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XTALK: a path-based approach for identifying crosstalk between signaling pathways

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Abstract

Motivation: Cells communicate with their environment via signal transduction pathways. On occasion, the activation of one pathway can produce an effect downstream of another pathway, a phenomenon known as crosstalk. Existing computational methods to discover such pathway pairs rely on simple overlap statistics.

Results: We present XTALK, a path-based approach for identifying pairs of pathways that may crosstalk. XTALK computes the statistical significance of the average length of multiple short paths that connect receptors in one pathway to the transcription factors in another. By design, XTALK reports the precise interactions and mechanisms that support the identified crosstalk. We applied XTALK to signaling pathways in the KEGG and NCI-PID databases. We manually curated a gold standard set of 132 crosstalking pathway pairs and a set of 140 pairs that did not crosstalk, for which XTALK achieved an area under the receiver operator characteristic curve of 0.65, a 12% improvement over the closest competing approach. The area under the receiver operator characteristic curve varied with the pathway, suggesting that crosstalk should be evaluated on a pathway-by-pathway level. We also analyzed an extended set of 658 pathway pairs in KEGG and to a set of more than 7000 pathway pairs in NCI-PID. For the top-ranking pairs, we found substantial support in the literature (81% for KEGG and 78% for NCI-PID). We provide examples of networks computed by XTALK that accurately recovered known mechanisms of crosstalk.

Availability and implementation: The XTALK software is available at http://bioinformatics.cs.vt.edu/~murali/software. Crosstalk networks are available at http://graphspace.org/graphs?tags=2015-bioinformatics-xtalk.

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Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction

Cells communicate with their environment through signal transduction pathways. Typically, a signaling pathway contains a well-defined set of receptors and transcription factors (TFs). Upon activation of the pathway's receptors, a series of signaling interactions activate the TFs in that pathway, thereby regulating the expression of target genes as a down-stream response. On occasion, these intra-cellular signal cascades result in off-target responses, commonly known as pathway crosstalk. More specifically, crosstalk occurs when the activation of the receptors of a specific signaling pathway results in the down-stream response being executed by the TFs of a different pathway (Housden and Perrimon, 2014). Such a down-stream response manifests itself through changes in the expression of the second pathway's target genes. Signaling pathway crosstalk may lead to several cancers (Elinav *et al.*, 2013; López-Otín and Hunter, 2010) and has also been implicated in host defense against pathogens in plants (Kunkel and Brooks, 2002).

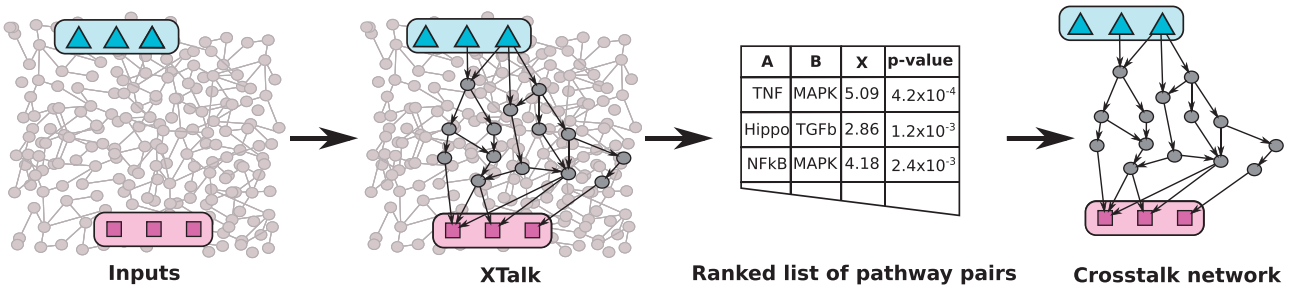

Fig. 1. Workflow for XTALK. XTALK takes as input a signaling network, a set of receptors in pathway *A* and a set of TFs from pathway *B*. XTALK enumerates *k* paths from the receptors to each TF, calculates a crosstalk statistic $\chi(A,B)$ and computes a *P* value representing the significance of crosstalk from pathway *A* to pathway *B*. XTALK also returns a crosstalk network representing the set of interactions responsible for the identified crosstalk. Triangles: receptors in pathway A; rectangles: TFs in pathway B

Computational methods have been developed to systematically predict crosstalk. The simplest approach, which we call nodes-in-common or NIC in this article, asks if two pathways share any proteins based on the observation that shared proteins may mediate crosstalk (Donato *et al.*, 2013; Knight and Knight, 2001; Taniguchi *et al.*, 2006). Hsu and Yang (2012) estimated pathway crosstalk based on similarities in Gene Ontology annotations. Interaction-based methods try to identify crosstalk by quantifying the connectivity between pathway pairs. Given a protein–protein interaction network, Li *et al.* (2008) and McCormack *et al.* (2013) linked two pathways *A* and *B* if more edges connected the proteins in *A* to the proteins in *B* than expected by chance in a randomly wired network. In the case when two pathways are known to crosstalk, Zielinski *et al.* (2009) developed a method that used a combined signaling network to identify the protein(s) responsible for crosstalk. A similar approach, biological process linkage networks (BPLN), linked two processes *A* and *B* if the proteins annotated to *A* were connected to more proteins annotated to *B* than expected by chance (Dotan-Cohen *et al.*, 2009). In this article, we selected BPLN as an exemplar of the interaction-based techniques.

These approaches have several drawbacks. The NIC methods cannot identify crosstalk when the two pathways have very few or no common proteins. For example, MAPK pathway and the Hippo pathway (Reddy and Irvine, 2013) crosstalk but share only a few proteins. More importantly, these methods treat a pathway simply as a set of proteins. While the edge-based approaches do use interactions between proteins, they fail to identify crosstalk when the members of pathway *A* do not interact with the members of pathway *B*, as in the case of crosstalk from the Hif-1 pathway to the Wnt pathway (Zhang *et al.*, 2013). More importantly, they do not consider the canonical structure of a pathway: a set of receptors connected via regulatory, signaling and physical interactions to a set of TFs. Therefore, they are unlikely to discover the mechanisms or the sequence of interactions that underlie pathway crosstalk.

To develop our method XTALK, we started by considering the biological definition of crosstalk. Crosstalk occurs between two signaling pathways *A* and *B* when the stimulation at the receptors of pathway *A* causes a cellular response downstream of pathway *B*, usually carried out by the TFs in *B*. We reasoned that there must be at least one directed path of signaling and regulatory interactions along which a signal can traverse from some receptor of pathway *A* to some TF in pathway *B*. We expected these paths to be short, so that the signal could travel rapidly to affect the response. Crosstalk can also be asymmetric: *A* may crosstalk with *B* (i.e. stimulation of *A*'s receptors controls at least one of *B*'s TFs) but *B* may not crosstalk with *A* (i.e. stimulation of *B*'s receptors has no effect on any of *A*'s TFs) as in the case of crosstalk from the insulin-like growth factor-I pathway to the leptin pathway (Ozbay and Nahta, 2008). Using these guiding principles, we developed XTALK to identify the *k* shortest paths from any receptor in pathway *A* to each TF in pathway *B* (Fig. 1), where *k* is a user-defined parameter. We implemented an efficient dynamic-programming based approach to exactly compute the statistical significance of the crosstalk. By design, XTALK reports the precise sequences of interactions and mechanisms that support the identified crosstalk between pathways.

To the best of our knowledge, databases of pathway crosstalk are currently unavailable for validation of methods such as XTALK. To overcome this challenge, we created a gold-standard set of directed pathway pairs that crosstalk. To create this dataset, we restricted our definition of crosstalk to only those events that occur when an interaction (e.g. activation, inhibition or binding) between two proteins mediates the crosstalk. We acknowledge that several other mechanisms of crosstalk exist, including protein–protein interaction events, feedback loops and when members of pathway *B* are downstream targets of TFs in pathway *A*. We plan to incorporate these mechanisms in the future. We studied over 400 publications for evidence of crosstalk between 272 pairs of pathways in the KEGG database Kanehisa *et al.* (2012). We found literature support for crosstalk between 132 pairs. These pairs formed our gold standard dataset.

We evaluated XTALK in several ways.

1. We used receiver operator characteristic (ROC) curves to compare XTALK, NIC and BPLN for the 272 pathway pairs that we had explicitly considered for inclusion in the gold standard (Section 3.1). XTALK achieved an area under the ROC (AUC) of 0.65, which was an improvement of 12% over its closest competitor BPLN.
2. We observed that the AUC of XTALK varied from one pathway to another (Section 3.2). Therefore, we studied the three pathways with the lowest AUCs. We found support in the literature for 9 out of 15 (60%) false-positive pathway pairs (Section 3.3).
3. We also considered XTALK 's results from an experimentalist's perspective by examining the top-three highest ranking pairs for each pathway. XTALK had a precision of 0.75 for these ranks. We were able to find support in the literature for seven false positives. Notably, in both this and the previous analysis, we were able to find the appropriate publications only after including proteins in XTALK 's crosstalk networks in our PubMed queries.
4. We expanded our analysis by applying XTALK to a comprehensive set of 658 signaling-related pathway pairs in the KEGG

database. We searched the literature for the top 27 pathway pairs among those that we did not consider for the gold standard. XTALK achieved 81% precision for these predictions (Section 3.5).

5. To assess the wider utility of XTALK, we applied it to more than 7000 pathways pairs in the NCI-PID database (Schaefer *et al.*, 2009). As in the case of the KEGG database, XTALK achieved high precision (78%) among the top-ranking pathway pairs.
6. Finally, we highlight the utility of the XTALK networks in recovering the known mechanisms of crosstalk and assisting in the manual curation of pathway pairs.

2 Algorithms

In this section, we describe XTALK (Section 2.1) and two other methods for estimating crosstalk: NIC, which uses the set of nodes in common between two pathways (Section 2.2), and BPLN, which is based on the set of nodes in one pathway that interact with nodes in the other pathway (Section 2.3). Supplementary Section 2 describes the datasets we use.

2.1 XTALK : average length of the *k* shortest paths

On the basis of the criteria outlined in Section 1, we define our measure of crosstalk as follows. Given two signaling pathways A and B, let R_A (respectively, R_B) denote the set of receptors in A (respectively, B). We define the sets T_A and T_B of TFs in the two pathways similarly. XTALK takes the following inputs: (i) unweighted, directed protein signaling network $G = (V, E)$, (ii) the sets R_A and R_B of receptors in A and B, respectively, and (iii) the sets T_A and T_B of TFs in the pathways. Let $\pi(r,t,k)$ denote the kth shortest path in G from receptor r to TF t and $d(r,t,k)$ denote the length of path $\pi(r,t,k)$. When $k=1$, $\pi(r,t,1)$ is the shortest path between r and t. We similarly define $\pi(R_A,t,k)$ as the kth shortest path from the set of receptors R_A to the TF t and the length of this path as $d(R_A,t,k)$; in this definition, we consider paths that start at any member of R_A. We now define the *crosstalk statistic* between pathways A and B as

$$\chi(A,B,k) = \frac{1}{k n_B} \sum_{t \in T_B} \sum_{l=1}^{k} d(R_A, t, l),$$

where $n_B = |T_B|$ and k is a user-specified parameter. If there were only $k' < k$ paths from a set of receptors to a TF, we considered only k' paths in the innermost summation.

In this formula, for each TF t in B, we compute the lengths of the k shortest paths in G from the receptor set R_A to t. We define the value of the crosstalk between A and B to be the average of these lengths, with the average taken over all the TFs in B that are reachable from at least one receptor in A. Note that this statistic is defined only if there is at least one path from a receptor in R_A to a TF in T_B. The identity of the receptors in R_A are not important, just as long as the signal can traverse (quickly) from *some* receptor in A to the TF in B. Smaller values of $\chi(A,B,k)$ are more indicative of crosstalk from A to B. In general, the statistic is asymmetric, i.e. $\chi(A,B,k) = \chi(B,A,k)$. In practice, to compute $\pi(R_A,t,k)$, we connect an artificial source node σ to each receptor $r \in R_A$ and calculate $d(R_A,t,k)$ as $d(\sigma,t,k) - 1$ using Yen's k-shortest loopless path algorithm (Yen, 1971).

We acknowledge that there are other ways of combining these shortest path lengths. We opted for this definition since it was simple, it satisfied our criteria, it was asymmetric and we could exactly compute its statistical significance. We designed a dynamic programming algorithm to quickly and exactly compute the statistical significance of $\chi(A,B,k)$ (Supplementary Section 1.1). We corrected all reported P values for testing multiple hypotheses (Benjamini and Hochberg, 1995).

2.2 Nodes common to pathways

The inputs to this method are (i) the proteins in pathway A and (ii) the proteins in pathway B. We compute the number of proteins present in both pathways and use the hypergeometric test to compute the statistical significance of this overlap. We refer to this method as NIC.

2.3 Edges crossing pathways

This approach considers whether two pathways connect via one or more protein–protein interactions and if these connections occur more frequently than expected at random, given an interactome. We modify the BPLN method (Dotan-Cohen *et al.*, 2009), which was originally developed to identify links between genes annotated to two (Gene Ontology) processes within undirected protein–protein interactions. In our context, the inputs to BPLN are (i) an unweighted, directed protein signaling network $G = (V, E)$ (as for XTALK), (ii) the proteins in pathway A and (iii) the proteins in pathway B. We compute the number of proteins q in pathway B, such that q is not a member of A and there is some protein p in pathway A, such that (p, q) is a (directed) edge in G. We compute the P value for this statistic using the hypergeometric test. We also consider a variant where we include q even when q is a member of pathway A.

2.4 Ranking pathway pairs

To establish a ranked list of pathway pairs for each method, we grouped the pairs by pathway A and optimized the AUC of each pathway A by considering multiple values of k (Supplementary Section 3.2). We ordered pathway pairs by increasing P value and assigned a rank $r_{A,B}$ to each pair. We then collated all pathway pairs first sorting by $r_{A,B}$ and then by the P value of the pair.

2.5 Crosstalk networks and visualization

As part of its calculations, XTALK produces a *crosstalk network*, which is the union of $k|T_B|$ shortest paths $\{\pi(R_A, t, l), t \in T_B, 1 \leq l \leq k\}$. This network represents the potential set of signaling interactions responsible for the crosstalk from pathway A to pathway B. We visualize our crosstalk network using GraphSpace, an internally developed graph-sharing website. We provide the networks computed by XTALK at http://graphspace.org/graphs?tags=2015-bioinformatics-xtalk. See Supplementary Section 1.2 for more details on GraphSpace.

3 Results

We studied the KEGG and NCI-PID databases. We divided all the pathway pairs we analyzed into three sets (Supplementary Sections 2.3–2.5): (i) the *KEGG curated set* containing the 272 pathway pairs we considered for inclusion in the gold standard; (ii) the *KEGG test set* containing 678 other pathway pairs and (iii) the *NCI-PID test set* containing 7254 pathway pairs. We performed several types of evaluations. *KEGG curated set:* First, we used it to compute AUC values for each algorithm (Section 3.1). We also computed the AUC values for each pathway individually (Section 3.2). Second, for the pathways with the lowest AUCs, we used XTALK's crosstalk networks to search the literature for support for false-positive predictions (Section 3.3). Third, in a complementary evaluation, we studied the false-positive pairs among the highest ranking results

for each pathway, since an experimentalist is likely to be interested in following such predictions for validation (Section 3.4). *Test sets:* We considered the highest-ranked pairs of pathways computed by XTALK for each of the two test sets (Section 3.5). We estimated the precision of XTALK by determining which pairs contained support in the literature for crosstalk. For these analyses, we only considered XTALK since it outperformed NIC and BPLN on the first analysis, as we show in Section 3.1. Finally, we discuss the crosstalk networks for specific top-ranked pathway pairs (Sections 3.6 and 3.7).

3.1 Comparison of XTALK, NIC and BPLN

We compared the performance of XTALK to that of NIC and BPLN on the basis of the AUC, the true-positive rate (TPR) and the false-positive rate (FPR) on both the `KEGG-family` and the `KEGG-protein` networks (Supplementary Section 2.1).

Figure 2 highlights the values of TPR10FPR, TPR30FPR and AUC for XTALK, BPLN and NIC on the `KEGG-family` and `KEGG-protein` networks. Several trends emerged from these plots. First, the NIC approach performed poorly on both networks (AUC of 0.50 and 0.48). Even at FPRs of 0.1 and 0.3, its TPR was at most 0.37. Second, on the `KEGG-family` network, BPLN showed a 16% improvement in AUC over NIC (Fig. 2). BPLN's TPR was larger than that of NIC for values of FPR between 0.1 and 0.9 (Supplementary Fig. S1). Third, XTALK outperformed both NIC and BPLN on both networks. The AUC of XTALK (0.65 on the `KEGG-family` network and 0.64 on the `KEGG-protein` network) was at least 12% higher than that of BPLN (0.58 on the `KEGG-family` network and 0.56 on the `KEGG-protein` network). On the `KEGG-family` network, XTALK achieved TPR10FPR of 0.30, an improvement of 100% over NIC (TPR10FPR of 0.14) and over BPLN (TPR10FPR of 0.15). XTALK dominated both NIC and BPLN for FPR up to 0.8 (Supplementary Fig. S1).

On both networks, the performance of NIC was similar to that of a random predictor, suggesting that it is neither necessary nor sufficient for two pathways to crosstalk if they share proteins. We also considered two variants of the NIC method that used only the receptors or only the TFs for each pathway. We observed nominal changes among all three variations (Supplementary Section 3.4.1). The improved performance of BPLN over NIC suggested that if multiple proteins in the two pathways interact with each other, these two pathways may have a higher propensity to crosstalk. In addition, for the variant of BPLN mentioned in Section 2.3, we observed a decrease in AUC for the variant of BPLN (Supplementary Section 3.4.2).

The AUC for each algorithm was marginally less on the `KEGG-protein` network than on the `KEGG-family` network. However, in the case of XTALK, paths in `KEGG-protein` crosstalk networks were much longer than in `KEGG-family` networks. We discuss the implications of these results in Section 4.

XTALK outperformed both NIC and BPLN. Moreover, XTALK did not show any bias toward reporting pathway pairs that contained many common nodes (Mann–Whitney P value 0.17 consider pairs with a rank of 1 or 2; see Supplementary Section 3.5). These results indicated that multiple short paths between receptors in A and TFs in B are very good predictors of crosstalk. XTALK achieved the best performance despite only using the receptors in A and the TFs in B as input. In contrast, NIC and BPLN required knowledge of all proteins annotated to both pathways. More importantly, XTALK returned paths capable of transmitting a signal from receptors to TFs, as opposed to a list of common proteins or a set of interactions connecting proteins in one pathway to those in the second. We demonstrate the usefulness of this property of XTALK in the remaining sections.

3.2 AUC value depends on pathway *A*

Upon examining XTALK results more carefully, we observed that the AUC values varied considerably from one pathway to another (Fig. 3). XTALK achieved an average AUC of 0.67 across all pathways and performed best on the toll-like receptor signaling pathway (AUC 0.86, 28% above average). Six pathways obtained an AUC above 0.70, while 10 pathways showed performance at or above average. Three pathways, TGF-β, Prolactin and Estrogen, achieved AUCs at or below random. We discuss these three pathways in Section 3.3. When we optimized k with respect to each pathway B, XTALK achieved a slightly smaller AUC of 0.60 (data not shown).

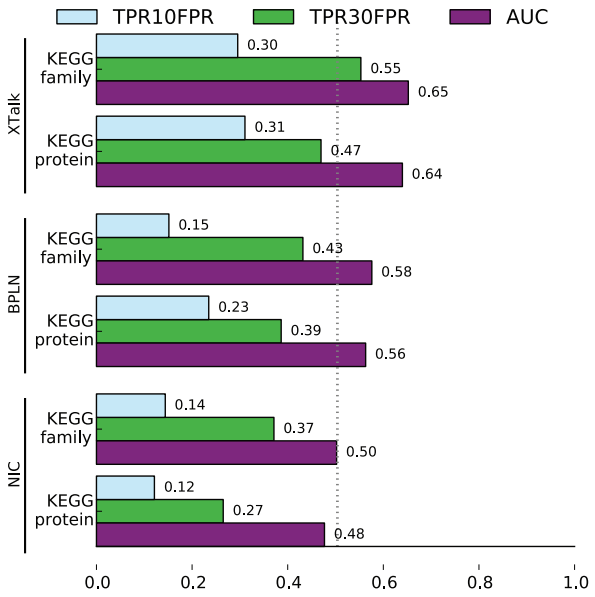

Fig. 2. Comparison of XTALK, NIC and BPLN. Bar plot highlighting the TPR at 0.10 FPR (TPR10FPR), TPR at 0.30 FPR (TPR40FPR) and the AUC. The vertical dotted line indicates the AUC for a random classifier

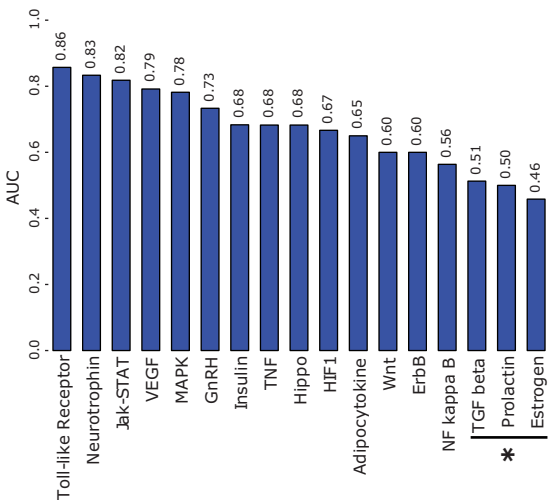

Fig. 3. Pathway-specific AUC values for XTALK on the `KEGG-family` network. The asterisk (*) denotes the three pathway pairs we discuss in Section 3.3

3.3 Literature support for false-positive pairs involving pathways with low AUC values

In Figure 3, the TGF-β, Prolactin and Estrogen pathways have the three lowest AUC values (0.51, 0.50 and 0.46, respectively). We considered all the false predictions (i.e. those pathway pairs not in the gold standard) involving one of these pathways as *A*. There were 15 such pathway pairs. For each of these pairs, we sought to identify publications that supported the crosstalk suggested by XTALK. To this end, we followed a two-step procedure. We first repeated the PubMed query originally used when establishing the gold standard dataset. If this query did not yield any publications to support the crosstalk, we then utilized the crosstalk network returned by XTALK. We identified proteins participating in paths for small values of *k* in this network. We either added these protein names to the PubMed query or replaced pathway names with the names of these proteins before repeating our search for relevant literature. Through these steps, we found literature support for 9 of the 15 false-positive pathway pairs (Table 1).

These nine pairs fell into three categories. (i) The paper supporting one pair was published in 2015, 1 year after we created the gold standard. (ii) We missed three pairs in the gold standard; we attribute these instances to the manual nature of our curation process. (iii) Notably, we discovered the papers supporting the remaining five pathway pairs only after we modified the PubMed query by including important proteins present in the crosstalk networks computed by XTALK. In other words, when we used simpler queries that involved only the names of the pathways and the word 'Crosstalk' or 'Pathway', these papers were either not present in the result or were ranked as being of low relevance by PubMed. Notably, each of these papers reported a path of interactions contained in XTALK 's crosstalk networks (Fig. 4). For example, the successful query used to identify the crosstalk from TGF-β to HIF1 was 'TGF beta HIF SMAD SP1'. The seventh path in the crosstalk network which connects the receptor TGFBR to the TF HIF1A (Fig. 4e) suggested this query. These results underscore both the considerable difficulties and subtleties in constructing a gold standard database of pathway crosstalk and the value of XTALK in discovering crosstalk events.

Table 1. Literature validation for false-positive pathways involving pathways with low AUCs

Pathway A	Pathway B	Literature support
Crosstalk network used to modify PubMed queries		
Estrogen	TNF	Xing *et al.* (2007)
Prolactin	Wnt	Zheng *et al.* (2011)
Prolactin	Neurotrophin	Sun *et al.* (2014)
Prolactin	GnRH	Hodson *et al.* (2010)
TGF-β	HIF1	Sanchez-Elsner *et al.* (2002)
Literature published after curation of gold standard		
Estrogen	Hippo	Zhou *et al.* (2015)
Confirmation missed during manual curation		
Estrogen	HIF1	Kazi *et al.* (2009)
Prolactin	VEGF	Clapp *et al.* (2009)
TGF-β	GnRH	Ying *et al.* (1986)

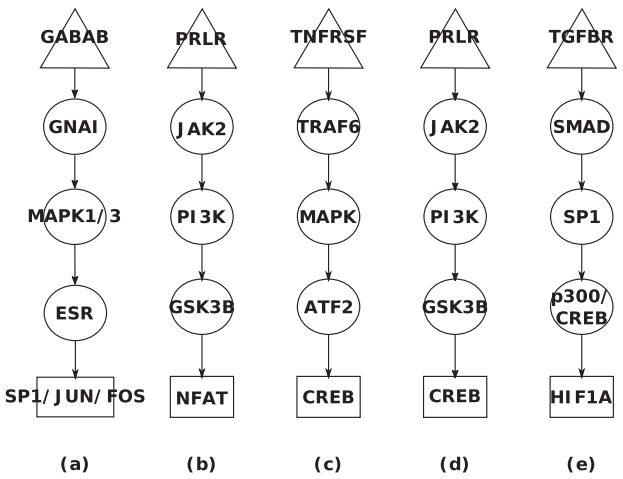

Fig. 4. Paths used to facilitate literature validation. (**a**) Estrogen to TNF, (**b**) Prolactin to Wnt, (**c**) Prolactin to Neurotrophin, (**d**) Prolactin to GnRH and (**e**) TGF-β to HIF1. Triangles: receptors (in pathway A); rectangles: TFs (in pathway B); circles: intermediate proteins. The protein SP1/JUN/FOS participates in both pathway A and pathway B

3.4 Performance of XTALK from an experimentalist's perspective

As a complementary way to evaluate the performance of XTALK, we took the viewpoint of an experimentalist, who we expect will study the highest-ranking pathway pairs for validation. To this end, we investigated top ranking pairs identified by XTALK from Section 3.1. Specifically, we considered pairs with a rank of 1, 2 or 3 for each pathway *A*. For these 51 pathway pairs, XTALK achieved a precision of 75%. In fact, all pairs with a rank of 1 were members of the gold standard dataset (i.e. true positives). Of the pathway pairs with a rank of 1, 12 involved MAPK as pathway *B*. It is not surprising that several pathways crosstalk with the MAPK pathway since many signaling responses converge on members of this pathway (Wagner and Nebreda, 2009). As a case study, we explore the crosstalk network for a rank 1 pathway pair that does not include MAPK in Section 3.6.

Next, we considered those top-ranking pathway pairs that were false positives. Table 2 summarizes these pairs. At the rank of 2 and 3, XTALK identified three and nine false-positive pathway pairs, respectively. We used the crosstalk networks returned by XTALK to assist in literature curation for these 12 false-positive predictions.

Table 2. False-positive predictions made by XTalk with a rank of 3 or less

Pathway A	Pathway B	Literature support
Rank 2		
NF kappa B	Neurotrophin	Li *et al.* (2001)
HIF1	Neurotrophin	Yin *et al.* (2012)
Prolactin	TLR	—
Rank 3		
VEGF	Estrogen	—
NF kappa B	TLR	Wardill *et al.* (2015)[a]
Jak-STAT	Neurotrophin	de Araujo *et al.* (2009)
Adipocytokine	TLR	—
TNF	TLR	Hayden and Ghosh (2014)
GnRH	TNF	—
MAPK	Wnt	Zhang *et al.* (2014)
TLR	Neurotrophin	Okun *et al.* (2011)
Estrogen	TNF	—

We provide a reference for all pathway pairs with literature support, with '—' indicating no literature support.

[a]The NFκB and Toll-like Receptor (TLR) pathways share a branch.

Table 3. Literature support for top-ranking pathway pairs in the KEGG test set

No. values of k	Pathway A	Pathway B	Literature support
13	mTOR	MAPK	Sunayama *et al.* (2010)
	p53 signaling	MAPK	Bragado *et al.* (2007)
	Hippo	Cell cycle	Hergovich and Hemmings (2012)
	Wnt	Cell cycle	Ille *et al.* (2007)
	TNF	Cytosolic DNA sensing	Konno *et al.* (2009)
	Melanogenesis	T cell receptor signaling	—
	Apoptosis	MAPK	—
	Jak-STAT	PI3K Akt signaling	Himpe and Kooijman (2009)
	Adipocytokine	T cell receptor signaling	Cassano *et al.* (2014)
	Hedgehog	Prolactin	—
12	FcγR-mediated phagocytosis	T cell receptor signaling	Gallo *et al.* (2010)
	TGF-β	Adherens junction	Willis and Borok (2007)
	Thyroid hormone synthesis	T cell receptor signaling	Mullins *et al.* (1995)
11	Oocyte meiosis	MAPK	—
	B cell receptor signaling	PI3K Akt signaling	Castello *et al.* (2013)
	NFκB	Cytosolic DNA sensing	Konno *et al.* (2009)[a]
	Progesterone-mediated oocyte maturation	MAPK	Cutini *et al.* (2009)
	Prolactin	PI3K Akt signaling	Belugin *et al.* (2013)
10	Calcium signaling	Wnt	Wu *et al.* (2012)[b]
	Cytokine cytokine receptor interaction	Jak-STAT	Donegan *et al.* (2015)
	Chemokine signaling	Estrogen	Sauve *et al.* (2009)
	PI3K Akt signaling	Chemokine signaling	Jin *et al.* (2015)
	ECM receptor interaction	PI3K Akt signaling	Yu *et al.* (2015)
	Cell adhesion molecule	PI3K Akt signaling	Ni *et al.* (2013)
	Gap junction	Wnt	Yu *et al.* (2012)
	Antigen processing and presentation	Estrogen	—
	VEGF	PI3K Akt signaling	Geng *et al.* (2015)

'—' indicates instances where we identified no evidence of crosstalk.
[a]The Cytosolic DNA sensing pathway is a branch of the NFκB pathway.
[b]The Wnt signaling pathway includes a branch of the calcium signaling pathway.

We identified publications supporting two of the three false-positive pairs at rank 2. Similarly for pathway pairs with a rank of 3, we identified literature to support five of the nine false-positive pairs. In Section 3.7, we discuss how the XTALK network helped to confirm the crosstalk from the NFκB to the Neurotrophin pathway.

3.5 Evaluating XTALK on the KEGG and NCI-PID test sets

Encouraged by the performance of XTALK on the curated set of pathway pairs, we extended our analysis to the KEGG test set of 658 pathway pairs (Supplementary Section 2.3). Since we did not have a gold standard for these pairs, we could not find values of k that optimized the AUC. Hence, we opted not to select any specific value of k. Instead, for each pathway A and for each value of k in the set $\{1-10, 20, 25, 50\}$, we ranked each pair (A, B) in increasing order of P value. Next, for each pair (A, B), we counted the number of values of k for which that pair had the smallest P value (i.e. rank 1) for pathway A. We ordered all pairs by this statistic. This methodology gave priority to those pathway pairs whose rank was relatively insensitive to a specific value of k.

We considered all pathway pairs that occurred with a rank of 1 for at least 75% of the values of k. In total, 27 pathway pairs met this criterion (Table 3). For each of these pairs, we queried PubMed to determine whether the pathways in the pair are known to crosstalk. We used key proteins from the crosstalk networks to find publications confirming five pathway pairs. This performance on the test set demonstrates the applicability of XTALK to novel pathway pairs.

To test XTALK on larger and alternative datasets, we applied it to the pathways in the NCI-PID database (Schaefer *et al.*, 2009) and a comprehensive human physical and regulatory interaction network (Supplementary Sections 2.1 and 2.5). The size of this dataset (115 pathways) precluded the creation of a new gold standard. This NCI-PID test set contained 7254 pairs. We applied the same validation procedure as for the KEGG test set. Of the 18 pathway pairs that met the ranking criterion, we found evidence in the literature for all but four pairs, yielding a precision of 78% (Table 4). We utilized the crosstalk network produced by XTALK for the validation of six pathway pairs.

3.6 Crosstalk from the Hippo to the TGF-β pathway

We turn our attention to the crosstalk network for a pathway pair present in the gold standard that was highly ranked by XTALK on the `KEGG-family` network. XTALK estimated the P value of the crosstalk from the Hippo pathway to the TGF-β pathway in the `KEGG-family` network as 1.3×10^{-3}, giving this pair a rank of 1 (with the Hippo pathway as pathway A). Figure 5 displays a simplified version of this crosstalk network connecting receptors in the Hippo pathway to each TF in the TGF-β pathway through $k = 10$ shortest paths; Supplementary Figure S5 displays the complete network, including all phosphorylation events.

In KEGG, the Hippo pathway shares receptors with the TGF-β pathway, e.g. TGFBR1 and TGFBR2. Surprisingly, KEGG represents these two proteins as a single node in the Hippo pathway but as individual proteins as well as a complex in the TGF-β pathway. On the basis of our definitions of receptors (Supplementary

Table 4. Literature support for top-ranking pathway pairs in the NCI-PID test set

No. values of *k*	Pathway A	Pathway B	Literature support
11	EphrinB EPHB pathway	PDGFR beta signaling pathway	Nakayama *et al.* (2013)
	ALK1 pathway	Regulation of Telomerase	Li *et al.* (2006)
	ATF 2 TF network	IL23-mediated signaling events	Piccaluga *et al.* (2014)
	Signaling events mediated by PTP1B	IL6-mediated signaling events	Owen *et al.* (2015)
	Nephrin Neph1 signaling in the kidney podocyte	PDGFR beta signaling pathway	—
	Wnt signaling network	Regulation of nuclear beta catenin[a]	Lien and Fuchs (2014)
	Insulin Pathway	PDGFR beta signaling pathway	Giri *et al.* (2012)
	Hepatocyte Growth Factor Receptor[b]	PDGFR beta signaling pathway	Kodama *et al.* (2000)
	CXCR4-mediated signaling events	Regulation of Telomerase	Qu *et al.* (2008)
	PDGFR alpha signaling pathway	CXCR4-mediated signaling events	Sciaccaluga *et al.* (2013)
	Regulation of Telomerase	CXCR4-mediated signaling events	—
	amb2 Integrin signaling	PDGFR beta signaling pathway	Bezuidenhout *et al.* (2009)
	Plasma membrane estrogen receptor signaling	PDGFR beta signaling pathway	Finlay *et al.* (2004)
	EphrinA EPHA pathway	PDGFR beta signaling pathway	Miao *et al.* (2001)
	Signaling events regulated by Ret tyrosine kinase	PDGFR beta signaling pathway	—
	IGF1 pathway	PDGFR beta signaling pathway	Ko *et al.* (1993)
	IL5-mediated signaling events	IL6-mediated signaling events	Burnham *et al.* (2014)
	ErbB4 signaling events	IL6-mediated signaling events	—

'—' indicates pairs where we identified no evidence of crosstalk.

[a]The full name is 'Regulation of nuclear beta catenin signaling and target gene transcription'.

[b]The full name is 'Signaling events mediated by Hepatocyte Growth Factor Receptor (c Met)'.

Section 2.2), we considered both the individual proteins as well as the node containing both of them to be receptors in the Hippo pathway. In our discussion below, we focus on the crosstalk network in its entirety rather than on individual paths within it.

The Hippo pathway is known to crosstalk with the TGF-β pathway [see Varelas and Wrana (2012) for a review of Hippo signaling]. Evidence in the literature indicates two distinct mechanisms of crosstalk: through activation and by inhibition. Our crosstalk network captures both phenomena. The activating crosstalk (yellow polygon in Fig. 5) occurs after TGFBR1/2 phosphorylates SMAD4 and SMAD2/3. Subsequently, WWTR1/YAP1 (both members solely of the Hippo pathway) binds to SMAD2/3 and SMAD4 to form a heteromeric complex (Mauviel *et al.*, 2011). After SMAD2/3/4 and WWTR1/YAP bind, the complex translocates to the nucleus and causes the transcription of TGF-β target genes (Varelas *et al.*, 2010).

To cause inhibitory crosstalk, YAP forms a complex with SMAD7 in the cytoplasm and represses TGFBR activity (Varelas and Wrana, 2012). Our crosstalk network includes both the complex formation between SMAD7 and YAP1 and the inhibition of TGFBR1/2 by YAP1 and by SMAD7 (pink polygon in Fig. 5). This negative crosstalk manifests under conditions of high cell density even in the presence of TGF-β, the ligand for the TGF-β pathway (Varelas and Wrana, 2012). High cell density activates the Hippo pathway, causing the formation of the YAP1/SMAD7 complex and subsequent inhibition of TGF-β receptors. This process decreases the expression of TGF-β target genes.

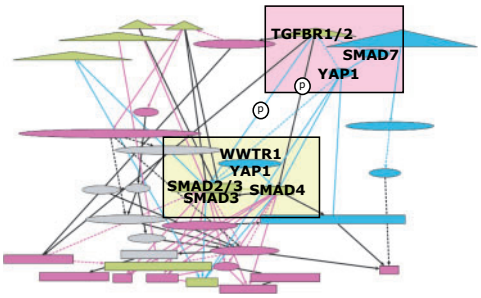

Fig. 5. Network computed by XTALK for crosstalk from the Hippo signaling pathway (**A**) to the TGF-β signaling pathway (**B**). Blue: proteins/edges in pathway *A*; purple: proteins/edges in pathway *B*; green: proteins/edges in both pathways; gray: proteins in neither pathway; black: edges annotated to neither pathway; triangles: receptors; rectangles: TFs. Undirected edges represent group/complex interactions. Solid/dashed edges represent activating/inhibitory interactions. The label '+p' denotes phosphorylation interactions discussed in the text. Please see the text for a discussion of the yellow and pink polygons

3.7 Crosstalk from NFκB to neurotrophin pathway

Using the `KEGG-family` network, XTALK identified three pathway pairs at a rank of 2 that were not in the gold-standard dataset (Table 2). Our original query using only pathway names did not provide conclusive evidence of crosstalk for any of these pairs. However, when we modified the queries to include the names of proteins involved in key interactions in XTALK's crosstalk networks, we succeeded in finding the appropriate publications. These results point to the usefulness of crosstalk networks.

We focus our discussion on the crosstalk from the NFκB to the Neurotrophin pathway. The NFκB signaling pathway is involved in a diverse set of functions, especially those related to the innate and adaptive immune response (Hayden and Ghosh, 2008). The Neurotrophin signaling pathway regulates neural processes such as neuronal survival, maintenance of axonal and dendritic networks and synaptic plasticity (Longo and Massa, 2013). The NFκB pathway contains three receptor families: Interleukin-1 receptors (IL1R), tumor necrosis factor receptor (TNFR) and toll-like receptor (TLR). The Neurotrophin pathway contains many TFs, including FOS, JUN, ATF and NFκB. In addition to a direct activation edge from TLR4 to NFKB1/RELA, the crosstalk network returned by XTALK includes signaling paths that start at IL1R1 and TNFRSF11A (RANK) (Fig. 6).

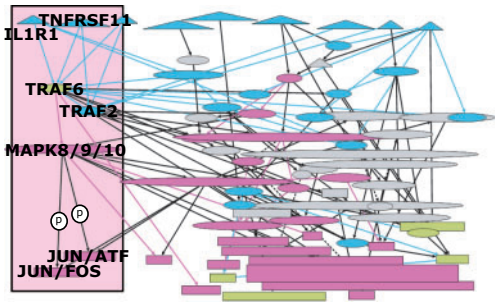

Fig. 6. Crosstalk network constructed by XTALK from the NFκB Pathway (**A**) to the Neurotrophin Pathway (**B**) using the `KEGG-family` network. See Figure 5 for details on node and edge colors and shapes. Supplementary Figure S6 displays the complete network

We first discuss the signaling paths beginning at the TNF receptor TNFRSF11A. The crosstalk network suggests that activated TNFRSF11A binds with either TRAF2 or TRAF6. TRAF2 and TRAF6 can themselves interact. TRAF6 activates MAPK8/9/10, which then activates JUN/ATF2, JUN/FOS and several other TFs. These heterodimers of the FOS and ATF family proteins with JUN are commonly referred to in the literature as AP1 (van Dam and Castellazzi, 2001). Lee *et al.* discovered a similar signaling path that explained the role of TNFRSF11A (RANK) in the activation of AP1 (Lee *et al.*, 2000). They proposed a path that started with TNFRSF11A, signaled through TRAF2 or TRAF6 until MAPK8/9/10, which regulates AP1 activity. The authors noted that the signaling from TRAF6 to MAPK8/9/10 was mediated through ASK1, MEKK1, NIK and SEK1. Our crosstalk network contained the core reactions involved in this signaling event. We did not recover interactions involving ASK1, MEKK1, NIK or SEK1.

Next, we discuss the signaling reactions that begin at IL1R and terminate at JUN/ATF2, JUN/FOS and other TFs. According to our crosstalk network, IL1R activates TRAF6, which activates MAPK8/9/10, thereby regulating AP1 activity. Many studies have suggested that IL1 signaling activates AP1 (Li *et al.*, 2001, 1999; O'Neill and Greene, 1998). The response of AP1 after IL1 signaling is mediated by TRAF6 (Li *et al.*, 1999). To the best of our knowledge, these interactions have not been documented in neural contexts.

4 Discussion

We have developed XTALK, a novel path-based approach to identifying pathway crosstalk. XTALK identifies crosstalk by computing several short paths along which an external signal can be transduced from the receptors of one pathway to the TFs of the other. When evaluated upon a literature-curated gold standard set of pathway pairs, XTALK achieved an AUC of 65%, a value much higher than node- and edge-based approaches (NIC and BPLN, respectively). We observed a high variance in AUC from one pathway to another. Focusing on the three pathways with the lowest AUCs, we succeeded in finding support in the literature for 60% (9 out of 15) false-positive pairs involving these pathways. Conversely, taking an experimentalist's perspective, we focused on false positives among highly ranked pairs and found support in the literature for 59% (7 out of 12) pairs. It is notable that search queries yielded the relevant publications only when we augmented the pathway names with key proteins that mediated the crosstalk. We learnt the identities of these proteins only after examination of the crosstalk networks. These results underscore both the considerable difficulties and subtleties in constructing a gold standard database of pathway crosstalk and the value of XTALK in discovering crosstalk events.

In the second analysis, we evaluated crosstalk on two test sets of pathway pairs, one from KEGG and the other from NCI-PID, that we did not consider for inclusion in the gold standard. The literature contained evidence for approximately 80% of pairs ranked highly by XTALK for each dataset. These results suggest that an experimentalist interested in a poorly studied pathway or one that is not in our gold standard may benefit from focusing experiments on the top-ranked predictions made by XTALK. Moreover, crosstalk networks can provide useful suggestions on the underlying mechanisms.

For the analysis of KEGG pathways, we used a signaling network derived from all relations in the KEGG database. We analyzed two versions of this signaling network: `KEGG-family` and `KEGG-protein`. The value of k for each pathway in the `KEGG-protein` was on average 14 times larger than that for the `KEGG-family`. During the construction of the `KEGG-protein` network, we expanded each interaction between two nodes in the `KEGG-family` network into a complete bipartite graph between the member proteins from each node in the `KEGG-family` (Supplementary Section 2). This transformation effectively increased the number of (short) paths between two proteins in the `KEGG-protein` network when compared with the corresponding families in the `KEGG-family` network. Consequently, the crosstalk networks computed upon applying XTALK to `KEGG-family` were much smaller and easier to interpret than for `KEGG-protein`.

Even more importantly, the paths in the `KEGG-protein` network can be less informative. As we illustrated in Section 3.6, the role of YAP (activating or inhibitory) differed based on the node in the `KEGG-family` network to which it belonged. Elucidating these context-dependent roles would not have been possible with the `KEGG-protein` network, which would have collapsed both versions of YAP into a single node. Notwithstanding XTALK's high precision on NCI-PID pathways, physical and regulatory interaction networks can suffer from similar problems. For these reasons, we encourage a move toward complex- or protein family level networks such as `KEGG-family`, since (i) they represent the underlying biology of signaling closely and (ii) the results with them are easier to interpret. This recommendation is in line with recent trends to explicitly represent protein families/complexes and reactions among them in network models (Fukuda and Takagi, 2001; Hu *et al.*, 2007; Klamt *et al.*, 2009; Ritz *et al.*, 2014).

Finally, we acknowledge that identifying pathway crosstalk remains a challenging problem. Although XTALK achieved higher AUC than NIC and BPLN, XTALK's performance may be further improved. There may be several reasons for this performance: (i) while creating the gold-standard dataset, we missed curating the correct publications documenting crosstalk for some pairs of pathway due to the inherent difficulty of phrasing the query correctly, as demonstrated in Section 3.3; (ii) the crosstalk between two pathways has not yet been discovered, e.g. a paper describing the crosstalk from the Estrogen to the Hippo pathway was published a year after we finished the gold-standard dataset (Zhou *et al.*, 2015); (iii) the background signaling network may not contain the interactions responsible for the crosstalk or (iv) as a community, we have an incomplete understanding about different characteristics of crosstalk.

We are actively considering ways to address these challenges and limitations. NLP-based methods can avoid the pitfalls of manual curation, especially if we train them on the specific sentences documenting crosstalk that we have included in our gold standard. We are considering extensions of XTALK that explicitly take into account the information on whether an interaction is activating or

inhibitory. It is also important to consider cell and tissue specificity. XTALK and its future versions promise to serve as powerful analytic methods to discover novel pairs of crosstalking pathways and the underlying mechanisms.

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