ARTICLE
Journal of Cellular Biochemistry 113:1820–1832 (2012)

Journal of Cellular Biochemistry

SMILE Silencing and PMA Activation Gene Networks in HeLa Cells: Comparison With Kidney Transplantation Gene Networks

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ABSTRACT

Recent findings indicated that the SMILE gene may be involved in kidney graft operational tolerance in human. This gene was found to be up-regulated in blood from patients with a well functioning kidney transplant in the absence of immunosuppression compared to other transplanted recipients with clinically different status. A microarray study of SMILE knock-down and phorbol 12-myristate 13-acetate (PMA) activation in HeLa cells was herein compared to our earlier analysis based on microarray data of kidney allograft tolerance and rejection in humans and in a rat model of allograft transplantation to determine possible new genes and gene networks involved in kidney transplantation. The nearest neighbors at the intersection of the SMILE knock-down network with the human tolerance/rejection networks are shown to be NPHS1 and ARRB2, the former (Nephrin) being involved in kidney podocyte function, and the decrease of the latter (Arrestin β2) being recently shown to be involved in monocyte activation during acute kidney allograft rejection in rat. Moreover, another one of the neighbors at the intersection of SMILE network and tolerance/rejection networks is XBP-1, that we report previously to be increased, at a transcript level, after ER stress in SMILE silenced cells. Finally, in this study, we also show that topological properties (both local and global) of joint SMILE knock-down network—tolerance/rejection networks and joint PMA activation network—tolerance/rejection networks in rat and human are essentially different, likely due to the inherent nature of the gene SMILE and the mitogen PMA, that do not act the same way on genes and do not interfere the same way on networks. We also show that interestingly SMILE networks contain more feed-forward loop (FFL) motifs and thus SMILE calls for a more fine-tuned genetic regulation. J. Cell. Biochem. 113: 1820–1832, 2012.

KEY WORDS: OPERATIONAL TOLERANCE; REJECTION; SMILE; HELA CELLS; KIDNEY; ALLOGRAFT; HUMAN; TMTC3

The state of operational tolerance in humans has been detected sporadically in some renal transplanted patients who stopped immunosuppressive drugs and retained a good renal function, demonstrating that allograft tolerance might exist [for a review,

Abbreviations used: ER, endoplasmic reticulum; FDR, false discovery rate; GEO, gene expression omnibus; MIAME, minimum information about a microarray experiment; PMA, phorbol 12-myristate 13-acetate; SAM, significance analysis of microarrays; siRNA, small interfering RNA; FFL, feed-forward loop.

M. Racapé, N. Bragazzi, S. Brouard, and C. Nicolini equally contributed to this work.

Grant sponsor: Ministero dell'Istruzione, Università e Ricerca (MIUR); Grant sponsor: Proteomics and Cell Cycle; Grant number: FIRB RBIN04RXHS; Grant sponsor: Nanobiosensors; Grant number: FIRB ITALNANONET RBPR05JH2P_003.

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Manuscript Received: 22 November 2011; Manuscript Accepted: 23 November 2011

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 1 December 2011

DOI 10.1002/jcb.24013 • © 2011 Wiley Periodicals, Inc.

Kasiske et al., 2000; Ashton-Chess et al., 2007, 2009]. Although the kidney is less susceptible to successful immunosuppressive drug withdrawal than the liver [Orlando et al., 2009; Lozano et al., 2011], there is now mounting evidence showing that kidney transplant recipients too can become operationally tolerant [Roussey-Kesler et al., 2006; Newell et al., 2010; Sagoo et al., 2010]. In search of biological signatures of "operational tolerance" in humans, we previously identified a list of 49 genes which were able to discriminate operationally tolerant patients from other cohorts of transplant patients and healthy individuals [Brouard et al., 2007]. The gene SMILE/TMTC3 was one of the genes found to be up-regulated in the blood of operationally tolerant patients and whose function was unknown [Racapé et al., 2011]. SMILE was found to interact with PDIA3, which has a crucial role in glycoprotein folding in endoplasmic reticulum (ER) [Zhang and Kaufman, 2008], in the loading of peptide on MHC class I [Santos et al., 2007] and which is over-expressed during ER stress. We showed that siRNA-mediated SMILE knock-down in HeLa cells induces a decrease in several types of transcripts involved in protein catabolism and proteolysis, particularly immunoproteasome subunits, suggesting that SMILE exerts its function via the proteasome pathway [Racapé et al., 2011]. This was confirmed by the facts that SMILE down-regulation and/or treatment with proteasome inhibitor (Bortezomib) induced dramatic ER enlargement and features of cellular injury and that Bortezomib inhibition of long-term cellular growth was strongly enhanced in SMILE siRNA-transfected cells. SMILE silencing was found to directly increase XBP-1 transcript expression after 6 h of Bortezomib treatment, while DNA microarray analysis revealed that SMILE down-regulation in HeLa cells affects secretory pathways, such as vesicle-mediated transport [Racapé et al., 2011].

Earlier, we outlined a microarray-based identification of key leader genes found respectively in blood from kidney transplanted patients with chronic rejection or operational tolerance by utilizing a non-statistical bioinformatics approach based on the identification of "key genes" either as those mostly changing their expression, or having the strongest interconnections [Sivozhelezov et al., 2008]. An informative picture emerges on the genes controlling the human transplant from the detailed comparison of these findings with the traditional statistical significance analysis of microarrays (SAM) and with the clinical study [Braud et al., 2008]. Some of these genes, such as BANK-1, a modulator of B-cell hyperactivation, have been confirmed through further studies using different techniques [Pallier et al., 2010]. In parallel, in another study, in a rodent model of kidney allograft, DNA microarrays were used to compare gene patterns in the kidney transplant from anti-donor anti-class II tolerated or untreated syngeneic rat in the Lewis 1W and Lewis 1A rat strain combinations [Jovanovic et al., 2010]. Statistical and non-statistical analysis combined with ab initio analysis, using the leader gene approach [Bragazzi et al., 2011] suggested that tolerance and rejection outcomes may be in large part correlated to the low expression variations of some genes, which can form a core gene network around specific genes, such as Rac1, NFKB1, RelA, AKT1, IKBKB, BCL2, BCLX, and CHUK. Through this model, we showed that AKT1 gene, WNT pathway and NO synthesis were strictly connected to each other, with AKT1 gene being the center of this complex network of interactions in kidney tolerance and rejection processes [Jovanovic et al., 2010].

The aim of the present study was to define networks of genes either modified by SMILE knock-down compared to control knock-down cells or phorbol 12-myristate 13-acetate (PMA)-activated cells compared to non-activated cells. Then, we compared these two networks with those found in our microarray studies in human [Sivozhelezov et al., 2008] and rat [Jovanovic et al., 2010].

MATERIALS AND METHODS

GENE EXPRESSION ANALYSIS IN HeLa CELLS USING DNA CHIPS

Gene expression microarray data representing three independent experiments from HeLa cells transfected 24 h with negative control or SMILE siRNA, or activated or not with 20 mM PMA during 6 h as previously described [Racapé et al., 2011] were submitted for our analysis. MIAME microarrays data were deposited in Gene Expression Omnibus Database (GEO record: GSE21886).

STATISTICAL AND NON-STATISTICAL ANALYSIS

SMILE silencing and PMA activation microarray derived data were treated separately. List of transcripts differentially expressed obtained from SAM are shown in Figure 1 for SMILE knock-down versus control and Figure 3 for PMA activation versus control. For each analysis, we arbitrarily fixed the false discovery rate (FDR) less than 0.5%. Networks displayed in Figures 1 and 3 were obtained using the STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string-db.org/).

Interaction networks from the genes involved in SMILE knock-down selected by SAM analysis (or alternatively Fisher Z-score or other known statistical approaches) resulted in large intractable networks (*not shown*). It is well-known that different statistical analysis can yield different results [Kim et al., 2006] and a need for combining statistical and biological significance is emerging, by combing the analysis of differentially expressed genes and systems biology approach [Draghici et al., 2007]. We performed non-statistical analysis on the different conditions of culture with or without activation based mostly on fold-changes that are in fact *biological* significance of change in expression level as previously described [Racapé et al., 2011]. After obtaining the SAM output, the columns containing d-scores and fold-changes were thus multiplied to select both statistically and biologically significant genes. We considered genes with both *statistical* and *biological* significant changes in expression: we performed a scatter-plot with the number of genes versus SAM d-score and log of fold-change and we picked those genes which resulted in a significant separation, selecting them for further bioinformatics analysis, this analysis being referred as semi-theoretical analysis. This approach was used to select genes included in the networks displayed in Figures 2, 4–6, and 8, and the Clusters of Orthologous Groups (COG) mode of STRING was used to extend these networks with family-wide and cross-species analogs.

TOPOLOGICAL ANALYSIS OF BIOLOGICAL NETWORKS

Networks have been proved a very useful tool for understanding cell functions [Barabási and Oltvai, 2004] and in the frame of the systems

biology they have been widely exploited for characterizing different cellular processes, among which also kidney allograft [see for review, Perkins et al., 2011]. As far as we know, this is the first time that topological and mathematical analysis have been used to compare the changes of two biological networks after a gene has been knocked-down.

Topological parameters of networks shown in Figures 4, 6, and 7 have been investigated using Cytoscape [Shannon et al., 2003], MAVisto [Schreiber and Schwöbbermeyer, 2005; Schwöbbermeyer and Wünschiers, 2012], and FANMODE [Wernicke and Rasch, 2007], and further mathematical analysis: we studied both global topology (density, heterogeneity, clustering coefficient, diameter, centrality index, number of neighbors, size, mean connectivity, degree, etc.) and local topology [small motifs, like SIM, feed-forward loop (FFL), MIM, etc.). These parameters are listed in Table II and discussed in details in the Results Section.

RESULTS

SMILE SILENCING IN HeLa CELLS

Genes selected with the SAM analysis form no visible physical interaction networks after SMILE silencing (Fig. 1). Therefore, we formed a semi-theoretical interaction network starting from the known interaction of SMILE with PDIA3 and added their neighbors based on family-wide and cross-species analogs (COG mode of STRING). The resulting map is shown in Figure 2. Genes significantly up-regulated are shown in red, down-regulated in blue, not changing their expression in green. Magenta lines denote physically observed interactions between the proteins encoded by the respective genes, cyan lines neighborhood in known regulatory or metabolic pathways. Using this method, we confirmed that indirect neighbors of TMTC3 are related to ER functioning and stress response genes (CANR, CALX, PDIA4, PDIA2, and ERP27) due to TMTC3 interaction with PDIA3 [Racapé et al., 2011].

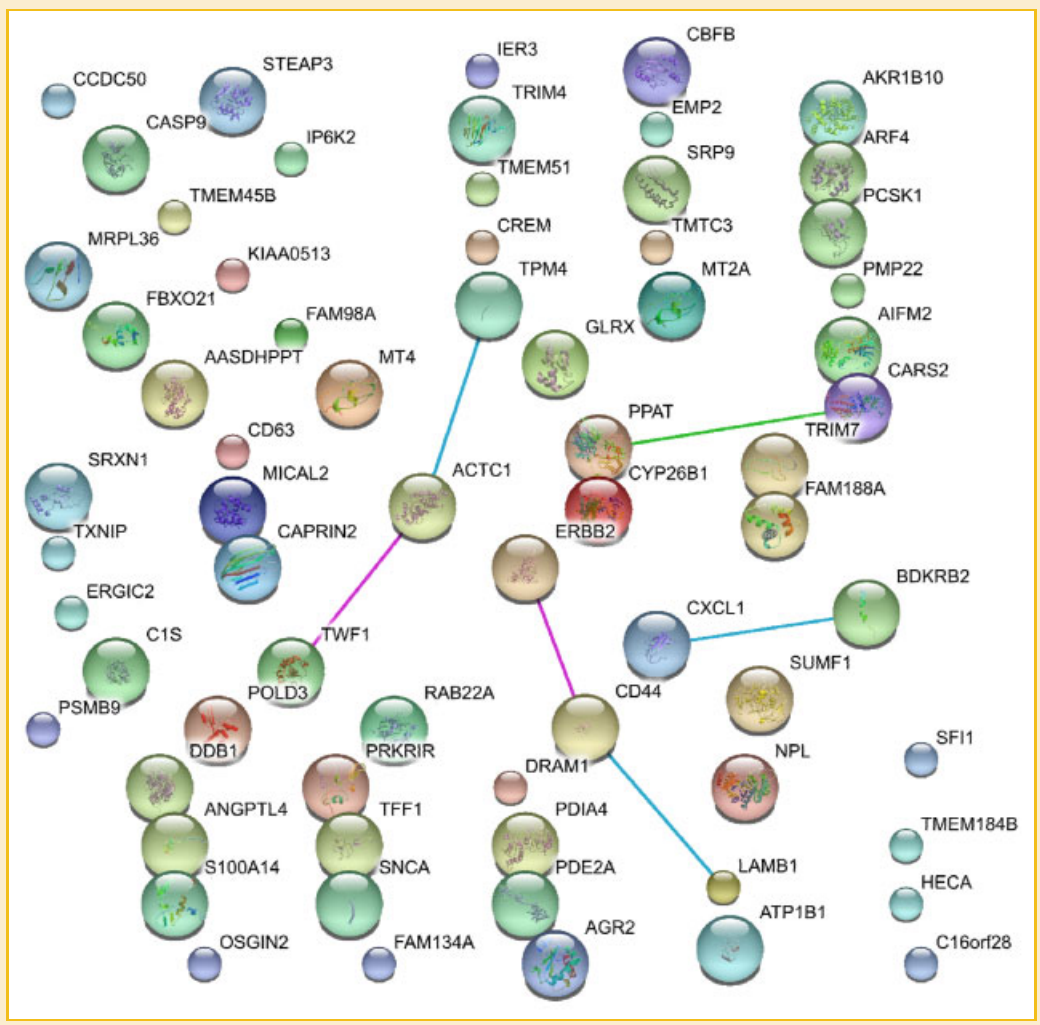

Fig. 1. Network connecting genes up- or down-regulated by SMILE knock-down, by microarray data for HeLa cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

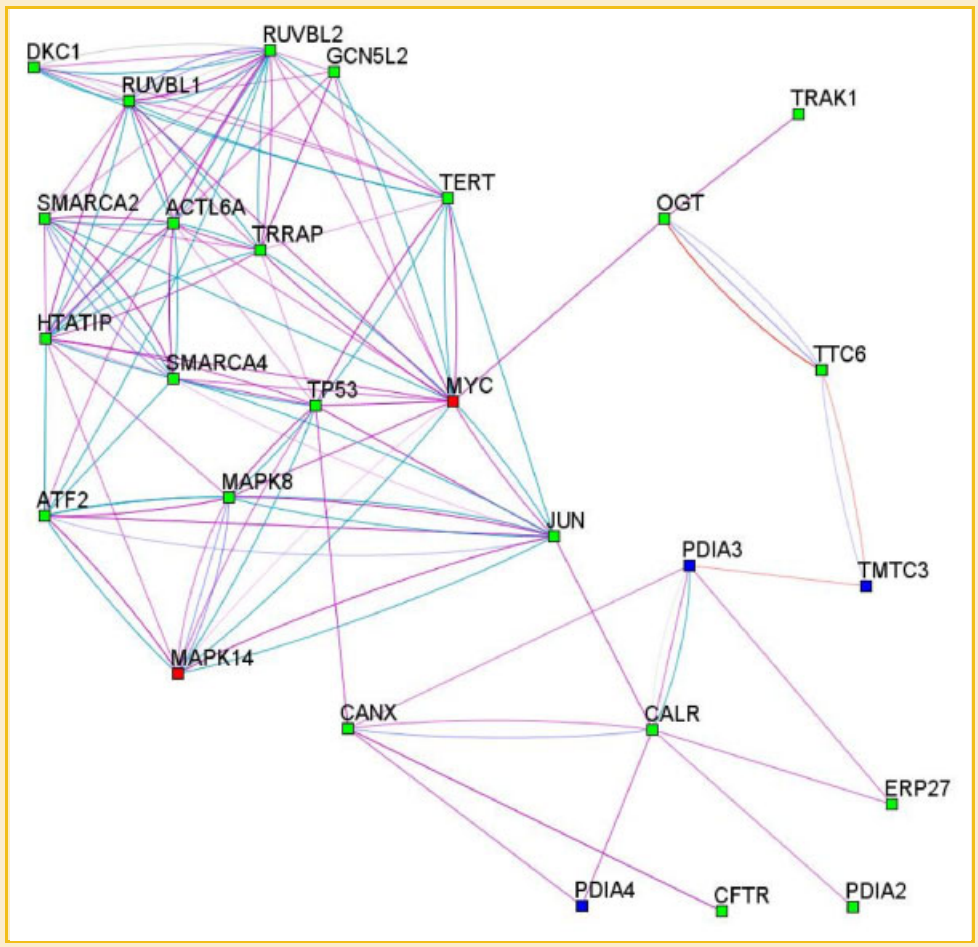

Fig. 2. Semi-theoretical network of SMILE silencing. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

HeLa CELLS AND PMA ACTIVATION

In contrast to SMILE silencing, gene highlighted from the SAM analysis resulted in a well-defined network following PMA activation (Fig. 3). This is likely due to extensive studies on PMA activation, leading to a more extended knowledge of genes involved in this activation than those involved in SMILE knock-down.

COMPARISON OF SMILE MODULATION AND PMA ACTIVATION NETWORKS WITH PREVIOUSLY IDENTIFIED NETWORKS IN KIDNEY TRANSPLANTED PATIENTS

SMILE modulation network and kidney transplantation in human. The two networks, semi-theoretical for SMILE silencing and expression-derived for PMA activation, were compared to the "pro-tolerance" and "pro-rejection" networks inferred from our previous studies in blood from patients with a kidney transplant [Braud et al., 2008; Sivozhelezov et al., 2008]. From Figure 4a, SMILE gene (TMTC3) is linked via PDIA3 to the network previously identified in the blood from human transplanted kidney recipients with a profile of chronic rejection (referred above as the pro-rejection network): namely STAT3, TNFRSF1A, IL2RB, LCK, VAV1, HNRPU, and their neighbors, as described in more details by Sivozhelezov et al. [2008], and circled in green in the Figure. SMILE gene relation to the network identified in the blood from human transplanted kidney recipients with a profile of operational tolerance (referred above as the pro-tolerance network; Fig. 4b) is less direct, although *one* gene, IFNγ is linked to SMILE again via the PDIA3 gene (circled in green in the Figure). There are five overlapping genes between SMILE knock-down network and the pro-tolerance and pro-rejection networks (four genes pro-tolerance: TP53, ATF2, JUN, and MAPK14 and one gene pro-rejection: HTATIP, Table I). We found that those five genes form their own network shown in Figure 5a.

PMA activation network and kidney transplantation in humans. For PMA activation and the human kidney transplantation (Fig. 6a,b), there is a sub-network of PMA activation most tightly connected to the pro-tolerance network, namely ERBB2, CD44, and IL4R (Fig. 6a). There is the same situation for the sub-network formed by genes RELA, NFKB1, and NFKBIA in comparison with the pro-rejection network (Fig. 6b).

Topological analysis of joint SMILE knock-down and PMA activation with tolerance and rejection networks. Before performing our topological analysis, we verified the scale-free behavior of our

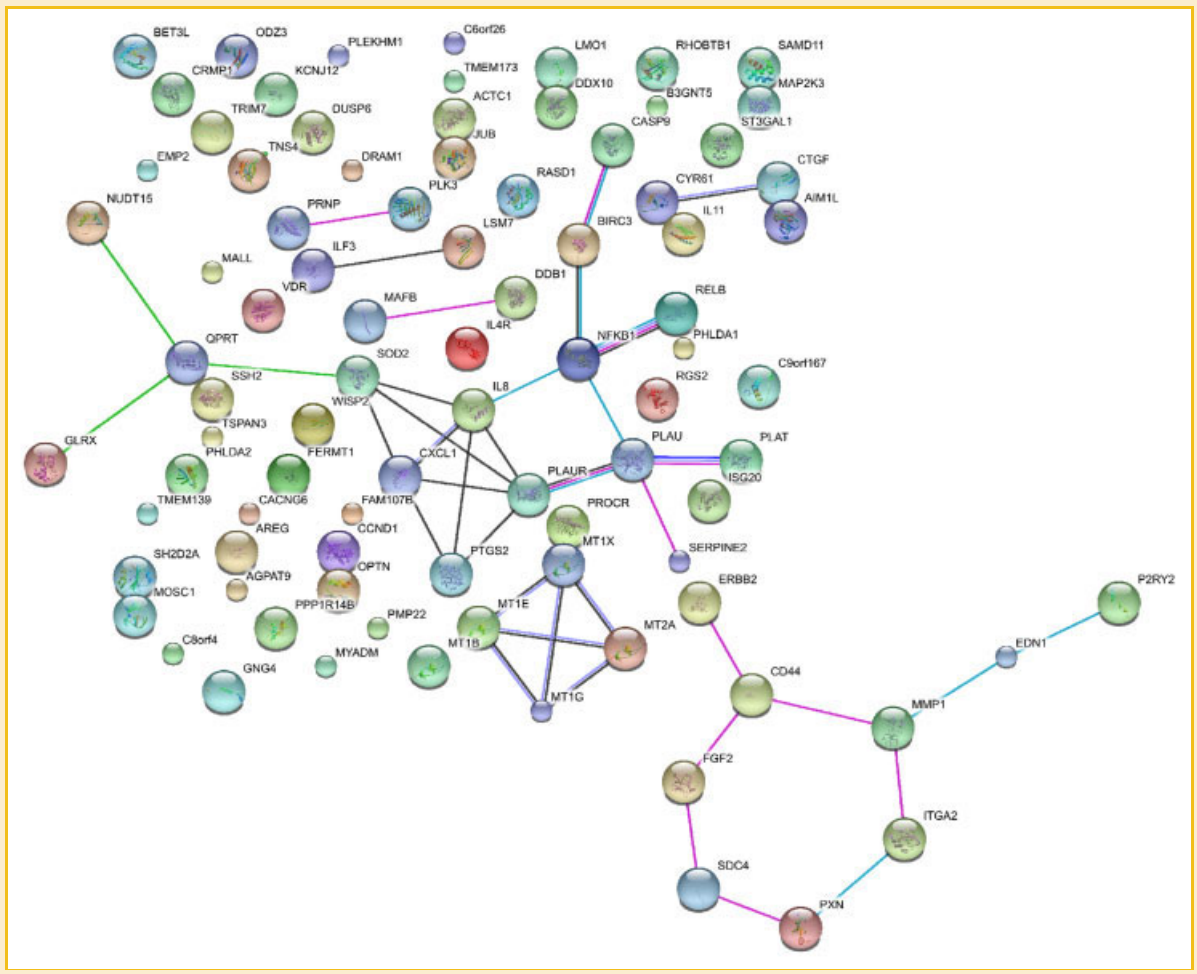

Fig. 3. Network connecting genes up- or down-regulated by PMA activation, by microarray data for HeLa cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

networks, since this feature has emerged as a peculiar and distinguishing characteristic of biological networks [Barabási et al., 2001].

From the "Density," the "Mean connectivity" and the "Clustering coefficient" rows of Table II, it appears that SMILE networks are denser and more clustered than PMA networks, each SMILE node having more neighbors than PMA. In contrast, PMA networks are more heterogeneous than SMILE networks. The centrality (tendency to form star-like rather than mesh-like topology) is small for all the networks (maximum is 1), which is typical of biological networks that rarely have a single superhub. Finally, size of the network (number of links) is higher for PMA networks. Other parameters (like diameter and degree) show little statistical variance or show an ambiguous trend (namely, degree is higher for PMA in the case of tolerance network, while being lower for PMA in the case of rejection).

Frequency distributions of node connectivity for rejection network + SMILE knock-down versus rejection network + PMA activation (Fig. 7a) showing statistically significant difference are also analyzed. Namely, higher connected (hub) genes occur more frequently in rejection network + SMILE knock-down than in rejection network + PMA activation. Instead, frequencies for tolerance network + SMILE knock-down versus tolerance network + PMA activation (Fig. 7b) show no statistically significant difference. We can conclude that SMILE knock-down more significantly affects the kidney transplant rejection network than PMA activation, while the tolerance network is affected to the same extent by SMILE knock-down and PMA activation.

As far as the local topology is concerned, we studied the small motifs composing the subgraphs of the network—using a well-established approach, comparing them against motifs in random networks and selecting only those motifs for which Z-score was higher than 2, whose *P*-value was lower than 0.05 and whose frequency was higher than five times. Our study showed that FFLs motifs [Mangan and Alon, 2003] are the core motifs of our networks and they are abundant in SMILE networks, suggesting that SMILE calls for more fine-tuned regulation.

COMPARISON OF SMILE MODULATION NETWORK AND PMA ACTIVATION WITH PREVIOUSLY IDENTIFIED NETWORKS IN A RAT MODEL OF KIDNEY ALLOGRAFT TOLERANCE

For the rat kidney transplant study, too few genes notably change expression to analyze pro-rejection or pro-tolerance network

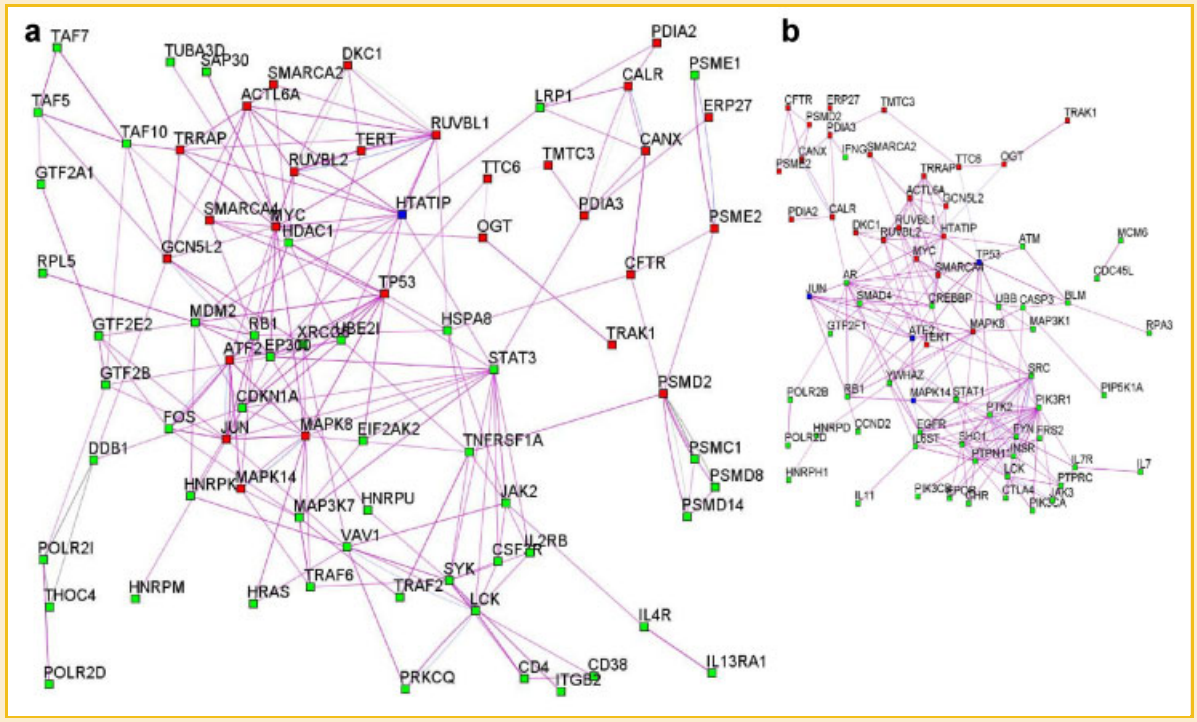

Fig. 4. Human kidney graft network pro-rejection (a) and pro-tolerance (b) combined with SMILE knock-down network for HeLa cells. In magenta are physically observed interactions between the proteins encoded by the respective genes. Green are pro-tolerance (*left*) and pro-rejection (*right*) genes from human kidney graft study; red is the SMILE silencing network; blue is overlap between the SMILE and the human kidney transplant networks. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Subnetwork of Our Network Formed by Genes Identified as Interaction Leaders in Our Previous Work on Human Graft, Which Overlaps With SMILE Silencing Network for HeLa Cells

Gene symbol	Gene annotation
MAPK14	Mitogen-activated protein kinase 14 [EC 2.7.11.24, mitogen-activated protein kinase p38 alpha (MAP kinase p38 alpha), cytokine suppressive anti-inflammatory drug-binding protein (CSAID-binding protein; CSBP), MAXinteracting protein 2 (MAP kinase MXI; responds to activation by environmental stress, pro-inflammatory cytokines, and lipopolysaccharide (LPS)] by phosphorylating a number of transcription factors, such as ELK1 and ATF2, and several downstream kinases, such as MAPKAPK2 and MAPKAPK5. Plays a critical role in the production of some cytokines, e.g., IL-6 (360 aa)
ATF2	Cyclic AMP-dependent transcription factor ATF-2 [activating transcription factor 2; cAMP response element-binding protein CRE-BP1, HB16]; transcriptional activator, probably constitutive, which binds to the cAMP-responsive element (CRE; consensus: 5′-GTGACGT[AC][AG]-3′), a sequence present in many viral and cellular promoters. Interaction with JUN redirects JUN to bind to CRES preferentially over the 12-*O*-tetradecanoylphorbol-13-acetate response elements (TRES) as part of an ATF2-c-Jun complex (505 aa)
TP53	Cellular tumor antigen p53 (tumor suppressor p53; phosphoprotein p53; antigen NY-CO-13); acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression (393 aa)
JUN	Transcription factor AP-1 (activator protein 1; AP1; proto-oncogene c-jun; V-jun avian sarcoma virus 17 oncogene homolog; p39); transcription factor that recognizes and binds to the enhancer heptamer motif 5′-TGA[CG]TCA-3′ (331 aa)
HTATIP	Histone acetyltransferase HTATIP (EC 2.3.1.48; 60 kDa Tat interactive protein; Tip60; HIV-1 Tat interactive protein; cPLA(2)-interacting protein); catalytic subunit of the NuA4 histone acetyltransferase complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histone H4 and H2A. This modification may both alter nucleosome–DNA interactions and promote interaction of the modified histones with other proteins which positively regulate transcription
NPHS1	Nephrin precursor (renal glomerulus-specific cell adhesion receptor); seems to play a role in the development or function of the kidney glomerular filtration barrier. May anchor the podocyte slit diaphragm to the actin cytoskeleton (1,243 aa)
ARRB2	Beta-arrestin-2 (Arrestin beta 2); regulates beta-adrenergic receptor function. Beta-arrestins seem to bind phosphorylated beta-adrenergic receptors, thereby causing a significant impairment of their capacity to activate G(S) proteins (421 aa)
XBP1	X-box binding protein 1; transcription factor essential for hepatocyte growth, the differentiation of plasma cells, the immunoglobulin secretion, and the unfolded protein response (UPR). Acts during endoplasmic reticulum stress (ER) by activating UPR target genes via direct binding to the UPR element (UPRE). Binds DNA preferably to the CRE-like element 5′-GATGACGTG[TG]N(3)[AT]T-3′, and also to some TPA response elements (TRE). Binds to the HLA DR-alpha promoter. Binds to the Tax-responsive element (TRE) of HTLV-I

Underlined are immediate neighbors of the network involved in kidney functioning and graft rejection.

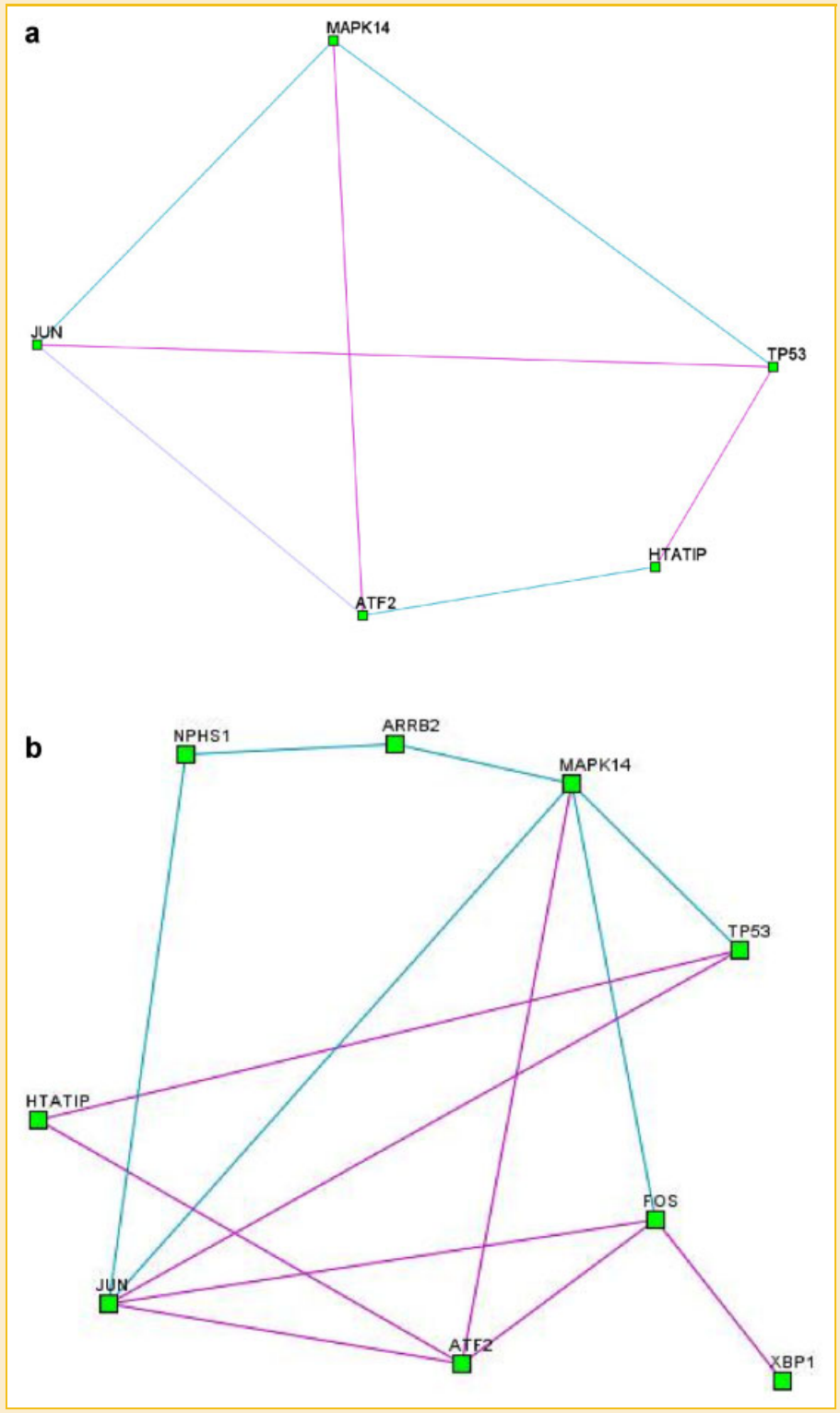

Fig. 5. a: A subnetwork of our network formed by genes identified as interaction leaders in our previous work on human graft, which overlaps with our new network on SMILE silencing for HeLa cells. b: Same subnetwork with its two immediate neighbors, arrestin beta2, nephrin, and X-box binding protein 1. Below, table containing descriptions of genes in the diagrams (found neighbors are in bold). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

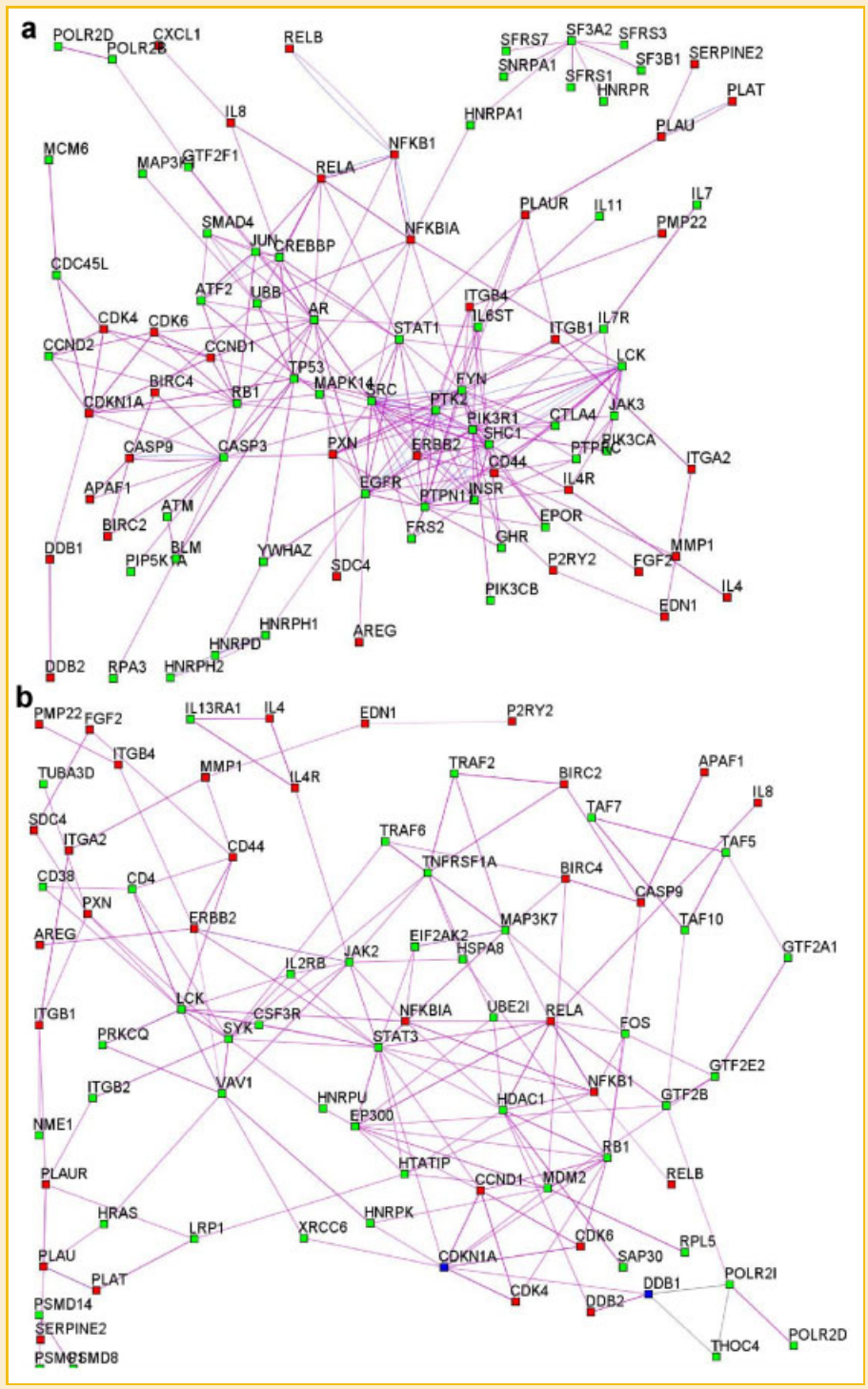

Fig. 6. Human kidney graft network pro-tolerance (a) and pro-rejection (b) combined with PMA activation network for HeLa cells. In magenta are physically observed interactions between the proteins encoded by the respective genes. Green are pro-tolerance (a) and pro-rejection (b) genes from human kidney graft study; red is PMA activation network; blue is overlap between the PMA activation and the human kidney transplant networks. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

separately. Besides, the setup of the microarray analysis (TOL/SYN and TOL/REJ) would render such analysis of separate networks biased. Therefore, we merged the tolerance and rejection gene lists. In Figure 8, the rat kidney transplant data are shown in combination with SMILE knock-down (Fig. 8a) and PMA activation (Fig. 8b). There is a sub-network of rat kidney transplant related genes interacting strongly with the SMILE network modulation (Fig. 8a, underlined in dotted line). These include RelA, CHUK, NFKB-1, NFKB-1A, and AKT1, having a significantly high number of links with SMILE sub-network. The same observation is even

TABLE II. Topology Parameters of Networks Shown in Figures Figure 4, 6, and 7

Parameters, samples	Tolerance SMILE	Tolerance PMA	Rejection SMILE	Rejection PMA	Rat SMILE	Rat PMA
Nodes	79	92	78	83	52	61
Density	0.062	0.053	0.064	0.047	0.087	0.05
Heterogeneity	0.826	0.926	0.687	0.758	0.639	0.691
Clustering coefficient	0.299	0.255	0.302	0.251	0.399	0.271
Diameter	8	8	8	9	6	9
Centrality index	0.173	0.159	0.121	0.14	0.174	0.138
Number of neighbors	4.861	4.848	4.949	3.831	4.462	3.016
Size	2.5	2.65	2.54	3.12	2.24	3.19
Mean connectivity	4.7	4.76	4.99	3.75	4.45	2.98
Degree	11.27	13.36	11.81	9.93	10.31	7.94
FFL subgraphs	20.00%	19.50%	17.00%	16.30%	18.20%	13.7%

more pronounced for PMA activation (Fig. 8b). Three of the genes mentioned above (NFKB-1, NFKB-IA, and RelA) even constitute an overlap between the rat transplant network and the PMA activation network.

Topological analysis of joint SMILE knock-down and PMA activation with tolerance and rejection networks from the rat data from Table II, also allowed to conclude that SMILE networks are topologically quite different than PMA activation networks. SMILE networks are denser, more clustered and connected, while being also more distributed. In contrast, PMA networks are less dense and more localized around specific genes.

Node connectivity also shows significant difference (Fig. 8c) as for the human data. Comparison graphs for average connectivities and clustering coefficient follow the same pattern (*not shown*). Considering that the rat networks are largely dominated by pro-rejection genes [Jovanovic et al., 2010], this leads to the same conclusion that SMILE knock-down more significantly affects the kidney transplant rejection network than PMA activation. Again from local topology analysis, we can see that SMILE network contains more FFL motifs than PMA network and this difference is even more strikingly true for rat compared to humans.

DISCUSSION

In this study, a microarray study of SMILE knock-down and PMA activation in HeLa cells was compared to our earlier analysis based on microarray data of kidney allograft tolerance and rejection in humans and in a rat model of heart allograft transplantation to determine possible new genes and gene networks involved in kidney transplantation. Based on bioinformatics analysis, we showed that at the intersection of the SMILE silencing gene network and earlier identified microarray-based pro-rejection versus pro-tolerance gene networks, five expression leader genes appear that form their own sub-network. These genes encode one transcription regulator, namely the kinase MAPK14, three transcription factors (JUN, ATF2, and TP53), and a histone acetyltransferase (HTATIP; Table I). This network has been expanded via STRING to its nearest neighbors as shown in Figure 5b. Also interesting are the two immediate neighbors of this subnetwork, Nephrin and Arrestin β2. Nephrin is a member of the immunoglobulin family of cell adhesion molecules that function in the glomerular filtration barrier in the kidney, being the structural component of the glomerular slit diaphragm [Ruotsalainen et al., 1999]. Arrestin β2 is known to have a significantly reduced expression in monocytes during kidney graft rejection as it has been recently demonstrated in human [Zakrzewicz et al., 2011] (Table I). Nephrin fosters activation of stress-activated protein kinase 38 and JUN which acts in complex with FOS [Huber et al., 2001]. In competition with the binding of Nephrin to the podocin, Arrestin β2 mediates Nephrin endocythosis and therefore its functioning reduction [Quack et al., 2006]. Note that the Arrestin β2 activation is via MAP kinase and down-regulates the TGF-β signaling pathway [Shenoy and Lefkovitz, 2011]. But most importantly, the network includes the protein XBP-1 already reported by Racapé et al. [2011] to be up-regulated in response to SMILE down-regulation, reinforcing these previous findings (Table I). FOS together with JUN forms a functional heterodimer with XBP-1 [Ono et al., 1991].

The comparison between the PMA activation network and the pro-tolerance network revealed that visually, the pro-tolerance network appears to be more tightly connected with the PMA activation network than the SMILE knock-down network, suggesting that tolerance may be associated to an activation profile. This will have, however, to be proven numerically, for example, by modifying our Leader Gene software [Bragazzi et al., 2011] to assign scores to *networks* and not to *individual genes*.

The other finding is that SMILE knock-down networks, irrespective of the context, show topological features strikingly different from the PMA activation networks. Indeed, analysis of the topological parameters of SMILE knock-down and PMA activation with transplantation networks in human shows increased density and clustering coefficient (that measures the degree to which nodes in a graph tend to cluster together, for example, average number of links between each gene's neighbors) of SMILE networks, meaning that SMILE networks are better connected and more clustered than PMA networks. Denser networks should be expected for SMILE because SMILE is a single gene while PMA activation may simultaneously activate or suppress many genes possibly unlinked to each other. In contrast, heterogeneity of PMA networks compared to SMILE networks suggests that there is a larger fraction of higher connected (hub) genes in PMA than in SMILE networks. In other words, SMILE knock-down leads to more evenly distributed effects on tolerance/rejection gene networks while PMA activation focuses on fewer but more highly connected genes. Finally, PMA networks

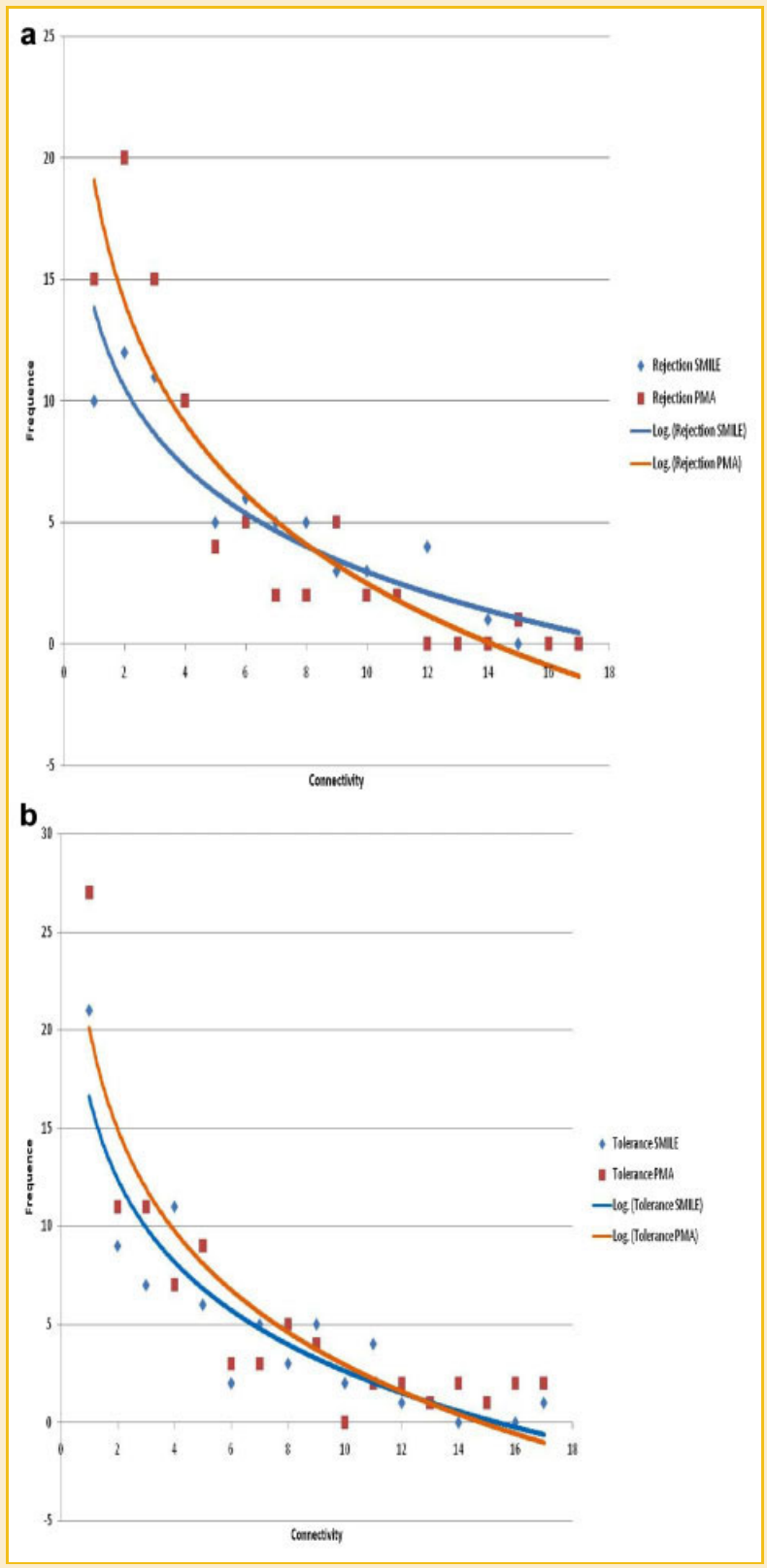

Fig. 7. Frequency distributions of node connectivity for rejection network in human + SMILE knock-down versus rejection network in human + PMA activation (a), and tolerance network in human + SMILE knock-down versus tolerance network in human + PMA activation (b). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

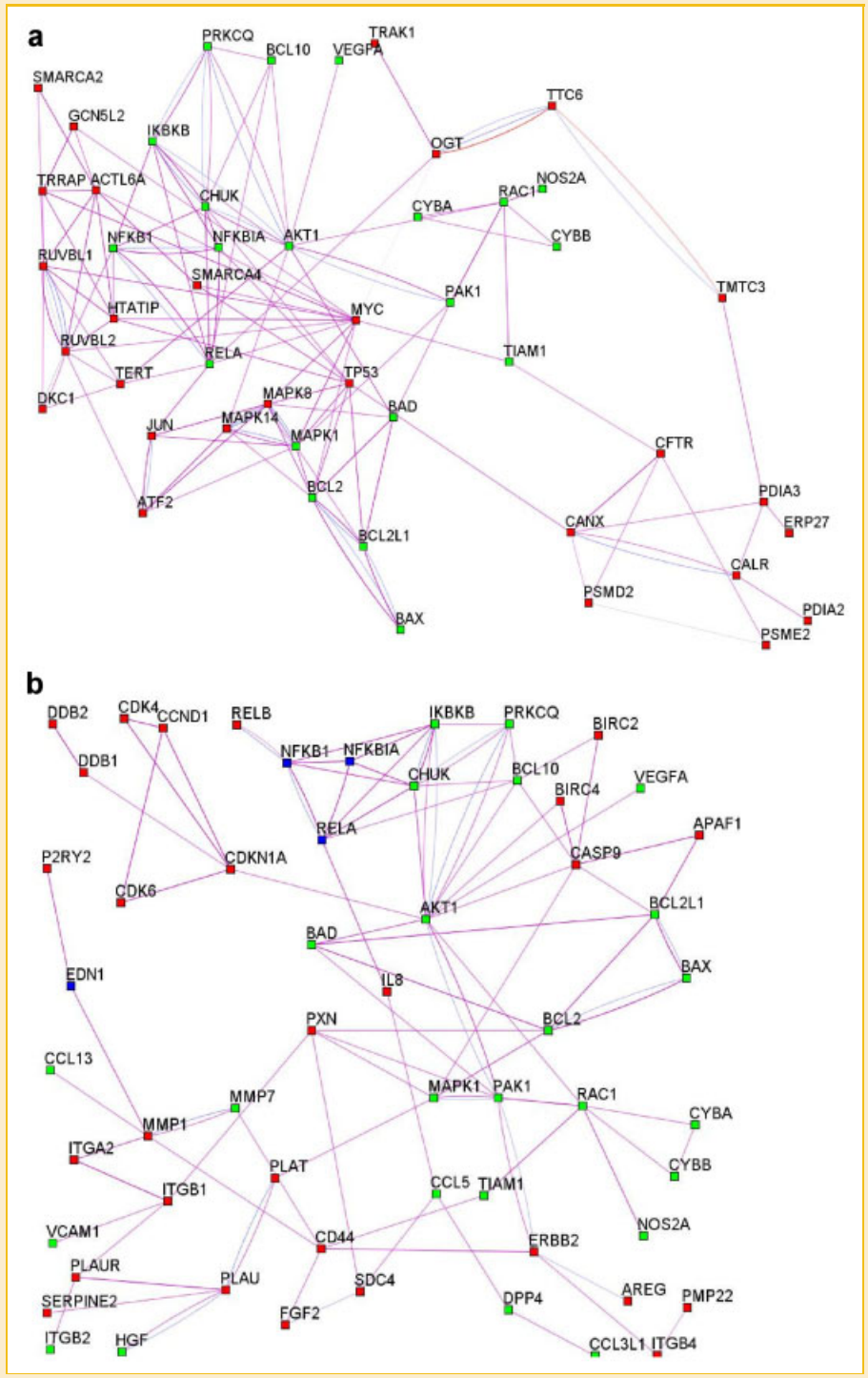

Fig. 8. Rat kidney transplant network (in green) is shown together with (both in red) SMILE knock-down (a) and PMA activation (b) for HeLa cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

display a bigger size of the networks (higher number of links). Larger number of links, however, does not mean better connection because links may be members of (partially) isolated subnetworks.

Another interesting observation is that SMILE networks contain more FFL motifs than PMA which means a more sophisticated genetic regulation. Interestingly, genes were found to be common between the two analyzed network combinations, SMILE knock-down network + rat transplant network and PMA activation network + rat transplant network (NFKB-1, NFKB-IA, and RelA). This allows concluding that common molecular mechanisms are involved in all three phenomena or that they are linked together through the same network. Besides, these results confirm our earlier

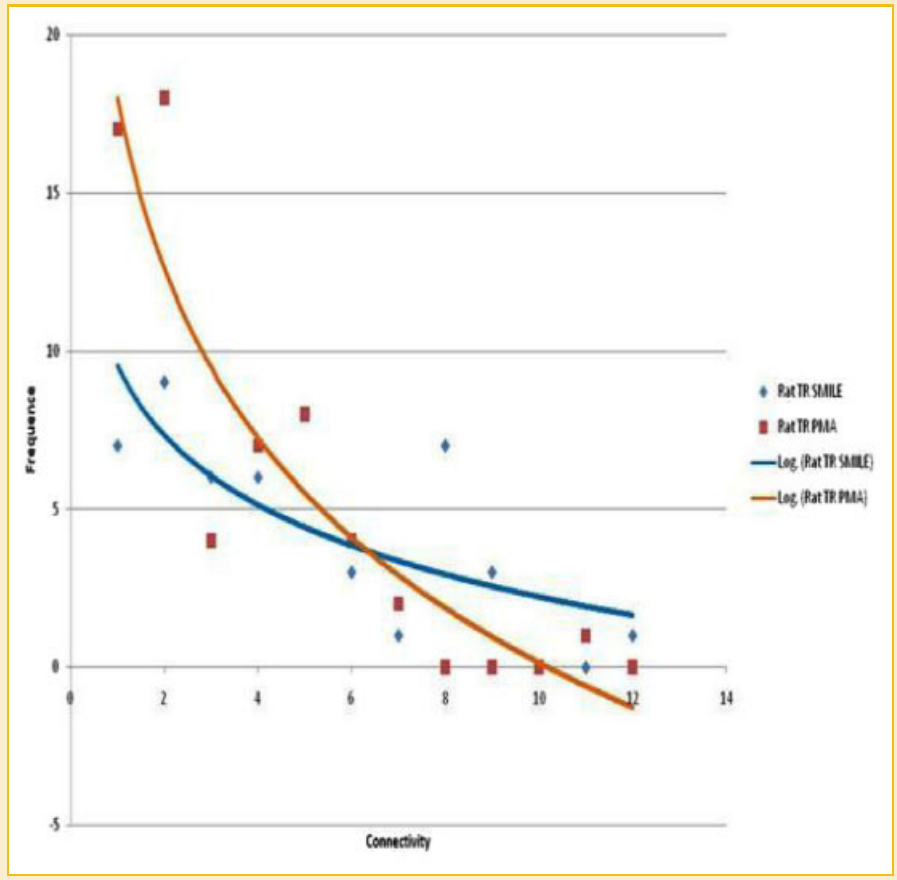

Fig. 9. Comparison of tolerance/rejection network in rat + SMILE silencing in HeLa cells versus tolerance/rejection network in rat + PMA activation in HeLa cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

finding on AKT1 gene in rat kidney transplant tolerance [Jovanovic et al., 2010], and this could be better investigated in further bioinformatics studies (Fig. 9).

ACKNOWLEDGMENTS

This study was supported by MIUR (Ministero dell'Istruzione, Università e Ricerca), Proteomics and Cell Cycle (FIRB RBIN04RXHS), and Nanobiosensors (FIRB ITALNANONET RBPR05JH2P_003), to Claudio Nicolini University of Genova as well as a MIUR grant for Funzionamento to Fondazione ELB.A. Nicolini.

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