Genome-Wide High Throughput Cell-Based Assay for Activators of NFkB

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1 Abstract

We demonstrate a systematic, cell-based, high throughput approach to the discovery of genes able to activate NFkB. Our assay was validated in four cell lines A375 (melanoma), HCT 116 (colon adenocarcinoma), MCF7 (breast cancer), and HEK-293 (embryonic kidney) with lymphotoxin, FADD, TRAF2, and TNF receptor super family 10B as known activators of NFkB. We optimized the transfection protocol to optimize the amounts of: i) the transfection reagent, ii) NFkB reporter DNA, iii) Renilla control DNA and iv) test DNA. Our methodology produced an excellent assay for the detection of activators of NFkB. We selected 18,000 clones encoding 14,000 different proteins (Unigene) from our library of 60,000 full-length cDNAs (22,000 unique proteins) for analysis in the four cell lines. We recovered more than 500 clones that gave the highest signals (>5 SD) and clustered these clones with respect to their activity across the four cell lines. The clustering annalysis allowed us to identify those genes that were active in all cell lines and those genes active only in one cell line. We recovered many of the well-known activators of the NFkB pathway including; i) receptor ligands, ii) receptors, iii) receptor associated proteins, iv) intracellular signaling proteins to v) transcription factors. We also discovered many proteins not previously identified or only recently identified in similar screens. These data demonstrate the validity of our approach to the screening and discovery of genes involved in the activation of NFkB. Our approach to high throughput, cell-based proteome screening is a facile route to the discovery and validation of genes involved in cell-biology.

2 Meso Scale Proteomics - Discovery Tools

Gene library
60,000 genes from cDNA clones
22,000 unique proteins
Ready for expression

Proteins labeled for in vitro studies

Expression in cells for cell based studies

In vitro Assays
- Antibody based
- Ligand-receptor
- Protein-protein
- Kinase
- Phosphatase
- Protease
- Ubiquitination

In vivo Assays
- Reporter based assay such as NFkB, CRE, SRE
- Indirect assays cAMP, caspases, cytokines
- FACS based assays for molecular markers

Introduction.

We built a library of cDNA clones from the mouse and human genomes that encode consensus, full-length protein sequences and allowed us to carry out functional screens across these genomes. The figure shows schema for the screens we completed. This poster describes one such screen based on a biological response: the activation of an NFkB reporter construct in a cellular system by transfection of individual genes from our library.

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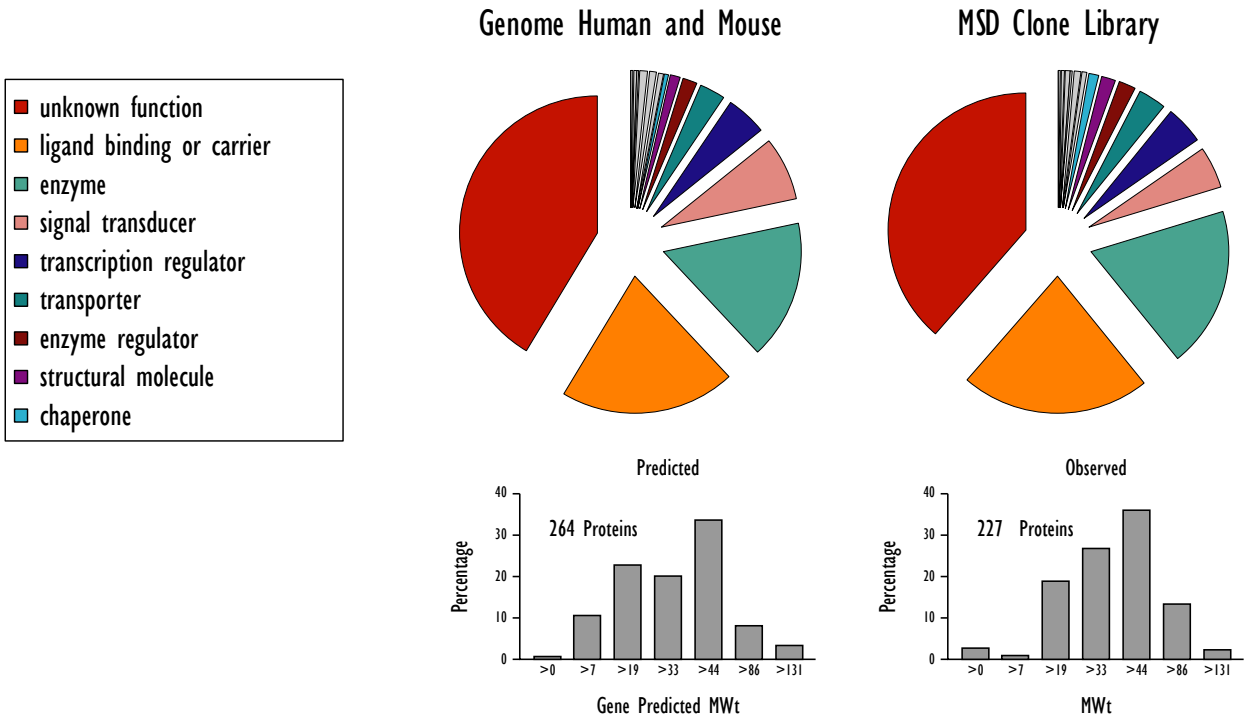
3 Functional diversity of the MSD clone library



The MSD library comprises cDNA clones isolated from a variety of tissues and cell lines that provided a comprehensive sampling of genes transcribed from the human and mouse genomes. The cDNAs were cloned into vectors that allowed their expression in cells and other systems. Clones were selected for our library using several criteria. First, we included the clone if its 5' sequence qualified it as full-length based on Genebank data. We also included clones in our library if they were from a unique Unigene cluster up to a maximum of 3 clones for any given cluster. We thus effectively normalized the clone collection to ensure an optimal representation of genes within the genome, avoiding the generation of a library with few genes in many copies.

We compared our collection of genes to human and mouse genome annotations based on gene onotologies (pie charts). This comparison generated a profile that largely matched the MSD library to these genomes suggesting a highly representative collection. We also expressed a sample of the library and demonstrated that we produced the expected gene products for >70% of the genes tested (see bar graphs) validating the functionality of the library. We tested the quality of the library in a number of functional screens including a search for tyrosine kinases. This screen that required the generation of active kinases from the library which occurred with a high degree of success other functional screens sought proteins interacting with phosphorylation sites on EGFR, IkBa and cRaf, or targeted protein substrates of the N-end rule ubiquitinylation pathway. We recovered most of the targeted activities in these screens that were known or predicted from the available literature. Other posters describing these screens are available at www.meso-scale.com.

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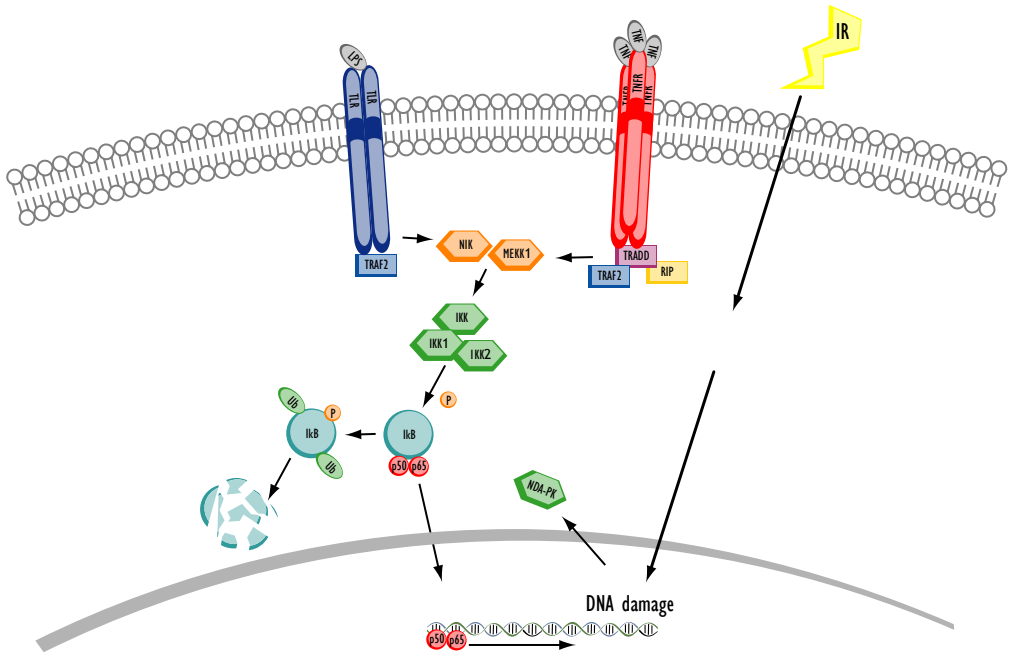
4 Cell based reporter assays: NFkB Signaling Pathway



5 Automation of assay

Media and transfection reagent

↓

DNA from library

↓

Media and reporter

↓

Incubate

↓

Add cells

↓

Harvest 12-60 h after transfection

↓

Read on HTS

The flow chart outlines the assay we developed for the detection of genes that activate NFkB. The gene of interest is co-transfected in with a plasmid encoding a reporter (luciferase in this case driven by a NFkB dependent promoter). The luciferase activity is used as a measure of the activation of NFkB due to the transfected gene. We also used a control vector containing the Renilla luciferase to allow for normalization of the transfection efficency.

This format was optimized using initial studies of transfection reagents and reporter vectors, followed by a statistical design of experiment approach.

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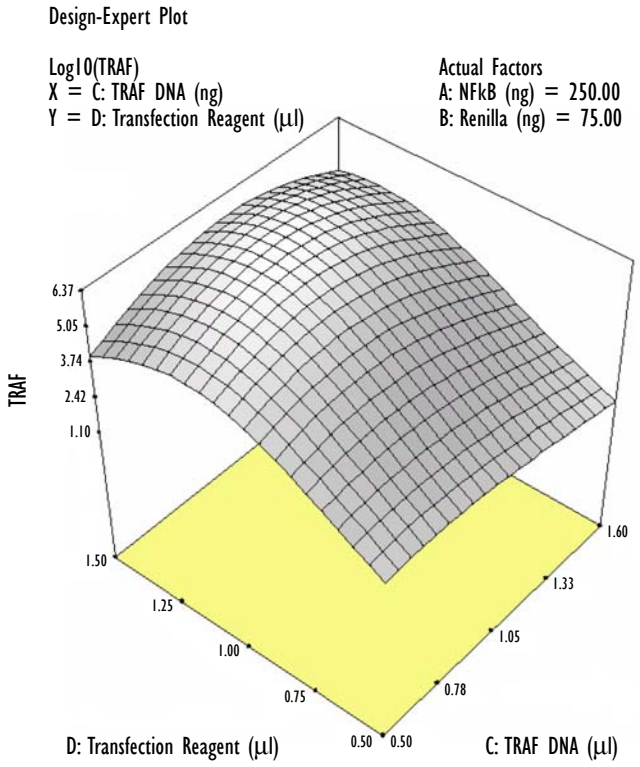
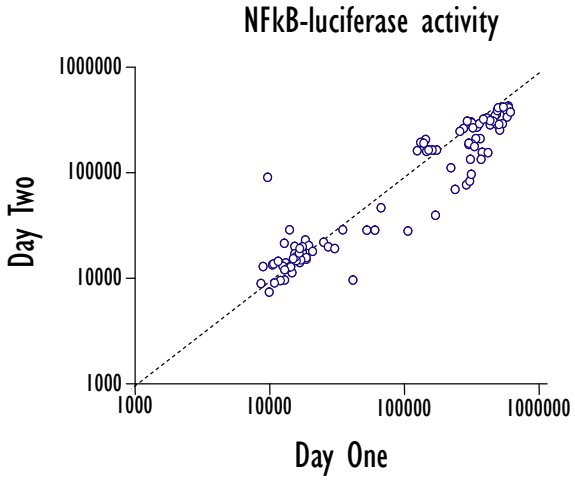
6 Assay optimization using Design of Experiment (Stat-Ease Inc.)

We employed statistical design of experiments using Design-Expert® software from Stat-Ease Inc to optimize various parameters of the experiments. We designed experimental approaches that evaluated multiple conditions simultaneously to optimize the transfection of cells with DNA made from our library. The response surface in the figure illustrates the approach. The plot shows that DNA from the human TRAF gene transfected into HEK293 cells best using 1.25µl of transfection reagent and 1ul of the TRAF DNA prep. We carried out similar studies (data not shown) with a representative number of other genes including both known activators of NFkB and well-established controls rigorously null for this pathway. The data set showed a range of optima for different genes, perhaps not unexpectedly, implying that any genome wide experiment would benefit from repeated measurement under varying conditions of transfection and cell culture. In what follows we used a single set of conditions.



7 Day to Day Variability



We also investigated the run-to-run and day-to-day variability of the screening workflow to establish the optimal conditions for the reagent preparations. Here we show an example of a comparison between two runs on successive days. We achieved very good day to day run correlations indicating conditions that worked well with an automation work flow.

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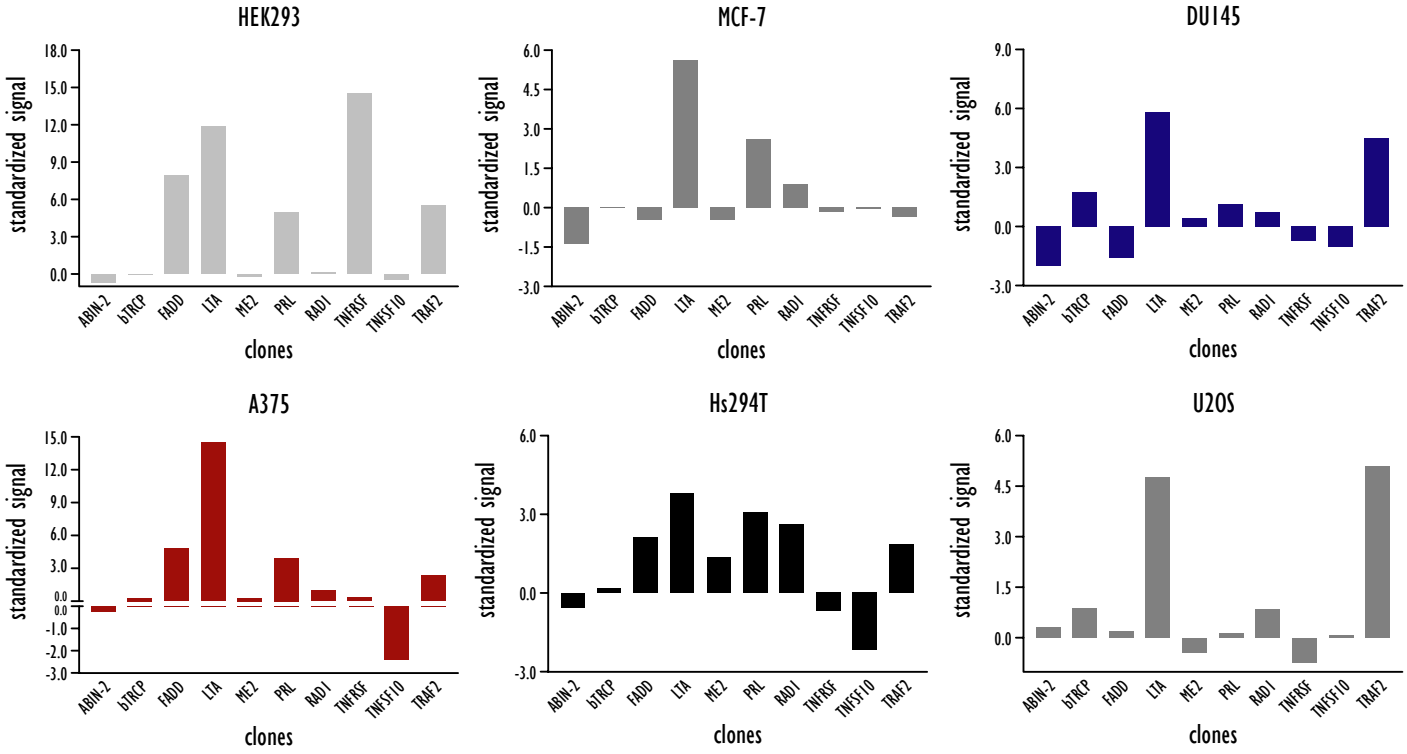
8 Cell line NFkB profiles with QC plate



Different cell lines responded differently to the known NFkB activators, as expected from their transfectability and their general characteristics. We investigated a number of cell lines for use in our screens. This figure shows examples of NFkB activation by genes on our control test plate. Each point is the average of 8 datum per gene tested. Cell-type specific responses were evident from a comparison of the activation profiles, an approach that negated the affects of the transfectability between cell lines. This comparison of activation profiles highlighted a number of differential effects with known NFkB activators. The biggest such differential effect occurred with TNFRSF (TNFRSF10B) that activated NFkB well in HEK 293 but failed to activate NFkB in all the other cell lines in this study. Similarly, the responses seen with TRAF2 were activation in HEK-293, U2OS and DU145 but little activation in MCF-7s. We selected HEK-293, MCF-7, A375 and HCT116 (not shown here) as our panel of cell lines for initial study of NFkB. We felt these cell lines gave us access to a range of interesting and relevant biologies while remaining sufficiently tractable in cell culture and transfections to allow a screening campaign.

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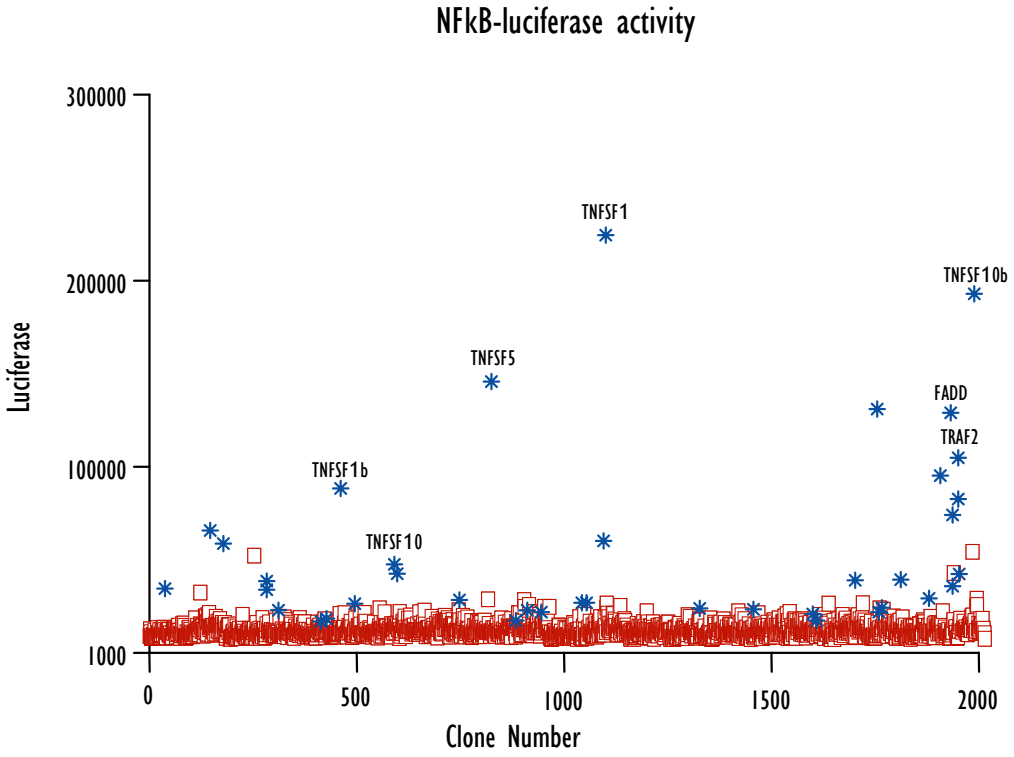
9 Detection Of Known Activators Of NFkB Mediated Transcription

We ran a pilot study of various reporter gene constructs to evaluate the potential of these constructs in a genome wide screen. Here, we show the results from this 'mini' screen of 2000 clones with an NFkB reporter construct, demonstrating the successful recovery of many known activators of NFkB. This "blinded" study validated that our methodology was able to establish known interactions in real systems and gave us confidence that the discoveries made using it would prove relevant.



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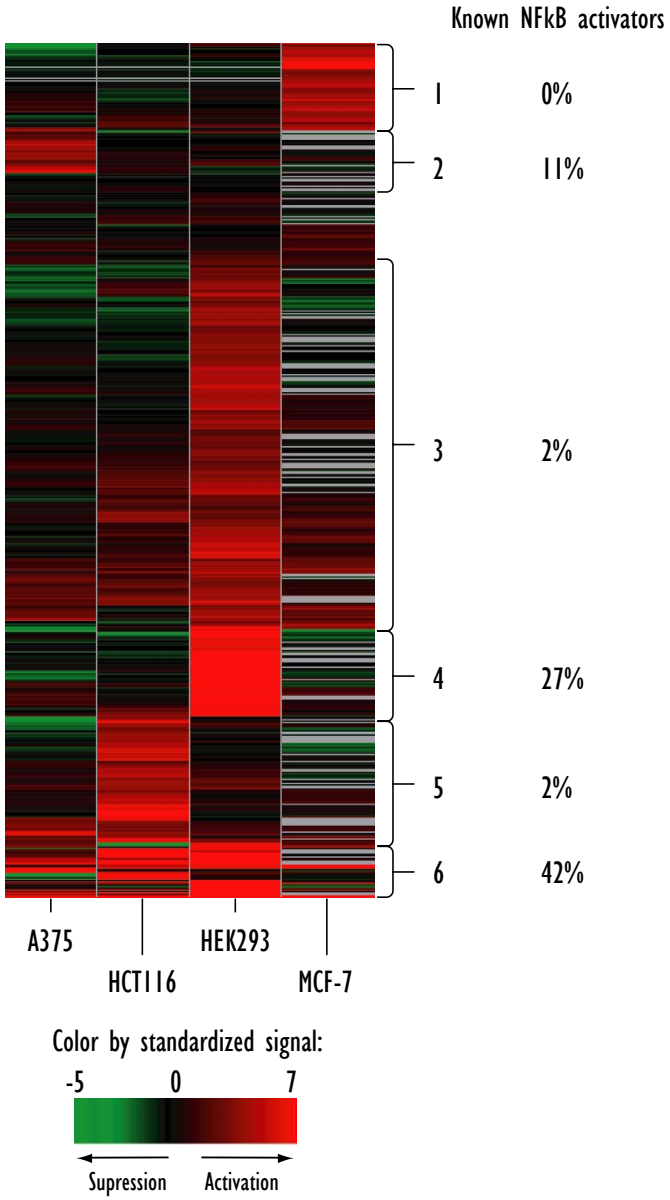
10 Heat map from NFkB screen

Hierarchical clustering of hits from NFkB screen

Our entire data set was queried for clones that gave a standardized signal (either uncorrected or corrected using the Renilla control) of > 5 in at least one of the four cell lines; 536 clones met this criterion. The standardized signal resulted from the ratio of the signal minus the median to the standard deviation of the inner 90% of the data from each assay plate ('standardized signal' = [signal – median] / 'standard deviation'). Data from the 536 clones were organized by hierarchical clustering based on the corrected standardized signals recorded across the four cell lines. Euclidean distance between the standardized signals was used as a metric, and the resulting clusters were ordered by their average standardized signal. The result is represented here as a heatmap where each clone is represented by a horizontal bar colored to reflect the corrected standardized signal recorded in each cell line, as shown by the legend (below). This analysis provided a simplified view of gene clusters involved in cell type specific modulations of the NFkB pathway. Annotation of these clusters is shown to the right of the heat map, where the percentage sign indicates the number of genes in the cluster already known as activators from the literature. Interestingly, activators of NFkB specific for the MCF7 and HCT116 cell types did not include many recognized activators of the NFkB pathway. This demonstrated the value of using multiple cell types in such screens and showed that very different response can occur for some genes involved in NFkB activation depending on the cell context. Our observations indicate that NFkB activation might be modulated in a specific way to achieve a therapeutic outcome without giving rise to systemic side effects. For example, the genes that appear to function in the activation of NFkB in MCF7 and HCT 116 should be interesting targets for the development of drugs aimed at treating breast and colon cancer respectively



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Selection of known NFkB activators recovered in our screen

The table illustrates the power of this type of screen for the dissection and discovery of signaling pathways. We successfully identified genes throughout the NFkB activation family, including receptor ligands; receptors to adapter proteins or receptor associated factors; other cytoplasmic mediators, and; the transcription factors that directly activate the NFkB reporter. In addition we recovered other known activators involved in cellular trafficking.

	Ligands	IL1A interleukin 1, alpha
		LTA lymphotoxin alpha (TNF superfamily, member 1)
	Receptors	TNFRSF1A tumor necrosis factor receptor superfamily, member 1A
		TNFRSF10B tumor necrosis factor receptor superfamily, member 10b
		Ltbr lymphotoxin B receptor
		LTBR lymphotoxin beta receptor (TNFR superfamily, member 3)
		TNFRSF10A tumor necrosis factor receptor superfamily, member 10a
		TNFRSF12A tumor necrosis factor receptor superfamily, member 12A
		TNFRSF5 tumor necrosis factor receptor superfamily, member 5
		LTBR lymphotoxin beta receptor (TNFR superfamily, member 3)
	Adapter proteins	Tirap toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
		Myd88 myeloid differentiation primary response gene 88
		Traf2 Tnf receptor-associated factor 2
		MYD88 myeloid differentiation primary response gene (88)
		MYD88 myeloid differentiation primary response gene (88)
		T2BP TRAF2 binding protein
		FADD Fas (TNFRSF6)-associated via death domain
	Cytoplasmic mediators	Bcl10 B-cell leukemia/lymphoma 10
		Card14 caspase recruitment domain family, member 14
		CARD14 caspase recruitment domain family, member 14
		C6orf4 chromosome 6 open reading frame 4
		CFLAR CASP8 and FADD-like apoptosis regulator
		IKBKE inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
		CARD4 caspase recruitment domain family, member 4
		CASP8 caspase 8, apoptosis-related cysteine protease
		PAK4 p21(CDKN1A)-activated kinase 4
	Transcription factors	Rela v-rel reticuloendotheliosis viral oncogene homolog A (avian)
		RELA v-rel reticuloendotheliosis viral oncogene homolog A, p65 (avian)
		RELA v-rel reticuloendotheliosis viral oncogene homolog A, p65 (avian)
	Trafficking	Vapa vesicle-associated membrane protein, associated protein A
		Ndfip1 Nedd4 family interacting protein 1

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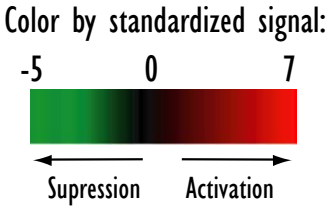
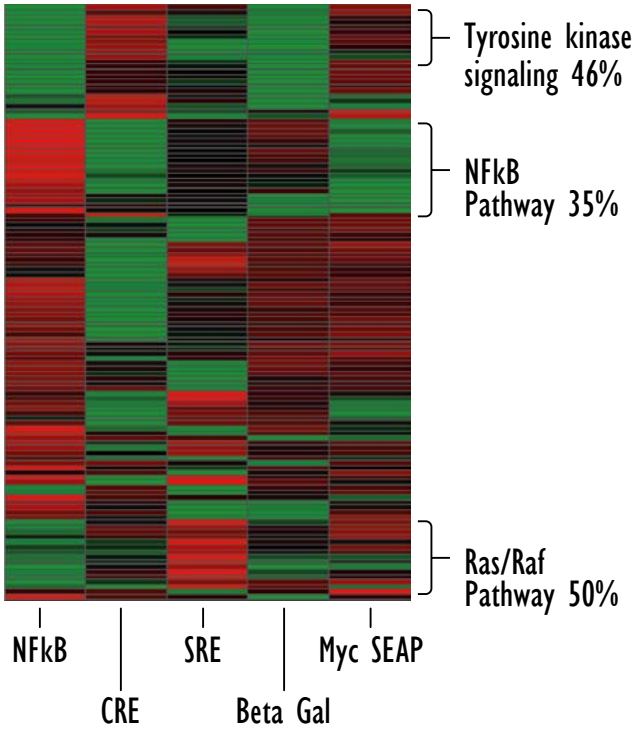
⓬ Pathway dissection using multiple reporters

Screens with multiple reporters offer potential to dissect and discover pathways. Functional screens of a genome can be further augmented using other reporter constructs in combination with the library and multiple cell lines. In this study, we combined a series of reporters and demonstrate, using a clustering methodology and a small subset of the data (approx. 2000 clones), the ability to see patterns in cellular pathways. The clusters of genes around the NFkB pathway, the Ras/Raf pathway and the tyrosine kinase signaling members indicate the additional utility that these multi-dimensional studies bring: Inter-experimental correlations allow the detection and selection of specific relationships from data that might otherwise be dominated by experimental or biological noise. These relationships are further critical to a successful effort to redact putative gene candidates into a smaller set of plausible targets.





⓭ Conclusions

We developed a rapid, sensitive and facile system for the detection of genes that activate NFkB. This format readily transferred to an automated system that allowed the screen to be carried out in a rapid and reproducible way. We identified ~500 genes from the 20,000 genes we tested that activate NFkB in at least one of the cell lines tested. From this list of genes we identified many known activators of NFkB, including receptor ligands, receptors, adaptor proteins, cytoplasmic signaling proteins and transcription factors. We have also developed a high throughput cell based assay for the discovery of small molecule inhibitors of NFkB activation by the genes from our screen like lymphotoxin et al (data not shown). Our protocol also allows us to transfect genes for many other cell based assays to identify genes in numerous cell signaling pathways. The correlations between these data sets provide real insights to the discovery and targeting of novel effectors of many cell biological pathways.

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