Mapping signaling pathway cross-talk in Drosophila cells

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Cellular communication is fundamental to all organisms and mediates numerous processes, such as cell fate decisions, proliferation, migration, and homeostasis. Remarkably, based on the types of ligands and signal transducers, only a limited number of pathways have been identified (1). These pathways include Notch, receptor tyrosine kinases, Wnt, Hedgehog, TGF-β, NF-κB, and JAK/STAT pathways. This limited set of pathways underscores that intricate signaling cross-talk must underlie most cellular interactions.

Signaling pathways are highly interconnected, and their cellular outcomes are context-dependent (2–6). Pathways can antagonize, enhance, or complement each other, and synergistic or inhibitory effects can dramatically change the nature of responses to a stimulus (7–9). Therefore, cells generate responses that are extremely diverse, explaining the emergence of a vast array of cell fate decisions and biological processes controlled by relatively few signaling pathways. In addition, pathway interactions are influenced by the signaling state of cells, and their specific outcomes can depend on previously acting signals (4, 10).

Although in vivo and in vitro examples of cross-talk between pathways have been well documented, we lack an integrative view of signaling incorporating the spatial and temporal interactions between pathways. Indeed, in most biological systems, we may know which pathways are required for a specific process, but we may not understand whether signals are processed sequentially or concomitantly and how pathways interact to produce the final outcome. For example, studies of the adult Drosophila midgut have revealed that most of the main signaling pathways are involved in homeostasis and/or regeneration (11). However, how they interact to promote proliferation or differentiation is poorly understood, because, in most cases, experiments lack the spatial and temporal information necessary to decipher whether signals are received concomitantly or whether, for example, one pathway regulates the activity of other pathways. Furthermore, in many cases, signaling pathway readouts are based on fluorescent reporters with low temporal resolution, making it difficult to evaluate whether a response is primary or secondary (12). Finally, most previous studies have been performed by activating signaling pathways to levels unlikely to be achieved physiologically, making it difficult to determine the biological significance of the regulated genes.

To obtain a systems level understanding of cross-talk mechanisms between the major metazoan transduction pathways, we examined the regulation of central components of signaling pathways, mostly ligands and receptors, in response to pathway activation. Specifically, we examined changes in gene expression in homogeneous cultured cells at a time scale allowing us to detect primary transcriptional targets (12–16). We selected Drosophila cell lines for these studies, because the signaling systems are simpler than in mammals. Importantly, in fly cell lines, most signaling pathways are not active under normal culture conditions (17). However, because these pathways can be activated by the simple addition of ligands or specific agonists (Materials and Methods), the transcriptional responses to various pathway activations can be readily studied with high temporal resolution.

Our analysis, focusing on the main limiting components for signal activation, demonstrates that activation of specific signaling pathways in cells often directly regulates the expression of ligand and receptor pathway components, highlighting sequential activation of pathways as a major mechanism of signaling cross-talk.

Significance

A key challenge in understanding cell communication is to characterize the coordinated activity of signaling pathways. A number of studies suggest that signaling pathways can regulate each other by direct control of ligand and receptor expression levels, triggering sequential signaling events in cells. To address the extent of sequential signaling, we profiled the transcriptional responses of ligand and receptor genes to single and combinatorial signaling pathway inductions in cell lines. Our analysis revealed that transcriptional regulation of genes encoding pathway components is a major level of signaling cross-talk, especially in the context of crosstalk of signaling pathways.


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cell lines expressing the relevant receptors (17) (Fig. L4). Then, we
analyzed changes in transcript levels of canonical target genes for
each pathway identified from the literature (18–24) using quanti-
tative PCR (qPCR), 30 min and 1 h after stimulation. In all cases,
the reporter genes showed the expected transcriptional responses,
validating the pathway stimulation methods (Fig. 1B).

Next, to determine the optimal time points at which to analyze
the transcriptional response to pathway activation, we performed a
time course analysis (0 min, 15 min, 30 min, 1 h, 2 h, and 6 h)
following stimulation of each pathway. We used the Nanostring
counter platform to analyze changes in gene expression, because it
is a robust and sensitive method effective for measurement of
low-abundance mRNAs, such as ligands (Dataset S1), for a rela-
tively large cohort of genes (25, 26). The method detects transcripts
with a high level of linearity, reproducibility, and accuracy, avoiding
biases introduced by amplification steps, such as for qPCR or
RNA-sequencing (RNA-seq) (Materials and Methods and Figs. S1
and S2). Although signaling cross-talk could potentially be medi-
ated through transcriptional regulation of any pathway component,
we focused our analysis on ligand and receptor expression, because
such expression is often the rate-limiting component of signaling
pathways (Dataset S1 and Nanostring probe sets in Dataset S2).

Gene lists were compiled based on previously reported pathway
ligands, receptors, and reporters (1, 4).

Analysis of the time courses revealed that the majority of changes
in ligand and receptor transcript levels were detectable at 30 min or
1 h (Fig. 1C). In addition, these time points are likely optimal for
detecting the expression of primary target genes (12, 13, 16, 27).

Direct Transcriptional Regulation of Pathway Components by Signaling
Pathways. To identify direct targets of the seven signaling path-
ways, we compared data from stimulated samples at 30-min and
1-h time points with data from unstimulated control samples. We
selected differentially expressed genes based on fold changes and
significance. The selected hits were divided into three categories
based on confidence (high confidence, mean fold ≥ 1.5 and P ≤
0.05; medium confidence, mean fold ≥ 2 in two-thirds of replications;
and low confidence, mean fold ≥ 1.5 in two-thirds replicates; hits
were called at 30 min and 1 h or using the combination of both
time points (Fig. 1D and Dataset S3).

For each pathway, we identified reporter, receptor, and ligand
genes that scored as hits (Dataset S4). With the exception of Thor
(Insulin pathway), all seven canonical reporters were identified as
targets of the expected pathways. The transcriptional response
following insulin stimulation was relatively weak, most likely due
to the presence of insulin in culture media that causes background
pathway activation in control samples. Nevertheless, although
Thor did not score as a hit due to our stringent criteria, transcript
levels were down-regulated as expected at both time points (0.76-
and 0.70-fold, respectively). Fourteen other pathway reporters
were also included in the dataset, yet relatively few of these re-
porters were responsive in the cell lines tested (Dataset S3), likely
due to contextual differences between the cell lines tested and the
tissues in which each of these reporters were originally identified.

Each pathway regulated at least one ligand gene, demonstrating
that ligand transcriptional regulation is likely a major mechanism
of pathway cross-talk (Fig. 1D and Dataset S4). In addition, there
were considerable differences between pathways in the number of
ligand and receptor genes regulated (Fig. 1D and Dataset S4), with
JNK (14 genes) being most prominent, followed by JAK/STAT (6
genes), Notch (5 genes), EGFR (5 genes), BMP (2 genes), Insulin
(2 genes) and, finally, Wnt with only one ligand regulated. Some of
these differences in response may be due to lower sensitivity in
detecting expression changes in genes that are already expressed
under control conditions. However, we found no correlation be-
tween fold induction of target genes and their basal expression
(Fig. S2), indicating that expression level is unlikely to explain the
differences in response between pathways or conditions. Alternatively,
differences in the number of target genes identified could reflect
the methods used to activate the pathways, which may result in
different levels of activation, although it is also possible that the
observed differences are inherent to the pathways analyzed.

Among the responsive pathway components for each assay were
often ligands of the stimulated pathway, suggesting that feedback
signal amplification may be a common output of signaling. For in-
stance, the strongest responding gene to EGFR pathway stimulation
was the ligand spitz (spz). Likewise, activation of the JAK/STAT, BMP,
and Wnt pathways increases the expression of their own ligands [un-
paired I (upd), decapentaplegic (dpp)], and wingless (wg), respectively.

As in the case of ligands, several signaling receptors were pri-
mary targets of pathways (Dataset S4). For example, Fas, Tollol, and
Pvr are all targets of JNK signaling (Fig. S3). However, receptor
inductions were generally weaker than ligand genes, and we did
not find any receptor genes involved in feedback regulation fol-
lowing single-pathway stimulations.

The hits also included examples of incoherent feedback motifs,
where both positive and negative regulators of the stimulated
pathway were simultaneously activated (Dataset S4). For example,
BMP signaling activated both the BMP ligand dpp and a negative
regulator of BMP signaling, Dad. Similarly, EGFR pathway
stimulation results in transcription of ligands and inhibitors of
EGFR, spi and aos, respectively. Further, activation of JAK/STAT
signaling stimulated the upd and upd3 ligands, as well as the

Fig. 1. Genes regulated by single-pathway inductions. (A) Reagents used to
stimulate each signaling pathway. (B) Expression changes of primary target
 genes at 30 min (light gray) and 1 h (dark gray). Bars represent the log2 fold
changes of induced versus control samples from two biological replicates. Error
bars indicate the minimum and maximum values. (C) Heat map showing ex-
pression changes of all genes included in the Nanostring code set at five time
points following pathway stimulations (15 min, 30 min, 1 h, 2 h, and 6 h).
Transcript levels are displayed as log2 fold change of normalized counts
compared with nontreated cells. (D) Volcano plot representing statistical sig-
nificance as a function of average fold change in gene expression for the
pathway stimulations indicated. Averages of 30-min and 1-h time points are
displayed in the graph to simplify visualization. Enlarged versions of single
assay plots are shown in Fig. S3.
negative regulator \textit{Socs36E}. Incoherent feedforward loops were also identified, where positive and negative regulators of other pathways were simultaneously regulated. For example, stimulation of the Insulin, EGFR, or JNK pathway caused activation of both JAK/STAT ligands and the JAK/STAT repressor \textit{Socs36E} (Fig. 1D and Dataset S4). Indeed, at least one incoherent feedforward or feedback loop was present among the targets of each pathway, with the exception of Notch and Wnt. We note, however, that incoherent feedforward loops have previously been identified downstream of Notch in another cell type (13, 14).

Responses to Combinatorial Stimulations. Because cells in tissues are often exposed to multiple ligands, we investigated the effects of combinatorial pathway stimulations on the expression of ligand and receptor genes. We used the same experimental setup as for single-pathway assays and performed combinatorial stimulations of five pairs of pathways: Insulin + JNK, JNK + JAK/STAT, Insulin + JAK/STAT, BMP + JNK, and BMP + JAK/STAT.

For all combinatorial stimulations, at least one known reporter gene for each activated pathway was transcriptionally affected (with the exception of \textit{Thor} following JAK/STAT + Insulin stimulation, which was reduced but did not meet the criteria to be classed as a hit), confirming the simultaneous activation of both pathways (Fig. 2 and Datasets S3 and S4). The majority of hits identified in single-pathway assays were also detected in combinatorial assays (Fig. 1D and Table 1). Interestingly, combinatorial stimulations of JAK/STAT and Insulin only regulated half of the hits identified from individual assays, indicating a possible inhibitory interaction between these pathways on the transcriptional induction of their respective target genes (Table 1 and Dataset S4).

Overall, combinatorial stimulations resulted in regulation of more genes than expected based on single stimulations, suggesting that several pathways have cooperative interactions (Table 1 and Dataset S4).

Validation of Target Genes by qPCR and RNA-Seq. To determine whether the target genes identified by Nanostring are robust, we used two orthogonal technologies to validate target genes from JNK and JAK/STAT single stimulations. For the JNK pathway, we selected six targets with varying levels of induction (\textit{dpp}, \textit{upd2}, \textit{upd3}, \textit{pyr}/2, \textit{pyr}, and \textit{ths}) and measured changes in expression following 30-min and 1-h stimulations using qPCR. The results correlated well with results from the Nanostring assays (correlation coefficient = 0.90), and all target genes were regulated by at least 1.5-fold in qPCR assays at one or both time points (Fig. 3A).

Next, we used RNA-seq to identify genes regulated following JAK/STAT pathway stimulation. Wild-type or \textit{STAT92E} mutant cells (28) were treated with control or Upd-conditioned media for 1 h before assessing changes in gene expression by RNA-seq. First, we combined results for the experimental and control treatments in wild-type cells and compared gene expression levels between RNA-seq and Nanostring assays performed under similar conditions. Results were highly reproducible, with a correlation coefficient of 0.86 (Fig. 3B), indicating that results from both approaches are robust. Next, we considered whether JAK/STAT target genes identified by Nanostring were also differentially expressed in the RNA-seq data. Of the nine target genes, four (\textit{ths}, \textit{pyr}, \textit{Ser}, and \textit{upd}) could not be reliably detected under at least one condition due to low expression levels. Of the remaining five genes, three (\textit{Socs36E}, \textit{Pip61F}, and \textit{Thor}) showed significant differential expression (\(P < 0.1\)) in the expected direction (Fig. 3C). Finally, we compared wild-type and \textit{STAT92E} mutant cells under control conditions and found that \textit{upd3} was significantly differentially expressed (Fig. 3C), suggesting that it is either an indirect target of the pathway or requires stronger stimulation to be regulated, consistent with its weak signal at early time points (Fig. 3S).

Finally, we used the qPCR results to validate that the Nanostring data are robust and the majority of target genes can be detected with multiple approaches.

Discussion

Signaling pathways are highly interconnected, and understanding how they cooperate to achieve specific biological outcomes is complicated by the diversity of mechanisms by which they can regulate each other (1, 4, 5, 29, 30). Secreted ligands and their receptors are key to the activity of signaling pathways, and the extent to which their transcriptional regulation is used to control signaling pathway activities is not fully appreciated. Here, we analyzed the immediate transcriptional response to pathway activation in homogeneous cell lines to address three basic questions in cell signaling with regard to ligand and receptor gene expression. First, what is the extent of transcriptional feedback regulation within a signaling pathway occurring at the level of ligands and receptors? Second, to what extent does one specific signaling pathway regulate the expression of ligands and receptors of other pathways? Third, how is the regulation of ligand and receptor expression modulated by costimulation of multiple signaling pathways? Our study, focusing on evolutionarily conserved metazoan signaling pathways, revealed intricate relationships between pathway activities and expression of ligand and receptor genes, suggesting that coordination of signaling pathway activities is more pervasive than previously thought. In addition, we identified many interactions that have previously been shown in in vivo systems or mammalian cells, suggesting that this level of signaling cross-talk is not specific to cultured \textit{Drosophila} cells.

Transcriptional Feedback Regulation Within a Signaling Pathway

Examination of the transcriptional regulation of ligands and receptors by their own pathways revealed that half of the pathways tested have positive feedback loops at the level of ligands. Specifically, induction of the BMP, EGFR, JAK/STAT, and Wnt pathways up-regulated the expression of their respective ligand genes \textit{dpp}, \textit{spi}, \textit{upd-upd3}, and \textit{vg} (Fig. 1D and Dataset S4). These observations from cell culture correlate well with previous in vivo studies, where positive feedback loops through ligand expression have been observed in a number of tissues for the JAK/STAT, Wnt, and EGFR pathways (23, 31, 32). For instance, the regulation of \textit{spi} by Ras signaling (a downstream component of the EGFR pathway) has been shown in vivo during embryonic stages (32). Interestingly, \textit{Spi} is produced as an inactive precursor and requires processing by Rho (Rho) to generate the active ligand (33). Expression of \textit{rho} is generally the limiting step in Spi-mediated signaling.
However, the transcriptional regulation of spi by EGFR signaling suggests that there may be situations in which Spi expression is limiting. Alternatively, Spi expression levels may be important to modify the range or strength of signaling rather than inducing the signaling event itself. Signal feedback via regulation of ligands has also been shown to play a central role in mammalian cells (8). Remarkably, transcriptional feedback loops were present in all combinatorial pathway inductions performed, and these feedback loops were mediated by multiple ligands in some cases (Dataset S4). For instance JAK/STAT + BMP stimulation up-regulated the three upd genes, although only two of them were induced by JAK/STAT stimulation alone.

Furthermore, incoherent feedback loops were also identified in our study as exemplified by the EGFR assay, where both spi and its antagonist aos were induced. Another case is in the context of JAK/STAT pathway activation, where both upd ligand and the Socs36E repressor were activated (Fig. 1D and Dataset S4).

Together, the results from our study indicate that autoregulation of signaling pathways via ligand transcription is a common mechanism found downstream of multiple pathways (Figs. 1D and 3D).
In addition, feedback loops appear to be more common following combinatorial pathway stimulations (Fig. 2), suggesting that these motifs may play an important role in the integration of signals by altering the strength, duration, and extent of signaling (34, 35).

Transcriptional Regulation Between Pathways. With the exception of the Wnt pathway, all individual and combinatorial pathway stimulations that we tested resulted in the regulation of ligands and/or receptors of other pathways, indicating that the transcriptional regulation of these components is a common mechanism by which pathways cross-regulate each other (Dataset S4).

The extent to which different pathways regulate ligands and receptors was variable. For example, JNK activation regulated 10 ligands and four receptors, whereas Wnt activation regulated only one ligand and no receptors. The response to JNK activation is consistent with its role in the Drosophila midgut, where JNK is thought to be stimulated immediately following tissue damage and multiple other pathways are regulated downstream of this initial signaling event (11, 36, 37). This variability in the response to stimulation may indicate inherent differences in the ability of each pathway to regulate other pathways sequentially. For example, the JNK pathway may act as a mediator signal that coordinates downstream signaling events, whereas pathways with lower connectivity may act as effectors rather than mediators. However, more extensive studies in a greater range of cellular contexts will be needed to investigate this possibility.

Finally, it has previously been suggested that pathways with more complex intracellular transduction cascades may have an increased potential for cross-talk (4). Whereas this possibility may be true for direct cross-talk between transduction cascades, we saw no such correlation at the transcriptional level.

Integration of Multiple Pathway Activities. In in vivo situations, cells are likely to be simultaneously exposed to ligands from multiple different pathways. For example, almost every core pathway described has been implicated in the response to tissue damage in the Drosophila midgut. However, it is currently unclear how these signals are integrated to produce the correct response.

The target genes regulated by combinatorial stimulations are not simply the sum of those target genes regulated by each pathway alone. Indeed, for all pathway combinations tested, there were examples of target genes that were absent from either pathway stimulation alone or genes regulated by one or both pathways alone that were not identified as hits following costimulation (Table 1). For example, following JNK and Insulin costimulation, 21 of the 45 targets of costimulation were not regulated by either JNK or Insulin alone. This result indicates that integration of signals from multiple pathways is not simply additive and more complex processing of these signals must be occurring.

Relevance of the Cell Line Interactions to the Study of Cell Signaling. The pathway interactions that we have identified from signaling pathway stimulation assays were summarized as a network of pathways to ligand and receptor links (Fig. 3D). Importantly, many of these connections have been previously identified in in vivo experiments. For example, studies in the Drosophila adult gut have documented that most signaling pathways are required for either regeneration or/and homeostasis (11, 36, 37). Activation of the JNK pathway in the injured or stressed midgut epithelium leads to expression of the ligands vein, decapentaplegic (dpp), Pfy, upd2, and upd3 from the enterocytes, enteroblasts, and visceral muscle, and subsequent activation of the EGFR, BMP, PVR, and JAK/STAT pathways. All of these ligands were identified by our JNK activation assay, indicating that our data are applicable to in vivo contexts.

In addition to the gut system, our results are reminiscent of the pathway interactions observed in tumor systems. For example, activation of the EGFR pathway leads to overproliferation of tissues in Drosophila and mammals, but the combinatorial activation of the JAK/STAT, EGFR, and JNK pathways has been shown to result in more aggressive tumors than EGFR alone (38). This conservation of signal integration suggests that studies of cross-talk in Drosophila may be applicable to understanding human diseases such as cancers. In particular, knowledge of interactions between signaling pathways will likely be important for understanding mechanisms of drug resistance in cancers.

Finally, although we have identified many transcriptional links between pathways, it is not clear from this study which of these events will lead to a change in signaling activity. Many of the transcriptional changes observed were weak (Dataset S3), and more sustained signaling or integration of other signals may be required to produce a biologically meaningful effect on the target pathway.

Materials and Methods

Pathway Stimulation Assays. Cell lines and cell culture. S2R+ (39), S2 (40), Kc167 (41), and STAT92E mutant (28) Drosophila cell lines were cultured using standard procedures. STAT92E mutant cells contain a frameshift deletion within the STAT92E gene and were previously shown to result in complete loss of detectible transcription factor activity (28).

Cell induction. To activate signaling pathways, we used the following: Insulin at 0.5 mg/mL (Sigma I6634) for the Insulin pathway, LPS from Escherichia coil at 25 mg/mL (G5583; Sigma) for the JNK pathway, recombinant Dpp protein at 33 μg/mL (159-dp-020CF, R&D Systems) for the BMP pathway, and 2 mM EDTA in PBS solution for the Notch pathway (42). For the Wnt, JAK/STAT, and EGFR pathways, conditioned media expressing Wg, Upd (Upd cDNA cloned into the pMK33 vector), or Spa protein were produced, respectively, from S2R+ cells transfected with the relevant plasmid, as previously reported (40, 43). Protein expression was induced with 0.5 mM CuSO4 and media were harvested after overnight incubation. For the control media, 0.5 mM CuSO4 was added overnight to untransfected cells. Supernatants from induced and control cells were collected, sterilized, and concentrated to optimize their efficiency for stimulation assays. For EGFR pathway assays, experiments were performed in S2 cells stably transfected with an inducible transgene encoding EGFR (43).

Analysis of Target Gene Expression Levels. Nanostring nCounter gene expression assays. To profile gene expression levels following signaling pathway induction, we used the Nanostring nCounter method. We designed a specific code set targeting 86 genes, including 40 ligands belonging to the main signaling pathways, 18 corresponding signaling pathway receptors, 21 previously characterized known transcriptional targets...
of the pathways, 3 additional components of signaling pathways (shaggy/E cadherin, rho, and Dia2), and 4 housekeeping genes. Genes encoding pathway components and reporters were identified using recent literature reviews (1). Nanostring probe sequences are listed in Dataset S2. Total RNA extracts way components and reporters were identified using recent literature reviews six different positive control probes. Next, for biological normalization, 10 Guide, 2001), whereby raw counts were normalized by the geometric mean of media/pdf/MAN_nCounter_Gene_Expression_Data_Analysis_Guidelines.pdf).

Determination of hits. Responsive genes for single and combinatorial treatments were determined using different criteria for high-, medium-, and low-confidence hits. For the high-confidence hits, genes with average fold change ≥ 1.5 and P ≤ 0.05 determined using t tests were selected. For the medium- and low-confidence hits, genes having a minimum of twofold and 1.5-fold similar trend expression changes in at least two of three biological replicates were selected, respectively.

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