

# Discovery Of Skeletrophin A Novel Ubiquitin E3 Ligase Regulator of NF-kappaB From A Genome-wide High Throughput Cell-based Assay

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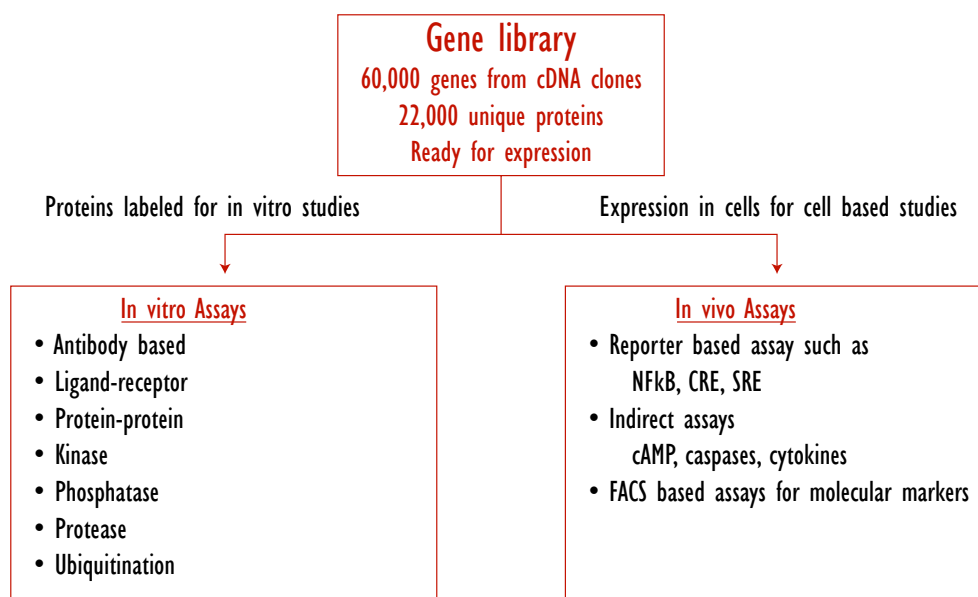
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# Discovery Of Skeletrophin A Novel Ubiquitin E3 Ligase Regulator of NF-kappaB From A Genome-wide High Throughput Cell-based Assay

## 1 Abstract

We discovered that skeletrophin, a novel ubiquitin E3 ligase, can be a potent cellular modulator of NF-kappaB. We used a systematic, genome-wide, high throughput cell-based assay to identify putative effectors of NF-kappaB by quantifying changes in the level of an NF-kappaB responsive reporter in response to individually transfected genes. This cell-based 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 NF-kappaB. Transfection protocols were optimized using a design of experiment statistical approach to select the amounts of i) the transfection reagent, ii) NFkB reporter DNA, iii) Renilla control DNA and iv) test DNA for the screen. 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 253 clones that gave the highest signals and clustered them with respect to their activity across the four cell lines. This clustering showed that some genes were active in all cell lines, while the effects of others specific, underscoring the contextual roles the. We recovered many of the well-known activators in the NF-kappaB pathway including i) receptor ligands, ii) receptors, iii) receptor associated proteins, iv) intracellular signaling proteins to v) transcription factors. In addition to these well known activators we also discovered many new activators. These newly identified activators of NF-kappaB were filtered further by the strength of activation, reproducibility and known biochemistry to select the most interesting set for further investigation. This set included the novel putative E3 ligase, skeletrophin. We explored expression patterns of skeletrophin and studied its effects on other cis-acting enhancer elements. We found that skeletrophin was both a potent inducer of NF-kappaB in the absence of other stimulation and a strong modulator of a priori activation of this pathway by TNF. These data in total demonstrate the validity of our approach to the screening and discovery of genes involved in the activation of NF-kappaB. We conclude that our approach to high throughput, cell-based screens through the proteome for activators of transcription represents a facile route to the discovery and validation of genes involved in this aspect of cell-biology.

## 2 Meso Scale Proteomics - Discovery Tools



### Introduction.

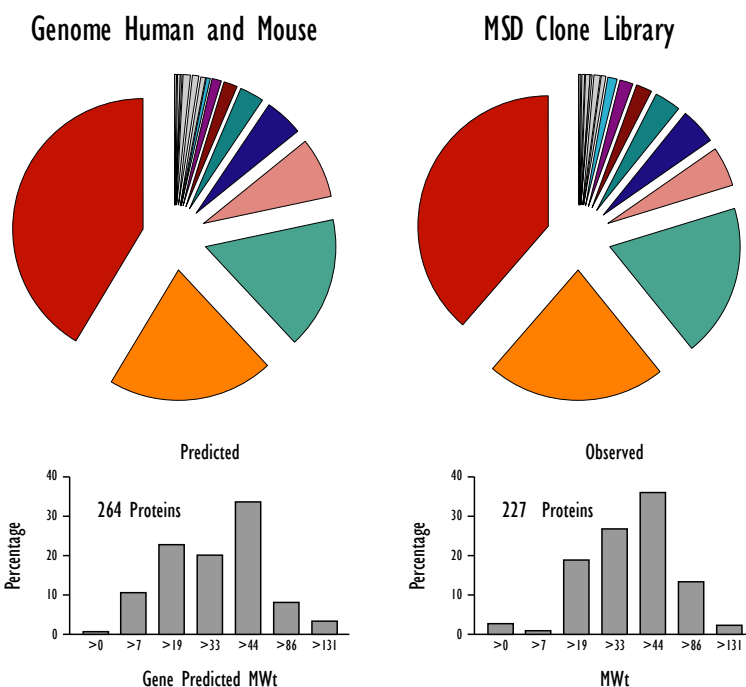
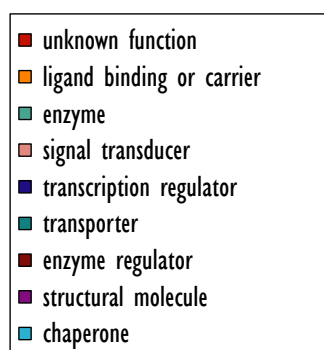
We built a library of cDNA clones from the mouse and human genomes that encode consensus, full-length protein sequences and allowed us to carry out functional screens across these genomes. The figure shows schema for the screens we completed. This poster describes one such screen based on a biological 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



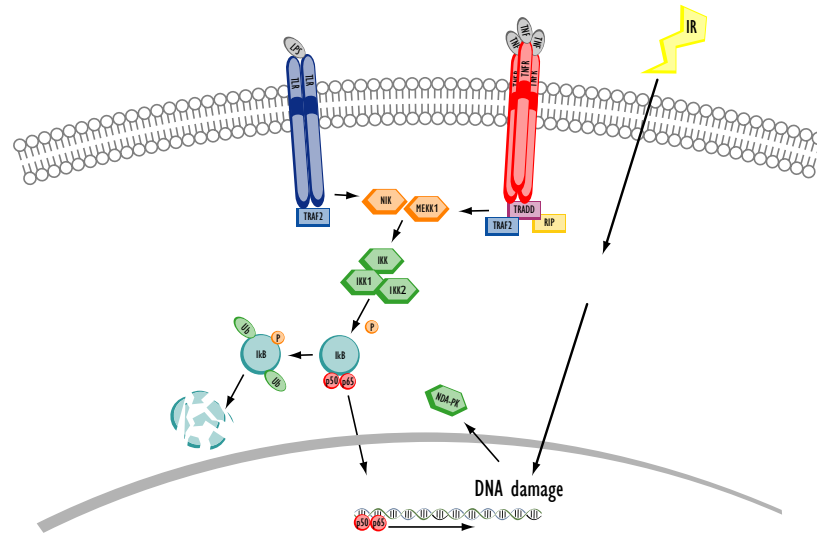
The MSD library comprises cDNA clones isolated from a variety of tissues and cell lines that provided a comprehensive sampling of genes transcribed from the human and mouse genomes. The cDNAs were cloned into vectors that allowed their expression in cells and other systems. Clones were selected for our 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 ontologies (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, I $\kappa$ B $\alpha$  and cRaf, or targeted protein substrates of the N-end rule ubiquitylation 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](http://www.meso-scale.com).

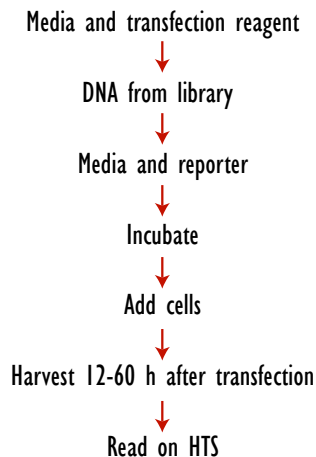


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## 4 Cell based reporter assays: NFkB Signaling Pathway



## 5 Automation of assay



The flow chart outlines the assay we developed for the detection of genes that activate NFκB. The gene of interest is co-transfected in with a plasmid encoding a reporter (luciferase in this case driven by a NFκB dependent promoter). The luciferase activity is used as a measure of the activation of NFκB due to the transfected gene. We also used a control vector containing the Renilla luciferase to allow for normalization of the transfection efficiency. 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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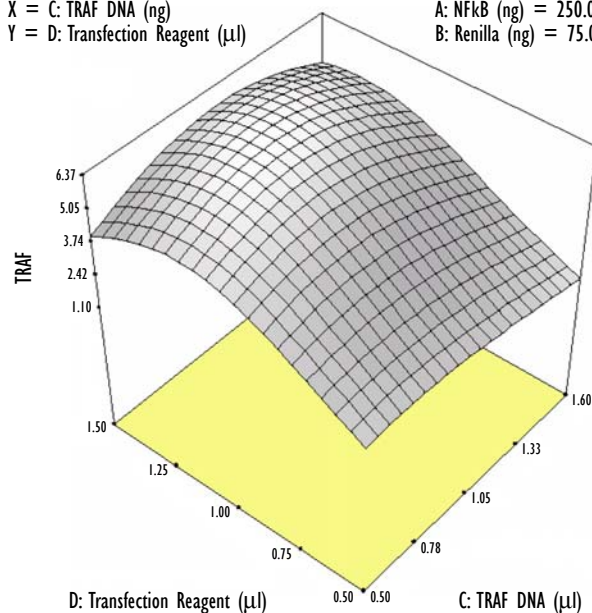
## 6 Assay optimization using Design of Experiment (Stat-Ease Inc.)

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 1µl 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 NFκB 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.

Design-Expert Plot

Log10(TRAF)  
X = C: TRAF DNA (ng)  
Y = D: Transfection Reagent (µl)

Actual Factors  
A: NFκB (ng) = 250.00  
B: Renilla (ng) = 75.00

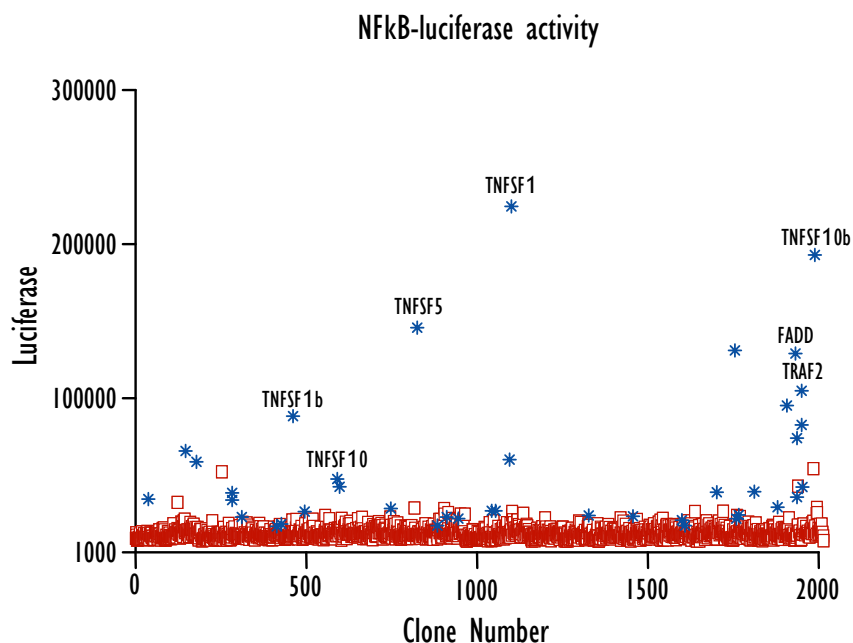


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## 7 Detection Of Known Activators Of NFkB Mediated Transcription

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.



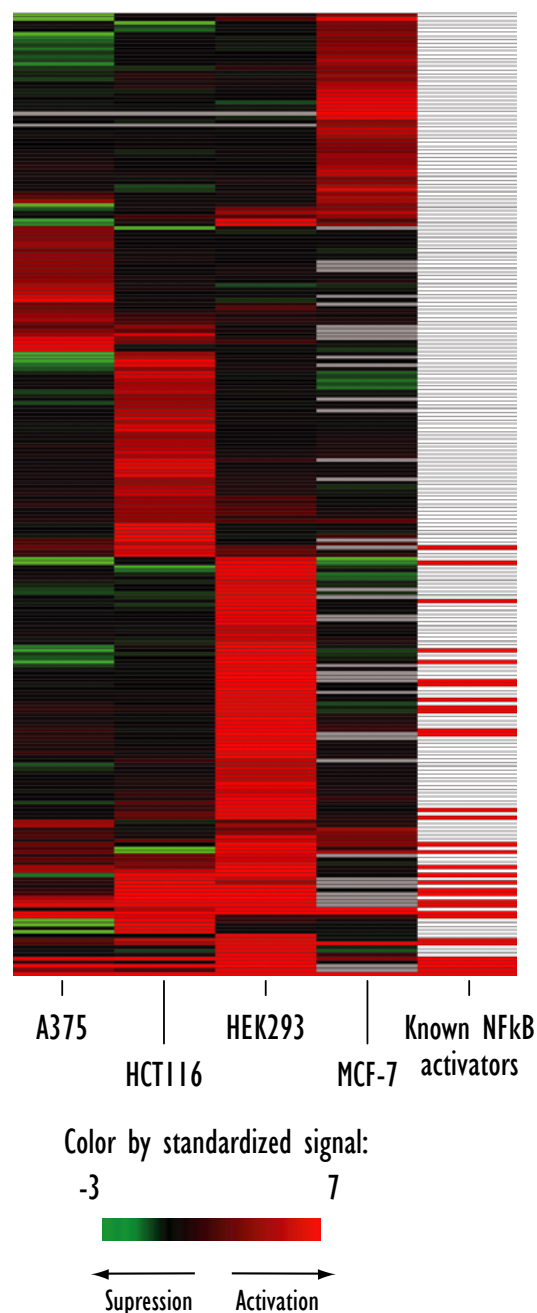
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## 8 Heat map from NFkB screen

Our entire data set was queried for clones that gave a standardized signal (corrected using the Renilla control) of  $> 4$  in A375,  $> 5.5$  in HEK293,  $> 4.5$  in HCT116 and  $> 4$  in MCF-7; 253 clones met these criteria. 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' =  $[\text{signal} - \text{median}] / \text{standard deviation}$ ). In this collection of hits 32 were already known to have a role in the NFkB pathway. Data from the 253 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. These data are represented here as a heat map where a horizontal bar colored to reflect the corrected standardized signal recorded in each cell line denotes each clone, as shown by the legend (below). This analysis provided a simplified view of gene clusters involved in cell-type specific, and more general, modulations of the NFkB pathway. Annotation of these clusters is shown to the right of the heat map, where the red band indicates a gene in the cluster known from the literature to be involved in the NFkB pathway. Interestingly, activators of NFkB specific for the A375, MCF7 and HCT116 cell types did not include many recognized activators of this pathway. This plot demonstrates the value of using multiple cell types in such screens and showed that very different responses 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 as has been seen for general inhibitors of this pathway. The genes that appear to function in the activation of NFkB in A375, MCF7 and HCT 116, for example, might be interesting potential targets for the development of drugs aimed at treating melanoma, breast and colon cancer respectively. The inhibition of NFkB activation in the cancer cell would be expected to increase its sensitivity to apoptosis induced during radiation and chemotherapy.

We analyzed the relative enrichment of the various gene ontologies (GO) in order to see if any general patterns of function were evident in the genes that stimulated or inhibited the NF-kappaB dependent expression. Analysis of the hits in HEK-293s demonstrated a clear association with apoptosis (enriched by 4.58 fold,  $p < 0.0001$ ), for example. Indeed, the collection of the most significant ( $p < 0.0003$ ) gene ontologies are all related to apoptosis and cell death: In order, these were: 1) apoptosis, 2) programmed cell death, 3) cell death, 4) death, 5) regulation of apoptosis, and 6) induction of apoptosis. We also see anti-apoptosis ( $p = 0.044$ ) ontologies in the enriched set. The indication that we have known activators of NF-kappaB is also represented in this collection by ontologies like as NIK-I-kappaB/NF-kappaB. The GO analysis of the inhibitors of NF-kappaB showed a significantly different pattern of ontologies although it still maintained the apoptosis theme. We see that in the top 10, 4 of the gene ontologies are NF-kappaB related based on the nuclear import although the statistical significance of these observations remains weak given the low number of genes in this cluster. Other, larger gene ontology clusters of interest with significant enrichment in the screen included receptor activity ( $p = 0.007$ ) and defense response ( $p = 0.02$ ). Using the same GO analysis with data from the colon tumor cell line HCT116 we see a significant shift in the spectrum of the gene ontologies having the greatest relative enrichment. The top 5 ontologies were 1) Defense response 2) Inflammatory response 3) innate immune response 4) response to biotic stimulus 5) response to wounding. The apoptosis collection was similarly represented but has been displaced by this collection of ontologies.



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## 8 Heat map from NFkB screen (con.t)

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. The breadth of protein types from nuclear to plasma membrane suggests that the screen was relatively unbiased towards one or another class or site of modulation.

Screens with multiple reporters offer the 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 clustering algorithm simply groups genes by their pattern of responsiveness across the entire set of reporters. The clusters around the NFkB pathway, the Ras/Raf pathway and the tyrosine kinase signaling members indicate the additional analyticity 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.

We selected Skeletrophin for further study from the list of novel activators of NFkappaB as a gene with little information regarding its biology. The Skeletrophin protein is 1,013 amino acids long and, according to SAGE data, does not generally appear highly expressed. Skeletrophin is interesting as it possesses two zinc finger, RING type domains (PS50135). The presence of these RINGs is a strong indication that Skeletrophin is an E3 ubiquitin ligase, given the data from numerous studies on proteins with these domains. The recovery of different clones of Skeletrophin reinforced that this gene was most likely a true hit from the screen and not a false positive. We carried out a study of the tissue specific expression of Skeletrophin using tissue blots (Table 1). These blots demonstrated elevated expression of Skeletrophin in certain leukemia's and carcinomas derived materials, in contrast to normal tissues where we did not see any strong tissue specific associations except that lung tissue was well represented. In normal tissues the highest levels of expressions occurred in material from spinal cord or bladder.

We recloned the Skeletrophin gene to attach an HA tag. In addition, we also generated a truncated version where we removed the carboxy terminal RING fingers. These genes were transfected into HEK293 cells to determine the nature of the molecular species that were expressed from these constructs. Expression of the full-length gene proved difficult and typically resulted in low levels of expression relative to other HA tagged genes. The truncation of the gene removing the RING finger domains resulted in elevated expression. The suggestion from this observation is that removal of the E3 ubiquitin ligase domains might be removing an auto-ubiquitination activity that controls the protein half-life in mammalian cells.

We discovered that Skeletrophin, in addition to its activation from basal levels of NF-kappaB, was also able to block NF-kappaB activation by TNF (see below) in HEK293 cells. This response was unexpected and indicated that Skeletrophin buffers NF-kappaB activation in some way in this model. This observation requires further study as does the role of the RING finger domains in the modulation of the NF-kappaB response.







We identified a novel regulator of NF-kappaB, Skeletrophin using our genome-wide system for the detection of genes that activate an NFkB responsive reporter. We determined that this gene may play a role in certain tumors based on its over expression in these tumors. We also showed that Skeletrophin blocked the activation of an NF-kappaB response stimulated by TNF further suggesting a possible role for this gene in cancer. We identified 253 other genes, in addition, from among the 20,000 genes we tested that also activated NFkB in at least one of the cell lines tested. Within this collection of activated genes we were able to identify clusters of genes that were specific to breast (MCF7), colon (HCT116) and melanoma (A375) tumors lines. In addition, we also found that the most significant gene ontologies enriched in the hit list were those associated with apoptosis. Our functional genomics method also allows us to discover genes involved in numerous other signaling pathways and biology. These approaches should also prove valuable in the determination of drug mechanisms of action, through pathway mapping and the general effects of differential activation of different reporters in response to biochemical inhibitors.



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## 9 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.

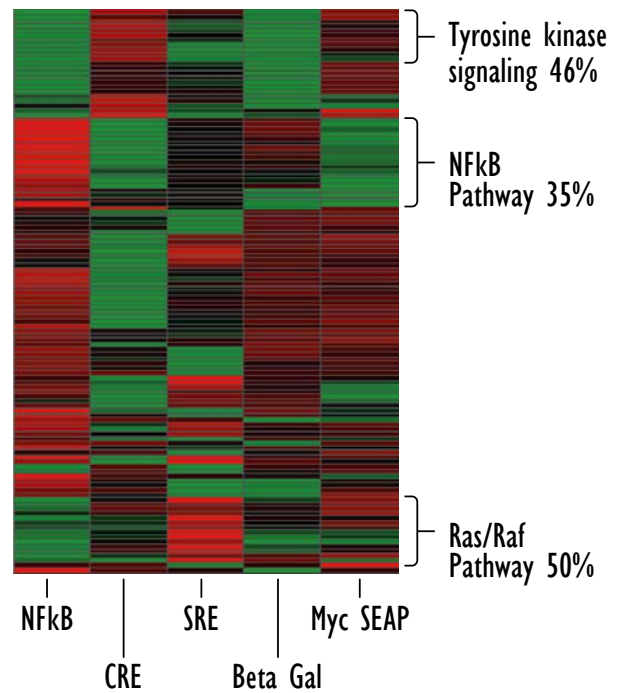
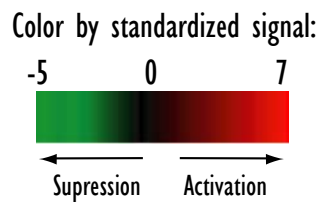
	<b>Ligands</b>	IL1A interleukin 1, alpha LTA lymphotoxin alpha (TNF superfamily, member 1)
	<b>Receptors</b>	TNFRSF1A tumor necrosis factor receptor superfamily, member 1A TNFRSF10B tumor necrosis factor receptor superfamily, member 10b Ltrb lymphotoxin B receptor LTBR lymphotoxin beta receptor (TNFR 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)
	<b>Adapter proteins</b>	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
	<b>Cytoplasmic mediators</b>	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
	<b>Transcription factors</b>	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)
	<b>Trafficking</b>	Vapa vesicle-associated membrane protein, associated protein A Ndfip1 Nedd4 family interacting protein 1



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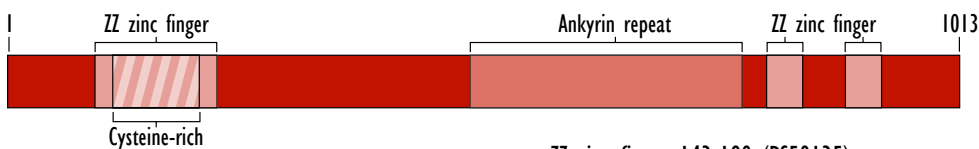
## 10 Pathway dissection using multiple reporters

Screens with multiple reporters offer the 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.



## 11 Skeletrophin

From the list of novel activators of we selected Skeletrophin a gene with little information regarding its biology for further study. The Skeletrophin protein is 1,013 amino acids long and from SAGE data does not appear to be highly expressed. Skeletrophin is interesting as it possesses two zinc finger, RING type domains (PSS0135). This is a strong indication that this is a classic RING finger E3 ubiquitin ligase based on the numerous studies with proteins containing these domains. In addition to both NF-kappaB activation and its domain structure, this gene was also recovered twice in our primary screen. The recovery of this gene multiple times demonstrated that this was most likely a true hit from the screen and not a false positive from the screen. To investigate this gene further we carried out a study of tissue specific expression using a tissue blot with the Skeletrophin (Table 1). This demonstrated that skeletrophin expression is elevated in certain leukemia's and carcinomas. In normal tissue we do not see any strong tissue specific associations, except that lung tissue is well represented. In normal tissues the highest levels were detected in spinal cord and bladder.



ZZ zinc finger 143-190 (PSS0135)  
 Cysteine-rich region, 149-176 (PSS0311)  
 Ankyrin repeat region (7), 522-836 (PSS0297)  
 Zinc finger RING type, 890-925  
 and 969-1002 (PSS0135)

TISSUE	LEVEL
chronic myelogenous leukemia, K-562	25.0
colorectal adenocarcinoma, W480	20.8
spinal cord	18.7
lung carcinoma, A549	18.0
bladder	17.1
rectum	15.8
atrium, right	12.9
fetal lung	12.1
lung	11.9
fetal thymus	11.6
adrenal gland	11.4
spleen	11.3
caudate nucleus	10.3
fetal brain	9.4

TABLE 1.



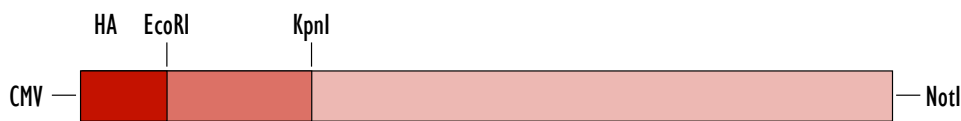
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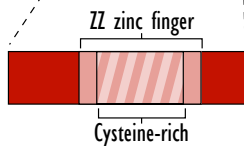
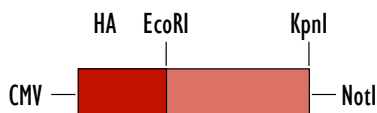
## 12 Skeletrophin expression

To further study the expression and interacting proteins we recloned the skeletrophin gene to attach an HA tag. In addition we also generated a truncated version where we had removed the COOH terminal RING finger E3 ubiquitin ligase. These genes were transfected to determine the nature of the molecular species that were expressed from these constructs. Expression of the full-length gene proved to be difficult and typically resulted in low levels of expression relative to other HA tagged genes. The truncation of the gene removing the RING finger E3 ubiquitin ligase domains resulted in elevated expression. The suggestion from this observation is that removal of the E3 ubiquitin ligase domains might be removing an auto-ubiquitination activity that controls the protein half-life.

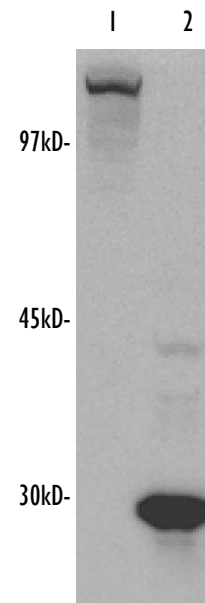
HA tagged full length skeletrophin



PCR ~800 bp skeletrophin N-terminus



Domain Structure



Expression of N-terminal HA skeletrophin

1- HA-FL Skeletrophin

2- HA-N-term Skeletrophin

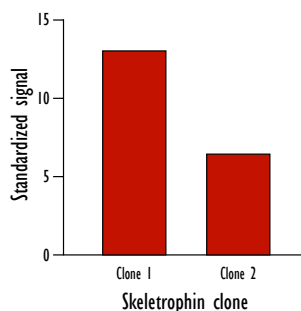


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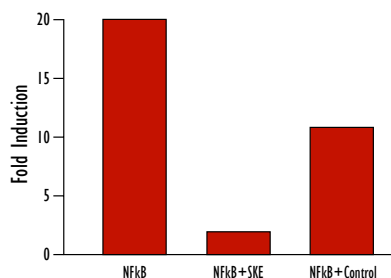
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## 13 Modulation of NF-kappaB activation by Skeletrophin

Activity of two Skeletrophin clones isolated from the NFkB screen



Skeletrophin inhibition of NFkB cis element activation following TNF stimulation



In follow up studies we discovered that Skeletrophin in addition to activation of basal NF-kappaB was able to block activation via TNF (see below). This response was unexpected and indicated that Skeletrophin is in some way buffering the NF-kappaB activation. Further studies are needed to dissect out the role to Skeletrophin in this buffering of NF-kappaB activation. The role of the RING finger domains in the modulation of the NF-kappaB response remains to be determined.

## 14 Conclusion

We discovered a novel regulator of NF-kappaB, Skeletrophin using our rapid, sensitive and facile genome wide system for the detection of genes that activate NFkB. We determined that this gene may play a role in certain tumors based on its over expression. Also we demonstrated that Skeletrophin was also able to block activation of a NF-kappaB response stimulated with TNF. In addition we identified 253 other genes from the 20,000 genes we tested that activate NFkB in at least one of the cell lines tested. With in this collection of activated genes we are able to identify clusters of genes that are specific to the breast (MCF7), colon (HCT116) and melanoma (A375) tumors line. In addition we also determined that the most significant gene ontologies enriched in the hit list were associated with apoptosis. Our functional genomics method also allows us to discover genes involved in numerous other signaling pathways and biology. These approaches are also valuable in the determination of drug mechanisms of action, through pathway mapping.

