, the illustrated regulatory mechanisms are inferred to have a more general function in determining progenitor fates.

expression of RTK pathway components and increased activation of MAPK. This effect of ectopic Wg or activated Arm is only transient but can be stabilized by the addition of activated Ras, further underscoring the interdependence of the two pathways. Dpp provides input to RTK/Ras signaling similar to that provided by Wg. Indeed, within the dorsal mesoderm, the intersection of the Wg and Dpp domains initially restricts Htl and Hbr expression, and the effect of ectopic Wg signaling on Eve is confined to the Dpp region (Carmena et al., 1998a; this work). Additional complexity to the control of RTK activity in this system derives from positive feedback regulation of the Ras/MAPK cascade and from reciprocal regulatory interactions between the Ras and Notch pathways (A. C., unpublished data).

The second mechanism by which Wg and Dpp contribute to the mesodermal RTK/Ras response occurs at the transcriptional level. Our analysis demonstrates that the *eve* MHE is directly regulated by dTCF, Mad, Tin, and Twi which function together with Ras-activated Pnt. Wg and Dpp directly influence progenitor identity through the action on the MHE of their respective downstream transcription factors, dTCF and Mad, as well as through their regulation of Tin and Twi (Bate and Rushton, 1993; Staehling-Hampton et al., 1994; Frasch, 1995; Wu et al., 1995), which also act directly on the MHE (Figure 7). These findings highlight the complex interrelationships that exist in this system among the prepattern signals (Wg and Dpp), the inductive signals (Htl and DER, RTK activators of the Ras pathway), the fate-determining transcription factors (dTCF, Mad, Twi, Tin, and Pnt), and a progenitor identity gene (*eve*).

Although not understood at a comparable molecular level, there are other developmental contexts in which signals may coordinately regulate both upstream components of RTK pathways and the transcription factors that directly cooperate with Ras-activated nuclear effectors to control target gene expression (e.g., Laufer et al., 1994; Stern et al., 1997; Ericson et al., 1998; Jiang and Sternberg, 1998; Szuts et al., 1998; Reifers et al., 2000). Such dual functions of convergent signals could prove to be a general mechanism for establishing the competence to respond in a tissue-specific manner to RTK inductive signaling.

Transcriptional Integration Mediates Ras Signaling Specificity

A characteristic feature of Ras signaling is its pleiotropy, raising the question of how specificity is achieved in particular developmental contexts (Rommel and Hafen, 1998; Tan and Kim, 1999). The present work highlights the importance of transcriptional integration in eliciting a tissue-specific response to an inductive Ras signal. Binding of Ras-activated Pnt to the *eve* MHE in the presence of the mesoderm-restricted factors Twi and Tin establishes a tissue-specific context for the inductive effects of Ras on heart and muscle progenitor formation. Additional specificity is achieved by the direct transcriptional inputs of Wg and Dpp to this enhancer. Such an integrative transcriptional mechanism could enable all mesodermal cells to interpret a set of combinatorial signaling codes that determine their individual identities (Carmena et al., 1998a). Although there may be a limited number of tissue-restricted and signal-activated transcription factors contributing to progenitor diversification, the complexity of such a mechanism could be increased by varying numbers and/or relative affinities of the corresponding binding sites in each cell type–specific enhancer. The particular organization of sites could also contribute to cellular specificity by promoting differential synergistic interactions among the cognate transcription factors. Such synergy occurs with the *eve* MHE, and may be a general feature of the regulation of genes governing progenitor fates.

Integration of signal-responsive and tissue-specific transcription factors occurs in the regulation of other *Drosophila* genes. For example, Vestigial is a tissue-specific selector protein that generates unique responses to several pleiotropic signals (Halder et al., 1998; Hepker et al., 1999). In the mesoderm, Twi and Tin may have analogous selector functions as essential germ layer–wide or region-restricted transcription factors that serve to integrate multiple signals. Whereas Tin functions with Pnt to specify dorsal Eve progenitors, different spatially-restricted transcription factors, such as the ventrally localized Paired domain protein Pox-meso (Bopp et al., 1989), may play a similar role in mediating responses to ventral Ras/MAPK signaling. We propose a general model in which distinct repertoires of tissue-specific and spatially-restricted transcription factors cooperate with specific combinations of signal-responsive transcriptional activators to generate unique progenitor identities in response to inductive RTK signaling. Evidence for a similar mechanism has recently been reported for the specificity of Ras signaling in *Drosophila* eye development (Flores et al., 2000 [this issue of *Cell*]; Xu et al., 2000 [this issue of *Cell*]).

Multiple Ets Domain Factors May Influence the Specificity of the Ras Response

Although mutations in the Ets domain activator Pnt cause only a partial loss of Eve progenitors and their progeny, mutation of the four Ets consensus sites in the MHE completely abrogates enhancer activity. Additional factors, possibly other positively-acting Ets proteins, may therefore contribute to *eve* regulation through these same sites. Several Ets proteins in addition to Pnt and Yan are expressed in the fly embryo (Chen et al., 1992), but their mutant phenotypes and expression patterns have not been well characterized. In the vertebrate nervous system, different signal-regulated Ets proteins help to define specific subsets of motor and sensory neurons (Lin et al., 1998). By analogy, the differential expression of Ets proteins could contribute to the specificity of Ras/MAPK signaling in *Drosophila* muscle and cardiac progenitors. Such a model would be consistent with the less severe loss of the P2 lineage as compared to the P15 lineage observed in *pnt* mutant embryos (Figure 2). Variation in Ets protein stability, binding affinity or capacity to interact with protein cofactors could provide an additional level of specificity to the RTK/Ras signaling response.

Concluding Remarks

The mechanisms by which cells acquire the competence to respond to multiple intercellular signals and by which these convergent inputs are integrated are fundamental to numerous developmental decisions. The present work illustrates how Wg and Dpp function as competence determinants by acting both upstream of and in parallel to a later inductive RTK/Ras signal. Wg and Dpp not only facilitate RTK/Ras activation but also coordinate the downstream transcription factors that directly mediate the acquisition of particular cell fates. In addition to being a marker of mesodermal progenitor identity, *eve* is also a direct target of the tissue-restricted and signal-activated transcription factors that cooperate to induce this identity. The ability to monitor Eve expression in different genetic backgrounds and to dissect the individual regulatory elements of the *eve* MHE has provided a comprehensive understanding of both the upstream signals and downstream nuclear effectors that control

myogenic and cardiogenic determination in a model organism. Moreover, this work has revealed a molecular basis for how competence, integration, and specificity of signaling responses are achieved in a well defined developmental context. Since the establishment of the Wg and Dpp expression domains has been traced to the earliest events that determine the embryonic body plan, our findings provide a framework that directly links these developmental processes to the later specification, patterning, and differentiation steps that occur during organ formation.

Experimental Procedures

Drosophila Strains and Genetics

The following mutant stocks were used: wg^{cx4}, wg^{IG22}, $pnt^{\Delta 88}$, and aop^{1} (*yan*). Ectopic expression was achieved using the Gal4-UAS system (Brand and Perrimon, 1993) and the following fly lines: *twi*-Gal4, UAS-*wg*, UAS-arm^{s10}, UAS-*dpp*, UAS-$Ras1^{Act}$, UAS-$pntP2^{VP16}$, UAS-yan^{Act}, and UAS-*twi*. Oregon-R was used as the wild-type reference strain.

Isolation of the *eve* MHE and Construction of Deletion Mutants

An 890 bp SphI/StuI fragment located 5.7 kb downstream of the *eve* transcriptional start site was subcloned with NotI linkers into pBSII-KS (Stratagene) to give pBS-M*eve*Not. To make Δ235, a BamHI site was introduced 235 bp downstream of the SphI site in pBSM*eve*Not by PCR and the product subcloned into pBS. For the remaining deletions, pBS-M*eve*Not was digested with NotI and either DraI (1-735) or PstI (1-546, Δ546), NotI linkers added, and the fragment subcloned into pBS. For Δ235-546, the Δ235 construct was digested with BamHI and PstI and the 0.3 kb fragment subcloned back into pBS. We refer to this construct as pBS-MHE. All constructs were subcloned into the P-element transformation vectors pETW-*lacZ* or pETW-nuc*lacZ*, which contain minimal heat shock gene promoters driving *lacZ* or nuclear-localized *lacZ*, respectively (A. M. M., unpublished data).

Analysis of Transcription Factor Consensus Sequences

The MHE was searched for the following consensus transcription factor binding sites using MacVector (Oxford Molecular, UK): dTCF, AAGATCAAAGG; Mad, GCCGNCGB; Twi, CANNTG; Tin, CTCAA GTGG; Ets, NSYGGAWRY.

Site-Directed Mutagenesis

Site-directed mutagenesis of the MHE was performed using the overlap extension method (Ho et al., 1989). The resulting PCR products were cloned, sequenced, and subcloned into pETW-nuc*lacZ*. The following italicized base pair changes were made (refer to Figure 4A for wild-type sequence): dTCF, CAG*TCGTCC*GC; Mad1, GTGG *AGTACT*; Mad2, GAGA*AGTAC*TGG; Mad3, AAGC*AGTACT*; Mad4, GTGG*AGTAAGTAC*AGA; Mad5, GATGCA*AGTACT*GC; Mad6, AAGC *AGTACG*; Twi1, C*CTTC*G; Twi2, *ACGCGT*; Tin1, TAC*CTCA*AA; Tin2, CTCCAC*CA*GG; Tin3, TTC*CTCA*CAC; Tin4, C*CTCA*AA; Ets1, *TAGG* CCGGAAGC; Ets2, GCATCCGG*CCTA*; Ets3, CATGG*CCTA*; Ets4, TCCGG*CCTA*.

P Element Transformation

w^{1118} or *yw* embryos were injected as previously described (Michelson et al., 1998a). At least four independent transgenic lines were analyzed for each construct. Lines having obvious enhancer-trap staining patterns were discarded. Although the MHE faithfully recapitulates endogenous *eve* expression throughout the period of progenitor specification, variable ectopic reporter expression was observed at later stages in many lines. The latter was not considered relevant to the present work, which focuses exclusively on early developmental events.

Protein Purification and Electrophoretic Mobility Shift Assays

His-tagged proteins were purified using Qiagen NTA-agarose according to the manufacturer's instructions; GST-tagged proteins were purified using glutathione-agarose (Sigma). GST-tagged Mad-MH1 (MadN, Kim et al., 1997) and a 6×His-tagged dTCF binding domain construct were the generous gift of S. Carroll. The Pnt Ets domain (aa 863-977) was cloned into pRSETA (Invitrogen) and expressed as a 6×His-tagged protein. The Tin DNA binding domain (aa 294-358) and full-length Twi and Yan were generated in vitro using the TnT coupled transcription/translation kit (Promega). Electrophoretic mobility shift assays employed end-labeled oligonucleotides that were run on 5% polyacrylamide gels in 0.5× TBE buffer.

Immunohistochemistry and Microscopy

Antibody staining and in situ hybridization of embryos were done as previously described (Carmena et al., 1998a). The following antibodies were used: α-β-galactosidase (Promega), 1:500; α-diphospho-MAPK (Sigma), 1:500; α-Eve (Frasch, 1995), 1:10,000; α-Htl (Michelson et al., 1998b), 1:10,000; α-Hbr (anti-Dof, Vincent et al., 1998), 1:200; and α-Runt (Kosman et al., 1998), 1:5000. Fluorescent staining was analyzed using a Leica TCS-NT confocal microscope and the separate channel images were composited using Adobe Photoshop.

Statistical Analysis

Data in Figure 2I were evaluated using a standard Z test. To test for synergistic versus additive effects of combined mutations in Figure 7A, a likelihood ratio test of independence was performed.

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References

Azpiazu, N., and Frasch, M. (1993). *tinman* and *bagpipe*—two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. Genes Dev. *7*, 1325–1340.

Bang, A.G., and Kintner, C. (2000). Rhomboid and Star facilitate presentation and processing of the *Drosophila* TGFα homolog Spitz. Genes Dev. *14*, 177–186.

Bate, M., and Rushton, E. (1993). Myogenesis and muscle patterning in *Drosophila*. C.R. Acad. Sci. Paris *316*, 1047–1054.

Baylies, M.K., and Bate, M. (1996). Twist: a myogenic switch in *Drosophila*. Science *272*, 1481–1484.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. Development *118*, 719–729.

Bopp, D., Jamet, E., Baumgartner, S., Burri, M., and Noll, M. (1989). Isolation of two tissue-specific *Drosophila* Paired box genes, *Pox meso* and *Pox neuro*. EMBO J. *8*, 3447–3457.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Brunner, E., Peter, O., Schweier, L., and Basler, K. (1997). *pangolin* encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. Nature *358*, 829–833.

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, F., and Michelson, A.M. (1998). Signalling by the *Drosophila* epidermal growth factor

receptor is required for the specification and diversification of embryonic muscle progenitors. Development *125*, 2075–2086.

Carmena, A., Bate, M., and Jiménez, F. (1995). *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. Genes Dev. *9*, 2373–2383.

Carmena, A., Gisselbrecht, S., Harrison, J., Jiménez, F., and Michelson, A.M. (1998a). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. Genes Dev. *12*, 3910–3922.

Carmena, A., Murugasu-Oei, B., Menon, D., Jimenéz, F., and Chia, W. (1998b). *inscuteable* and *numb* mediate asymmetric muscle progenitor cell divisions during *Drosophila* myogenesis. Genes Dev. *12*, 304–315.

Chen, T., Bunting, M., Karim, F.D., and Thummel, C.S. (1992). Isolation and characterization of five *Drosophila* genes that encode an ets-related DNA binding domain. Dev. Biol. *151*, 176–191.

Clandinin, T., DeModena, J., and Sternberg, P. (1998). Inositol trisphosphate mediates a RAS-independent response to LET-23 receptor tyrosine kinase activation in *C. elegans*. Cell *92*, 523–533.

Downward, J. (1998). Ras signalling and apoptosis. Curr. Opin. Genet. Dev. *8*, 49–54.

Ericson, J., Norlin, S., Jessell, T.M., and Edlund, T. (1998). Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Development *125*, 1005–1015.

Flores, G.V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M., and Banerjee, U. (2000). Combinatorial signaling in the specification of unique cell fates. Cell *103*, this issue, 75–85.

Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. Nature *374*, 646–467.

Fujioka, M., Emi-Sarker, Y., Yusibova, G.L., Goto, T., and Jaynes, J.B. (1999). Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. Development *126*, 2527–2538.

Gabay, L., Seger, R., and Shilo, B.-Z. (1997). In situ activation pattern of *Drosophila* EGF receptor pathway during development. Science *277*, 1103–1106.

Gisselbrecht, S., Skeath, J.B., Doe, C.Q., and Michelson, A.M. (1996). Heartless encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. Genes Dev. *10*, 3003–3017.

Halder, G., Polaczyk, P., Kraus, M.E., Hudson, A., Kim, J., Laughon, A., and Carroll, S. (1998). The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in *Drosophila*. Genes Dev. *12*, 3900–3909.

Hepker, J., Blackman, R.K., and Holmgren, R. (1999). Cubitus interruptus is necessary but not sufficient for direct activation of a wing-specific *decapentaplegic* enhancer. Development *126*, 3669–3677.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene *77*, 51–59.

Imam, F., Sutherland, D., Huang, W., and Krasnow, M.A. (1999). *stumps*, a *Drosophila* gene required for fibroblast growth factor (FGF)-directed migrations of tracheal and mesodermal cells. Genetics *152*, 307–318.

Jiang, L.I., and Sternberg, P.W. (1998). Interactions of EGF, Wnt and HOM-C genes specify the P12 neuroectoblast fate in *C. elegans*. Development *125*, 2337–2347.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., and Laughon, A. (1997). *Drosphila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic. Nature *388*, 304–308.

Klämbt, C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of midline glial cells. Development *117*, 163–176.

Kosman, D., Small, S., and Reinitz, J. (1998). Rapid preparation of a panel of polyclonal antibodies to *Drosophila* segmentation proteins. Dev. Genes Evol. *208*, 290–294.

Lai, Z.C., and Rubin, G.M. (1992). Negative control of photoreceptor development in *Drosophila* by the product of the *yan* gene, an ETS domain protein. Cell *70*, 609–620.

Laufer, E., Nelson, C.E., Johnson, R.L., Morgan, B.A., and Tabin, C. (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. Cell *79*, 993–1003.

Lawrence, P.A., Bodmer, R., and Vincent, J.-P. (1995). Segmental patterning of heart precursors in *Drosophila*. Development *121*, 4303–4308.

Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessell, T.M., and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell *95*, 393–407.

Maloof, J.N., and Kenyon, C. (1998). The Hox gene lin-39 is required during *C. elegans* vulval induction to select the outcome of Ras signaling. Development *125*, 181–190.

Michelson, A.M., Gisselbrecht, S., Buff, E., and Skeath, J.B. (1998a). Heartbroken is a specific downstream mediator of FGF receptor signalling in *Drosophila*. Development *125*, 4379–4389.

Michelson, A.M., Gisselbrecht, S., Zhou, Y., Baek, K.-H., and Buff, E.M. (1998b). Dual functions of the Heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. Dev. Genet. *22*, 212–229.

O'Neill, E.M., Rebay, I., Tjian, R., and Rubin, G.M. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell *78*, 137–147.

Pai, L.-M., Orsulic, S., Bejsovec, A., and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in *Drosophila*. Development *124*, 2255–2266.

Rebay, I., and Rubin, G.M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell *81*, 857–866.

Reifers, F., Walsh, E.C., Leger, S., Stainier, D.Y., and Brand, M. (2000). Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). Development *127*, 225–235.

Rommel, C., and Hafen, E. (1998). Ras—a versatile cellular switch. Curr. Opin. Genet. Dev. *8*, 412–418.

Ruiz Gomez, M., and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. Development *124*, 4857–4866.

Rushton, E., Drysdale, R., Abmayr, S.M., Michelson, A.M., and Bate, M. (1995). Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. Development *121*, 1979–1988.

Sackerson, C., Fujioka, M., and Goto, T. (1999). The *even-skipped* locus is contained in a 16-kb chromatin domain. Dev. Biol. *211*, 39–52.

Sekelsky, J., Newfeld, S., Raftery, L., Chartoff, E., and Gelbart, W. (1995). Genetic characterization and cloning of *Mothers against dpp*, a gene required for *decapentaplegic* function in *Drosophila melanogaster*. Genetics *139*, 1347–1358.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. Nature *372*, 783–786.

Stern, C. (1954). Two or three bristles? Am. Sci. *42*, 213–247.

Stern, H.M., Lin-Jones, J., and Hauschka, S.D. (1997). Synergistic interactions between bFGF and a TGFβ family member may mediate myogenic signals from the neural tube. Development *124*, 3511–3523.

Su, M.T., Fujioka, M., Goto, T., and Bodmer, R. (1999). The *Drosophila* homeobox genes *zfh-1* and *even-skipped* are required for cardiac-specific differentiation of a *numb*-dependent lineage decision. Development *126*, 3241–3251.

Szuts, D., Eresh, S., and Bienz, M. (1998). Functional intertwining of Dpp and EGFR signaling during *Drosophila* endoderm induction. Genes Dev. *12*, 2022–2035.

Tan, P.B., and Kim, S.K. (1999). Signaling specificity: the RTK/RAS/MAP kinase pathway in metazoans. Trends Genet. *15*, 145–149.

Tan, P.B., Lackner, M.R., and Kim, S.K. (1998). MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. Cell *93*, 569–580.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. Cell *88*, 789–799.

van der Geer, P., Hunter, T., and Lindberg, R.A. (1994). Receptor-protein tyrosine kinases and their signal transduction pathways. Ann. Rev. Cell Biol. *10*, 251–337.

Vincent, S., Wilson, R., Coelho, C., Affolter, M., and Leptin, M. (1998). The *Drosophila* protein Dof is specifically required for FGF signaling. Mol. Cell *2*, 515–525.

Wu, X., Golden, K., and Bodmer, R. (1995). Heart development in *Drosophila* requires the segment polarity gene *wingless*. Dev. Biol. *169*, 619–628.

Xu, X., Yin, Z., Hudson, J.B., Ferguson, E.L., and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. Genes Dev. *12*, 2354–2370.

Xu, C., Kauffmann, R.C., Zhang, J., Kladny, S., and Carthew, R.W. (2000). Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the *Drosophila* eye. Cell *103*, this issue, 87–97.