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Reciprocal Regulatory Interactions between the Notch and Ras Signaling Pathways in the *Drosophila* Embryonic Mesoderm

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Convergent intercellular signals must be precisely integrated in order to elicit specific biological responses. During specification of muscle and cardiac progenitors from clusters of equivalent cells in the *Drosophila* embryonic mesoderm, the Ras/MAPK pathway—activated by both epidermal and fibroblast growth factor receptors—functions as an inductive cellular determination signal, while lateral inhibition mediated by Notch antagonizes this activity. A critical balance between these signals must be achieved to enable one cell of an equivalence group to segregate as a progenitor while its neighbors assume a nonprogenitor identity. We have investigated whether these opposing signals directly interact with each other, and we have examined how they are integrated by the responding cells to specify their unique fates. Our findings reveal that Ras and Notch do not function independently; rather, we have uncovered several modes of cross-talk between these pathways. Ras induces Notch, its ligand Delta, and the epidermal growth factor receptor antagonist, Argos. We show that Delta and Argos then synergize to nonautonomously block a positive autoregulatory feedback loop that amplifies a fate-inducing Ras signal. This feedback loop is characterized by Ras-mediated upregulation of proximal components of both the epidermal and fibroblast growth factor receptor pathways. In turn, Notch activation in nonprogenitors induces its own expression and simultaneously suppresses both Delta and Argos levels, thereby reinforcing a unidirectional inhibitory response. These reciprocal interactions combine to generate the signal thresholds that are essential for proper specification of progenitors and nonprogenitors from groups of initially equivalent cells.

Key Words: **signal integration; myogenesis; cardiogenesis; EGF receptor; FGF receptor; Notch; Ras; *Drosophila* development; cell fate specification; equivalence group.**

INTRODUCTION

Intercellular signaling plays a critical role in proliferation, migration, specification, differentiation, and survival of cells during metazoan development. Signals can either stimulate or inhibit a given process, and the same signal can have distinct effects in different developmental contexts, even within the same organism. Multiple signals can simultaneously influence a common response, the net result reflecting integration of the individual inputs by the target cells (Jordan *et al.*, 2000). Each signal also may be subject to positive and/or negative feedback regulation, which fine tunes its output both spatially and temporally (Freeman, 2000; Perrimon and McMahon, 1999). While the biochemical details of individual signal transduction cascades have been well characterized, the potential for regulatory cross-talk among signaling pathways as an integrative mechanism for generating response specificity and precision has not been as extensively investigated.

The specification of muscle and heart progenitor cells in

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the *Drosophila* embryo has emerged as an excellent system for studying the interplay among various developmental signals (reviewed by Baylies and Michelson, 2001; Frasch, 1999). After the initial subdivision of the embryonic mesoderm into somatic and visceral components, the former is held in an uncommitted state by the inhibitory Notch (N) pathway (Artavanis-Tsakonas *et al.*, 1999). In the case of at least some muscle progenitors, this inhibition is relieved by the Wnt family member, Wingless (Wg), by an as yet unknown mechanism (Brennan *et al.*, 1999). The combination of Wg and Decapentaplegic (Dpp), a BMP superfamily member, defines a dorsal mesodermal region revealed by *lethal of scute* (*l'sc*) expression that is competent to respond to a subsequent inductive signal mediated by two receptor tyrosine kinases (RTKs)—the *Drosophila* EGF receptor (DER; Buff *et al.*, 1998) and the Heartless (Htl) FGF receptor (Michelson *et al.*, 1998b)—which act through the common Ras/MAPK cascade (Carmena *et al.*, 1998a). RTK activation restricts *l'sc* expression to clusters of equivalent cells from which individual progenitors are singled out under the opposing influences of positive Ras activity and a second, lateral inhibitory function of N (Baker and Schubiger, 1996; Bate *et al.*, 1993; Carmena *et al.*, 1995, 1998a; Corbin *et al.*, 1991; Fuerstenberg and Giniger, 1998; Park *et al.*, 1998a). Each progenitor then divides asymmetrically to form sibling founder cells which express muscle identity genes, such as *even skipped (eve), Krüppel* (Kr), *S59/slouch* (*slo*), and *nautilus* (*nau*; Baylies and Michelson, 2001; Frasch, 1999). The founder cells seed the formation of individual muscles by attracting other myoblasts to fuse with them (Ruiz Gomez *et al.*, 2000). These so-called fusion competent myoblasts derive from N-inhibited equivalence group cells that fail to acquire a progenitor fate (Bour *et al.*, 2000). N once again plays several critical roles, regulating both the distinct fates of the two founders arising from the same cell division (Carmena *et al.*, 1998b; Park *et al.*, 1998b; Ruiz Gomez and Bate, 1997) and the subsequent differentiation of these cells (Bate *et al.*, 1993; Fuerstenberg and Giniger, 1998).

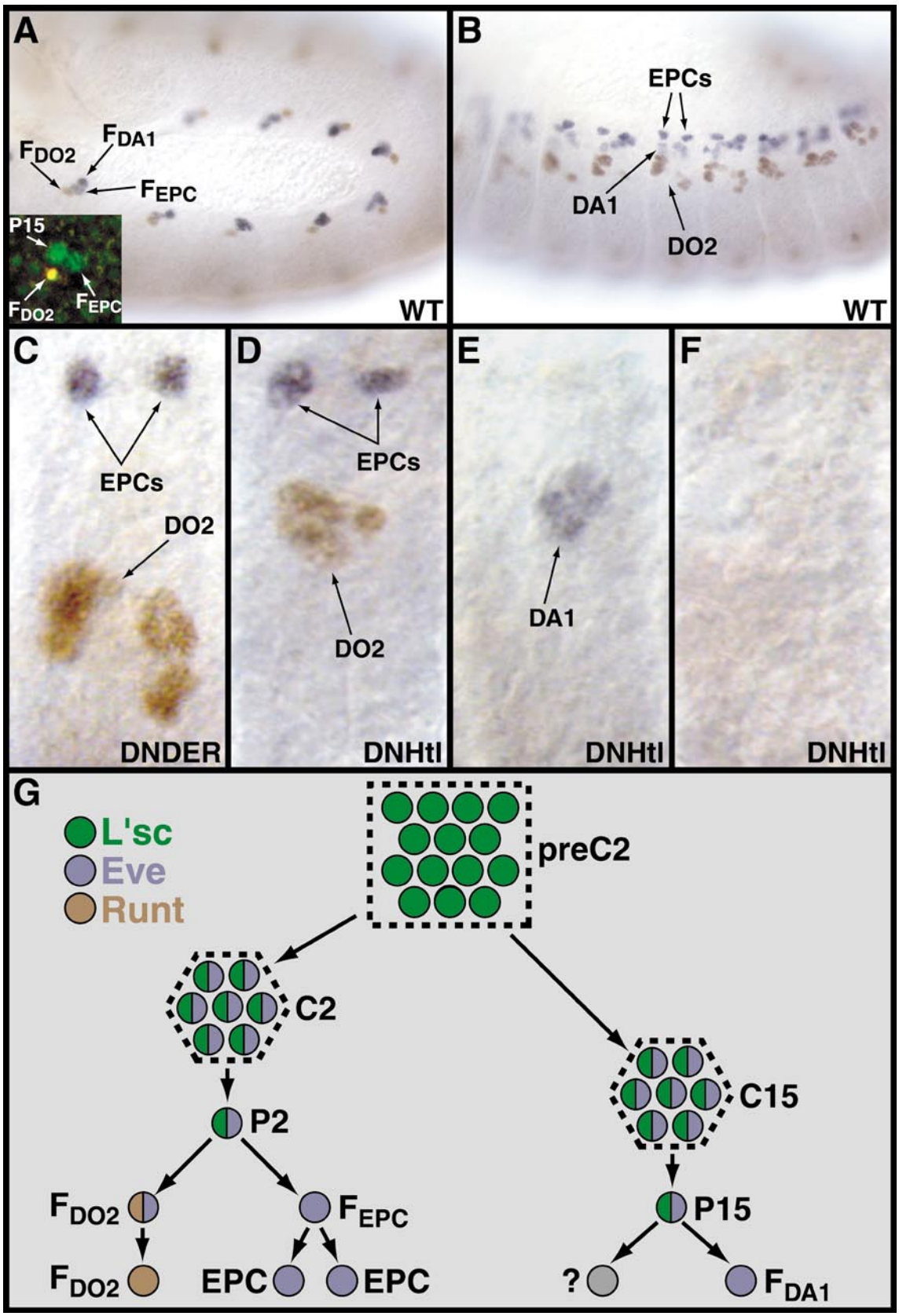
Although the distinct roles of multiple positive and negative signals involved in the determination of muscle and heart progenitors have been delineated, the nature of their interactions remains less well understood. Transcriptional convergence has recently been shown to be an important mechanism for integrating the positive inputs of Wg, Dpp, and Ras (Halfon *et al.*, 2000), and it has been suggested that Wg can relieve early N inhibition of progenitor fates (Brennan *et al.*, 1999). However, it is not known how Ras and N act in opposition during the singling out of a progenitor from an equivalence group. Based on the temporal and spatial pattern of MAPK activation, it is known that RTK/Ras signaling overlaps with N function as a progenitor segregates (Carmena *et al.*, 1998a). Here, we have investigated the opposing functions of Ras and N and report that there are intricate reciprocal interactions between these signals. Ras activates expression of the N ligand, Delta (Dl), as well as expression of the RTK signaling components, Htl (Michelson *et al.*, 1998b), Heartbroken (Hbr, also known as Stumps and Dof; Imam *et al.*, 1999; Michelson *et al.*, 1998a; Vincent *et al.*, 1998), and Rhomboid (Rho; Buff *et al.*, 1998; Freeman, 2000). This results in amplification and reinforcement of the inductive Ras signal within the prospective progenitor and, due to the nonautonomous effect of Dl, increases N inhibitory signaling in adjacent cells of the cluster. Complementing these effects, N downregulates Dl expression, upregulates its own expression, and blocks the positive autoregulatory Ras feedback loop in cells not destined to assume a progenitor fate. Furthermore, we have found that Argos (Aos), a diffusible inhibitor of DER (Freeman *et al.*, 1992), synergizes with Dl and N in mediating lateral inhibition. As a result of these multiple interactions and feedback loops, emerging progenitor and nonprogenitor cells from each cluster acquire distinct signaling activities that promote their respective fates.

MATERIALS AND METHODS

Drosophila Strains and Genetics

The following mutant stocks were used: Dl^{X} and $aos^{\Delta 7}$. Ectopic expression was achieved with the Gal4-UAS system (Brand and

FIG. 1. Lineages of two dorsal muscles and pericardial cells. Embryos of the indicated genotypes were stained for Eve (blue) and Runt (brown) expression at early stage 12 (A) or stage 14 (B–F). Lateral views are shown in this and subsequent figures, with anterior left and dorsal up. (A) Confocal micrograph (inset) of the dorsal mesoderm of one hemisegment from a late stage 11 wild-type (WT) embryo stained for Eve (green) and Runt (red). Coexpression of both proteins is shown in yellow. Runt is first expressed in the founder of muscle DO2 (F_{DO2}) when Eve is still present in this cell. At a slightly later stage, Eve is no longer detected in F_{DO2} but persists in its sibling, the founder of two pericardial cells (F_{EPC}), and in the founder of muscle DA1 (F_{DA1}). (B) Runt and Eve expression persists, revealing the tissues to which each founder contributes: two dorsal somatic muscles (DO2 and DA1) and two pericardial cells (EPCs) per hemisegment. (C–F) The phenotypes of embryos in which DER or Htl activity is disrupted support the lineage relationships of the progenitors, P2 and P15. In embryos expressing a dominant negative form of DER (DNDER) under *twi-Gal4* control, DA1 is not detected, but both DO2 and EPCs are always present (C). In embryos expressing a dominant negative form of Htl (DNHtl) under the control of both *twi-Gal4* and *Dmef-Gal4*, both P2 and P15 lineages are affected. The incomplete penetrance of the DNHtl phenotype is revealed by: loss of only DA1 (D), both EPCs and DO2 (E), or all three cell types (F). Of note, muscle DO2 and the EPCs are always both absent or both present in a given hemisegment of DNHtl embryos, consistent with these cells deriving from a common progenitor. (G) A diagrammatic representation of P2 and P15 lineages.



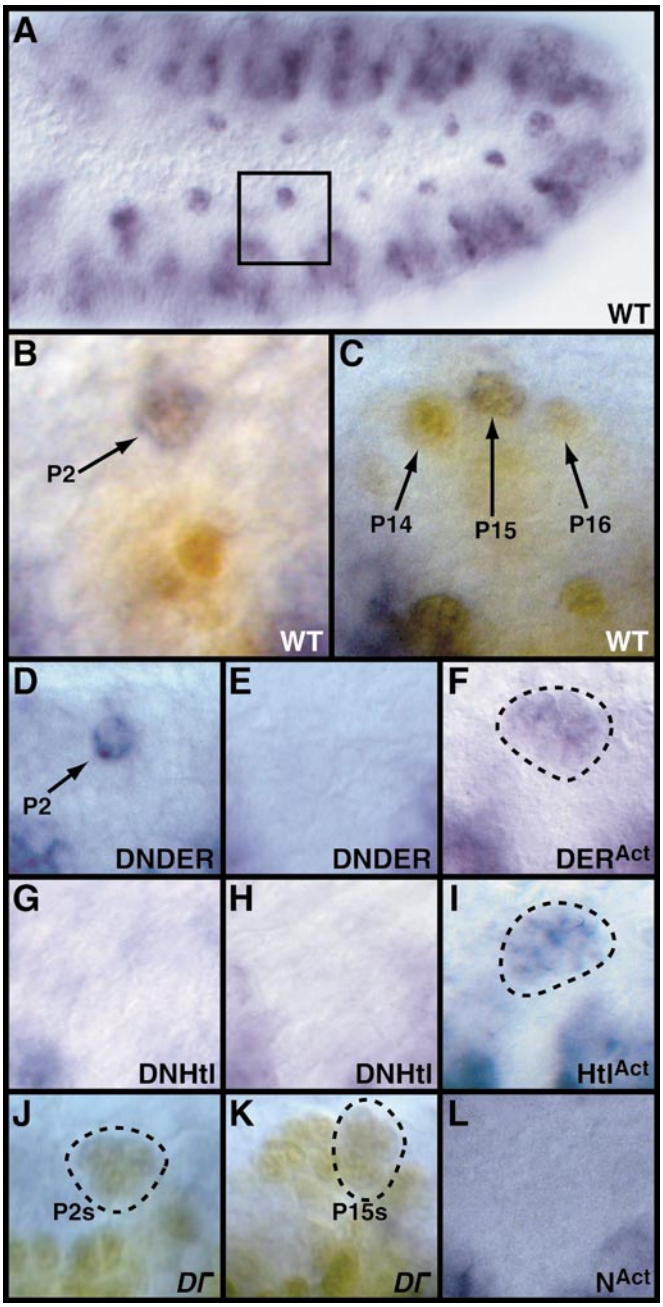


FIG. 2. Expression of *aos* in P2 and P15 depends on both Ras and N signaling pathways. (A) *In situ* hybridization pattern of *aos* in a stage 11 WT embryo. The square highlights the dorsal mesoderm of one hemisegment, as shown enlarged in (B–L). (B, C) Double *aos in situ* hybridization (blue) and anti-L'sc antibody staining (brown) showing *aos* expression in P2 and P15 in WT embryos. (D, E, G, H) Consistent with the characterized effects of DER and Htl on P2 and P15 specification, *aos* is detected in P2 (D) but not in P15 (E) in embryos expressing DNDER, whereas *aos* expression is not detected in either P2 (G) or P15 (H) in embryos expressing DNHtl. (F, I) In embryos expressing activated forms of DER (F) or Htl (I), *aos* is expressed in additional dorsal mesodermal cells. (J, K) In *Dl* mutant embryos, *aos* expression (blue) persists along with L'sc (brown) in all equivalence group cells from which P2 and P15 derive. (L) In embryos expressing an activated form of N, no *aos* mesodermal expression is found. *twi-Gal4* was used in all experiments, except those involving DNHtl, in which case a line containing both *twi-Gal4* and *Dmef-Gal4* was used.

Perrimon, 1993) and the following fly lines: *twi-Gal4, Dmef-Gal4, UAS-DNDER, UAS-DNHtl, UAS-DER^{Act}, UAS-Htl^{Act}, UAS-N^{Act}*, and *UAS-Su(H)^{VP16}, UAS-Ras1^{Act}, UAS-pntP2^{VP16}* (kindly provided by C. Klämbt). A single *twi-Gal4* driver was used for all *UAS* experiments, except those involving *UAS-DNHtl* (Figs. 1 and 2) and those involving *UAS-Su(H)^{VP16}* and *UAS-Ras1^{Act}* in which N expression was examined (see Fig 5). In the latter cases, a combination of *twi-Gal4* and *Dmef-Gal4* was employed. Oregon-R was used as the reference wild-type strain. Combinations of mutations and P-element transgenes were generated by standard genetic crosses. Balancer chromosomes containing different *LacZ* transgenes were used for identification of homozygous mutant embryos.

Immunohistochemistry and Microscopy

Embryo fixation, antibody staining, and *in situ* hybridization were carried out by standard protocols (Carmena *et al.*, 1998a; Tautz and Pfeifle, 1989). The following primary antibodies were used: rabbit α-Eve, 1:3000 to 1:10,000; mouse α-Eve, 1:25; mouse α-Dl, 1:40; mouse α-β-galactosidase (Promega), 1:500 to 1:2000; rabbit α-Hbr/Dof, 1:200; rabbit α-Htl, 1:2000; guinea pig α-Kr, 1:2000; rat α-L'sc, 1:400; mouse α-diP-MAPK (Sigma), 1:400; guinea pig α-Runt, 1:8000; and rabbit α-N, 1:400. For immunostaining with the α-N antibody, embryos were fixed by using the heat-methanol method (Tepass, 1996). In addition to the N immunofluorescence, N histochemical staining was done simultaneously by using identical conditions for wild-type, Su(H)^{VP16}, and Ras1^{Act} embryos in order to clearly compare the intensities of N staining. This experiment was done twice with the same result. Fluorescent images were recorded by using a Zeiss LSM 510 confocal microscope and assembled by using Adobe Photoshop.

Cell Counts and Statistical Analysis

Muscle and heart cells were quantitated as previously described (Buff *et al.*, 1998; Michelson *et al.*, 1998b). Cell counts were done on at least 57 embryos of each genotype. The phenotypic effects of individual mutations in various genetic backgrounds were tested for significance with pairwise comparisons of genotypes by a standard Z test. To evaluate synergistic vs additive effects of combined mutations, a likelihood ratio test of independence was performed (Halfon *et al.*, 2000).

RESULTS

Muscle and Heart Cells Arise from the Same Progenitor

Two progenitors characterized by expression of the segmentation gene *eve* are specified in a distinct temporal

order in the *Drosophila* embryonic mesoderm (Carmena *et al.*, 1995, 1998a,b). Progenitor 2 (P2) develops first and divides asymmetrically to give rise to two founder cells, one specific for a pair of persistently Eve-positive heart-associated or pericardial cells (EPCs) in every hemisegment and a second of previously undetermined identity. This second founder coexpresses Eve along with the gap gene Runt, with Eve levels rapidly fading but Runt persisting as development proceeds (Fig. 1A). By the time that Eve is evident in the EPCs, Runt labels a single somatic muscle, dorsal oblique muscle 2 (DO2; Fig. 1B). Runt is also detected in the muscle DO2 precursor during germband retraction (data not shown). The second Eve progenitor, P15, forms later than P2 and divides asymmetrically to yield the founders of dorsal acute muscle 1 (DA1) and another cell whose fate cannot be followed since a specific, stably expressed marker for it is unavailable (Figs. 1A and 1B).

To further substantiate the lineage relationships among these progenitors and founders, we utilized prior observations related to RTK signaling dependence of P2 and P15 specification: whereas P15 requires the activities of both DER and Htl, only Htl is involved in P2 formation (Buff *et al.*, 1998; Carmena *et al.*, 1998a; Michelson *et al.*, 1998b). In this way, targeted mesodermal expression of a dominant negative form of DER strongly blocked formation of DA1 but not the EPCs (Fig. 1C). Also, consistent with DO2 and EPC founders being the progeny of P2, DO2 development, like that of the EPCs, was not affected by dominant negative DER (Fig. 1C). Additional support for the sibling relationship between the DO2 and EPC founders derived from the analysis of targeted expression of a dominant negative form of Htl. Under conditions in which early mesoderm migration is not perturbed, dominant negative Htl generates an incompletely penetrant phenotype in which different hemisegments lose derivatives of P2, P15, or both progenitors (Michelson *et al.*, 1998b). With such partial inhibition of Htl activity, muscle DO2 and the EPCs were consistently either both present or both absent from any given hemisegment; in no cases did one of these cell types develop without the other, as expected for cells derived from a common progenitor (890 hemisegments analyzed; Figs. 1D–1F, and data not shown). In contrast, muscle DA1 frequently formed in the absence of muscle DO2 and the EPCs, consistent with its derivation from an independent progenitor (Fig. 1E). Taken together, these data establish that the EPC and DO2 founders are sibling cells of the P2 division, whereas the other Eve-expressing muscle founder arises from a different progenitor.

These lineage relationships and associated marker gene expression patterns are summarized in Fig. 1G. We note that this model differs from one derived on the basis of clonal analysis in which it was proposed that the two Eve-positive mesodermal cell types originate from the same progenitor (Park *et al.*, 1998b). This discrepancy may relate to the fact that muscles form by sequential cell fusions involving both founders and fusion-competent cells of potentially different parental cell origins, thereby confounding the interpretation of clonal analysis in which the cytoplasm of a single myotube is labeled by the lineage tracing marker.

Aos Inhibition of Progenitor Specification

Given DER involvement in muscle progenitor specification, we determined whether the DER antagonist Aos also participates in this process. Aos is expressed sequentially in mesodermal clusters C2 and C15 and later persists in the respective progenitors (Figs. 2A–2C, and data not shown). Aos expression is lost from C2 and P2 when Htl but not DER activity is inhibited (Figs. 2D and 2G), whereas in C15 and P15, Aos expression is both DER- and Htl-dependent (Figs. 2E and 2H). The finding that Aos expression relies on both RTKs is supported by the observation that Aos is still expressed in P2 in *spitz (spi)* mutants in which DER but not Htl signaling is impaired (data not shown). Constitutive activation of either DER or Htl leads to more Aos-expressing cells, an effect that is due to activation of the common Ras/MAPK pathway by each of these RTKs (Figs. 2F and 2I). Aos expression also is influenced by neurogenic gene function: Aos expression is maintained in the additional P2 and P15 cells specified in *Dl* mutant embryos, while Aos expression is suppressed by a constitutively active form of N (Figs. 2J–2L). Thus, Aos mesodermal expression responds to both the positive and negative signals involved in Eve progenitor determination.

To assess the potential role of Aos in muscle and heart development, we examined the effect of *aos* loss-of-function on specification of Eve progenitors and founders. In stage 11 *aos* null mutant embryos, some, but not all, hemisegments exhibit increased numbers of Eve-positive mesodermal cells (Figs. 3A and 3B). By double labeling with antibodies directed against Eve and Kr—a muscle identity gene that is expressed in the P15 but not the P2 lineage (Buff *et al.*, 1998; Carmena *et al.*, 1998b)—we determined that both EPC and DA1 founders are duplicated in the absence of *aos* function (Figs. 3C–3F). Consistent with this finding, a significant increase in differentiating DA1 muscles and EPCs was detected in *aos* mutant embryos at later developmental stages (Figs. 3G, 3H, 4A, 4B and 4D). In agreement with the common progenitor origin of muscle DO2 and the EPCs, DO2 also was duplicated in a significant number of hemisegments in *aos* mutant embryos (Figs. 4A and 4C). The formation of these supernumerary muscle and cardiac cells is DER-dependent, as expected for loss of a DER-specific antagonist, even though DER normally is involved only in muscle DA1 development (see below). Furthermore, the extra Eve-positive cells that form in the absence of *aos* derive from the same equivalence groups from which the normal progenitors segregate (Figs. 3A–3F). These findings suggest that Aos provides, albeit weakly, a lateral inhibitory signal involved in progenitor specification.

Synergistic Lateral Inhibition by Aos and Dl

The Eve progenitor phenotype associated with loss of *aos* function resembles that of neurogenic mutants (Carmena *et al.*, 1998a; Fig. 3I); that is, all cells of a given equivalence group are specified as progenitors, consistent with a loss of lateral inhibition. However, neither null *Dl* nor null *aos* mutants exhibits a fully penetrant Eve progenitor phenotype: in each case, some hemisegments have a wild-type number of Eve cells, although consistently more affected hemisegments are seen in *Dl* than in *aos* mutants (Figs. 3B and 3I). This suggests that the lateral inhibitory functions of these two signals are partially redundant, with the classic neurogenic pathway having the predominant effect. Thus, we examined the development of Eve cells in an *aos Dl* double mutant. In the absence of both gene products, all hemisegments were equally affected and the Eve clusters appeared even larger than in either single mutant (Fig. 3J). Additional experiments revealed that activation of Eve expression is found not only in the normal Eve equivalence groups but also in cells of equivalence groups that are immediately adjacent to those from which P2 and P15 are derived (data not shown). Thus, *aos* and *Dl* cooperate in mediating inhibitory effects on progenitor identity that extend beyond the limits of a single equivalence group (see Discussion).

The synergy between *aos* and *Dl* in this context is underscored by the strong genetic interactions exhibited by these genes. Heterozygosity for a null *Dl* allele or homozygosity for a null *aos* allele each was associated with an increase in the number of DA1 and DO2 muscles ($P < 0.002$); the number of EPCs was also affected in *aos/aos* ($P < 0.001$) but not in *Dl*/+ embryos ($P \approx 0.08$; Fig. 4A). Significantly, there was a marked dominant enhancement by *Dl* of the *aos* loss-of-function phenotype for all three cell types ($P < 0.001$ for DA1 and DO2; $P < 0.02$ for the EPCs) and, at least for muscles DA1 and DO2, this represented a true synergy between the individual mutations ($P < 0.001$; Fig. 4A).

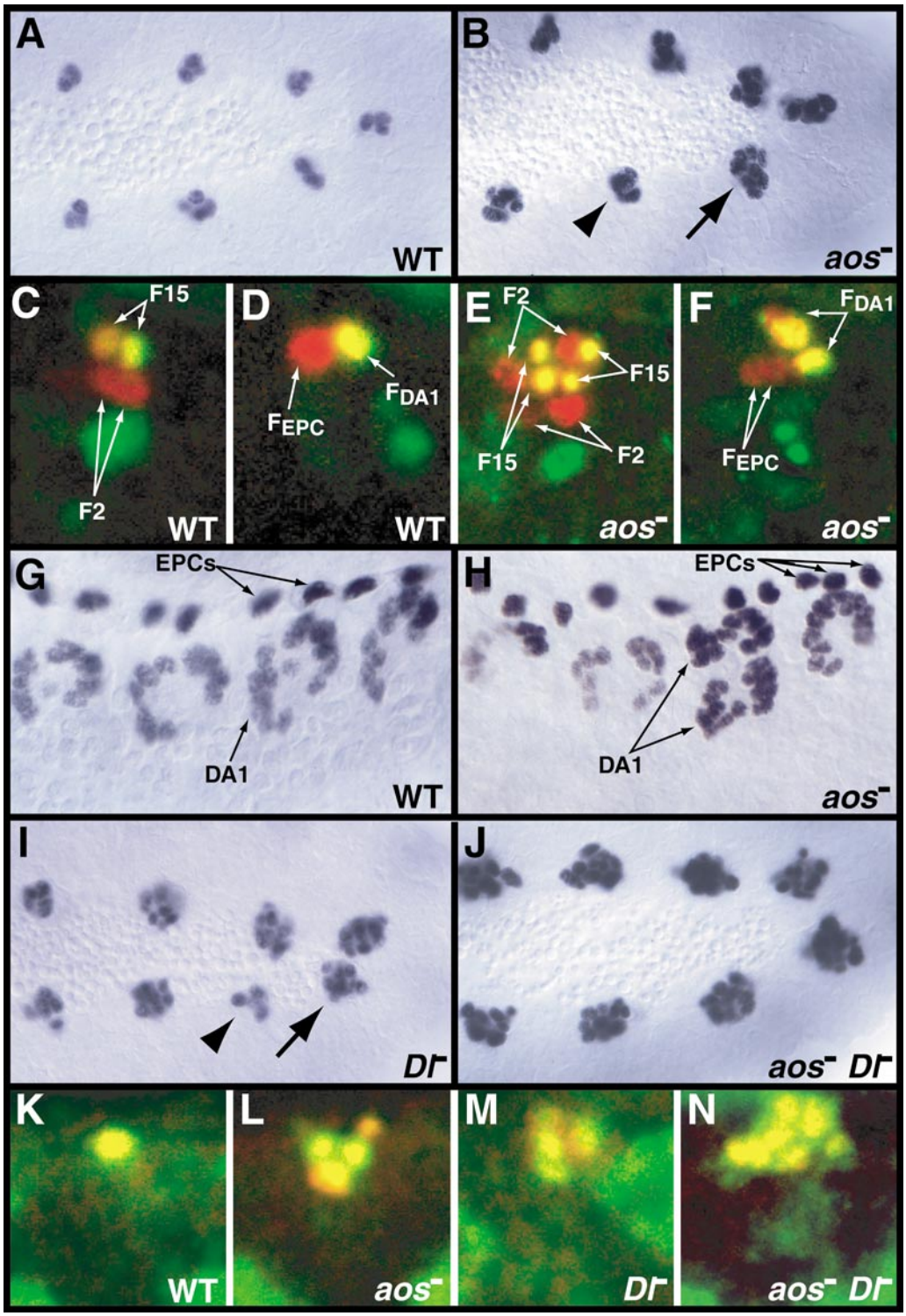
A further connection between neurogenic and RTK signaling was revealed by the ability of *Dl* to dominantly suppress the inhibitory effect of a dominant negative form of DER on the development of muscle DA1 ($P < 0.001$; Fig. 4B). As expected for a DER antagonist, an *aos* null allele also suppressed the effects of DNDER on DA1 formation, but only when the *aos* allele was homozygous ($P < 0.001$; Fig. 4B). Interestingly, although neither DO2 nor the EPCs is dependent on DER for its formation, both cell types are increased in the absence of *aos* function ($P < 0.001$; Figs. 4A, 4C, and 4D; see above). Moreover, this effect is markedly suppressed by DNDER ($P < 0.001$; Figs. 4A, 4C, and 4D), suggesting that Aos plays an important role in preventing basal or spontaneous DER activity (see Discussion). In summary, *aos* and *Dl* interact genetically both with each other and with *DER* during mesodermal progenitor specification, consistent with the involvement of both Aos and Dl in the common process of lateral inhibition.

Given the similarity of the neurogenic and *aos* progenitor specification phenotypes, as well as the *aos-Dl* and *Dl-DER* genetic interactions, we next examined expression of the diphosphorylated form of MAPK (diP-MAPK; Gabay *et al.*, 1997)—a measure of Ras activity—in both *aos* and *Dl* mutant embryos. As expected for loss of a DER inhibitor, MAPK activation is maintained in each of the additional P2 and P15 progenitors that form in *aos* mutant embryos (Fig. 3L, and data not shown). MAPK activation also persists in the additional Eve progenitors that form in both *Dl* single and *aos Dl* double mutant embryos (Figs. 3M and 3N), suggesting that not only Aos but also neurogenic signaling is capable of blocking the Ras/MAPK pathway. As noted below, a constitutively active form of N has the predicted inhibitory effect on MAPK activation (see Fig. 6I). These findings provide additional evidence supporting the antagonism between neurogenic and RTK/Ras signaling during specification of mesodermal Eve progenitors.

Reciprocal Regulation of Dl by Ras and N

We next determined the overall mesodermal expression of Dl, as well as the expression of this protein in Eve equivalence groups and progenitors. Dl expression is modulated in the mesoderm of early stage 11 wild-type embryos, becoming largely restricted by late stage 11 to the domain that gives rise to both heart and somatic muscle (Fig. 5A). Analysis during progenitor formation revealed that Dl exhibits a highly dynamic pattern of expression (Figs. 5D–5G). Initially, Dl was detected at the periphery of all cells of the Eve C2 equivalence group (Fig. 5D). As progenitor selection

FIG. 3. Aos and Dl cooperate in progenitor selection by inhibiting Ras signaling. (A, B, G, H) Embryos stained for Eve expression at stage 11 (A, B) and stage 14 (G, H). In *aos* null mutant embryos, additional Eve progenitors are specified in some (arrow, B) but not all (arrowhead, B) hemisegments. As a result, more EPCs and DA1 muscles are detected at later stages (G, H). (C–F) Confocal micrographs of individual dorsal mesoderm hemisegments from late stage11/early stage 12 WT (C, D) or *aos* mutant (E, F) embryos double stained with anti-Eve (red) and anti-Kr (green) antibodies. In WT (C), Kr is coexpressed with Eve in P15 progeny (F15) but is not detected in P2 progeny (F2). Kr persists along with Eve in F_{DA1} (derived from F15; D). In *aos* mutant embryos, additional founders from both P2 and P15 lineages are specified (E, F). (I) Most (arrow) but not all (arrowhead) hemisegments in *Dl* mutant embryos exhibit an increased number of Eve progenitors. (J) The *aos, Dl* double mutant shows a fully penetrant phenotype with increased numbers of Eve progenitors in all hemisegments. (K–N) Confocal micrographs of individual hemisegments from stage 11 embryos of the indicated genotypes, double stained with anti-Eve (red) and anti-diP-MAPK (green). In *aos* or *Dl* mutant embryos, diP-MAPK persists in the extra progenitors, with a fully penetrant phenotype seen in the *aos, Dl* double mutant.



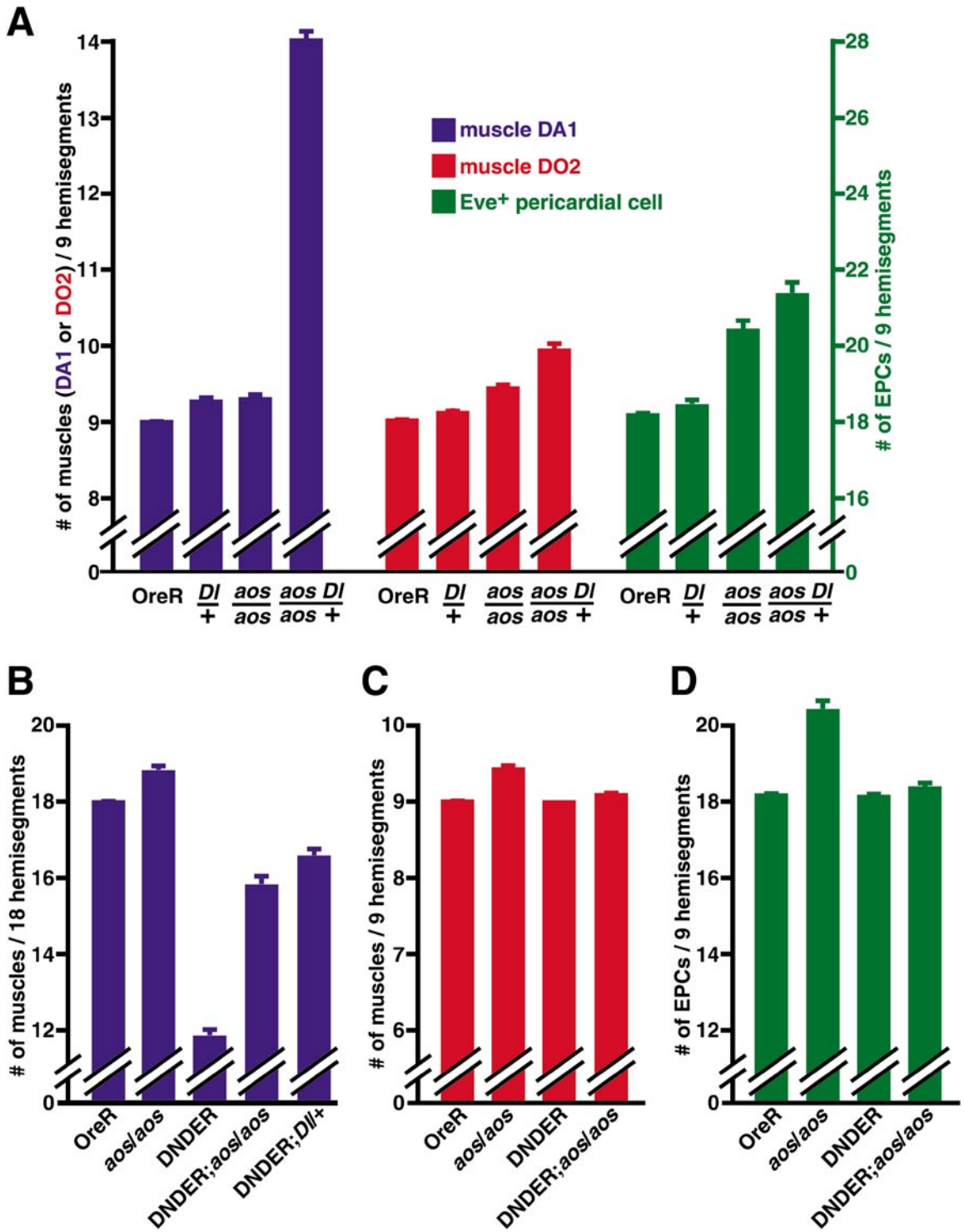


FIG. 4. Genetic interactions between *aos*, *Dl*, and *DER* during muscle and heart progenitor specification. (A) Quantitation of the number of muscles and EPCs formed in WT and the indicated mutant backgrounds reveals a strong synergy between *aos* and *Dl*. (B–D) Genetic interactions occur between *aos* and *DER* as well as between *Dl* and *DER* for development of muscle DA1 (B), muscle DO2 (C), and the EPCs (D). Note that although DER is not required for DO2 or EPC development, specification of both cell types is affected in *aos* null mutant embryos and this phenotype is suppressed by DNDER. Expression of DNDER was driven by *twi-Gal4* in all experiments. See text for description of statistical parameters.

proceeds, Dl levels appear to increase in one cell of the cluster (arrowhead in Fig. 5E), and then becomes restricted to the single Eve-positive P2 progenitor (Fig. 5F). Dl eventually fades from this cell and a similar expression pattern is recapitulated during formation of progenitor P15 (Fig. 5G).

Constitutive Ras activation leads to a generalized increase in Dl expression (Fig. 5B). This effect was more readily evident at single cell resolution when embryos were double labeled with Eve and Dl antibodies. Coincident with the failure to segregate individual Eve progenitors under the influence of activated Ras, Dl is maintained at high levels in all or most dorsal mesodermal cells in which Eve is expressed (Figs. 5H–5K). In contrast, constitutive N activation results in a generalized suppression of Dl expression (Fig. 5C).

N Expression Is Stimulated by Ras and N

Given the changes in Dl expression during Eve progenitor specification, we next determined if N responds to Ras and N signaling. In wild-type embryos, N is uniformly expressed throughout the mesoderm when the Eve C2 cluster first appears (Fig. 5L). As progenitor specification ensues, N remains widely expressed, including in the emerging P2 progenitor and in C2 cells from which Eve is lost (Fig. 5M). By the time P2 is fully singled out and ready to divide, N is found at the highest level in this cell (Fig. 5N; note arrow in the red channel shown in panel N″).

The above results suggest that N expression may be positively regulated by both Ras and N. To further evaluate this possibility, we examined the effect of constitutive activation of each pathway on N expression. Activated Ras stimulates a generalized increase in mesodermal N expression, as does a constitutively activated form of the downstream N effector, Suppressor of Hairless [Su(H); Figs. 5O and 5P]. These findings confirm that N is subject both to positive feedback control and to positive cross-regulation by Ras. Moreover, both the suppressive effect of N on Eve progenitor formation and the activating effect on its own production are Su(H)-dependent processes.

Positive Autoregulation of the RTK/Ras Pathway

In addition to the downstream Ras effector, diP-MAPK, proximal components of both the Htl and DER RTK pathways are localized in the mesoderm of wild-type embryos during Eve progenitor specification. These include the Htl FGFR itself, the FGFR-specific signal transducer Hbr (Imam *et al.,* 1999; Michelson *et al.,* 1998a; Vincent *et al.,* 1998) and Rho, a transmembrane protein involved in DER activation (Buff *et al.,* 1998; Freeman, 2000; Michelson *et al.,* 1998a; Figs. 6A–6D). Given that constitutive Ras signaling induces extra RTK-dependent progenitors and leads to MAPK hyperactivation within these cells (Carmena *et al.,* 1998a; Fig. 6E), we tested if expression of upstream signaling components is also affected by Ras. Additional cells expressing Htl, Hbr, and Rho are indeed induced under the influence of activated Ras. This effect was most evident for the two FGF pathway components, with Rho showing a less marked response (Figs. 6F–6H). This suggests that *htl, hbr,* and *rho* undergo positive autoregulation during progenitor specification, thereby enabling the fate-inducing RTK signal to be amplified in a cell autonomous manner.

N Inhibits the RTK/Ras Positive Feedback Loop

Loss of neurogenic gene function leads to an overproduction of muscle and heart progenitors, whereas constitutive activation of N suppresses formation of these cells (Baker and Schubiger, 1996; Bate *et al.,* 1993; Corbin *et al.,* 1991; Fuerstenberg and Giniger, 1998; Park *et al.,* 1998a). Given the effect on MAPK activation of loss of *Dl* function (Fig. 3M), we investigated the consequences of constitutive N activation on Ras signaling and the expression of RTK pathway components. When an activated form of N was expressed throughout the mesoderm, not only was Eve progenitor formation inhibited, but also diP-MAPK, Htl, Hbr, and Rho expression were suppressed (Figs. 6I–6L). Thus, N is capable of interfering with RTK/Ras signaling by blocking the Htl- and DER-positive feedback loops.

Balance between Ras and N Signaling Determines Cell Fate

The reciprocal phenotypes associated with constitutive Ras and N activation enabled us to further investigate the opposing influences of these pathways on muscle and heart progenitor formation. To this end, both effectors were expressed simultaneously in the mesoderm. Under these conditions, a mixed Eve progenitor phenotype was observed (Figs. 7A, 7B, 7D, and 7E). In some hemisegments more Eve cells formed, consistent with a predominating effect of activated Ras; in some hemisegments no such cells developed, suggestive of a greater influence of activated N; and in others a normal number of Eve cells was present, as if the opposing effects of Ras and N were appropriately balanced, as must occur in wild-type embryos (Fig. 7E). This experiment was repeated with all four pairwise combinations of two independent insertions each of *UAS-Ras1*^{Act} and *UAS-N*^{Act}. In each case, a qualitatively similar result was obtained. However, the relative frequencies of the activated Ras- and activated N-predominating hemisegments varied for each combination, consistent with position effect variability in the strengths of the individual transgene insertions (data not shown). In agreement with this interpretation, two copies of activated Ras were capable of completely reversing the effect of one copy of activated N (data not shown).

We also examined the expression of diP-MAPK, Htl, Hbr, and Rho in embryos expressing both *UAS-Ras1*^{Act} and *UAS-N*^{Act}. As seen for Eve, expression of each component varied among hemisegments, reflecting a primary influence of either activated Ras or activated N (Figs. 6M–6P). Col-

lectively, these findings reveal that the inductive Ras and lateral inhibitory N pathways compete with each other during muscle and heart progenitor specification, and that a requisite balance must be achieved in order for the proper number of progenitors to be determined.

N Inhibition Is Not Reversed by the Ras Effector, Pnt

The nuclear effects of *Drosophila* RTK/Ras signaling are mediated through the phosphorylation by MAPK of two Ets domain transcription factors, Yan and the P2 isoform of Pointed (Pnt; Brunner *et al.*, 1994; O'Neill *et al.*, 1994). Phosphorylation of Pnt converts it to a transcriptional activator which is directly involved in the regulation of mesodermal *eve* expression (Halfon *et al.*, 2000). If N acts solely by blocking MAPK activation, for example by stimulating expression of a MAPK phosphatase (MKP) as has recently been described for *Caenorhabditis elegans* vulva development (Berset *et al.*, 2001), then a constitutively activated form of Pnt should efficiently reverse the inhibitory effect of N on Eve progenitor formation. Like constitutive Ras, constitutive Pnt on its own induces additional Eve progenitors (Halfon *et al.*, 2000; Figs. 7A–7C). However, unlike Ras, Pnt is incapable of reversing the effect on Eve of activated N (Figs. 7E and 7F). Thus, N cannot solely be acting via a MKP to oppose Ras/MAPK signaling in the *Drosophila* embryonic mesoderm.

DISCUSSION

Muscle and heart progenitor specification in *Drosophila* embryos is orchestrated by the complex interplay of multiple intercellular signals. We focus here on mechanisms by which the inductive Ras signal acts cooperatively and competitively with the inhibitory N pathway in the segregation of a single progenitor from an equivalence group. The key features of this system include positive and negative feedback regulation leading to signal amplification and attenuation, reciprocal cross-talk, and combinatorial synergy between separate inhibitory signals. Together, these regulatory mechanisms generate unique signaling thresholds that determine specific cellular fates.

Autoregulation of the RTK and N Pathways

Autoregulation of a signal transduction cascade can cause either enhancement or attenuation of the transduced signal, depending on whether the feedback loop acts positively or negatively (Freeman, 2000; Perrimon and McMahon, 1999). Both types of feedback control occur during the Ras- and N-mediated specification of Eve mesodermal progenitors.

Ras activation leads to increased expression of several proximal components of both the FGFR and EGFR pathways that serve to amplify and/or prolong both fate-inducing RTK/Ras signals in the emerging Eve progenitors. A similar amplification of EGFR signaling occurs via induction of Rho during *Drosophila* oogenesis (Wasserman and Freeman, 1998) and mesothoracic bristle formation (Culí *et al.*, 2001), and via upregulation of EGFR expression during *C. elegans* vulva development (Wang and Sternberg, 2001). The present analysis also uncovers a positive feedback mechanism for inductive FGFR signaling, in this case via increased expression of not only the Htl receptor but also its specific signal transducer, Hbr (Fig. 8A). Interestingly, the data suggest that the downstream components may respond to different thresholds of Ras activity as Rho exhibits a less robust response than either Htl or Hbr to Ras activation.

A negative feedback loop occurs in the DER pathway through autoactivation of the inhibitory ligand, Aos (Golembo *et al.*, 1996). We show that Aos cooperates with Dl to block the progenitor-inducing Ras signal in both adjacent and more remote cells of the cluster (see below). Aos could also exert a late inhibitory effect on the progenitor by terminating the inductive DER signal as Spi levels decrease following the establishment of cellular identity. Consistent with this possibility, MAPK activation fades from the singled out progenitor prior to its asymmetric division, suggesting that prolonged RTK signaling does not occur (Carmena *et al.*, 1998a).

Positive and negative feedback also occur during N function in the mesoderm. N activation both downregulates its ligand Dl and upregulates its own expression, thereby enhancing the potential for inhibitory signaling in cells not destined for the progenitor fate. Together, these opposing changes in Dl and N expression produce a unidirectional inhibitory signal emanating from the prospective progenitor and directed toward the adjacent nonprogenitor cells (Fig. 8A). Similar feedback mechanisms regulate the N pathway in the *Drosophila* embryonic CNS (Kunisch *et al.*, 1994), adult PNS (Heitzler *et al.*, 1996; Heitzler and Simpson, 1991; Parks *et al.*, 1997) and wing vein-forming cells (de Celis *et al.*, 1997; Huppert *et al.*, 1997), and also apply to the N receptor-ligand combinations controlling gonadal and vulval cell fates in *C. elegans* (Greenwald, 1998; Wang and Sternberg, 2001).

Cross-talk between the RTK/Ras and N Pathways

(A) Competition between Ras and N. Competitive cross-talk between Ras and N is manifest by the ability of the latter to block the expression of proximal components of the two RTK pathways—namely Htl, Hbr and Rho—as well as to prevent the associated activation of MAPK. An antagonistic relationship between the RTK and N pathways is also revealed by the strong genetic interaction between *Dl* and *DER*, in agreement with previously reported genetic studies (Price *et al.*, 1997; Verheyen *et al.*, 1996). Collectively, these results establish that the RTK and N pathways are not simply acting in parallel to exert opposing influences on progenitor specification; rather, N must be interfering with the generation and/or transmission of the inductive RTK signal. This effect could occur at multiple

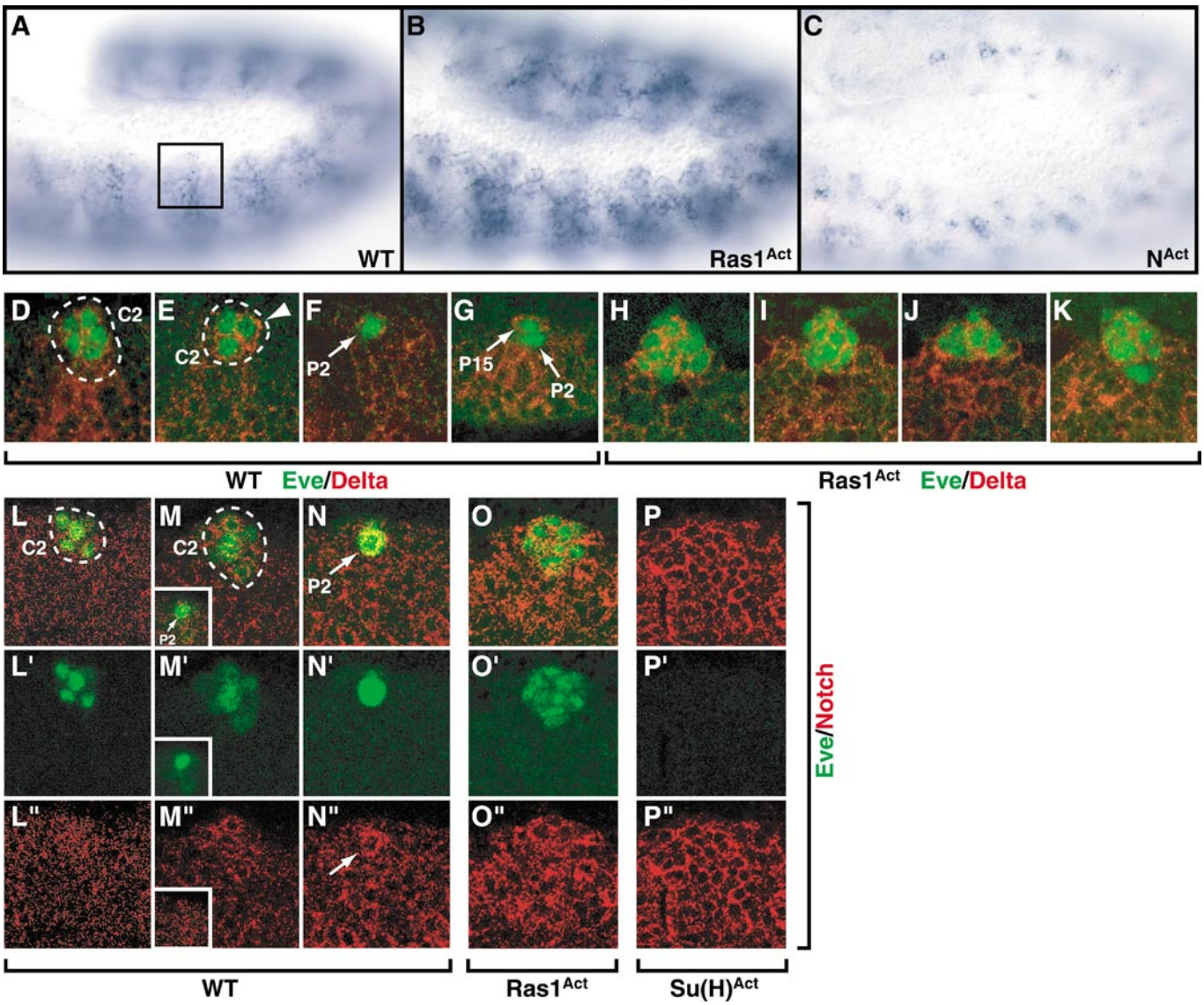


FIG. 5. Regulation of Dl and N expression by Ras and N pathways. (A–C) Late stage 11 embryos of the indicated genotypes stained for Dl expression. In WT embryos (A), Dl is modulated along the anteroposterior axis into alternating high and low expression domains. The rectangle (A) represents the area of an individual hemisegment that is enlarged in (D–P) Dl expression is upregulated by Ras activation (B) and is repressed by activated N (C). (D–K) WT embryos (D–G) and embryos expressing an activated Ras (H–K) double stained for Eve (green) and Dl (red). In WT, Dl is detected along with Eve in the C2 equivalence group (D) and is subsequently upregulated in one cell of this cluster, the prospective P2 progenitor (arrwowhead, E). Dl initially persists but later disappears from P2 (F, G). A similar dynamic pattern of Dl expression is observed during P15 specification (G, data not shown). In response to activated Ras, Dl persists along with Eve in all cells of both clusters C2 and C15 throughout the time during which P2 and P15 are normally singled out (H–K). (L–P) WT embryos (L–N) and embryos expressing activated forms of Ras (O) or Su(H) (P) stained for Eve (green) and N (red) expression. The two channels are shown separately and merged in the upper row. At C2 stage, N is uniformly expressed in the mesoderm (L) and remains in all C2 cells as P2 is selected (M). Inset in (M) shows a confocal section at the level of the emerging P2 from the same hemisegment as the indicated C2. After P2 is singled out, it expresses N at the highest level (arrow in N″); compare with the level of N expression in the emerging P2 (inset in M″). Activation of either Ras or Su(H) in the mesoderm causes a generalized increase in mesodermal N expression (O, P). In addition, Eve is completely suppressed by activated Su(H) (P). *twi-Gal4* was used in all ectopic expression experiments, except those shown in (O) and (P), where a line containing both *twi-Gal4* and *Dmef-Gal4* was employed.

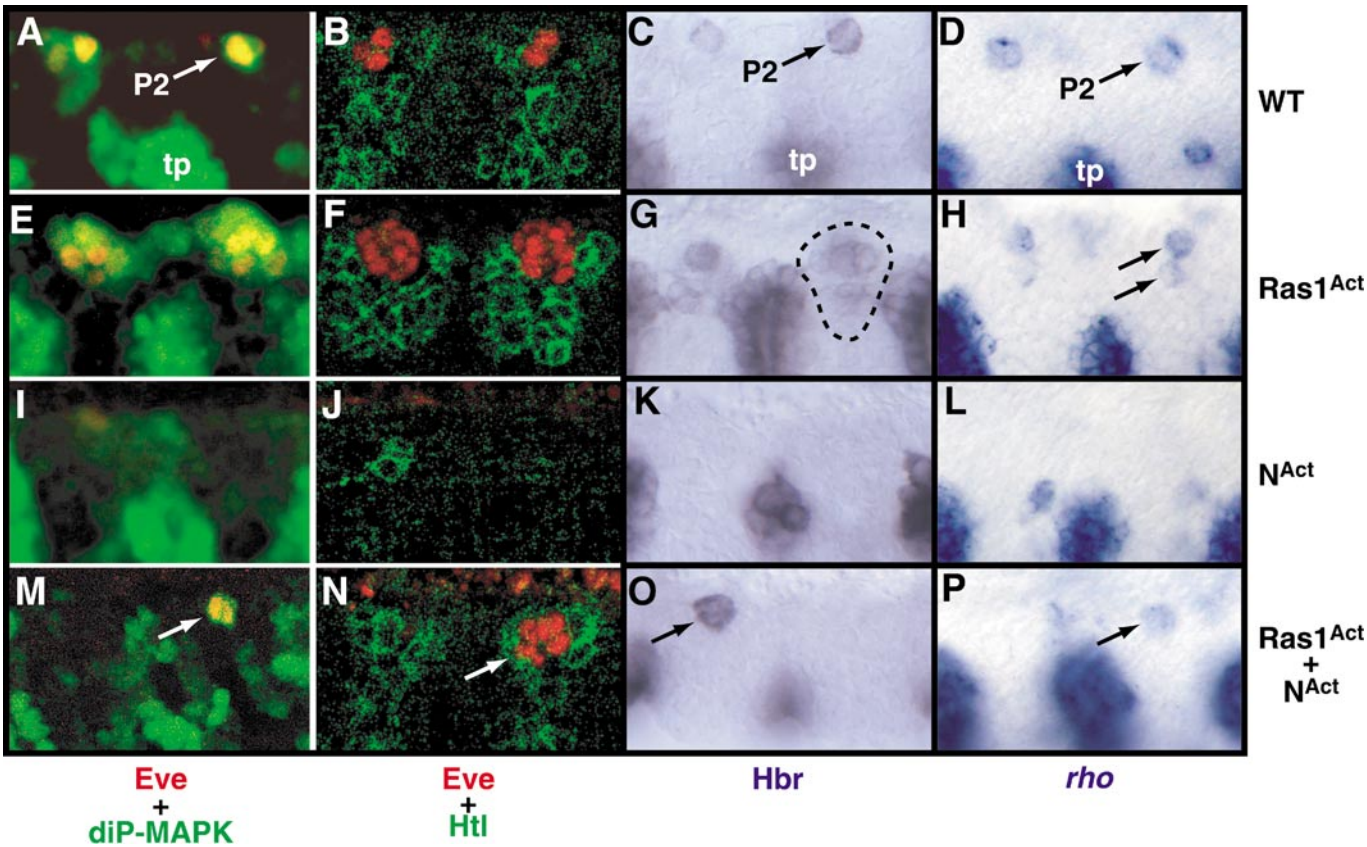


FIG. 6. DER and Htl are subject to positive feedback regulation that is inhibited by N signaling. Each panel shows the dorsal mesoderm of two hemisegments of a stage 11 embryo. WT embryos (A–D) and embryos expressing activated forms of Ras (E–H), N (I–L), or both Ras and N (M–P) immunostained for Eve (red) and diP-MAPK (green) (A, E, I, M), for Eve (red) and Htl (green) (B, F, J, N), for Hbr (C, G, K, O) and for *rho* RNA (D, H, L, P). In Ras^{Act} embryos, additional cells express diP-MAPK, Eve, Htl, Hbr, and *rho*, indicating that Ras activates a positive feedback loop that amplifies the signaling output of both the EGF and FGF receptors. N inhibits this positive autoregulation by downregulating Htl, Hbr, *rho*, and diP-MAPK expression. When both activated constructs of N and Ras were expressed at the same time, an intermediate phenotype was found (also see Fig. 7E). *twi-Gal4* was used for all ectopic expression experiments. tp, tracheal pit.

levels. The ability of activated N to at least partially block MAPK activation induced by constitutive Ras argues that N functions downstream of Ras. An additional direct effect of N on expression of Ras-responsive target genes cannot be excluded, particularly since Enhancer of split repressors are involved in the specification of progenitor cell fates (Corbin *et al.*, 1991; unpublished results). Such targets could include *eve* itself, or, given positive autoregulation of RTK signaling, one or more RTK pathway components.

During *C. elegans* vulva development, Lin-12/N inhibits EGFR activity by stimulating the expression of a MAPK phosphatase (Berset *et al.*, 2001). This is an attractive explanation for the effect of N observed here. However, while stimulation of a MAPK phosphatase could contribute in part to N inhibition of Ras signaling in the *Drosophila* embryonic mesoderm, it cannot be the only explanation since a constitutively activated form of Pnt is completely unable to reverse the activity of constitutive N. This is in marked contrast to the substantial reversal of N exhibited by activated Ras and occurs even though Pnt is a major Ets domain activator involved in RTK-dependent *eve* regulation (Halfon *et al.*, 2000).

To account for the differential abilities of constitutive Ras and Pnt to compete effectively with constitutive N, we favor the idea that an additional, as yet uncharacterized, Pnt-independent function of Ras may be a target of N inhibition. Hence, there exist at least four potential sites of competitive interaction between these pathways: (1) direct regulation of target gene enhancers by pathway-specific transcriptional activators and repressors; (2) regulation of MAPK phosphorylation; (3) inhibition by N of RTK pathway component expression; and (4) an additional level of Pnt-independent cross-talk between Ras and N. These mechanisms modulate the relative flux through the competing Ras- and N-dependent processes and determine which pathway predominates, thereby achieving a critical threshold for a given cell fate (Fig. 8B). The importance of relative activity thresholds is underscored by our results

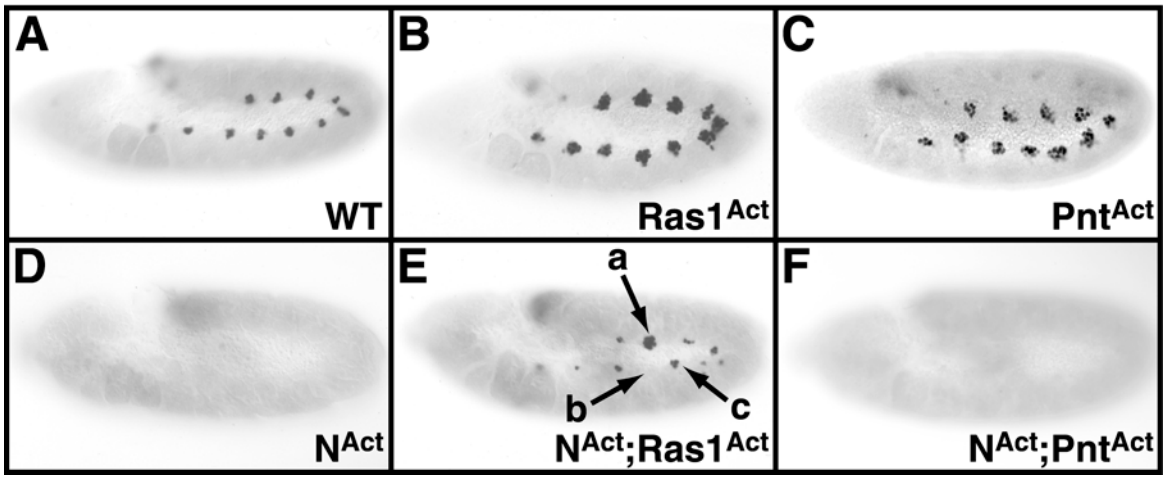


FIG. 7. An activated form of Pnt cannot overcome N inhibition of the Ras signaling pathway. Each panel shows a stage 11 embryo of the indicated genotype stained for Eve expression. (A–C) Both constitutive forms of Ras and Pnt lead to a similar increase in Eve-expressing cells. (D) N activation suppresses mesodermal Eve expression. (E) Simultaneous constitutive activation of N and Ras causes an intermediate phenotype in which some hemisegments have excess Eve progenitors (a), some have no Eve progenitors (b) and some have a normal number of Eve cells (c). (F) In contrast to activated Ras, activated Pnt is unable to overcome N-mediated inhibition of Eve progenitor specification. *twi-Gal4* was used for all ectopic expression experiments.

with different combinations of activated Ras and N insertions in which different numbers of Eve-expressing cells were induced, presumably reflecting slight fluctuations in the relative strengths of each pathway in individual cells (Fig. 7, and data not shown). Similar levels of control may underlie the antagonistic effects of Ras and N in other developmental contexts (Culf *et al.*, 2001; de Celis *et al.*, 1997; Ikeya and Hayashi, 1999; Kumar and Moses, 2001; zur Lage and Jarman, 1999).

(B) Cooperation between Ras and N. Although the net effect of Ras and N signaling in the present system is the result of their antagonistic relationship, several forms of cooperative cross-talk also occur. For example, Ras activation induces the expression of Dl. Since the Ras signal is amplified by a positive feedback loop, this has the effect of biasing Dl expression to the emerging progenitor, thereby generating a polarized, nonautonomous inhibitory signal that acts on adjacent cells of the cluster. Aos is also a target of Ras activation, and Aos acts synergistically with the neurogenic pathway to block inductive RTK signaling. Thus, through its effects on the two inhibitory ligands, Dl and Aos, Ras cooperates with N to insure that only one cell segregates as a progenitor from each equivalence group (Fig. 8C).

Further cooperation is evident in the N-mediated downregulation of Dl and Aos in prospective nonprogenitors, a combination of negative feedback and cross-talk that effectively prevents neighboring cells from sending an inhibitory signal to the emerging progenitor. Yan is yet another Ras-dependent component that reinforces the effect of N: when MAPK is suppressed in cells in which N is active, Yan is a functional repressor that blocks progenitor fate (Halfon *et al.*, 2000; Fig. 8A). Other examples of cooperation between Ras and N signaling include mammalian cell tumorigenesis (Fitzgerald *et al.*, 2000) and photoreceptor specification in the *Drosophila* eye (Cooper and Bray, 2000; Tomlinson and Struhl, 2001).

One seemingly paradoxical signaling interaction is N expression upregulation by Ras. Since Ras output is amplified in the progenitor, N protein might be expected to decrease in this cell, thereby restricting lateral inhibition to the appropriate direction (de Celis *et al.*, 1997; Huppert *et al.*, 1997; Levitan and Greenwald, 1998; Parks *et al.*, 1997). However, increased N in the Eve progenitor does not actually affect the polarity of lateral inhibition because the activating ligand, Dl, is downregulated by N in the adjacent nonprogenitors. Of further relevance, Dl may inhibit N activity when the two proteins are expressed in the same cell (de Celis and Bray, 2000; Jacobsen *et al.*, 1998). Moreover, upregulation of N normally occurs very late in progenitor specification, as opposed to Dl which increases in one cell of the cluster at an earlier stage (compare Figs. 5E and 5N). Lastly, increased N has independent biological significance in progenitors since N is required for an asymmetric division that immediately follows the specification of these cells. In this respect, the response of the N receptor to Ras activation is an efficient, anticipatory "feed forward" mechanism for insuring that this cell division is appropriately regulated.

(C) Establishing a balance between Ras and N. The progressive changes in Ras and N signaling in the present system are summarized in Fig. 8B, and the cellular events corresponding to each stage are depicted in Fig. 8C. Our model emphasizes that, while clusters of equivalent cells

begin with the same signaling repertoires, they acquire distinct biochemical states which uniquely determine progenitor and nonprogenitor identities. Most importantly, this complex circuitry drives the reciprocal alterations in Ras and N activities toward the requisite thresholds that are essential for determining these fates. What biases one cell in an equivalence group toward the imbalance in Ras and N signaling that initiates the entire mechanism remains an open question. One possibility is that localized expression of the RTK ligands may provide the initiating event. Similarities of other developmental systems reinforce the general relevance of these conclusions (Culí *et al.*, 2001; de Celis *et al.*, 1997; Ikeya and Hayashi, 1999; Levitan and Greenwald, 1998; Spencer *et al.*, 1998; Wilkinson *et al.*, 1994; zur Lage and Jarman, 1999).

Synergy between Two Distinct Inhibitory Signals

The reinforcing effects of Aos and Dl are essential since neither is fully capable of insuring that only one progenitor segregates from each Eve cluster. Furthermore, simultaneous loss of both inhibitory pathways leads to the formation of additional Eve cells within the competence domain but outside of the normal Eve equivalence groups. This suggests that the combined actions of both inhibitors prevent the spreading of the inductive signal beyond the normal cluster boundaries, as might occur through positive feedback of Rho expression and the associated increase in secreted Spi production. Such a remote inhibitory mechanism is particularly relevant to Aos which is hypothesized to act at a longer range than Spi (Freeman, 2000). Of note, synergistic inhibition by Aos and the N pathway has not been observed in other systems (Freeman, 1994).

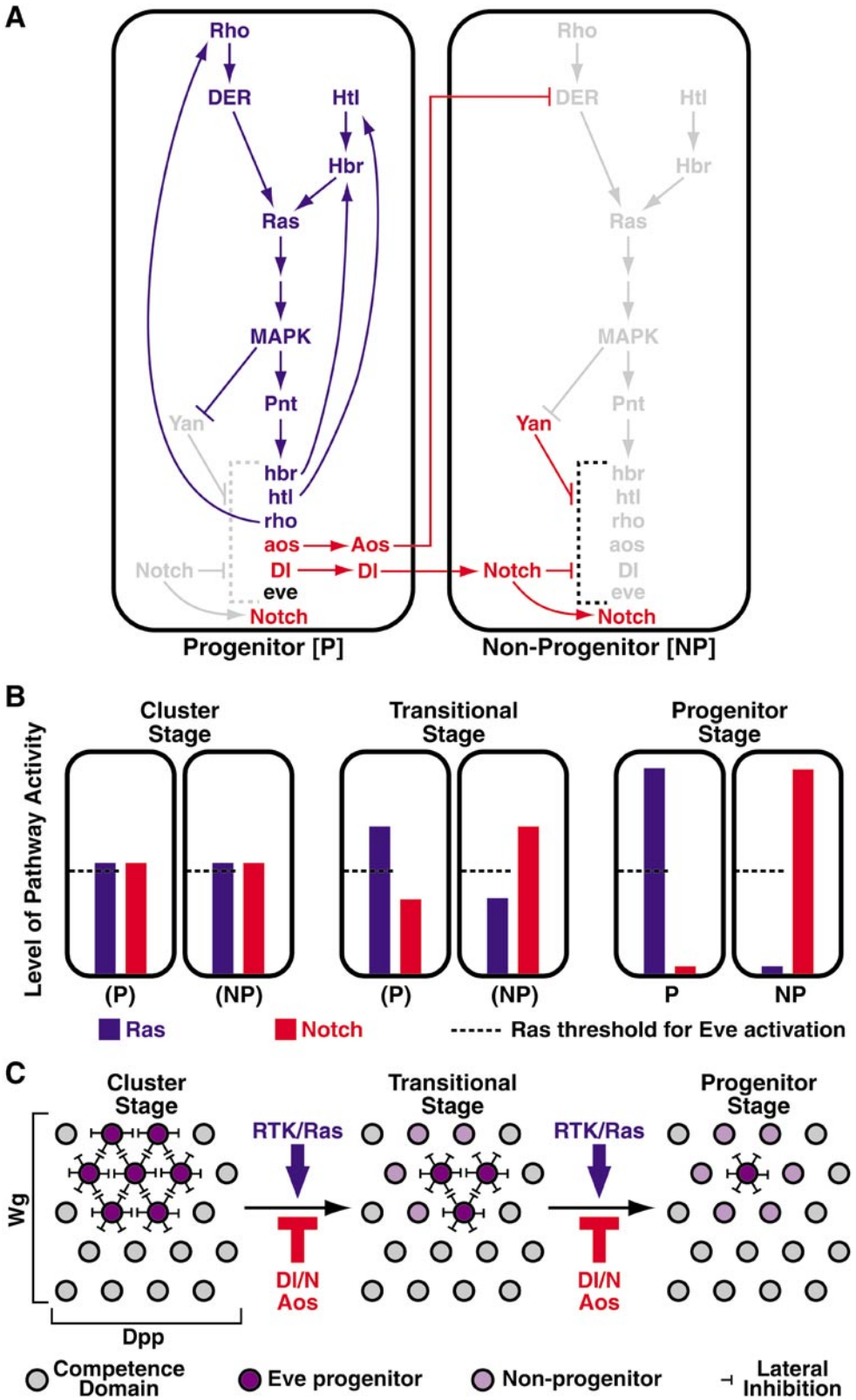
Our results also revealed an effect of Aos on Htl- but not DER-dependent C2 cluster development. Although this could be interpreted as indicating a role for Aos in the inhibition of the Htl FGFR, we favor the idea that Aos is actually blocking basal and/or spontaneous levels of DER activation in C2 cells (Schweitzer *et al.*, 1995). This interpretation is supported by our finding that a dominant negative form of DER suppressed the effect of *aos* loss-of-function not only in DER-dependent C15, but also in C2 which does not require DER for its specification (Carmena *et al.*, 1998a). In this cluster, the requisite Aos expression is dependent on Htl activity.

Another advantage of combining Aos and Dl relates to their differing properties. Aos is a secreted inhibitor capable of acting over several cell diameters (Freeman *et al.*, 1992), whereas Dl—although subject to proteolytic processing (Klueg *et al.*, 1998; Qi *et al.*, 1999)—is generally considered a membrane-bound ligand requiring cell contact for its activity (Heitzler and Simpson, 1991). If a progenitor emerges from the center of a cluster such that it is in close proximity to all of its initially equivalent neighbors, then Dl alone might be sufficient for the segregation of only one progenitor. However, if a progenitor forms on the periphery of a cluster, then the addition of Aos would compensate for the inability of Dl to inhibit its more distant neighbors. Thus, two distinct modes of lateral inhibition have complementary and reinforcing functions.

CONCLUSIONS

The involvement of RTK/Ras and N pathways in the specification of Eve muscle and heart progenitors exemplifies the complex regulatory interactions that can occur between two antagonistic signaling pathways acting in concert. Our findings demonstrate that these signals do not function independently, converging only at the most distal step leading to a particular biological response. Rather, their effects are intertwined at multiple levels to form an integrated network of cross-talk nodes and feedback loops. The combination of cell autonomous and nonautonomous components of both pathways affords the high degree of regulatory versatility and specificity required to generate the

FIG. 8. Model for feedback and cross-talk in the Ras and N signaling pathways during muscle and heart progenitor specification. (A) A singled out progenitor (P) and representative nonprogenitor (NP) from the same equivalence group are shown after fate acquisition is complete. In the P cell, EGF and FGF receptor signaling via the Ras/MAPK cascade predominates, whereas N signaling suppresses this pathway in NP cells. RTK signaling is amplified by a positive feedback loop which upregulates Htl, Hbr and Rho expression in P cells. Additional Ras targets include *aos, Dl, N,* and the muscle identity gene, *eve*. Aos and Dl act nonautonomously to inhibit neighboring cells, Aos as a direct DER antagonist and Dl as the N ligand. Pnt and Yan are MAPK-responsive Ets domain transcriptional activator and repressor proteins, respectively. Yan is suppressed in P and activated in NP cells where it cooperates with Aos and N/Dl to block positive RTK output. Although not indicated, N could in part function by inhibiting MAPK activation. Note that multiple sites of cooperation and competition between the Ras and N pathways generate reciprocal, self-reinforcing changes in the signaling circuitry of individual equivalence group members. (B) Diagrammatic representation of the temporal changes of Ras and N levels throughout the process of progenitor specification. N levels at the cluster stage are arbitrary: Ras levels at this stage exceed the threshold for Eve activation During the transtion stage, Ras and N activities change reciprocally in P and NP cells as a consequence of the feedback and cross-regulatory interactions illustrated (A). By the end of this process, Ras activity predominates in P and N activity predominates in NP cells. (C) Diagrammatic representation of the dorsal region of the mesoderm where Eve progenitors are specified. The intersecting domains of Wg and Dpp define a region competent to respond to subsequent Ras signaling. Ras is locally activated in a cluster of cells within this competence domain (an equivalence group), and Eve becomes expressed in all cells of this cluster in response to Ras. Through the mechanisms shown (A), initially small differences in Ras levels among equivalence group cells are rapidly amplified in the prospective progenitor. Positive and negative signals in each panel are depicted in blue and red, respectively.



polarized signaling activities that distinguish progenitors from their nonprogenitor neighbors. These interactions are especially remarkable since, once initiated, they propagate into self-sustaining cascades that differentially drive equipotent cells to their individual fates. Of further significance, the mesodermal cells produced by these mechanisms give rise to the differentiated derivatives that compose the stereotyped structures of the embryonic heart and body wall muscles. Thus, the signaling circuitry uncovered here not only establishes the finely tuned balance between the inductive and inhibitory influences which coordinately generate progenitor cell patterns, but also sets the stage for subsequent morphogenetic events.

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