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Generation of mouse ES cell lines engineered for the forced induction of transcription factors

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Here we report the generation and characterization of 84 mouse ES cell lines with doxycycline-controllable transcription factors (TFs) which, together with the previous 53 lines, cover 7–10% of all TFs encoded in the mouse genome. Global gene expression profiles of all 137 lines after the induction of TFs for 48 hrs can associate each TF with the direction of ES cell differentiation, regulatory pathways, and mouse phenotypes. These cell lines and microarray data provide building blocks for a variety of future biomedical research applications as a community resource.

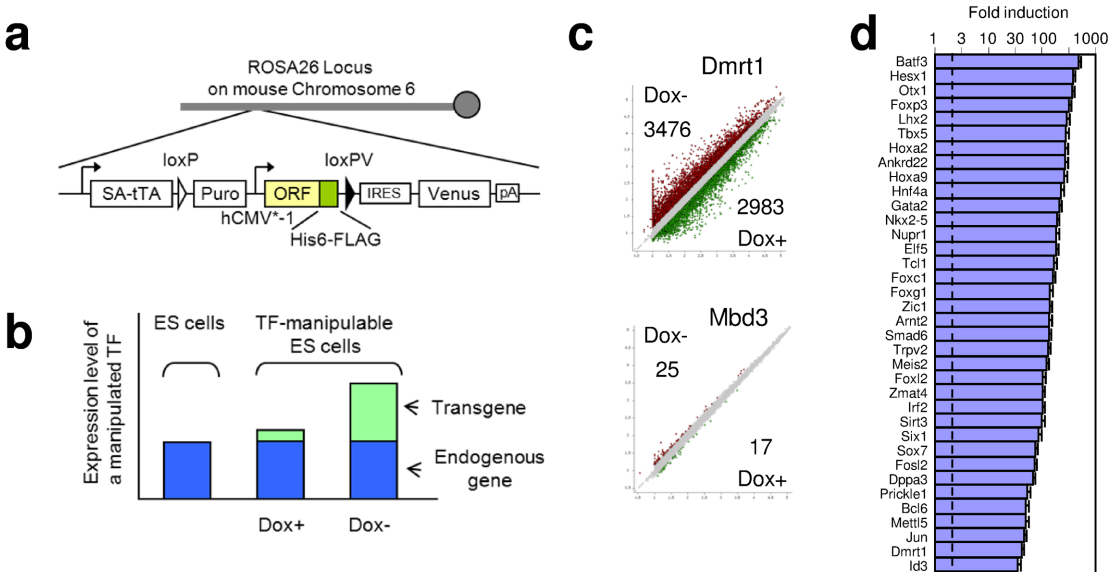
Mammalian genomes encode 1,500–2,000 transcription factors (TFs)[1], which cross-regulate one another to form the network of TFs. The network controls the transcriptome of cells, thereby defining the identity of cells. A powerful approach to deciphering such a complex network is the systematic perturbation of individual TFs followed by global gene expression profiling[2].

Results

Here we report the generation of mouse embryonic stem (ES) lines, each of which has been engineered by integrating an expression cassette of a specific transcription factor (TF) into the ubiquitously expressing Rosa26 locus (Fig. 1a)[2]. The Rosa26 locus[3] drives relatively uniform expression of the exogenous copy (transgene) of a TF, which is repressed by doxycycline (Dox) and can be induced in Dox- cell culture conditions (Fig. 1b)[4]. Combined with the 53 ES lines reported previously[2], we present a total 137 ES cell lines. The majority of the manipulated genes were TFs, which were selected from a set of high-priority genes involved in critical functions in mouse ES cells and their differentiation[5]. To ensure the quality of these ES cell lines, we implemented vigorous QC steps that have been described previously in detail[2]. As a part of the characterization of these ES cell lines, we carried out global gene expression profiling by DNA microarrays 48 hours after TF induction (Fig. 1c; GEO accession number, GSE31381). The induction of a TF was confirmed by qRT-PCR (Fig. 1d, Supplementary Table 1 for primer pairs). The effect of TF induction on the transcriptome of mouse ES cells was highly variable (Fig. 1e; Supplementary Table 2). On a scale of the number of genes significantly changed in expression (FDR ≤ 0.05, fold change ≥ 1.5), the top 10% of studied TFs changed 4676 genes on average (e.g., Dmrt1), whereas the bottom 50% of TFs caused significant changes in expression in only 54.5 genes on average (e.g., Mbd3) (Fig. 1c, d).

To further characterize the transcriptome alterations caused by each TF, we compared our microarray data with 3 public databases: the gene expression profiles of many mouse organs/tissues at The Genomics Institute of the Novartis Research Foundation (GNF) (ver. 2 & 3)[6,7], the Genetic Association Database (GAD) on gene sets associated with mouse phenotypes[8], and the MSigDB database (ver. 3) of gene sets associated with signaling pathways and cellular functions[9]. Because the GNF database is quantitative and the two other databases are qualitative, we used different methods to quantify association: correlation of median-subtracted log-transformed gene expression values for the GNF database, and Parametric Analysis of Gene Expression (PAGE)[10] for the GAD and msigdb databases (see Supplementary Methods).

A comparison of our microarray data with the GNF database showed that the induction of a TF in ES cells often initiates the differentiation of ES cells into specific cell types as soon as 48 hr later, when cells do not yet exhibit any overt phenotypes (Fig. 2 for GNF ver. 3; Supplementary Fig. 1 for GNF ver. 2). For example, the transcriptome



e

TF name	Up	Down
Aes	29	18
Aff1	27	22
Ankrd22	4	5
Arnt2	25	11
Ascl1	1401	951
Ascl2	1483	1009
Ash2l	42	61
Atf3	351	175
Atxn1	21	2
Batf3	15	15
Bcl6	677	961
Cbx8	43	188
Cdx2	3219	2938
Cdyl	9	4
Cdyl2	33	451
Ctbp2	192	31
Ctnnb1	2	1
Dedd2	1	0
Dlx3	1763	1379
Dmrt1	3476	2983
Dnmt3b	12	0
Dppa3	24	1
Dppa5a	7	10
Eed	2	0
Elf1	1145	946
Elf5	398	781
Ell2	18	13
Eomes	1453	1823

TF name	Up	Down
Esx1	2941	2445
Ets1	408	450
Etv1	34	15
Etv3	245	93
Etv5	38	44
Fbxo15	0	8
Fem1b	65	17
Fgfbp1	2	1
Fhl2	0	0
Fosl2	470	387
Foxa1	1379	1274
Foxc1	1440	889
Foxg1	1783	1637
Foxj2	22	0
Foxl2	2120	1624
Foxn3	13	65
Foxp3	773	686
Gadd45a	488	293
Gata2	2756	2684
Gata3	2205	2075
Gbx2	1411	1282
Grhl2	313	211
Hesx1	493	661
Hmga2	57	27
Hnf4a	868	696
Hoxa2	168	138
Hoxa9	402	367
Hsf2bp	38	43

TF name	Up	Down
Id1	124	224
Id3	14	38
Inppl1	15	2
Irf2	677	407
Jarid1a	11	0
Jarid2	2	0
Jmjd2c	10	0
Jun	1826	1477
Klf3	676	702
Klf4	2478	2320
Klf9	455	591
Lass2	4	3
Lhx2	548	456
Mbd3	25	17
Mef2c	752	350
Meis2	53	59
Mettl5	1	0
Mkrn1	5	2
Msc	765	667
Mybl2	205	209
Myc	443	944
Mycn	195	101
Myod1	1319	991
Nanog	241	165
Nkx2-5	2603	2042
Nr0b1	150	195
Nr2f1	535	387
Nr2f2	961	874

TF name	Up	Down
Nr5a2	795	816
Nrip1	1685	1163
Nsbp1	10	8
Nupr1	18	0
Ostf1	1	8
Otx1	355	110
Otx2	850	407
Pdlim1	13	6
Pou5f1	818	587
Prickle1	0	0
Rest	0	3
Rhox6	203	69
Rxra	557	337
Sall4	299	420
Sap30	11	7
Sfpi1	1303	1372
Sfrs6	126	49
Sirt3	3	16
Six1	63	81
Smad1	2	6
Smad4	2	0
Smad6	27	11
Smad7	362	293
Sox11	1329	1403
Sox15	122	161
Sox2	1635	1663
Sox7	530	557
Sox9	3001	2509

TF name	Up	Down
Stat3	7	0
Stra13	20	9
Sub1	0	0
Suz12	11	24
T	689	710
Tbx3	22	19
Tbx5	614	731
Tcea3	28	20
Tcf3	3101	2644
Tcf4	333	149
Tcfap2c	816	682
Tcfcp2l1	315	312
Tcl1	33	201
Tgif1	484	598
Tgm2	0	5
Trpv2	1	2
Tuba1a	11	4
Txlng	1	4
Ugp2	39	50
Whsc2	0	2
Zfand3	4	1
Zfp57	31	26
Zic1	704	528
Zmat4	4	5
Zscan4c	993	977

Figure 1 | Induction of transcription factors (TFs) in ES cells: (a) plasmid structure that includes loxP recombination sites, puromycin resistance gene, open reading frame (ORF) of a TF with hCMV promoter followed by His6-FLAG tag; (b) schematic diagram showing the expression of transgenic TF induced in Dox- conditions; (c) examples of scatterplots of gene expression in Dox- versus Dox+ condition. Green and red dots indicate genes that are differentially expressed with statistical significance (FDR<0.05, change >1.5 fold); (d) Increase of transcription factor expression after the induction of a transgene, as measured by qPCR (Dox- vs. Dox+); results from two biological replicates (3 technical replicates each); error bars (S.E.M.; ANOVA); and dashed line = 2 fold change; (e) a list of TFs and the number of genes up- or down-regulated by the induction of the TF (FDR<0.05, change >1.5 fold) (Supplementary Table S2).

of ES cells shifted toward a neural profile after the induction of Sox9, Foxg1, Klf3, or Pou5f1; toward endoderm after the induction of Hnf4a, Gata2, Gata3, or Esx1; and toward skeletal muscle and heart after the induction of Myod1 or Mef2c. Similarly, the transcriptome of ES cells shifted toward hematopoietic cell lineages after the induction of Sfpi1, Elf1, or Irf2; and toward T-cells and thymocytes after the induction of Elf5 or Tgif1. Interestingly, TFs associated positively with transcriptome changes toward specific lineages showed a negative association with those toward different cell lineages (Fig. 2). For example, TFs associated with transcriptome changes toward neural tissues were negatively associated with those toward hematopoietic lineages (e.g., Sox9 and Foxg1 in Fig. 2), and *vice versa* (e.g., Irf2, Elf1, Sfpi1 in Fig. 2). These data suggest that TF networks are organized to cross-regulate as if different tissue lineages are mutually exclusive.

A comparison of our microarray data with the GAD database identified associations of TF's with mouse phenotypes (Fig. 3). Many newly identified associations are consistent with published data. For example, Hoxa2 was associated with the pancreatic alpha and beta cells[11]; Foxc1, with hair follicle/shaft[12,13]; and Sox11 with skeletal defects[14]. A comparison of our microarray data with the msigdb database identified the association of each TF with specific cells and pathways (Fig. 4). For example, Smad6 was associated with keratinocytes[15]; Myod1, with alveolar rhabdomyosarcoma[16]; and Hnf4a, with lipoproteins[17].

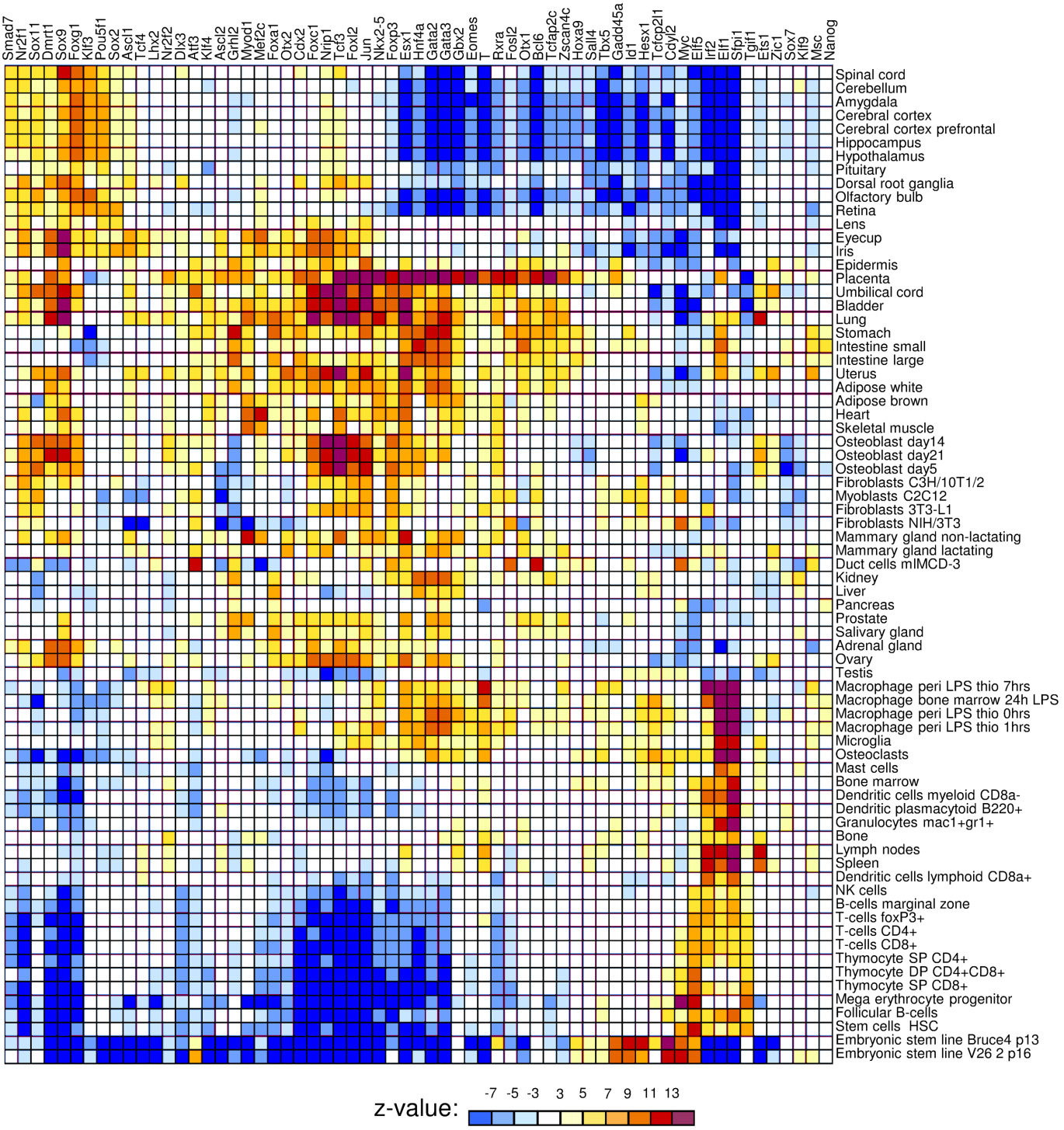


Figure 2 | **Correlation of gene expression response to the induction of TFs with tissue-specific gene expression from the GNF ver. 3 database[7].**

Discussion

The collection of mouse ES cell lines reported here are freely available to the research community (http://esbank.nia.nih.gov/index.html). The analysis presented here can help researchers select ES cell lines suitable for their own research programs. For example, these TF-manipulable ES cell lines can be used to study the complex mechanisms of ES cell differentiation toward specific lineages. These ES cell lines are also adaptable to a variety of experiments and analyses, as shown in our previous report[2]. For example, each TF is C-terminally tagged with His6-FLAG, which simplifies studies of TF localization, protein-protein interactions, and protein-DNA interactions[2]. Further mining of the microarray results reported here as well as additional experiments with provided ES cell lines and their derivatives will yield more insight into gene regulatory networks. Carrying out similar experiments for more regulatory proteins (ideally for all TFs and additional signaling proteins) should give increasingly complete information to comprehend gene regulation in mammalian cells and organs.

Methods

Derivation of transgenic ES cell lines. ES cell lines with inducible TF transgenes were derived from MC1 mouse ES cells (129S6/SvEvTac), passage 17. Cells were cultured

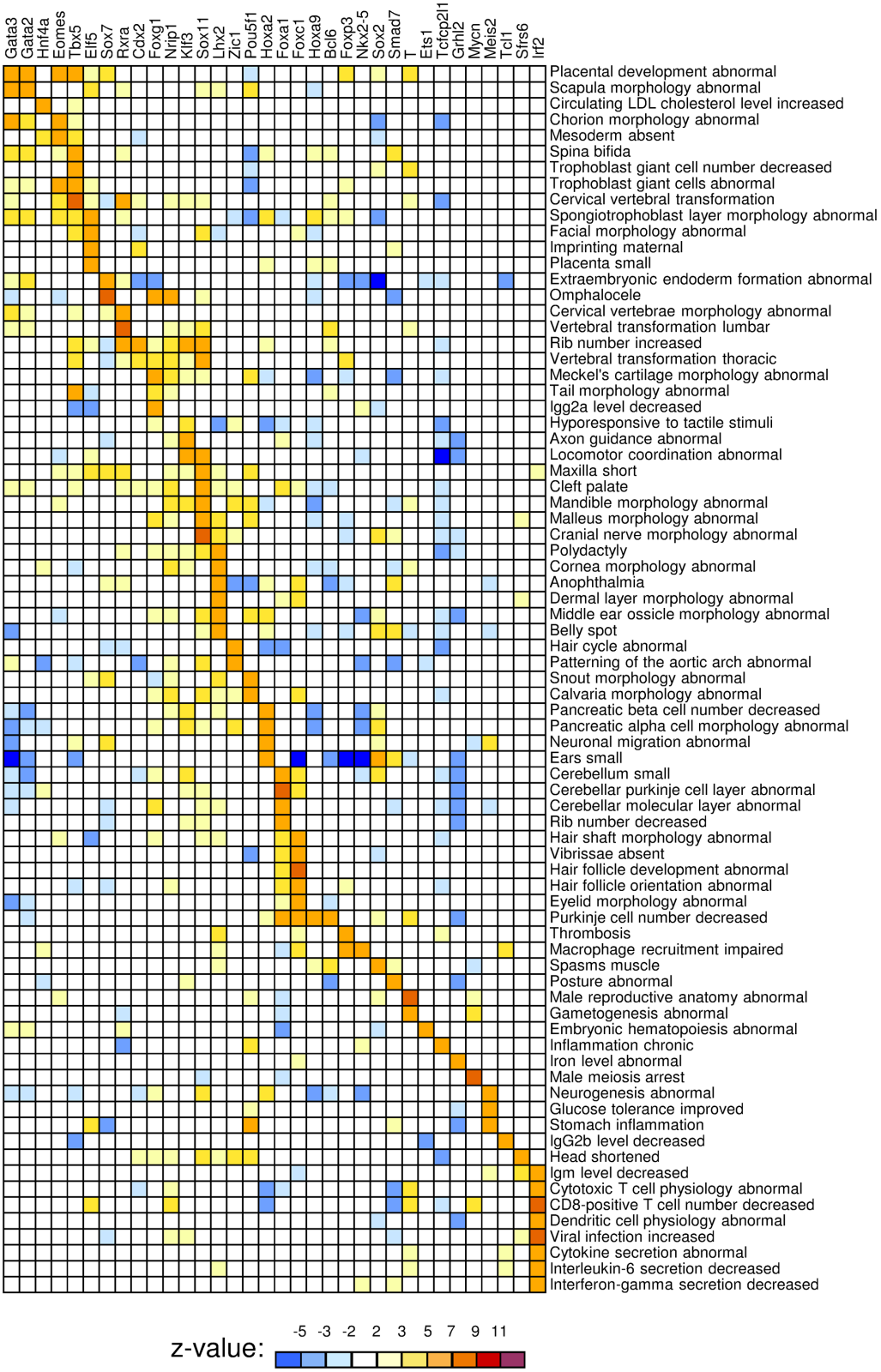


Figure 3 | Enrichment of gene sets associated with mouse phenotypes from GAD database[8] among genes that were upregulated (positive) or downregulated (negative) after the induction of various TFs.

in DMEM with 15% FBS and LIF on feeder cells. Cells were electroporated with a linearized pMWROSATcH vector and selected by hygromycin B. Knock-in for ROSA-TET locus was confirmed by southern blotting. For exchange vectors, PCR amplified ORFs were subcloned into pZhcSfi that was modified to express a His6-FLAG tagged protein and puromycin resistance gene. ES cells were co-transfected with a sequence verified exchange vector and pCAGGS-Cre and selected by puromycin in the presence of doxycycline (Dox). Isolated clones were tested for Venus expression, hygromycin B susceptibility, transgene RNA expression, genotyping for Cre mediated integration, and mycoplasma contamination.

Gene expression analysis of cells with induced TFs. ES cells (passage 25) were cultured in the standard LIF+ medium with Dox+ on a gelatin-coated dish throughout the experiments. Cells from each cell line were split into 6 wells and the media was changed 24 hr after cell plating: 3 wells with Dox+ medium, and 3 wells

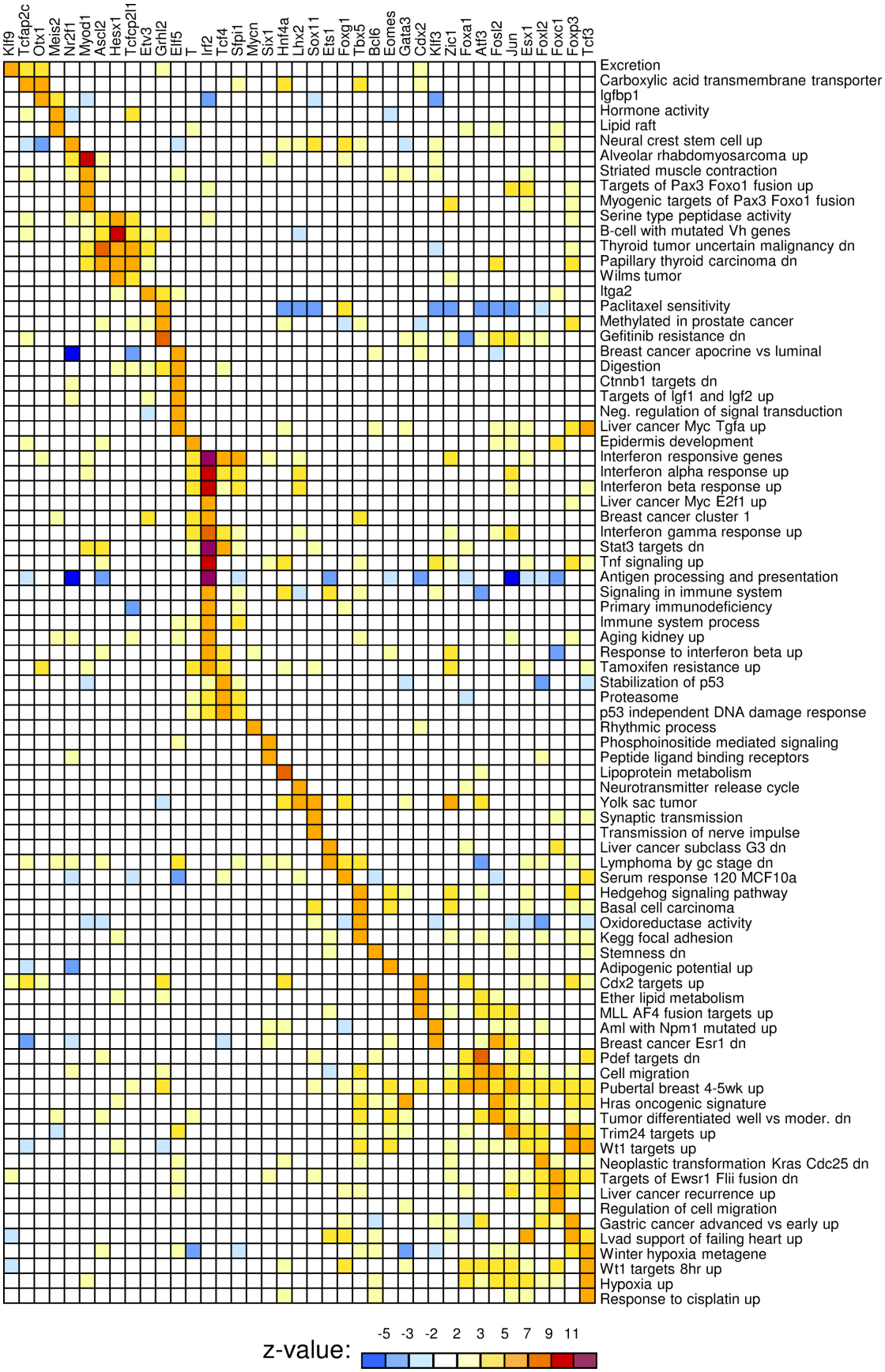


Figure 4 | **Enrichment of gene sets associated with various functions and signaling pathways from msigdb ver. 3 database[9] among genes that were upregulated (positive) or downregulated (negative) after the induction of various TFs.**

with Dox- medium to induce transgenic TFs. Dox was removed via washing 3 times with PBS at 3 hour intervals. Total RNA was isolated by TRIzol (Invitrogen) after 48 hr, and two replications were used for real time qPCR (see primers in Supplementary Table S1) and for microarray hybridization. RNA samples were labeled with total RNA by the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). For most TFs, we hybridized Cy3-CTP labeled sample from Dox- medium together with a Cy5-CTP labeled sample from Dox+ medium. But for 7 TFs we labeled samples from Dox- and Dox+ with Cy3, and hybridized them independently with a Cy5-labeled reference target, which is a mixture of Stratagene Universal Mouse Reference RNA and MC1 cells RNA (this method requires a double number of arrays). Analysis showed that both methods produce results of comparable quality. Targets were hybridized to the NIA Mouse 44K Microarray v3.0 (Agilent, design ID 015087)[18]. Slides were scanned with Agilent DNA Microarray Scanner. All DNA Microarray data are available in Supplementary Table S2, at GEO/NCBI[19] (http://www.ncbi.nlm.nih.gov/geo; accession number GSE31381), and at NIA Array Analysis software[20] (http://lgsun.grc.nia.nih.gov/ANOVA).

Normalization of microarray data and detection of outliers. Two methods of array hybridizations were used in this study: (1) RNA extracted from cells with induced transcription factors (TFs) (cultured in Dox- conditions) and from controlled cells (cultured in Dox+ conditions) were Cy3 labeled and all hybridized on separate arrays together with reference RNA labeled with Cy5; and (2) RNA extracted from cells with induced TFs (Dox-) were labeled with Cy3 and hybridized together with RNA from control cells (Dox+) which were labeled with Cy5. The second method does not use reference RNA. Data processing depended on the method of hybridization. Potential Cy3/Cy5 bias in microarrays with the hybridization of Dox- vs. Dox+ samples was removed by normalization to the median logratio of gene expression change in all TF-manipulation experiments. The details of the method are available in Supplementary Information.

Statistical analysis of microarray data. For statistical analysis we used NIA Array Analysis, which estimates the False Discovery Rate (FDR) to account for multiple hypothesis testing[20]. Response of genes to the knockdown of TFs was measured as a logratio (i.e., difference between means of log-transformed intensities) between manipulated (Dox-) and control (Dox+) cells. We considered gene expression change as significant if logratio was significantly different from zero (FDR < 0.05) and the change of expression was >1.5 fold.

Correlation with tissue-specific gene expression. Association of gene expression changes induced by TF manipulation with tissue-specific gene expression was evaluated based on the correlation between our microarray results with the GNF database[7]. Correlation was estimated between gene expression responses to TF manipulation (logratio of Dox- vs. Dox+) and median-centered log-transformed gene expression in various tissues from GNF database (ver. 2 and 3). Because the importance of genes in ES cells and adult tissues may be different and different platforms of microarrays used in these studies are not 100% compatible, we applied correlation analysis to a subset of genes that are highly expressed and dynamic in both data sets. We selected 10,000 genes in each database with the highest score equal to the product of average log-expression and standard deviation of expression (after induction of various TFs or in different tissues), and then took the intersecting portion of 5,595 genes for GNF ver. 3 (5,295 genes for ver. 2). Then, correlation values and corresponding z-values were estimated based on this subset of genes. The matrix was sorted using hierarchical clustering, TMEV, ver 3.1[21].

Analysis of gene set enrichment. Enrichment of target genes in subsets of genes that are upregulated or/and downregulated following the manipulation of the TF is quantified using a modified Parametric Analysis of Gene Enrichment (PAGE)[10]. PAGE is based on the comparison of the average expression change in a specific subset of genes, xset, with the average expression change in all genes, xall:

$$z = (\mathrm{xset} - \mathrm{xall}) * \mathrm{sqrt}(\mathrm{nset})/\mathrm{SDall} \quad (1)$$

where nset is the size of the gene set and SDall is standard deviation of expression change among all genes. We modified this method by applying equation (1) to the subset of N top upregulated and another subset of N top downregulated genes rather than to all genes combined, which allowed us to detect the enrichment of the same gene set among both upregulated and downregulated genes. The value of N = 5000 was selected experimentally because it appeared that the enrichment of genes with TF binding sites is always limited to the top 5000 upregulated or downregulated genes. The probability distribution of expression change within subsets of N upregulated and downregulated genes is not normal; however, because we compare averages for large sets of genes (usually, nset is >50), the probability distribution of these averages is close to normal based on the central limit theorem[22]. Thus, it is reasonable to use equation (1) as an approximation. In the case when both up-regulated and down-regulated genes were enriched in a specific functional gene set, we subtracted the smaller z-value from both z-values. The matrix of z-values was first sorted using hierarchical clustering, TMEV, ver 3.1[21], and then manually converted to a semi-diagonal form.

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Author contributions

LSC, YP, AN, JSC, HY, LVS, LX, HGH, MT, EM, BYB, GM, and UB carried out the experiments. AAS, YQ, DD, and MSHK carried out the data analysis. MSHK conceived the project. DLL, DS, and MSHK supervised the project. AAS and MSHK wrote the manuscript with inputs from all authors. All authors reviewed the manuscript.

Additional information

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