A Bioinformatics Approach to Defining Gene Signaling Pathways

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Abstract

An improved understanding of gene signaling pathways and mechanisms involved in cancer continues to be a necessary feat to further cancer research and yield effective cancer treatments. Many cancers involve the misregulation of transcription factors E2f, c-Myc, FoxM1, and Stat family members, which are responsible for regulating many genes in the cell. While some gene targets of these transcription factors are known, many more are yet to be discovered. Identification of all the direct gene targets will provide not only the unidentified pathways, but also a better idea of the interconnectedness of the misregulated pathways in cancer.

The project employs an information retrieval and integration approach to identify all the potential direct targets of E2fs, c-Myc, FoxM1, and Stat in order to understand the relationship between these regulators as well as their respective roles in cancer. As the medical community delves further into researching cancer treatments, there is an increasing demand for an effective and reliable tool that assimilates massive amounts of existing information regarding relevant cancer pathways and provides easy access through a single portal. This project aims to mine data from existing public repositories and ongoing experiments to process and calibrate it to a certain standard, so that searching for details about transcription factors and potential targets will be easier.

The data is categorized in multiple ways to make queries and searches more efficient. Every data entry has information regarding the source of the experiments, type of target gene regulation, experimental variables, and confidence score. The confidence score assigned to each essentially classifies the gene as a high or low stringency target gene. Currently, manual retrieval of data has yielded entries corresponding to about 3000 target genes amassed from over 600 publications concerning E2fs, Myc, Stat, and FoxM1. We have also computationally extracted

data from relevant supplementary microarray raw data files. Furthermore, we aim to improve the precision of the confidence score given to each target gene by utilizing the influx of all new relevant data. The collective data obtained will serve to provide researchers with comprehensive information on gene-signaling pathways in various cancers with the goal of improving current research, instigating new ideas, and identifying potential targets of cancer treatments.

Introduction

Cancer encompasses a number of diseases that result from the abnormal and uncontrolled proliferation of cells. The basic mechanism by which cancer arises involves genetic mutations that eventually lead to misregulated signaling pathways. A "direct transcriptional target" of a transcription factor is a gene whose regulatory DNA sequences are physically bound by the transcription factor. Transcription factors themselves are DNA-specific binding proteins that control the rate of gene expression. They can act as activators or repressors leading to the upregulation or downregulation of genes, respectively. Within a cell, transcription factors engage in complex formation with other molecules including co-activators, co-repressors, co-factors, and histones that together bind to target genes and engage the transcriptional machinery. E2Fs, c-Myc, Stat, and FoxM1 are examples of such transcription factors.

E2f and c-Myc

Many cancers are known to have mutations in tumor suppressor genes and transcription factors. One such tumor suppressor gene is retinoblastoma (RB) which encodes a nuclear protein (Rb) that exhibits cell cycle control in the G1 and G2 phases[1]. Deletion or inactivation of both RB alleles plays an essential, rate-limiting role in retinoblastoma and osteosarcomas that arise within families that carry a mutated RB gene. Previous studies in mouse models have shown that Rb has dual roles in gating cell cycle progression and promoting cellular differentiation[2].

E2F is a large transcription family consisting of three activators: E2F1, E2F2, E2F3a, and six repressors: E2F3b, E2F4-8. In the biological system, Rb sequesters E2F to regulate entry of cell cycle. The role of Rb as an indirect regulator of E2F target gene expression has also been demonstrated in previous studies[3]. Numerous cancer types involve the misregulation of the Rb/E2F pathway, underlining the importance of identifying the entire cadre of E2F target genes

and in gaining a comprehensive understanding of the pathway[3].

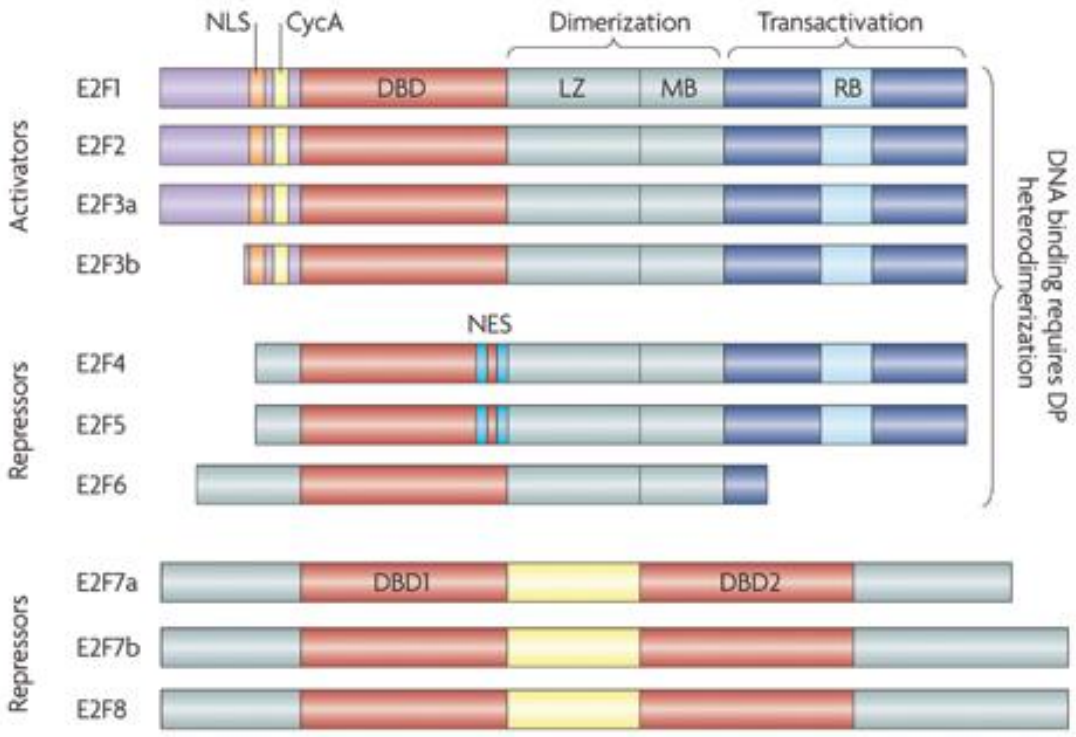

Figure i: E2f family members.[15]

Rb also regulates the activities of other classes of the transcription factors involved in processes of cell proliferation and differentiation[4]. Thus, Rb is significant not only as a tumor suppressor but also as a mediator in gene communication[4]. Another potential transcription factor target of Rb is c-Myc, which regulates about 15 percent of human genes in cancer cells, including those involved in cell division, cell growth, and apoptosis[5]. c-Myc, a specific member within the 3-member Myc transcription family, is found to be constitutively expressed and activated in many cancers, leading to the misregulation of many genes in the human body[6]. However, whether Myc activity is impacted with an Rb mutation is unknown.

While both E2F and Myc transcription factors are misregulated in cancer, if and how these transcription factors communicate and collaborate in cancer initiation and progression

remains to be determined. Therefore, the relationship between the E2F and the c-Myc signaling pathways needs further analysis. Moreover, the identification of direct transcriptional targets of E2F and Myc will serve to further understand the relationship between these two pathways in the context of cancers with Rb mutations.

FoxM1

FoxM1 is a unique transcription factor known to be involved in cell proliferation and cell cycle progression[7]. It is a member of the forkhead box family of helix-turn helix proteins and in humans consists of three members including FoxM1a, b, and c. Endogenous FoxM1 is upregulated at the S and G2/M phases in the cell. Accordingly, FoxM1 regulates the expression of many G2/M genes, such as mitotic cyclins, that are essential for proper mitotic cell division and chromosome stability[8]. Therefore it is unsurprising that FoxM1 null-embryos result in embryonic lethality due to high polyploidy in cells of the heart and liver[9].

FoxM1 is frequently upregulated in many human cancers. Though it is known to play a key role in tumor initiation, growth, and progression, the mechanism by which FoxM1 initiates tumorigenesis is still unknown[7]. FoxM1 is also regulated by proliferative and anti-proliferative signals and proto-oncogenes and tumor suppressors, specifically p53[10]. Since these factors are often mutated in cancers, FoxM1 expression is directly affected, thus leading to the misregulation of a large number of genes.

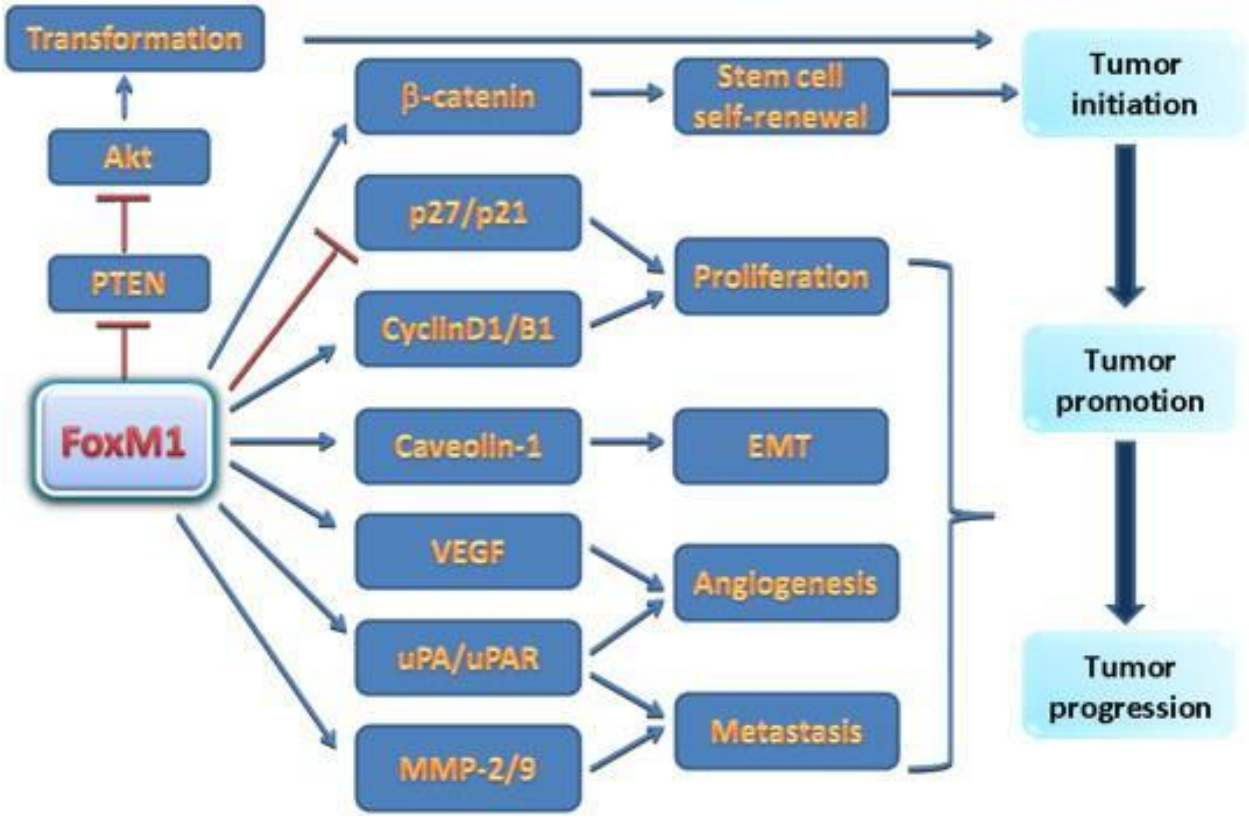

Figure ii: FoxM1 targeted pathways.[16]

Studies have shown that c-Myc, E2f1, and E2f3 are FoxM1 targets[11]. Therefore, elucidating the pathways involving FoxM1 and other transcription factors can provide researchers with a better understanding of tumorigenesis.

Stat

Another transcription factor that is known to be misregulated in cancer is Stat. Signal transducer and activation of transcription (Stat) consists of a family of latent cytoplasmic transcription factors including Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. They are downstream effectors of cytokine and growth factor receptor signaling. Constitutively activated Stat proteins have been detected in many cancers and primary tumors[12]. More specifically, constitutively activated Stat3 is known to play key role in oncogenesis and tumor angiogenesis [13]. In cancers, uncontrolled signaling of both Stat3 and Stat5 has been demonstrated to contribute

to cell proliferation and prevent apoptosis.

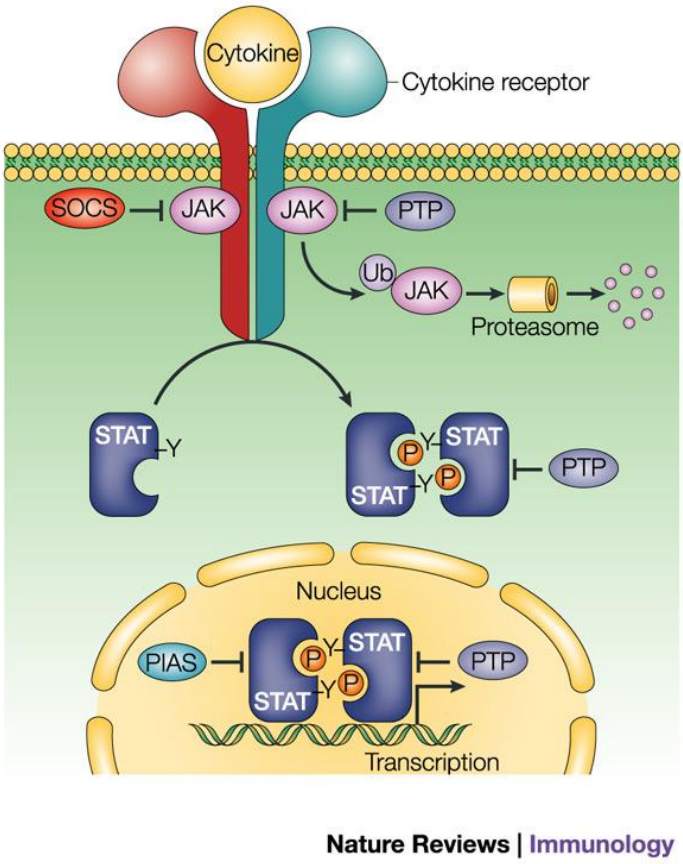

Figure iii: Regulation of JAK-STAT signaling in the immune system.[17]

While Stat3 has already been utilized as a potential target of cancer treatments [14], there is still a need to elucidate downstream pathways regulated by all Stat proteins, including Stat3. This is necessary in order to understand the role of Stat proteins in oncogenesis and tumor initiation. Moreover, the relationship between Stat gene signaling pathways and those of other transcription factors such as E2fs, and c-Myc, and FoxM1 is still unknown and necessitates investigation.

The database: what, why, and how

Database web sites for identifying target genes already exist but the criteria used to define them are unclear and often unknown to the user. Also, most of these sites are not openly available to the scientific community and are quickly outdated. As a result, the information

offered by these sites is limited and unconvincing to most researchers. The proposed project presents a clear and precise way to classify and understand ‘gene targets’ by taking advantage of the whole publicly available literature.

Our database assimilates large amounts of information from all published articles pertaining to specific transcription factors and presents it in a consistent format. Before the data is exported to the database, it is first analyzed in-depth and formatted. The goal is to have standardized data, taken from credible sources, with comparable parameters. These parameters are indexed in such a way that multiple types of searches result in the most relevant results and details being displayed. We have also implemented a ranking algorithm that lists the results from most relevant to more generic entries corresponding to the search.

Transcription factors act differently in different environmental conditions. For example, their activity varies depending on the species and tissues. Such specific information about regulation is often undocumented in database sites. The lack of tissue/cell-type specific information is one reason why pathway misregulation in the context of Rb is still not understood. Since transcription regulation is highly tissue specific, the classification of transcription family members, experimental species, tissues, and cell lines used in experiments can improve researchers’ understanding of site-specific cancers.

In our database, experiment variables are taken into consideration while defining genes. For each gene listed, the database provides the user with the source of the publication, transcription factor family member, species, tissue/cell line, type of experiment, number of experimental replicates, experimental control, quality/fold change, type of regulation, and figure number. We have also integrated an “alias match” which would pull up entries listed under a gene “alias” when searching for a specific target gene.

Moreover, there is a need to differentiate between direct and indirect targets due to the fact that direct targets can regulate indirect target genes through transcription or other mechanisms. Due to the need for such differentiation, the database has an added functionality that yields a confident direct target gene list for each specific transcription factor family member queried by the user. The classification is based on the confidence score of the gene, which is in turn based on score values assigned to the different experiments and other criteria used to identify and evaluate target genes, including experimental replicates and the number of available papers specific to the gene of interest. The user can query on any of the parameters and even sort the results based on gene confidence score, experiment strength, and fold change.

With such detailed and reliable information, cancer researchers will likely turn their attention to this database to seek information on direct targets of their favorite transcription factors being studied. Researchers can utilize this database to confirm their own data and make educated predictions for future experiments. By making this site publicly available, cancer researchers will also be inclined to initiate new research avenues and return information to the online site developed here that pertain to their new research findings.

The basis of cancer is genetics and while this research area is advancing at a rapid pace, the mechanisms involved in cancer initiation and progression have lagged behind. This project will not only provide insights into the genetic basis of cancer but also lead to the identification of potential targets of therapeutic cancer treatments. Identification of the genes involved in pathways is a preliminary step in understanding cancer development and proliferation. However, such identification will also be of use to all researchers since a complete understanding of the pathways and mechanisms within a cell is yet to be attained. Therefore, this database aims to supply knowledge that is applicable to all fields of biological research.

Supporting Data

The information in the database necessitates validation. And so, we have compared data sets with each other to not only ensure that the data is standardized but to also elucidate any similarities or differences between gene-signaling pathways of different transcription factors. Currently, all four transcription factors share 43 common target genes. E2f123 and c-Myc share 336 common targets. Additionally, we have compared multiple microarray data sets including one from Rb KO mouse intestinal epithelial cells with the E2f data set and another from colorectal cancer (CRC) patients with all transcription factor data sets. Comparison of the misregulated genes in the tumor sample with the confident gene targets of all four transcription factors showed that a majority of the confident target genes were, in fact, misregulated in the colorectal cancer tumors.

Materials and Methods

Database content:

Specific information was chosen to be listed on the database including: gene name, transcription factors family member, PubMed ID, species, tissue/cell line, experiment, control, replicates, quality/fold change, regulation, and figure number.

Experiments chosen for analysis:

The following assays were chosen and scored to determine and annotate the likelihood of a gene being a direct transcriptional target: Nuclear Run-on Assay[18], Chromatin immunoprecipitation coupled with real-time PCR (ChIP-PCR)[19], ChIP-seq[20], ChIP-chip[21], ChIP[22], ChIP-re-ChIP[23], Electromobility Shift Assay (EMSA)[24], qPCR[25], Microarray expression platforms (Affymetrix), Reporter Gene Assay[26], Reverse transcriptase PCR (RT-PCR)[27], Northern Blot[28], Reverse Northern Blot, RNase Protection Assay (RPA)[29], ELISA[30], and Western Blot[31].

Experiment Scoring:

The experiments were divided into two groups: binding and expression. Therefore, they were scored separately. By analysis and collaboration with researchers, we have developed a scoring matrix for all experiments featured in our database. The confidence score associated with each experiment represents the degree of confidence that we associate with a gene if it is labeled as a direct target gene by that experiment. Each experiment received a score out of 1 based on its methodology and sensitivity to fold change. Binding experiments: ChIP-seq (0.8), ChIP (0.8), ChIP-re-ChIP (0.6), EMSA (0.4). Expression experiments: Nuclear Run-on Assay (1), ChIP-PCR (.8), ChIP-chip (0.7), Reporter Gene Assay (0.6), qPCR (0.6), Microarray (0.6), RPA (0.5),

RT-PCR (0.5), Northern Blot (0.5), Reverse Northern Blot (0.5), ELISA (0.4), and Western Blot (0.4).

Analysis of published articles:

Published articles pertaining to each transcription factor were obtained mostly through PubMed. The experiments presented in the papers were analyzed and the results organized and recorded using a spreadsheet. Microarray soft files were obtained from GEO Data Sets and analyzed. The information was then exported onto the database.

Confident potential target gene list:

The expression experiments (each entry) were filtered so only those that yielded a fold change of |fold change>1.5| were chosen. Each row was assigned a raw score, which is the confidence score of the experiment associated with that row. Then an average score of each gene in the data set was obtained. The genes were then filtered so that only those that yielded an average score value > 0.5 and those that appear more than once in the data set were labeled as confident targets genes. By choosing only genes that appear at least twice in a dataset, we are ensuring that multiple variables and not just fold change contribute to the confidence of the target gene list.

Results

A sample image of the beta prototype database is shown in Figure 1. Currently, we have entries for 349 unique published articles for E2fs, 234 for Myc, 160 for Stat proteins, and 84 for FoxM1 on the database. Pathway overlap between the target gene lists of all four transcription factors has shown that there are 43 common genes (Figure 2A, B). There are 1636 unique gene entries for E2fs, 4973 for Myc (c-Myc, N-Myc, and L-Myc), 1951 for FoxM1, and 1272 for Stat proteins. Each transcription factor family also has a different number of experiment types based on the available publications (Figure 3).

An ongoing project in the Leone lab will utilize this web-based program to identify transcriptional targets of E2F and Myc during intestinal development and cancer. Deletion of E2F1-3 or c-Myc in the small intestine can rescue the over-proliferation of intestinal cells in an Rb-deficient gut by preventing the misregulation of hundreds of genes. Simultaneous deletion of E2F1-3 and c-Myc disrupts the structure in villi by misregulation of gene expression. Microarrays have suggested that the transcriptional pathways of both E2F1-3 and c-Myc are distinct, but may have significant overlap. The expectation is that E2F and c-Myc will co-bind and co-regulate key G2/M cell cycle related target genes such as Ccna2, Cdc20, and Ccnb1 as well as G1/S genes including Pcna and Cdc6, explaining how these two transcription factor pathways may be regulated by a single tumor suppressor protein (Rb). Overlap between 2945 E2f123 targets and 7570 c-Myc targets shows 1417 targets which are common to both (Figure 4). Many of the 1417 common targets are key cell cycle regulators.

Supporting E2f data using Rb KO expression data:

Rb is a known direct regulator of E2f in the cell. Previous studies[32] have shown that when Rb is knocked-out, E2f and E2f gene targets are misregulated. Therefore, we performed

microarray analysis of mouse intestinal epithelial cells with Rb knockout to obtain data sets on misregulated E2f target genes. Microarray analysis revealed 701 misregulated genes with a |fold change > 1.5| and a p value < 0.05. Comparison of these misregulated genes with the confident targets genes of E2f showed that 170 out of 851 targets genes were commonly misregulated. We also compared the list of these misregulated genes with the list of confident targets genes of Myc and found that 235 out of 2790 targets genes were commonly misregulated.

Tumor Microarray:

Microarray analysis of colorectal cancer tissue (CRC) and normal tissue from human patients[33] has yielded 6293 misregulated genes with a |fold change > 1.5| and a p value < 0.05. We compared these misregulated genes to the genes in the confident target gene list of the four transcription factors. The confident target gene list was initially filtered so only overexpression data sets were taken into consideration. Comparison showed that 327 out 841 E2f targets, 1149 out of 2790 c-Myc targets, and 24 out of 67 FoxM1 targets were commonly misregulated. None of the 17 targets of Stat proteins were misregulated in the cancer tissue.

Discussion

The purpose of our transcription factor database is to elucidate commonly misregulated pathways in cancer through the identification of direct transcriptional target genes of transcription factors. Our database employs significant criteria such as scoring to define a gene as a confident direct target. In addition to this, our database is the first of its kind to use all of the publicly available information pertaining to specific transcription factors, analyze the information, and present it in a user friendly format. Based on the current mass of data it is difficult to determine pathway overlap between all four transcription factors simultaneously. However, certain transcription factors such as E2f123 and c-Myc do share many direct targets, suggesting possible coregulation of genes and similar signaling pathways within the cell. With the future influx of data including large data sets such as microarrays, the pathway overlap between E2fs and c-Myc will likely become clearer.

To gain a better understanding of the signaling pathways of both E2fs and c-Myc under the context of Rb, we compared their confident target genes with misregulated genes in an Rb knockout environment. Comparison has shown that only a portion of the E2fs and c-Myc targets are misregulated in the Rb knockout environment. The overlap supports a portion of confident targets genes on the database. On the other hand, the lack of overlap could tell us something about specificity of gene signaling in such an environment. While the complete signaling pathways between Rb, E2fs, and c-Myc are still unclear, the valid trends presented in that database can be used to support and characterize the relationship between the tumor suppressor and both transcription factors.

Because E2fs, c-Myc, FoxM1, and Stat are known to be upregulated in most cancers, we compared the confident target gene list only from overexpression data to the list of misregulated

genes in the tumor sample. As expected, we found that many of the confident direct targets of E2fs, c-Myc, and FoxM1 were misregulated in the tumor samples. However, the 17 confident targets of Stat proteins were not misregulated in the CRC tissue samples. The lack of sufficient data on Stat proteins could be the primary reason why there is no overlap between genes. Another possibility is that Stat proteins and Stat signaling pathways have higher tissue specificity than expected. Nevertheless, the comparison partially supports the data presented in the database. By supporting the data sets on the database and by continually updating the information, researchers can be sure that the information presented is significant and reliable. And so, we believe that cancer researchers will likely turn to our database for the latest information of direct targets genes. In the future, we hope to study and provide data on many more cancer-specific transcription factors in order to better understand the complexities of cancer.

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Figure Legends

Figure 1: A screenshot of the current beta prototype database website.

Figure 2: (A) The data presented in this figure takes into account all gene entries regardless of experimental variables and confidence score. (B) The 43 gene targets are not confident gene targets.

Figure 3: The microarray number has been cut off for all transcription factors. Actual values are: 4589 (E2f), 12334 (c-Myc), 2404 (Stat), and 3473 (FoxM1).

Figure 4: Only overexpression data is taken into account. Diagram does not take into consideration confident target gene list.

Figures

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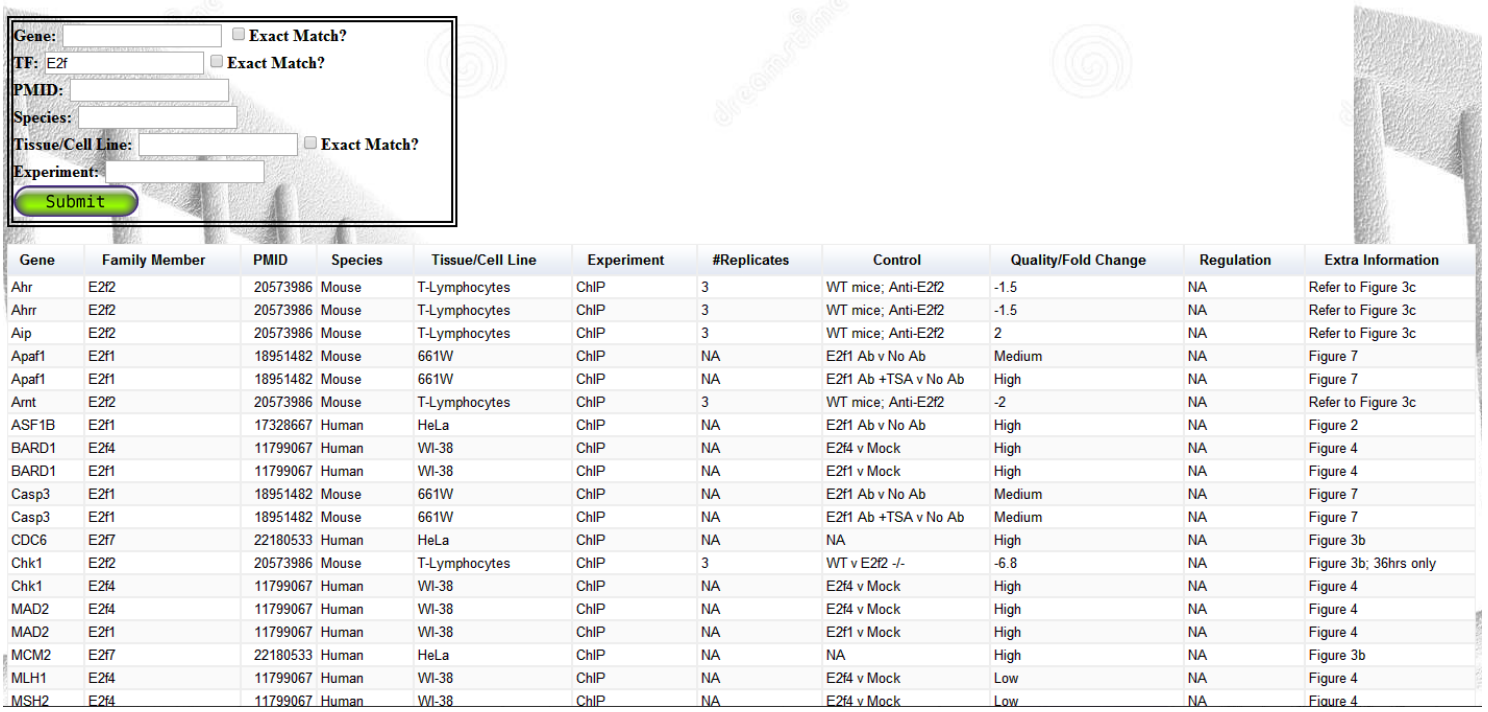

Gene	Family Member	PMID	Species	Tissue/Cell Line	Experiment	#Replicates	Control	Quality/Fold Change	Regulation	Extra Information
Ahr	E2f2	20573986	Mouse	T-Lymphocytes	ChIP	3	WT mice; Anti-E2f2	-1.5	NA	Refer to Figure 3c
Ahrr	E2f2	20573986	Mouse	T-Lymphocytes	ChIP	3	WT mice; Anti-E2f2	-1.5	NA	Refer to Figure 3c
Aip	E2f2	20573986	Mouse	T-Lymphocytes	ChIP	3	WT mice; Anti-E2f2	2	NA	Refer to Figure 3c
Apaf1	E2f1	18951482	Mouse	661W	ChIP	NA	E2f1 Ab v No Ab	Medium	NA	Figure 7
Apaf1	E2f1	18951482	Mouse	661W	ChIP	NA	E2f1 Ab +TSA v No Ab	High	NA	Figure 7
Arnt	E2f2	20573986	Mouse	T-Lymphocytes	ChIP	3	WT mice; Anti-E2f2	-2	NA	Refer to Figure 3c
ASF1B	E2f1	17328667	Human	HeLa	ChIP	NA	E2f1 Ab v No Ab	High	NA	Figure 2
BARD1	E2f4	11799067	Human	WI-38	ChIP	NA	E2f4 v Mock	High	NA	Figure 4
BARD1	E2f1	11799067	Human	WI-38	ChIP	NA	E2f1 v Mock	High	NA	Figure 4
Casp3	E2f1	18951482	Mouse	661W	ChIP	NA	E2f1 Ab v No Ab	Medium	NA	Figure 7
Casp3	E2f1	18951482	Mouse	661W	ChIP	NA	E2f1 Ab +TSA v No Ab	Medium	NA	Figure 7
CDC6	E2f7	22180533	Human	HeLa	ChIP	NA	NA	High	NA	Figure 3b
Chk1	E2f2	20573986	Mouse	T-Lymphocytes	ChIP	3	WT v E2f2 -/-	-6.8	NA	Figure 3b; 36hrs only
Chk1	E2f4	11799067	Human	WI-38	ChIP	NA	E2f4 v Mock	High	NA	Figure 4
MAD2	E2f4	11799067	Human	WI-38	ChIP	NA	E2f4 v Mock	High	NA	Figure 4
MAD2	E2f1	11799067	Human	WI-38	ChIP	NA	E2f1 v Mock	High	NA	Figure 4
MCM2	E2f7	22180533	Human	HeLa	ChIP	NA	NA	High	NA	Figure 3b
MLH1	E2f4	11799067	Human	WI-38	ChIP	NA	E2f4 v Mock	Low	NA	Figure 4
MSH2	E2f4	11799067	Human	WI-38	ChIP	NA	E2f4 v Mock	Low	NA	Figure 4

Figure 1: Sample image of the beta prototype database website.

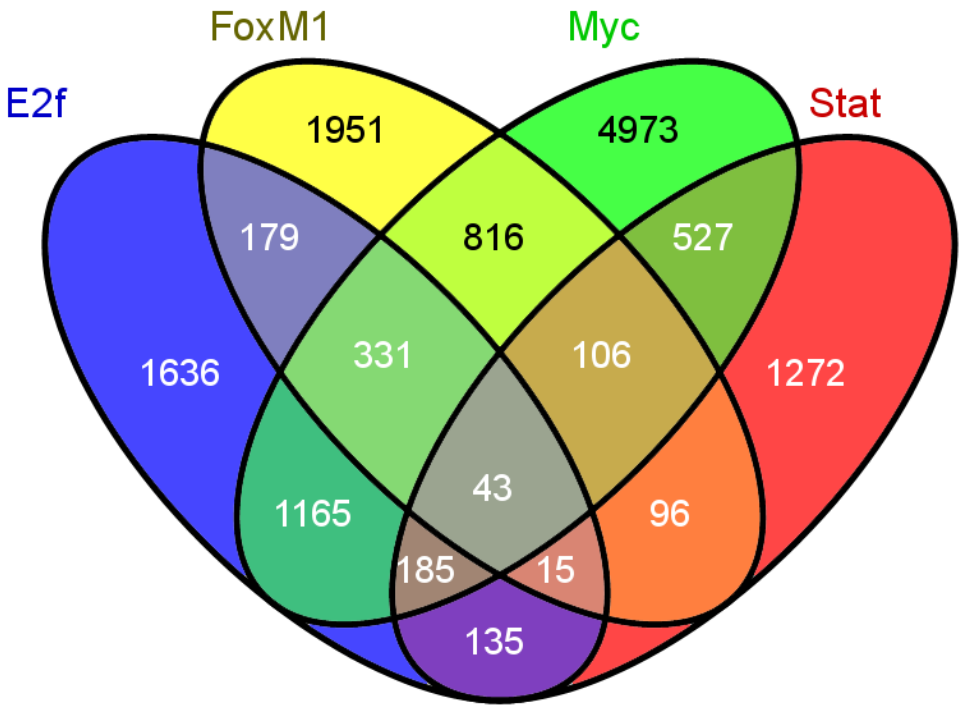

Figure 2A: Target gene overlap between transcription factors E2fs, Myc, FoxM1, and Stat proteins.

CASP3	HMMR	CD44	SOX2	WWC1	CCND1	P27	DDX10
CDC25A	CALM2	DCLRE1A	PRKD3	ABCB7	ZMYND8	P53	
MYC	MMP16	KIF23	NOL11	AP1G1	KIF2A	MDM2	
RECQL	TPP2	CSRP2	SIRPA	APCDD1	RBL1	CCR2	
C-MYC	UPA	NUP160	TBC1D15	IL1RN	PIK3CA	DLGAP5	
P21	PLK4	FOXM1	UBR7	IGF1	NOLC1	NUPL1	

Figure 2B: 43 gene targets common to transcription factors E2fs, Myc, FoxM1, and Stat proteins.

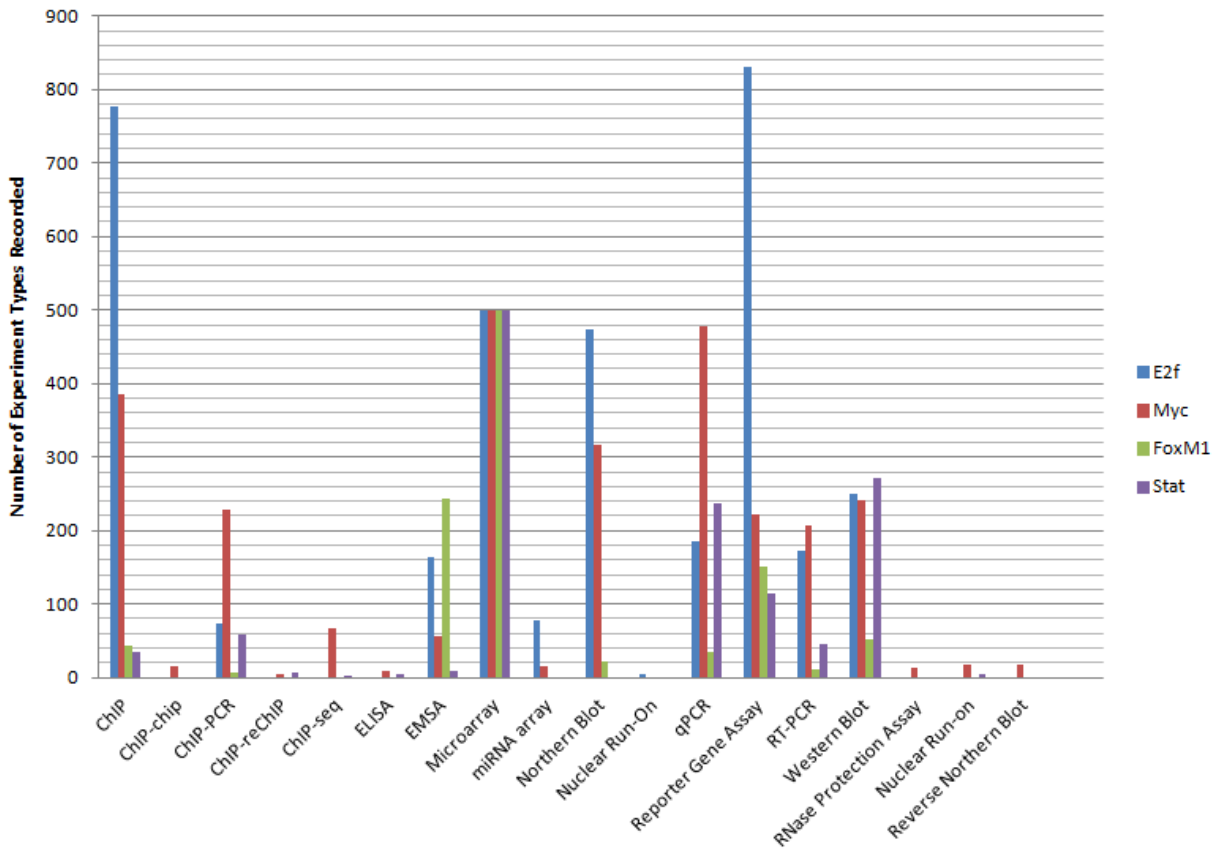

Figure 3: Number of different experiment types per transcription factor family.

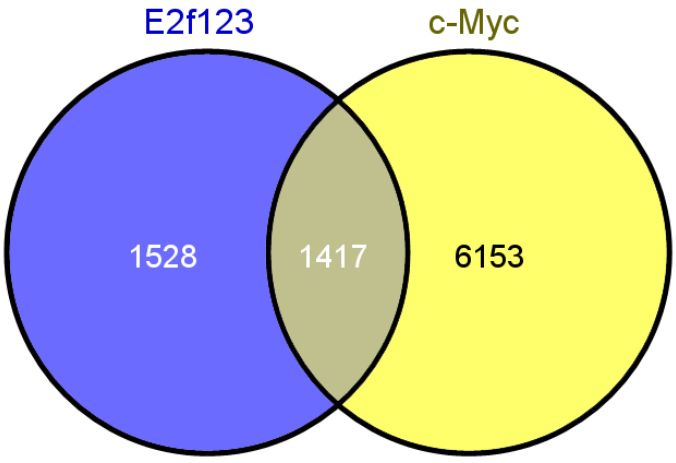

Figure 4: Target gene overlap between transcription factors E2f1,2,3 and c-Myc.

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