Nodal points and complexity of Notch-Ras signal integration

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Metazoans use a handful of highly conserved signaling pathways to create a signaling backbone that governs development. How these few signals have such a versatile action likely depends upon the larger-scale network they form through integration, as exemplified by cross-talk between the Notch and receptor tyrosine kinase (RTK) pathways. We examined the transcriptional output of Notch–RTK cross-talk during *Drosophila* development and present in vivo data supporting a role for selected mutually regulated genes in signal integration. Interestingly, Notch–RTK integration did not lead to general antagonism of either pathway, as is commonly believed. Instead, integration had a combinatorial effect on specific cross-regulated targets, which unexpectedly included numerous core components of the RTK and other major signaling pathways (TGF-β, Hh, Jak/Stat, nuclear receptor and Wnt). We find the majority of Ras-responsive genes are also Notch-responsive, suggesting Notch may function to specify the response to Ras activation.

receptor tyrosine kinase | cell signaling | signal cross-talk

Metazoans use a surprisingly small number of highly conserved signaling pathways to pattern a wide array of highly diverse body plans (1–3). These same pathways control the development of morphologically dissimilar tissues and organs that comprise complex multicellular animals by providing spatial and temporal cues that influence the transcription of downstream effectors, ultimately governing cell differentiation, migration, proliferation, and death. How these highly conserved signals can have such a versatile developmental action is a basic biological question of significance to development, evolution, and the pathogenesis of numerous diseases where dysregulation of these signals is a feature. It seems clear that the developmental action of signals through any one of the fundamental signaling pathways depends on the larger-scale network they form through their integration (3). Learning how signaling pathways are interlinked is thus of fundamental importance. Here, we examine the effects of cross-talk between Notch and receptor tyrosine kinase (RTK)/Ras/MAPK (henceforth RTK), 2 signaling pathways of major importance, which may prove a useful paradigm for understanding pathway integration generally.

Previous studies involving different developmental contexts and metazoan species uncovered numerous processes influenced by Notch and RTK, demonstrating that integration of these signals is vital to cell fate regulation (3–5). For the vast majority of cases, the interrelationship between Notch and RTK appears antagonistic (3, 4). Developmentally, Notch signaling provides a mechanism to limit specific cell fates to a single cell within a group of initially equivalent cells, and in many contexts Notch activation restricts cells to an uncommitted fate (6). In contrast, RTK signaling has a positive influence on developmental commitment and acts to induce cells to follow specific differentiation programs (5, 7). Such signal integration must involve sets of genes that directly or indirectly respond to both pathways, acting as nodal points to integrate their effects on development. However, the extent and complexity of this network, although important, are unknown.

In the present study we adopted a genome-wide approach to identify points of signal integration by exploring the combined transcriptional output of the 2 pathways. Genes identified in this way can be direct or indirect transcriptional targets, an important fact given that both may have crucial roles in development. Our analysis of identified targets, corroborated by genetic interaction studies, reveals the scope and complexity of Notch–RTK cross-talk and a surprising degree of pathway interconnectedness.

Results

To identify nodal points that interlink the Notch and RTK pathways, we examined the transcriptional output of each during *Drosophila* embryonic development and identified common transcriptional targets. Pathway activation was achieved through ubiquitous GAL4-mediated expression of activated Notch (*UAS-N*^{act}) or through an activated form of the RTK signal mediator Ras1 (*UAS-Ras1*^{V12}), either alone or in combination, under the control of the *armadillo* (*arm*) promoter, which drives GAL4 (*arm-GAL4*) at moderate physiological levels (8, 9). Consistent with a low resulting level of signal activation, development of transgenic embryos continued into the very late stages of embryogenesis, well beyond the time at which transcriptional effects were analyzed (data not shown). Embryos of each class and controls were collected at two 1-hour time points, representing distinct yet continuous developmental contexts. The transcriptional effects of pathway activation in 3 biological replicates per end point were analyzed by Affymetrix microarrays. Differential expression was defined as a greater than 1.5-fold change in transcript levels that met stringent statistical criteria (*Materials and Methods*).

Transcriptional Consequences of Notch and Ras Activation in 2 Developmental Contexts. A total of 681 genes were differentially expressed at either time point in samples where pathways were activated singly or in combination relative to GAL4-only controls [Fig. 1*A* and supporting information (SI) Dataset S1 (all differentially expressed)]. From those, 578 Notch-responsive genes were identified in 2 ways: through comparisons of activated Notch samples with GAL4-only controls and through comparisons of samples expressing both activated Notch and Ras with those expressing activated Ras alone [Fig. 1*B* and Dataset S1 (Notch responsive)]. This allowed us to identify targets of the pathway not detected through comparisons with controls. A similar approach revealed 163 Ras-responsive genes within the 681 gene set

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) Database, www.ncbi.nlm.nih.gov/geo (accession no. GSE11203).

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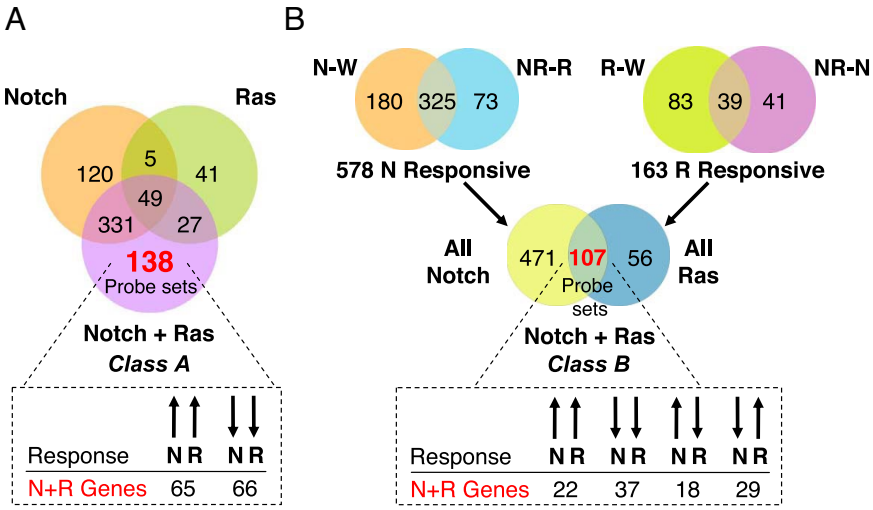

Fig. 1. An overview of identified transcriptional targets. (*A*) Venn diagram of probe sets responding to Notch^{act} (Notch), Ras1^{V12} (Ras), or both transgenes in combination (Notch + Ras) in comparisons with w^{1118}; *arm-GAL4* controls. Number of probe sets within each category is listed. A total of 138 probe sets representing 131 *Class A* mutually Notch- and Ras-responsive genes are indicated. Patterns of response to Notch and Ras are indicated in the dashed box by arrows representing up- or down-regulation. The number of genes with each response is shown. (*B*) Venn diagrams showing Notch (N-W) and/or Ras (R-W) targets identified in comparisons either with w^{1118}; *arm-GAL4* controls (W) or in dual-transgenic Notch^{act}-Ras1^{V12} (NR) samples compared with Ras1^{V12} (NR-R) or Notch^{act} (NR-N) expressed singly (*Materials and Methods*). A total of 107 probe sets representing 106 *Class B* genes with a response to both Notch and Ras are indicated. Patterns of response to Notch and Ras are indicated as in *A*.

[Dataset S1 (Ras responsive)]. These results were validated by RT-PCR for a select panel of targets (*Materials and Methods*). The Notch-responsive genes include Ras targets and vice versa, and both groups include numerous known transcriptional targets (Fig. S1).

A classic criterion for determining functional kinship between genes relies on genetic interactions. Validating our approach, among all 681 genes we identified 51 known genetic interactors of *Notch*, *Epidermal Growth Factor Receptor* (*EGFR*), and/or *Ras1*, a statistically significant enrichment (Fisher exact test, P value = 1.45×10^{-19}; Fig. S2). Notably, the majority of these interactions were uncovered in the *Drosophila* eye or wing, contexts distinct from those we analyzed.

Mutually Notch-Ras-Regulated Genes. We reasoned that genes that integrate the biological effects of Notch and RTK include those that respond to both pathways. Such mutually regulated genes were identified in 2 ways (*Materials and Methods*). First, we identified genes that responded only when both pathways were simultaneously activated [*Class A* genes; Fig. 1*A* and Dataset S1 (*Class A* additive Notch-Ras)]. Second, we identified genes that responded both to Notch and to Ras when singly activated [*Class B* genes; Fig. 1*B* and Dataset S1 (*Class B* shared Notch-Ras)]. By using these criteria, we identified 131 *Class A* and 106 *Class B* genes (Fig. 1, boxes), which include a small overlapping set. Of all such mutually regulated genes, 54 had well-characterized alleles. Remarkably, 23 of those were known genetic interactors of *Ras1*, *EGFR*, or *Notch* or its ligand, *Delta* (*Dl*). Moreover, among these 23 were 6 that have been reported to interact with both pathways, consistent with the notion they may serve as nodes to integrate the pathways.

***Class A* Genes Include Notch and RTK Pathway Regulators That Can Act as Integrating Nodal Points.** Among genes that respond to the simultaneous activation of Notch and RTK signaling (*Class A* genes), we identified RTK pathway components, including *GTPase activating protein 1* (*Gap1*) and *Map kinase phosphatase 3* (*Mkp3*), and the Notch pathway regulator *fringe* (*fng*). Previous studies indicated that *Mkp3* and *Gap1* antagonize RTK signal transduction (7, 10). *fng* is a known signal modulator of the Notch pathway that increases the sensitivity of the receptor to Delta (Dl) (11). Thus, these targets, which respond additively to Notch and Ras (Fig. 2 *A–D*), may serve as nodal points that function to increase Notch and decrease RTK responsiveness in cells receiving both signals. The developmental consequence of this relationship may bias cells receiving both signals toward a Notch-dependent repression of cell fate commitment (Fig. S3).

To test this model, we examined whether modifying transcript levels of these genes has an impact on sensory organ precursor (SOP) cell fate determination, a process where Notch and Ras act in opposition. Because Notch functions to limit the formation of the SOP to a single cell in the proneural field, and ectopic Ras activation results in supernumerary SOP formation (12, 13), modulating transcript levels of an integrating node might affect cell fate specification. Using the *scabrous-GAL4* driver (*sca-GAL4*) (14), we increased levels of *Gap1*, *fng*, or *Mkp3*, and each blocked SOP specification (Fig. 2 *E–J*); phenocopying increased Notch signaling and decreased RTK signaling (15), consistent with the model (Fig. S3). We note that *Mkp3*, which had never before been associated with Notch activity in *Drosophila*, displayed extensive genetic interactions with a variety of Notch pathway components, consistent with a broad role for *Mkp3* in Notch signaling (Fig. 2 *K–T*).

The Pervasive Effects of Notch Activation on RTK Signaling. Because *Class A* genes respond to the simultaneous activation of both pathways, to further examine integration we identified 106 genes that responded to Notch and Ras when activated separately (*Class B*). Surprisingly, *Class B* genes included 65% of all identified Ras targets (Fig. 1*B*, overlap). Given the reported predominance of Notch/RTK antagonism (4, 5), coexpression of Notch and Ras could be expected to lead to a general mutual antagonism, in which coactivation of one pathway attenuates the transcriptional output of the other. Instead, we found that integration involved significant cooperativity, which predominated across a complex set of additive and antagonistic effects. Of all Notch- or Ras-responsive genes identified, 34% (233/681) were coregulated, and of these only a minority (47/233, or 20%) responded to Notch and Ras in an opposing fashion.

Remarkably, we found that Notch and Ras show complex interactions at multiple levels of signal transmission and transduction. These included the mutual or reciprocal transcriptional regulation of Ras pathway components by Notch and vice versa (Fig. 3*A* and Fig. S4). Ras-responsive genes included the Notch ligand *Dl* (16) and the *Class A* gene *fringe*. Genes regulated by Notch, either singly or in combination with Ras, included the RTK ligand *spitz*; the regulators of *spitz* processing, *rhomboid* and *Star*; RTK receptors *EGFR*, *heartless*, and *breathless*; and intracellular antagonists of RTK signal transduction *sprouty*, *Gap1*, *Mkp3*, and, as previously reported, *anterior open* (*aop*) (17) (Fig. 3*A* and Fig. S4). Notably, components of other fundamental signaling pathways, including Transforming Growth Factor-β, Hedgehog, Nuclear Receptor, and Wnt/wingless, were among identified targets of Notch and Ras, and mutual targets of both (Fig. S4). Such cross-influence provides a

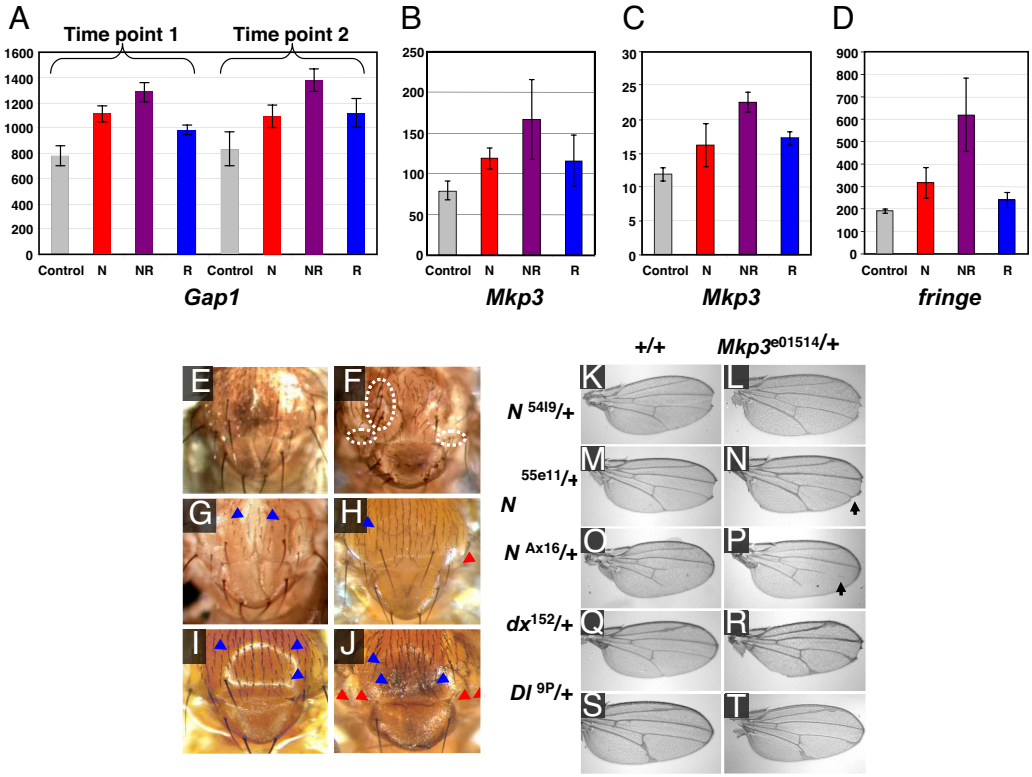

Fig. 2. The *Class A* genes *Gap1*, *Mkp3*, and *fringe* are Notch-Ras responsive. (*A*) Signal levels for *Gap1* (probe set *151669_at*) in controls, Notch^{act} (N), Notch^{act}/Ras1^{V12} dual transgenics (NR), and Ras1^{V12} (R) at both time points. Error bars indicate 95% confidence intervals. Additive Notch-Ras transcriptional influence on *Gap1* is observed. (*B*) Signal levels for *Mkp3* probe set *141660_at*. (*C*) Signal levels for *Mkp3* probe set *149030_at*. In both *B* and *C*, an additive Notch-Ras transcriptional influence on *Mkp3* was observed during time point 2. No response was detected during the first time point. (*D*) Signal levels for *fringe* (probe set *143664_at*) show an additive Notch-Ras transcriptional influence during the second time point only. In *E–J*, increased levels of *Gap1*, *Mkp3*, and *fringe* phenocopy both Notch activation and decreased RTK signaling. (*E*) The notum of a wild-type fly. (*F*) Notum of a *sca-GAL4*, *UAS-Ras1^{V12}* fly reared at 18 °C. Supernumerary machrochaete are observed in individuals with nearly complete penetrance for thoracic and posterior alar bristles, indicated by dotted lines. (*G*) *Notch^{Abruptex16}* notum reared at 25 °C. Loss of thoracic machrochaete is seen (blue arrowheads) at high penetrance. (*H*) Notum of a *sca-GAL4*, *UAS-Gap1* fly reared at 25 °C. Missing thoracic machrochaete are observed (blue arrowhead) with a penetrance of 14.9% at 25 °C and 87.3% at 29 °C. Missing posterior alar bristles are also observed at high penetrance (red arrowhead). Neither *sca-GAL4* nor *UAS-Gap1* parental lines displayed abnormalities at either temperature. (*I*) Notum of a *sca-GAL4*, *UAS-fng* fly reared at 29 °C. Missing thoracic machrochaete are observed (blue arrowheads) with a penetrance of 98%. *UAS-fng* parental lines are wild type in the absence of GAL4. (*J*) Notum of a *sca-GAL4*, *UAS-Mkp3* fly reared at 29 °C. Missing thoracic machrochaete are observed (blue arrowheads) with a penetrance of 100%. Missing posterior alar bristles also are observed at high penetrance (red arrowheads). *UAS-Mkp3* parental lines are wild type in the absence of GAL4. (*K–T*) *Mkp3* interacts genetically with Notch pathway components. (*K*) A control wing heterozygous for the null *Notch* allele N^{54L9} (N^{54L9}/+). (*L*) N^{54L9}/+; *Mkp3^{e01514}*/+ transheterozygotes show a weak enhancement of the Notch wing margin defect. (*M*) A control wing heterozygous for the null *Notch* allele N^{55e11} (N^{55e11}/+). (*N*) N^{55e11}/+; *Mkp3^{e01514}*/+: arrow points to region of enhancement. In panels *O–R*, tests were performed in a *C96-GAL4* background. (*O*) A wing from a Notch gain-of-function allele *Notch^{Abruptex16}*/Y male. (*P*) In *Notch^{Abruptex16}*/Y; *Mkp3^{e01514}*/+, arrow indicates suppression of the L4 vein loss. (*Q*) *deltex^{152}*/Y. (*R*) *deltex^{152}*/Y; *Mkp3^{e01514}*/+: enhanced deltex phenotype. (*S*) *Delta^{9P}*. (*T*) *Delta^{9P}*; *Mkp3^{e01514}*/+: enhanced wing deltas are observed. *Mkp3^{e01514}*/+ wings were wild type (data not shown).

possible mechanism for the functional intermeshing often seen for these pathways (3).

Because these findings raise the possibility that Notch activity may help specify Ras output, we examined the impact of Notch coactivation on genes that responded only to Ras but not Notch when activated singly. Interestingly, nearly half of such Ras targets were *Class B* genes, responding differently to Ras when Notch was coactivated. As with all *Class B* genes, the effect of Notch on these varied and antagonistic and additive influences was seen in comparable proportions [Fig. 1*B* and Dataset S1 (*Class B* shared Notch-Ras)]. We note that the canonical RTK target *pointed* (*pnt*) was not influenced by Notch, nor were most canonical Notch targets by Ras, suggesting that our findings cannot be trivially explained as a consequence of coexpression. Overall, these data are consistent with a role for Notch in providing RTK with output specificity (Fig. 3 *B* and *C*). The observed impact of Notch activity on Ras output may involve influences on RTK core component transcription (Fig. S4). However, because the RTK target *pnt* does not respond to Notch activity and the impact of Notch on other targets shows no consistent pattern, other mechanisms of integration appear important.

The Identification of Novel Genetic Nodal Points Among Coregulated Genes. Because mutually regulated genes included several known to interact genetically with *Notch*, *Ras*, and/or *EGFR*, we sought to identify additional Notch/Ras-regulated genes capable of modifying phenotypes associated with both pathways. We tested 16 such genes with no prior genetic link to Notch for their ability to affect wing phenotypes associated with mutations in the Notch pathway. Of these, 15 novel Notch pathway interactors were identified (Table S1 and Fig. S5), including 9 known to interact with either *Ras* or *EGFR* (www.flybase.org). The majority of genes tested interacted genetically with components of both pathways, in a developmental context distinct from the embryo where such response was identified. This observation strongly supports the general relevance of these genes to both pathways and their potential role as integrating nodal points in multiple contexts. Additional novel Notch pathway genetic interactors were identified among genes that responded either to Notch or Ras alone (Table S1 and Fig. S6), including the *ets-domain lacking* and *spire*, which interact genetically with RTK pathway components.

Core Nodal Points Include the COE Transcription Factor *knot*. Nodal points that act across multiple contexts may constitute "core" elements that create a general framework for cross-talk. Given the widespread importance of Notch–RTK cross-talk to metazoans, such elements may be evolutionarily conserved. We sought to identify genes that responded to Notch and Ras across the 2 developmental contexts defined by our 2 embryonic time points to uncover nodal points of general importance to cross-talk. In all, 28 such genes were identified (Fig. 4*A*). We subsequently confirmed the differential expression for 3 of these genes by RT-PCR (Fig.

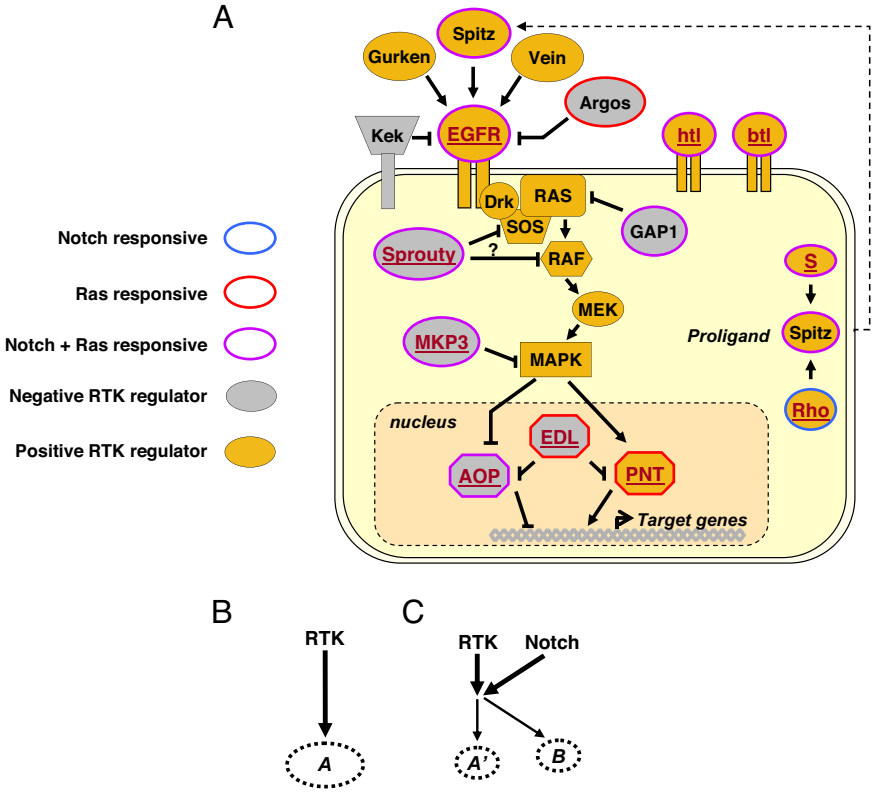

Fig. 3. Notch and Ras have an impact on RTK signaling through their mutual regulation of RTK core components. (*A*) The RTK signal transduction mechanism is shown. Proteins depicted with orange filled symbols mediate RTK activation and signal transduction. Arrows indicate a positive effect on signaling. Proteins in depicted with gray filled symbols oppose RTK signaling. Blunted arrows indicate points of antagonism. Proteins outlined in red responded to Ras activation in our study, those outlined in blue responded to Notch, and those in purple responded to both Notch and Ras. Known or novel genetic interactors of both pathways are indicated by underlined red text. In *B* and *C*, Notch signaling can help specify RTK transcriptional output. (*B*) In the absence of Notch signaling, activation of Ras leads to transcription effect A, as indicted. (*C*) Upon coactivation with Notch, transcriptional output downstream of Ras activation is partially respecified. Although some canonical targets remain uninfluenced (transcription effect A′), other targets show an altered response (transcription effect B). Thus, Notch activation may play an important role in specifying transcriptional effects downstream of Ras activation.

4*B*). Also among these 28 genes was *aop*, a known target of Ras in multiple contexts and of Notch in the *Drosophila* eye imaginal disk (17). Moreover, these 28 genes included 3 novel Notch genetic interactors: *knot*, *heartless*, and *twist* (Fig. 4 *C–F* and Table S1), all of which have been documented to display genetic interactions with *Ras* or *EGFR* (www.flybase.org). Together, these findings strongly support the relevance of these 28 genes to Notch-Ras signal integration.

knot, a *Collier/Olf/EBF* (COE) family transcription factor, was of particular interest, because its *Xenopus* homolog, *XCoe2*, is involved in primary neuron specification, where its activity is subject to regulation by Notch-mediated lateral inhibition (18). Indeed, the possibility that other COE family members function in binary cell fate decisions across species has led to the speculation that there is an evolutionary conserved link between COE family members and Notch signaling (19, 20). We tested whether *knot* defines a node that integrates the action of Notch- and Ras-mediated signals by using a genetic approach.

Previous work has shown that *knot* interacts genetically with *EGFR* (15). *knot* also acts to molecularly repress *EGFR* and activate *Notch* transcription in the *Drosophila* wing (21, 22), supporting a link between *Notch*, *Ras*, and *knot*. Our genetic tests corroborate the relevance of *knot* to the *Notch* pathway (Fig. 4 *C* and *D*). In addition, Notch represses *knot* transcription during the specification of certain proneural clusters, a process wherein both genes function (20). To determine whether *knot* is capable of influencing Notch and Ras cross-talk in a specific cell fate decision, we increased *knot* transcript levels during SOP determination by using *sca-GAL4* (14) and compared the resulting phenotype to that of *sca-GAL4*-driven activated Ras (Fig. 4 *G–I*). Although of lesser severity, ectopic *knot* expression closely phenocopied the effect of activated Ras (Fig. 4 *H* and *I*) or loss of Notch activity (13) upon thoracic macrochaete. However, $Ras1^{V12}$ expression resulted in additional supernumerary presutural and humeral bristles not seen in ectopic *knot* individuals.

To further examine the role of *knot* in bristle development, *knot* loss-of-function clones were generated by using the FLP/FRT system (23). In clones lacking *knot* expression, failure of SOP specification was observed at low penetrance (<5%) (Fig. 4*J*). This is consistent with phenotypes resulting from reduced RTK signaling (13) or increased Notch activity using a Notch gain-of-function allele, $N^{Abruptex16}$ (Fig. 2*G*). Thus, *knot* may act as a nodal point of Notch-Ras signal integration in 4 separate developmental contexts. This suggests *knot* is involved generally in cell fate commitment downstream of Notch-Ras cross-talk (Fig. 5*I*).

Discussion

Although evidence of cross-talk between Notch and Ras and its significance is widespread (3), surprisingly few studies have been specifically designed to address signal integration. Using transcriptional profiling and our ability to manipulate the activation of Notch and RTK signaling in transgenic animals, we established that Notch and Ras have a complex interrelationship and are intertwined to an unanticipated degree.

The unexpected finding that the majority (65%) of Ras targets respond to Notch activation underscores the extraordinary extent of integration between Notch and Ras and also raises the possibility that Notch activity may help to specify Ras output. The mechanism that allows the multitude of different RTK inputs to have distinct biological effects through a shared signal transduction cascade, which involves the activation of Ras and other intermediaries, remains an important question (24, 25). Output specificity has been thought to involve differences in the precise subcellular localization of Ras activation, the duration of signaling, or its strength (25). Our study suggests the possibility that specificity can also be provided by additional signals that integrate with the RTK pathway to instruct the cellular response. An effect of Ras activation on Notch output was evident, although less pronounced, and did not involve canonical Notch targets.

Beyond specifying output, our data support a model wherein cells receiving both signals are more responsive to Notch than RTK as a result of biases mediated through genes they mutually regulate (Fig. S3). Such biases may influence cell fate but also RTK ligand production. Here, it is potentially significant that although Notch and Ras converge to increase transcript levels of the RTK ligand

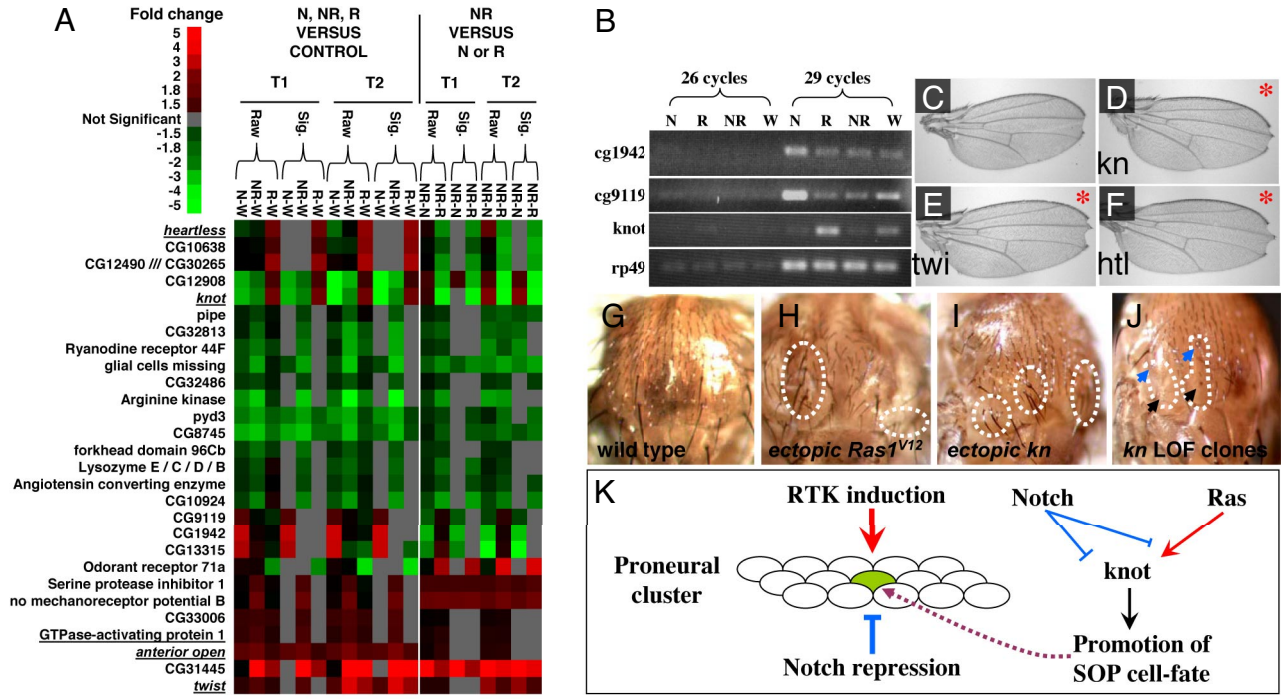

Fig. 4. Core targets of Notch-Ras coregulation. (*A*) Complete linkage cluster of *Class A* or *B* Notch-Ras targets mutually regulated at both time points. Transcriptional response is indicated as in Fig. 3. Ras pathway genetic interactors are underlined, and known (*anterior open*) and novel *N* genetic interactors are italicized. (*B*) Confirmation by RT-PCR of differential expression for *CG1942*, *CG9119*, and *knot* in Ras1^{V12} (R), Notch^{act} (N), dual Notch^{act}-Ras1^{V12} (NR), and nontransgenic GAL4 only (W) samples. *Rp49* was used in parallel as a control. All RNA were from time point 1. Cycles of PCR amplification are indicated. (*C*) Control wings heterozygous for a null *Notch* allele, N^{54L9} (N^{54L9}/+), display a 38% penetrance. In *D–F*, wings are heterozygous for both N^{54L9} and the listed gene. The penetrance of the Notch wing nicking phenotype is shown in parentheses. For calculations, wings displaying any margin defect are scored as mutant. (*D*) *knot*^{1}/+: enhanced (82%). (*H*) *twist*^{1}: enhanced (77%). (*I*) *heartless*^{AB42}: enhanced (100%). *Knot*, *twist*, and *heartless* are known genetic interactors of *Ras* or *EGFR* (red asterisks). *knot* functions in a Notch-Ras-responsive cell fate switch. In *G–J*, dashed lines indicate regions of affected machrochaete specification. (*G*) Notum of a wild-type fly. (*H*) Notum of a *sca-GAL4*, *UAS-Ras1*^{V12} fly reared at 18 °C. Supernumerary machrochaete observed with nearly complete penetrance for thoracic and posterior alar bristles. (*I*) Notum of a *sca-GAL4*, *UAS-knot* fly reared at 25 °C. Supernumerary machrochaete are observed at 58.5% penetrance for thoracic bristles and 100% for anterior postalar bristles. Both *sca-GAL4* and *UAS-knot* parental lines had rare bristle abnormalities (*18*). (*J*) *hs-flp122*; *FRT42D kn*^{KN4} *pwn*/*FRT 42D ubi-gfp* flies after 30 min at 38 °C at 48–72 h AEL (*Materials and Methods*). Loss-of-function *knot* clones show bristle defects at <5% penetrance. Clone boundary is marked with dashed line. Bristles marked with *pawn* (*pwn*) are indicated by blue arrows. Black arrows indicate adjacent machrochaete showing incomplete development where instead of bristle, a small, empty socket formed. This phenotype was not seen in control *pwn* homozygotes (data not shown). (*K*) Overall, these results suggest a model wherein *knot* acts a nodal point downstream of Notch-Ras integration to influence the outcome of cell fate decisions.

spitz and of *Star*, a positive regulator of Spitz processing, Notch increases transcript levels of *rhomboid*, which is responsible for Spitz cleavage and secretion (Fig. 3*A*). Such convergence may make cells receiving both signals produce and release more Spitz, which may ultimately affect RTK activation in neighboring cells, while they themselves are less responsive to RTK ligands.

The mutually regulated genes we identified proved a surprisingly rich source of novel genetic interactors, and for many, relevance to cross-talk was validated in vivo. Our experimental approach was designed to identify nodal points regardless of their direct or indirect regulation by Notch and Ras. However, we note that 57 genes that responded to Notch or Ras identified here were found to be direct transcriptional targets of Notch in an independent study (ref. 42 and S. Bray, personal communication), a statistically significant enrichment for such targets (Fisher exact test, $P = 4.72 \times 10^{-23}$).

We are particularly interested to examine the degree to which nodal points defined here are conserved across species and, within species, across developmental contexts. Such conserved nodal points, which we term *hypernodes*, are of particular importance, because these may represent a minimal framework upon which organ formation and even speciation may depend (3, 26). The interesting possibility remains that, like Notch and RTK, all of the major signaling networks regulating development are profoundly interconnected and that the underlying circuitry governing such networks is conserved across tissues, and even across species.

Materials and Methods

***Drosophila* Strains and Genetics.** The following lines were used: *UAS-Notch*^{act} (27); *UAS-Ras1*^{V12} (28); *UAS-kn* and *kn*^{KN4} lines were kindly provided by James Mohler (22); *arm-GAL4* (9); *C96-GAL4* (29); *sca-GAL4* (30); *edl*^{k06602}, *Cas*^{k03902}, and *esg*^{k00606} (31); and *Mkp3*^{e01514} (32). All other mutant fly strains are described in flybase (www.flybase.org). A line containing both *UAS-Notch*^{act} and *UAS-Ras1*^{V12} was generated by recombination. Fly culture and crosses were carried out according to standard procedures at 25 °C unless otherwise noted. For qualitative genetic interactions to be scored, consistent independent assessments by two experimenters were required. Loss-of-function *knot* clones were generated by using FLP recombinase (33) in *hs-flp 122*; *FRT42D kn*^{KN4} *pwn*/*FRT42D ubi-gfp* after 30 min at 38 °C at 48–72 h after egg lay (AEL).

Sample Preparation. *UAS-Notch*^{act} and *UAS-Ras1*^{V12} were expressed ectopically under GAL4 control (34). Homozygous *armadillo-GAL4* virgin females were crossed to homozygous *UAS-N*^{act}, *UAS-Ras1*^{V12} and *UAS-Notch*^{act}, and *UAS-Ras1*^{V12} males. Homozygous *armadillo-GAL4* virgin females were crossed to *w*^{1118} males as controls. Intracellular Notch (Notch^{act}) is an activated form of the Notch receptor (27), and Ras1^{V12} mimics RTK pathway activation (28). *UAS-Notch*^{act} and *UAS-Ras1*^{V12} are third-chromosome inserts. A homozygous viable *UAS-Notch*^{act}, *UAS-Ras1*^{V12} recombinant line was recovered by scoring for increased mini-*w*$^{+}$ expression. Embryos were collected on apple juice plates for 1 h; incubated at 25 °C for 5 h and 30 min for the first time point and 6 h and 45 min for the second time point; dechorionated by treatment with 50% Clorox bleach at room temperature for 1 min with agitation; washed with 0.5× PBS, 1% Tween; and rinsed with deionized, distilled H_2O. Embryos then were shock frozen and stored in liquid nitrogen at 5.75 and 6.75 h, respectively, AEL for the first time point and 7 and 8 h, respectively, AEL for the second. All collections were performed in

parallel sets but staggered to equalize the length of embryo submersion in liquid. Each collection contained ≈500 embryos. Frozen embryos were ground by pestle in the TRIzol reagent (Invitrogen). RNA was extracted via standard methods, with the inclusion of an additional TRIzol extraction before RNA precipitation to increase RNA purity. Total RNA was prepared from 3 independent experiments for each described embryonic genotype at each of the 2 time points.

Microarray Statistical Analysis. RNA samples were subjected in triplicate to analysis by Affymetrix high-density oligonucleotide arrays using the DrosGenome1 array (Affymetrix) that contains 14,010 probe sets specific to 13,108 *Drosophila* genes. Probe synthesis and microarray hybridization were performed according to standard Affymetrix protocols. External standards were included to control for hybridization efficiency and sensitivity. Following washing, the chips were scanned with a Hewlett-Packard GeneArray laser scanner. Signal levels were obtained and statistical analysis performed by using the GC-Robust MultiArray expression measure (GC-RMA) (35) and LInear Modes for MicroArray (LIMMA) data packages and the affylmGUI graphical user interface (36) in the R programming environment (37). Additional analysis was performed by using Excel (Microsoft). For data visualization, ''Cluster'' was used to perform complete-linkage hierarchical clustering by expression pattern using uncentered correlation of ratio data in all columns as a similarity metric, and heat maps were generated by using ''TreeView'' (38). For a gene to be included in this study it must have passed set criteria for differential expression in at least 1 time point in comparisons between experimental and GAL4-only control embryos. Recent studies of gene expression using LIMMA have used the empirical Bayesian Log of Odds of differential expression factor (B) to identify differentially expressed genes (39, 40). In our analysis we compared several different statistical approaches and found that using GC-RMA with LIMMA and thresholds of $B > 0$ and fold changes >1.5 to determine differential expression most enriched for known targets of Notch and Ras (data not shown). A total of 711 probe sets met these criteria, representing a maximum of 681 unique genes. Comparisons between samples singly transgenic for Notch or Ras and samples expressing both were preformed upon these 711 probe sets posthoc. LIMMA computes a false discovery rate (FDR)-corrected *P* value (41). For comparisons where differential expression met our chosen criteria, greater than 99% showed an FDR *P* value below 0.05. Overall $B > 0$ served as more stringent criteria than FDR-corrected *P* value alone and, most importantly, was better at identifying known gene responses. Transcript level change was confirmed by RT-PCR for >95% of a panel of differentially expressed targets (Fig. 4*B* and data not shown).

Confirmation of Differential Expression by RT-PCR. A total of 1 μg of total RNA was used for cDNA synthesis. RT-PCR was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the supplied protocol. To ensure amplification was not derived from contaminating genomic DNA, an RT minus control was included. A total of 0.5 μL of the RT reaction was used for PCR. All specific primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify a product of ≈250 base pairs matching the target sequences used for the Affymetrix DrosGenome1 array. All PCR reactions were overlaid with mineral oil and performed for 16, 19, 22, and 26 cycles to ensure linear amplification. After 2 min of denaturation at 94 °C, each cycle consisted of 30 seconds at 94 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C, followed by 5 min at 72 °C. The products were visualized by 1.5% agarose gel electrophoresis. The gene, primers used, and product size are indicated as follows: *knot*, ttcattttgagcgaaccactt and ttttgcggctaagttctgct, 349 base pairs; CG1942, ccgtatgctcagcaagtcaa and tcgaaaatgtccacctctcc, 225 base pairs; CG9119, cactggcgaacagaacttca and ccagttgctgaaggagaagg, 289 base pairs; and *Rp49*, atgctaagctgtcgcacaaa and gacaatctccttgcgcttct, 254 base pairs.

GEO Access. The array data from this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE11203.

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