Although genome-wide proteomic approaches are rapidly improving, the most widely available and cost-effective genome-wide expression data is still collected at the mRNA level. These experiments are carried out using either microarrays or next-generation sequencing. The idea is to first infer the most significant transcription factors, protein complexes and putative protein kinases that are likely responsible for the observed changes in genome-wide gene expression. By combining data from chromatin immuno-precipitation-exome sequencing (ChIP-seq/chip) and/or position weight matrices (PWMs), protein–protein interactions and kinase–substrate phosphorylation reactions, we demonstrate how we can identify regulatory mechanisms responsible for genome-wide changes in gene expression. The idea is to first infer the most likely transcription factors that regulate the differences in gene expression, then use protein–protein interactions to connect the identified transcription factors using additional proteins to build transcriptional regulatory subnetworks centered on these factors and finally use kinase–substrate protein phosphorylation reactions to identify and rank candidate protein kinases that most likely regulate the formation of the identified transcriptional complexes.
As an alternative to ChEA, we used TRANSFAC (Matys et al., 2006) and JASPAR (Portaלה-Casamar et al., 2007) listing putative target genes for each transcription factor for human or mouse by scanning the promoter sequences. We kept all individual entries from both databases even though for some transcription factors there are more than one PWM. For JASPAR we used the JASPAR Core.

The ChEA or the GMT file created from TRANSFAC and JASPAR were used to analyze lists from mRNA expression profiling by performing gene-list enrichment analysis with the Fisher’s exact test using the ChEA or the PWMs dataset as the prior biological knowledge gene-list library. ChEA and PWMs each have their own advantages and disadvantages. ChEA is created from empirical observations in different cell types and conditions. On one hand, ChEA considers the chromatin state of the cell under a specific condition, which is not done by PWMs and may produce more specific overlapping genes with fewer false positives. However, the ChEA approach may miss hits for transcription factors if the examined expression is derived from completely different cell types or the transcription factor is missing from ChEA. Another advantage of the PWM GMT library is that it provides more coverage for factors. For example, TRANSFAC contains 830 mouse and 1113 human matrices for about ∼300 transcription factors, whereas the ChEA database currently only has 159 factors.

2 METHODS

2.1 Identifying differentially expressed genes

The first step of the X2K computational approach is a standard procedure where differentially expressed genes (mRNAs) are identified. Such sets of genes can originate from experiments that profiled cells under different conditions, during different stages of differentiation, from tissues of different patients or different cell-lines. The identified sets of differentially expressed genes can then be grouped into up or down subgroups, clusters of genes that behave similarly across different perturbations, or gene modules that behave similarly over a time course. The outputs from such analyses produce sets of unranked lists of genes. For microarray experiments we performed here, MA5.0−processed data from the gene expression omnibus (GEO) database was used. Quantile normalization was then required for cross-assay comparisons. Following normalization, differentially expressed genes were identified via the R statistical package LIMMA (Smyth, 2004).

2.2 Identifying upstream transcription factors

Once sets of differentially expressed genes are identified, these gene lists can be fed into the transcription factor inference module of X2K using the tool and database ChIP-seq/chip Enrichment Analysis (ChEA) (Lachmann et al., 2010) or PWMs to obtain a list of transcription factors that are the likely upstream regulators of the identified differentially expressed gene set. The ChEA database and software contains manually extracted results of transcription factor/target-gene interactions from ChIP-seq/chip experiments applied to human or mouse cells. This database currently contains a network of 361 299 interactions, manually extracted from 157 publications, describing the binding of 159 transcription factors to their target genes with fewer false positives. However, the ChEA approach may miss hits for transcription factors if the examined expression is derived from completely different cell types or the transcription factor is missing from ChEA. Another advantage of the PWM GMT library is that it provides more coverage for factors. For example, TRANSFAC contains 830 mouse and 1113 human matrices for about ∼300 transcription factors, whereas the ChEA database currently only has 159 factors.

2.3 Connecting transcription factors with protein–protein interactions

Most analyses that attempt to link gene expression changes to upstream regulators stop at the step of promoter analysis, or attempt to infer pathways directly from differentially expressed genes. However, X2K further ‘connects’ the identified transcription factors using networks of experimentally reported protein–protein interactions or protein complexes. Genes2Networks (G2N) is command-line and web-based software that we developed in the past to connect lists of mammalian genes/proteins in the context of background mammalian signalome and interactome protein networks (Berger et al., 2007). The background protein–protein interactions network we use in X2K is made of experimentally determined mammalian interactions collected from 18 databases/datasets and currently contains 24 036 proteins connected through 389 959 interactions. The input to the program is a list of human Entrez gene symbols and background protein interaction networks, while the output is a subnetwork made of ‘intermediate’ proteins that ‘connect’ the ‘seed’ list of genes/proteins. This is achieved by finding all shortest paths between all pairs of seed nodes with a specified maximum path length and then adding additional interactions between intermediates. Different settings allow for filtering interactions from background networks by limiting the number of interactions from a specific protein, each type of interactions or only including interactions that are reported more than once. Once transcription factor-centered complexes upstream of differentially expressed gene modules are identified, using the G2N module of X2K, we identify the protein kinases that are most likely responsible for the transcription factor complexes’ formation and functional regulation.

2.4 Identifying protein kinases upstream of transcriptional complexes

Once we build a subnetwork/protein complex that connects the identified transcription factors to each other, we convert this subnetwork to a list of proteins and feed it as input to the Kinase Enrichment Analysis (KEA) (Lachmann and Ma’ayan, 2009) module of X2K. KEA is web-based and command-line software with an underlying database that provides users with the ability to link lists of mammalian proteins with the protein kinases that likely phosphorylate them. The system draws from several available kinase–substrate databases to compute kinase enrichment probability based on the distribution of kinase–substrate proportions in the background kinase–substrate database compared with the protein kinases found to be associated with an input list of proteins using the Fisher’s exact test. Using information available in the public domain, we reconstructed a mammalian kinase–substrate network. The kinase–substrate interactions are from the

annotated genes for these two organisms. The program Pathway, provided by TRANSFAC, was used to scan promoter sequences. We kept all individual entries from both databases even though for some transcription factors there are more than one PWM. For JASPAR we used the JASPAR Core.

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2.5 The X2K software

All together, starting from a set of differentially expressed genes, we end up with protein kinases, transcription factors and protein complexes that are putative regulators of the inputted differentially expressed genes. The X2K system was developed as an open source Java desktop application and is available at http://www.maayanlab.net/X2K with documentation. The underlying code for X2K was developed using the Java 6 SDK under the Eclipse IDE. Using an Apache Maven build process, command-line and Swing GUI versions are packed into an executable JAR with all the necessary background files included. User can unpack the JAR to access the background files and network files in different formats, including networks that can be visualized with Cytoscape (Shannon et al., 2003), SNAVI (Ma’ayan et al., 2009), Pajek, or yEd. User manual is available as supporting materials.

3 RESULTS

3.1 Application of X2K to recover drug-targeted pathways from gene expression signatures

To demonstrate how X2K can be used to infer upstream regulators given gene expression changes, we first applied the tool to analyze expression data from the Connectivity Map (CMAP) (Lamb et al., 2006). The CMAP database developed by the Broad Institute is a large dataset of mRNA microarray gene expression profiles made from experiments where four different types of human cancer cell lines were treated with many single FDA-approved drugs and then gene expression was measured after 6 hours. CMAP contains 6100 perturbations with 1309 single drugs, where compounds were applied in different concentrations, to different cell types, or other variable experimental conditions. Using CMAP, we examine if the known drug target proteins fall within the subnetworks created by the intermediate steps of X2K. We omitted G-protein coupled receptors (GPCR) targeting drugs because X2K is not designed to recover those. First, we extracted the top 500 upregulated and bottom 500 downregulated genes from CMAP for each drug perturbation experiment based on the ranked gene lists provided for download from the CMAP website. We then entered these lists as input into the X2K pipeline. Once we collected all the transcription factors, protein complexes and protein kinases based on gene expression changes induced by the different drug perturbations, using the default settings of X2K, we asked whether the genes/proteins appearing in these pathways are enriched in known drug targets reported in DrugBank.

We show that ∼15–17% of the time we can recover the drug target in pathways created by X2K using ChEA or TRANSFAC/JASPAR. The TRANSFAC/JASPAR option is slightly better in recovering targets as compared with ChEA. Interestingly, targets can be recovered directly within the differentially expressed genes better than by chance but with much less recall and specificity as compared with X2K. Having targets appearing in differentially expressed genes more than by chance was previously reported by Iskar et al. (2010), which is consistent with our findings. In addition, targets can be found in pathways constructed directly from the differentially expressed genes, but this procedure too has less recall and specificity as compared with X2K. Other statistical controls show that targets can be found in randomly generated gene lists of 500 human genes ∼2–3% of the time, and in pathways created from randomly generated lists of genes ∼7% of the time. Hence, X2K is capable of recovering drug targets from gene expression better than other methods. More parameter tuning, as well as expansion and improvement of the databases quality and coverage used by X2K are expected to improve performance. This is reserved to future studies.

3.2 Application of X2K to obtain a global view of cellular differentiation

The X2K method can be applied globally to map the putative upstream ‘regulatory state’ of mammalian cells by comparing and contrasting the subnetworks generated by the program across different cell types and cell states. Our hypothesis is that given a set of samples from genome-wide expression data across many cell types and experimental conditions, we can correctly infer the activity patterns of the upstream transcription factors and protein kinases across samples to obtain a global picture of cell regulation across multiple regulatory layers (Supplementary Fig. S1). Such activity patterns can be approximated by enrichment analyses applied to the weighted expression of differentially expressed gene modules. This approach can also be used to validate whether X2K is identifying a set of transcription factors and protein kinases that are unique to specific cell types and experimental conditions. Developing an initial approach to achieve this goal, we first analyzed 44 samples...
from genome-wide expression data collected from embryonic stem cells induced to differentiate toward different lineages as well as several other terminal cell types all collected and previously analyzed by other studies (Aiba et al., 2009). The gene expression data matrix was subjected to an iterative consensus agglomerative clustering algorithm with within-module-coherence threshold of 0.7 and merging threshold set to 0.8 (Qu et al., 2011). As a result, 300 expression modules were identified, but only 49 modules had a hundred or more genes, and these modules were retained for further analysis. Upstream transcription factors enriched for each module were computed using CMA. An enrichment significance matrix $M$ was then generated with entries $m_{ij}$ representing the -log10(p-value) of the enriched transcription factor $j$ for module $k$. A pseudo activity matrix $P$ was then generated with $p_{ij}$ representing pseudo activity for transcription factor $j$ in sample $i$ calculated as follows: $p_{ij} = \max(m_{ij}) \times a_{ij} \times e_{ij} \times i_{ij}$ where $a_{ij}$ is the mean expression of module $k$ in sample $i$, $e_{ij}$ is the expression level of the transcription factor and $i_{ij}$ is a Boolean indicator function that checks if the transcription factor is expressed above average in the sample. Hence, the pseudo activity $p_{ij}$ is composed of the binding score for the transcription factor, the average mRNA expression of the regulated module and the expression level of the transcription factor in the sample (Supplementary Fig. S1). To visualize the preservation of ordering of the samples across regulatory layers for the 44 cell types and conditions, we implemented four data visualization methods: (i) Principle Component Analysis (PCA) (Supplementary Fig. S2); (ii) Minimum Spanning Trees (MST), implemented with a modified script based on the recently published sample discovery (SPD) package (Qu et al., 2011) (Supplementary Fig. S3); (iii) hierarchical clustering (Supplementary Figs S4 and S5); and (iv) our Grid Analysis of Time-series Expression (GATE) software (MacArthur et al., 2010), repurposed to have each hexagon representing a cell type (Fig. 3).

The GATE software takes as input a data table, where rows are variables and columns are measurements. The software uses simulated annealing to arrange variables on a hexagonal grid based on correlations between variables across all measurements. In our case, the variables are cell types or tumor samples, and the columns, representing measurements, are inferred pseudo-activity levels of transcription factors and protein kinases. Similar to the way we compute pseudo-activity for transcription factors, we can identify the upstream protein kinases enriched for each module using the command-line version of X2K and the same steps performed for the transcription factors. Consequently, by using the upstream regulatory transcription factors and protein kinases activity patterns, the landscape of samples can be correctly time-ordered and samples of the same subtype are closer to each other than to other subtypes. To test whether the preserved ordering of samples across regulatory layers arises by chance, we applied the same procedure to shuffled data (Supplementary Fig. S6). We quantified the preservation of the ordering by an objective error function, counting the times neighbors of each node are preserved, and clearly saw that the ordering is far from random ($P$-value <10$^{-15}$, two-tailed t-test for both transcription factors and protein kinases). Enriched transcription factors with relatively high predicted activity in the pluripotent stem cells are generally known factors such as Oct4/Pou5f1, Nanog and Sox2. In comparison, enriched lineage commitment regulators are predicted to be active in more differentiated cell types. For example, Gata4 is a known master regulator for the endoderm lineage and correspondingly displays high pseudo activity scores in late-stage endoderm cells. These results can be used to characterize the upstream regulatory profile of the 44 different cell types. This approach can be used to tune the parameters and datasets used by X2K to validate the approach by setting the thresholds that best preserve the ordering of samples and recovering the already known transcription factors and protein kinases for cell types.

3.3 Application of X2K to unravel regulatory mechanisms of subtypes of breast cancer

The inherent inter-patient heterogeneity of breast cancer motivates the identification of unique molecular signatures of the disease at the individual patient level. The ability to identify molecular regulatory differences at the genome, transcriptome, gene-regulome and kinase levels for particular cancer subtypes may enable us to better tailor and optimize therapeutics for individual patients. To achieve this, we illustrate the utility of X2K to uncover putative upstream regulatory mechanisms from previously published gene expression data collected from a large cohort of breast cancer tumors. We show that subtype similarities in gene expression can be grouped and visualized based on the pseudo-activity scores of upstream transcription factors and protein kinases that likely regulate differentially expressed genes in the breast cancer subtypes. Specifically, X2K was applied to analyze a publicly available breast cancer gene expression dataset from fresh frozen tissues of 327 patients that were randomly selected from a group of diagnosed individuals between 1991 and 2004 at the Koo Foundation Sun-Yat-Sen Cancer Center (Kao et al., 2011). In the original study, the cancer tissues were categorized into six subtypes based on differential gene expression signatures. Based on OncotypeDX (Paik et al., 2004) and MammaPrint (van’t Veer et al., 2002) signatures, the risk for
Indeed, the TGF-β (SMAD4), are predicted to be upregulated in the high-risk subtypes. With identified enrichment for NANOG and SOX2, the high risk to be downregulated in many cancers (Raaphorst, 2005). Together with probe-set consolidation, hierarchical clustering, and gene co-expression module identification. X2K was applied to co-expressed modules, where each module consisted of a list of genes whose expression profiles correlated across all patients. The detailed procedure and parameters are similar to those applied above for analyzing the 44 stem cells and their differentiated progeny. The hexagonal grid folds on itself to form a torus such that hexagons at the edges are close to hexagons from the opposite side.

3.4 Application of X2K for reanalyzing expression data collected from hippocampal neurons and activated hepatic stellate cells in liver fibrosis

Lastly, in two additional case studies, we applied the X2K approach to analyze data from prior studies that applied microarray genome-wide gene expression analyses to investigate two commonly studied mammalian systems: (i) investigating differences in gene-expression profiles collected from hepatic stellate cells (HSCs) in liver fibrosis; and (ii) detecting upstream regulatory pathways by reanalyzing microarray data collected from hippocampal neurons treated with bicuculline during development. For the HSCs case study, we reanalyzed gene expression profiles to investigate regulatory mechanisms of hepatic fibrosis. Hepatic fibrosis is a scar-forming response to liver damage often due to chronic liver disease. In fibrogenesis, HSCs become activated and differentiate into extra-cellular-matrix-producing myofibroblasts. To better understand the gene expression changes that occur during such a process, De Minicis et al. (2007) conducted a microarray study to examine differences in gene expression profiles between cultured and in vivo-activated HSCs. Using the study as a source of microarray data for X2K, we identified the putative upstream transcription factors, intermediate proteins and protein kinases that may regulate the fibrosis response of HSCs. The transcription factor Tcf3 was predicted as a top candidate and this is supported by studies investigating the anti-fibrogenic role of Wnt signaling in the pro-fibrogenic response, as a loss of adipogenic transcriptional regulation has been shown to be important for HSC activation (She et al., 2005). Among the predicted kinases are members of the ribosomal s6 kinase (RSK) family of serine/threonine kinases that can phosphorylate C/EBPbeta, an adipogenic transcription factor known to regulate collagen type I expression. RSK-mediated phosphorylation of C/EBPbeta at Thr217 appears to be crucial for the progression of fibrosis; Rsk inhibition led to regression of fibrosis in CCI4-treated mice, and increased activation of RSK and phosphorylated C/EBPbeta both were found in activated HSCs of liver fibrosis patients (Buck and Chojker, 2007). Hence, it appears that X2K was able to correctly identify known upstream regulators based on the differentially expressed gene alone.
For the next case study, we reanalyze data relevant to long-term potentiation (LTP). Zhang et al. (2007) examined gene expression changes in neonatal mouse hippocampal neurons undergoing induction of rhythmic network activity. Reanalysis of this data using X2K has recaptured the transcription factor CREB and the protein CaMK4 as important upstream regulators. Activation of CREB is heavily implicated in the literature (Hardingham et al., 1999). In addition, the calmodulin-dependent kinase (CamK4) was also recovered from the X2K analysis. The link between CamK4 and CREB dependent transcription is well established (Matthews et al., 1994; Sun et al., 1994). Following this link to N-Methyl-D-aspartic acid (NMDA) receptor activation is clearly through calcium signaling. It was shown that CamK4 activation is important for different forms of LTP that depend on NMDA receptor activation (Kang et al., 2001). Activity-dependent increases in intracellular calcium, likely through voltage-gated calcium channels, affect increases in nuclear calcium where CamK4 is preferentially localized. Hence, the X2K pipeline is capable of recovering known pathways and likely predicting pathways not known to be involved before. More details about the two case studies from this section are available as Supplementary Material and as part of the X2K online documentation.

4 DISCUSSION

The X2K pipeline presents a new rational approach to identify and rank upstream regulators that are responsible for observed changes in gene expression collected at the genome-wide scale from mammalian cells. The approach, applied to datasets such as CMAP, has the potential to rapidly advance drug target discovery and help in unraveling drug mechanisms of action. The application to mapping transcription factor profiles and kinase profiles of many individual cell types, i.e. different cells during lineage commitment or tumors from patients, can be used to obtain a global view of the axis of cell signaling networks across many cell types or to compare individual patients for suggesting appropriate pharmacological interventions.

In addition, specific applications to common studies that examine genome-wide gene expression under two conditions, such as the two case studies we presented for HSCs and hippocampal neurons, can benefit from X2K analysis for generating hypotheses for further functional experiments following the global expression profiling.

While currently the X2K method uses only protein/DNA interactions, protein-protein interactions and kinase-substrate reactions, other types of data could be added. For example, histone modifications, microRNAs and other types of post-translational modifications could be incorporated into the pipeline. While more sophisticated enrichment analyses tests could be implemented, i.e. gene set enrichment analysis (GSEA) (Subramanian et al., 2005), and better parameter tuning can be achieved by cross-validation, the initial application of the approach shows great promise. The X2K approach is useful for data integration across layers and the reuse of prior knowledge within newly acquired expression datasets linking expression changes to upstream regulation. Another limitation of the method is the assumption of independence between regulators and targets when applying the ChEA or KEA steps. It is known that the kinase and transcriptional regulome networks are made of tightly coupled protein kinases regulating other kinases and transcription factors regulating other transcription factors. Several recent studies considered such interactions for transcription factors (Asif and Sanguinetti, 2011; Novershtern et al., 2011). Such interdependencies could be added to the X2K analysis where these two regulatory networks could be dynamically modeled. Regardless of these limitations and future directions, the current application of X2K presents an advancement toward our ultimate goal of understanding mammalian cell signaling networks from a global perspective at a molecular level of resolution.

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