Analysis of diverse regulatory networks in a hierarchical context shows consistent tendencies for collaboration in the middle levels

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Gene regulatory networks have been shown to share some common aspects with commonplace social governance structures. Thus, we can get some intuition into their organization by arranging them into well-known hierarchical layouts. These hierarchies, in turn, can be placed between the extremes of autocracies, with well-defined levels and clear chains of command, and democracies, without such defined levels and with more co-regulatory partnerships between regulators. In general, the presence of partnerships decreases the variation in information flow amongst nodes within a level, more evenly distributing stress. Here we study various regulatory networks (transcriptional, modification, and phosphorylation) for five diverse species, *Escherichia coli* to human. We specify three levels of regulators—top, middle, and bottom—which collectively govern the non-regulator targets lying in the lowest fourth level. We define quantities for nodes, levels, and entire networks that measure their degree of collaboration and autocratic vs. democratic character. We show individual regulators have a range of partnership tendencies: Some regulate their targets in combination with other regulators in local instantiations of democratic structure, whereas others regulate mostly in isolation, in more autocratic fashion. Overall, we show that in all networks studied the middle level has the highest collaborative propensity and coregulatory partnerships occur most frequently amongst midlevel regulators, an observation that has parallels in corporate settings where middle managers must interact most to ensure organizational effectiveness. There is, however, one notable difference between networks in different species: The amount of collaborative regulation and democratic character increases markedly with overall genomic complexity.

coregulatory partnerships | hierarchy | middle managers | autocracy | democracy

In the cell, gene regulation is mediated by specialized regulators that regulate the amount or activity of their targets. For example, transcription factors (TFs) regulate the expression of target genes (TGs) by binding to their regulatory regions. Similarly, by virtue of phosphorylation, kinases regulate the activity of their targets in a posttranslational manner by adding phosphate groups to certain amino acids. These interactions can be modeled by networks with edges pointing away from regulators to their targets (1–4).

Previously, regulatory networks have been arranged into more intuitive structures like pyramidal hierarchies with the regulatory edges (chain of command) pointing downward to obtain more insight into their architecture. There have been comparisons between corporate and biological hierarchies to demonstrate strikingly similar organization (5, 6). Rearrangement into hierarchies has also been used to identify functional modules and global regulators by network decomposition approach (7). It has been shown that distinct topological units (called origons) at the root of these hierarchies are significantly affected by environmental signals (8). These origons have been shown to be responsive at various stages of adaptation of *Mycobacterium tuberculosis* allowing a gradual progression of network under both replicative (growth) and nonreplicative (dormancy) states (9). Evolutionary analysis of *Escherichia coli* showed that transcriptional networks tend to grow by expansion of existing hierarchical layers, rather than addition of new layers (10). More recently, a study of TF dynamics and network architecture showed that top-level TFs in the hierarchy of yeast are relatively abundant, long-lived, and noisy whereas middle-level TFs are more well-connected and involved in higher number of GO processes (11).

In this study, we build upon the idea that depending upon the layout of regulatory edges, there are two aspects of regulation (12). In an autocracy, few top regulators influence their own set of targets directly or through a chain of influence (Fig. 1*A*). A social example of this kind would be a military hierarchy where general officers (such as general or lieutenant general) command over their own field grade officers (colonel, major, etc.) who in turn, are in charge of company grade officers (captain and first lieutenant). In a pure democracy, many genes exert regulatory influence on all other genes and the response is the concerted action of hundreds of genes (Fig. 1*B*). An example of this would be professional organizations such as a club or a scientific collaboration network without any apparent chain of command. Whereas an autocracy organizes into a neat hierarchy with well-defined levels but lacks comanagement or co-control, a democracy displays much more comanagement without well-defined levels or a clean hierarchy. It should be noted here that terms like democracy and autocracy used in this study do not exactly match the political science notions; they are defined based on analogies from Bar-Yam et al. (12).

Both autocratic and democratic scenarios are extremes and cells operate under an intermediate situation demonstrating a high degree of comanagement and coregulation with an architecture that can be organized into hierarchies (Fig. 1*C*). An example of this scenario would be a law firm formed by partners that have a well-defined place in the hierarchy and manage a common set of staff members such as associates and paralegals, which in turn share a team of legal assistants and interns.

In general, the presence of cross-regulation decreases the difference in information flow between nodes within a level, resulting in stress being more evenly distributed across the network. In an autocratic hierarchy (Fig. 1*A*), all regulatory information from the top regulators (*Squares*) to the circles pass through a specific midlevel regulator (*Triangles*). Thus, if a particular

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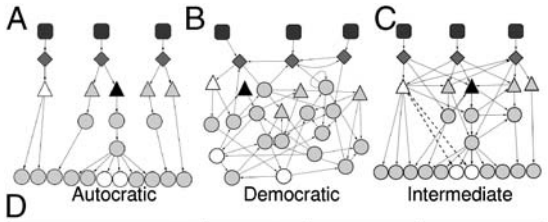

D

	Autocratic	Democratic	Intermediate
Betweenness (△)	1.03	3.6	3.3
Betweenness (▲)	4.1	1.08	3.4
Var. Betw. (triangles)	2.1	0.58	1.74
Var. Betw. (all)	2.9	1.4	1.9
$D_{Net\text{-}collab}$	0	0.91	0.71

Fig. 1. Regulatory architectures. (*A*) Autocratic organization with a clean hierarchy but no coregulation. (*B*) Democratic organization with no apparent hierarchy but many coregulation instances. (*C*) Intermediate architecture with a hierarchical skeleton and a high degree of coregulation. (*D*) Various properties for different components of the network: Normalized betweenness (Betw.), its variance (Var. Betw.), and $D_{Net\text{-}collab}$. Because there are many more edges in a democracy than an autocracy (between the same number of nodes), we normalize the betweenness of each node by diving it by the average value of betweenness over the entire network. $D_{Net\text{-}collab}$ is the average of D^i_{collab} over the entire network; $D_{Net\text{-}collab} = \langle D^i_{collab} \rangle_i \ \forall\ i \in N$. D^i_{collab} is defined as the fraction of targets that are coregulated by at least one other regulator.

midlevel regulator has many direct reports, it will become a major bottleneck. In contrast, in the more democratic layouts shown in Fig. 1 *B* and *C*, there are many paths from the top regulators to the bottom resulting in a distributed stress and less severe bottlenecks. In particular, in Fig. 1*A*, the addition of two cross-regulatory edges between the empty triangle and the empty circles reduces the normalized "betweenness" of the black triangle to 3.4 (it was previously 4.1) and also increases that of empty triangle to 3.3 (previously 1.03), thus decreasing the variation of betweenness in the middle level and evenly distributing the information flow. This has been shown for some larger instantiations of simulated hierarchies in Fig. S1 and *SI Text*.

In this paper, by reorganizing the biological networks into simple intuitive hierarchies, we examine their coregulation patterns for their similarities with comanagement collaborations in corporate hierarchies and show that both kinds of hierarchies bear close resemblances with one another. We essentially focus on multi-input motifs (MIMs) where a group of nodes together coregulate another set of nodes (2, 13) and place these motifs (and others such as feed-forward loops) in a hierarchical context following the chain of command.

Results

We analyzed cotranscription networks in five evolutionary diverse species for which the data available is reasonably abundant: *M. tuberculosis*, *E. coli*, yeast, rat, mouse, and human. The modification and phosphorylation network, however, are appreciably available only for human and yeast, respectively; for others they are very sparse. Fig. 2*A* provides the size of different networks for all species. We constructed coregulatory network by placing an edge between two regulators if they shared at least one target gene. Edges that were less significant than random were removed (Fig. 3*A*; see *Materials and Methods*). As used in previous studies (14, 15), this step only forms the most significant partnership associations.

Reorganization Into Hierarchies. We fractionated the regulators into three levels using a simple technique based on in-degrees (number of a node's regulators, Fig. 3*B*). In the top level are those regulators that do not have any incoming edges; they only have

A

	Regulators	Targets	Interactions
Sc_Ph	179	350	4,736
Hs_Mo	518	1,218	2,782
Hs_Tr	156	3,032	6,896
Rr_Tr	91	461	1,092
Mm_Tr	144	1,092	2,403
Sc_Tr	157	4,410	12,873
Ec_Tr	160	1,420	3,123
Mt_Tr	50	741	937

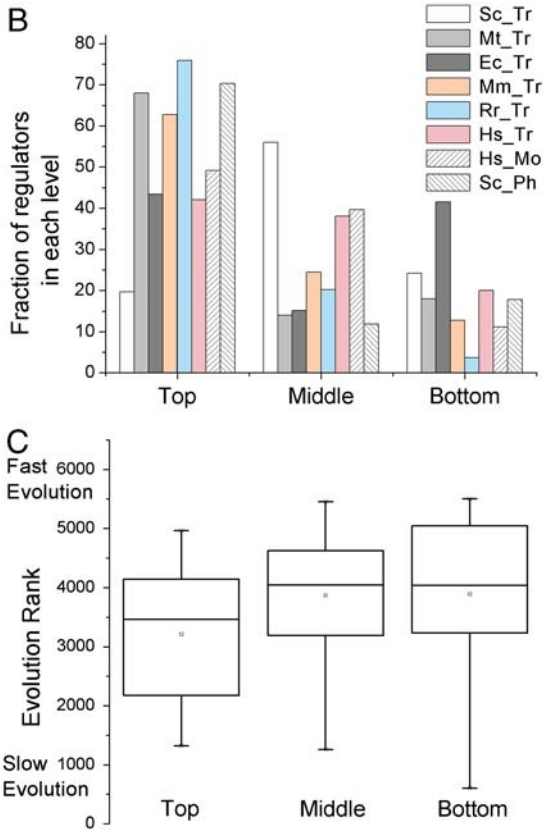

Fig. 2. Network sizes, relative population of different levels, and evolution rates. (*A*) Number of regulators, targets, and interactions in each network. Network names use the following notations: initials of the scientific name of the species (such as Sc for Yeast and Rr for Rat) followed by the kind of network (Tr for transcription, Mo for modification, and Ph for phosphorylation regulatory networks). (*B*) Relative population of the top, middle, and bottom level for each network. (*C*) Evolution rates of different levels for Yeast regulatory hierarchy (Sc_Tr).

outgoing regulatory edges (top managers). Those regulators that are regulated by other regulators and also regulate other regulators were designated to the middle level (middle managers). By this definition, all the loops in the network lie in the middle level. Finally, the bottom level consisted of the remaining regulators that were only regulated by other regulators (junior managers) and their targets are the nonregulator genes at the very bottom. Most of these hierarchies are not pyramidal (Fig. 2*B*).

Hierarchy Is Rationalized In the Context of Protein Function. To ascertain the biological relevance of assignment of regulators to

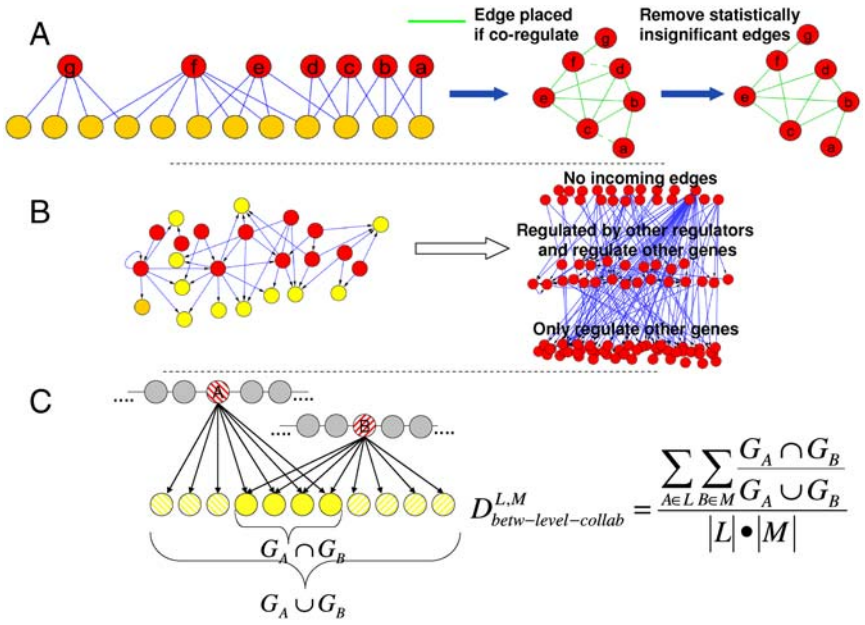

Fig. 3. Obtaining coregulation network and analysis of hierarchical structure of regulatory networks. (*A*) Beginning with regulatory network, we will first place an edge between two TFs (or kinases) if they coregulate (or cophosphorylate) at least one common target gene. Only those edges that are more probable than random will be retained (*Solid Green Edges* in the last network; see *Materials and Methods* for details). (*B*) Reorganization of regulatory networks into hierarchies. In the top level will be those regulators that do not have any incoming edges. Those regulators that are regulated by other regulators but also regulate other regulators will be designated to the middle level. Finally, the bottom level consisted of the remaining regulators that are only regulated by other regulators. (*C*) Investigation of coregulation tendencies between different levels in the hierarchy. We define degree of collaboration between two levels *L* and *M* using the intersection and union of target genes between all possible pairs of regulators from the two levels normalized for the size of each level.

different levels, we examined the prominent gene ontology (GO) cellular process categories of regulators in each level (they, of course, all have the GO molecular function of transcription). For example, in the case of *E. coli*, for the top level, the two most significant GO categories are response to stimulus and stress response. These categories are reasonable for top regulators, as responding to these stimuli involves a number of regulatory steps downward. So, these regulators receive these stimuli and respond to it by starting downstream regulatory processes. Most of the regulators in the middle level are involved in processes such as signal transduction and cellular metabolism. These involve a lot of cross talk and interregulatory interactions, which is reasonable for regulators in the middle level that are regulated by and regulate other regulators. The majority of regulators in the bottom level are involved in amino acid and carbohydrate catabolic processes, which are mostly stand-alone functions. These regulators directly bind to their target genes and carry out a specific process. GO annotation analyses, from the Database for Annotation, Visualization, and Integrated Discovery tool (16) for all other networks reveal similar observations (Fig. S2). The different levels also relate roughly to previous classification of proteins into distributors, integrators and workhorse proteins (*SI Text*) (17). We also investiaged the evolutionary patterns of transcription factors in different layers and found that top-level TFs evolve the slowest whereas bottom-level TFs show the hightest rate of evolution (Fig. 2*C* and *SI Text*).

Autonomous vs. Collaborative. Regulators can be divided into two categories based on the number of their coregulatory partners. Some regulators have only a small fraction of their target genes that are coregulated by other regulators (such as LexA in Fig. 4*A*). These autonomous regulators regulate a majority of their target genes in isolation. They represent a local instantiation of an autocratic structure. On the other hand, collaborative regulators regulate a large fraction of their target genes in combination with other regulators (such as FhlA in Fig. 4*B*) and represent local instantiations of democratic structure. For a given regulator, to distinguish an autonomous from collaborative mode, we calculate the fraction of target genes that are coregulated by at least one other regulator. This fraction, which we call "degree of collaboration" for a node i (D^i_{collab}) is close to 0 for autonomous regulators and is close to 1 for collaborative ones. Not surprisingly, GO annotation analysis for these regulators reveals that collaborative regulators are enriched in processes like sensory transduction and various signaling pathways (e.g., MAPK and TGF-β) that require them to interact with other regulators in the system whereas autonomous regulators are commonly involved in stand-alone processes like degradation and phosphorylation (Fig. S3 and Fig. S4).

To investigate if there is any bias toward autonomous or collaborative regulation in different species, we plotted the range of collaborative fraction of each node (D^i_{collab}) using a box-plot representation (Fig. 5). We observe that more complex species (such as human and rat) are shifted significantly (P-values listed) toward higher values of this ratio than the less complex ones (such as *E. coli* and yeast). This suggests that regulators tend to be more collaborative among higher species. Similarly, degree of collaboration for a pair of nodes, i and j, can be defined as

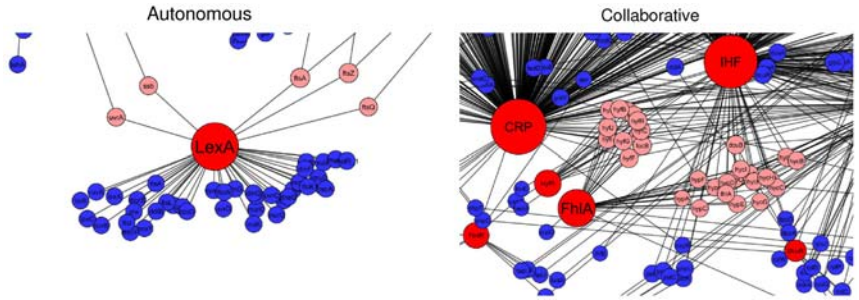

Fig. 4. Autonomous vs. collaborative hubs. The genes that are regulated by >1 TF are colored in light pink; the rest are in blue.

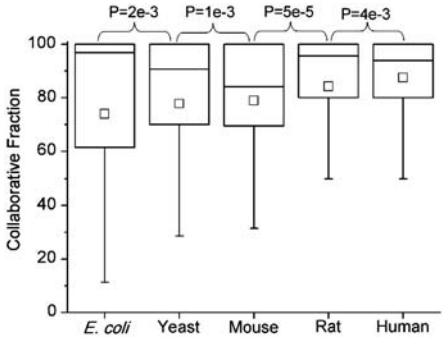

Fig. 5. Box-plot of the collaborative fraction (on the Y-axis) defined as the ratio of coregulated and total target genes for every regulator for all five transcriptional regulatory networks. P-values for each of the neighbors are calculated using the two sample Wilcoxon test with the null hypothesis that the distribution for species on the right (higher) is greater than that for the previous one (lower species). In other words, a low P-value indicates that the distribution for the higher species is shifted significantly toward higher values than the lower one. The smaller square corresponds to the mean of the distribution and is essentially $D_{\text{Net-collab}}$, degree of collaboration averaged over the entire network (*SI Text*).

$D^{i,j}_{\text{pair-collab}} = \frac{G_i \cap G_j}{G_i \cup G_j}$ where G_a is the set of targets of regulator a. Fig. S5 plots the histogram of $D^{i,j}_{\text{pair-collab}}$ and shows that a fairly good fraction of $D^{i,j}_{\text{pair-collab}}$ lies between the extremes of 0 and 1, suggesting that cellular regulatory hierarchies are intermediates between autocratic ($D_{\text{collab}} \sim 0$) and democratic ones (high D_{collab}).

Propensity of Each Level to Be Collaborative. We next wanted to determine which hierarchical level has the highest collaborative propensity. For this purpose, we define degree of collaboration for a level L as the average of the D^i_{collab} for all nodes i in level L, $D^L_{\text{Level-collab}} = \langle D^i_{\text{collab}} \rangle_i \ \forall\, i \in L$. Not surprisingly, we found that in all five species, the middle level showed the highest propensity to be collaborative (Fig. 6*A*). In other words, it is the target genes of the middle level that are coregulated by other regulators the most.

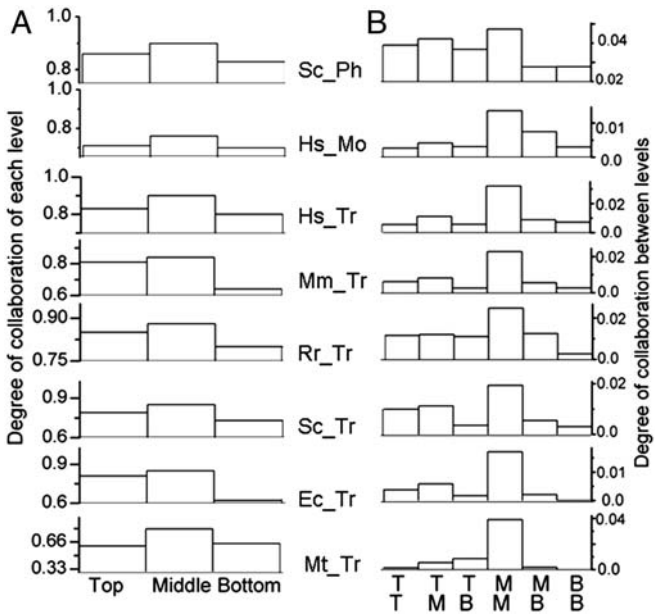

Fig. 6. Collaborative tendencies of and between various levels. (*A*) Normalized collaborative propensity for each level, $D^L_{\text{Level-collab}}$. (*B*) Degree of collaboration between different levels, $D^{L,M}_{\text{betw-level-collab}}$. Network names indicated in the middle follow the same notation as in Fig. 2.

Coregulation Collaborations Within and Across Different Levels. Next, we examined which two levels have highest coregulation tendencies between them. To investigate inter- and intralevel coregulation patterns, we defined degree of collaboration between the levels L and M as

$$D^{L,M}_{\text{betw-level-collab}} = \frac{\sum_{A \in L} \sum_{B \in L} \frac{G_A \cap G_B}{G_A \cup G_B}}{|L| \bullet |M|}$$

where G_i is the number of genes regulated by regulator i, and $|X|$ is the total number of regulators in level X (Fig. 3*C*). $D^{L,M}_{\text{betw-level-collab}}$ is essentially the ratio of the number of genes coregulated by two regulators (from the same or different levels) and the union of their target genes summed over all such pairs of regulators from the two levels. Note that it is normalized for the size of each level. $D^{L,M}_{\text{betw-level-collab}}$ varies between 0 (no coregulatory collaborations between two levels) and 1 (all the targets of either level are regulated by the other level and also vice versa). A higher $D^{L,M}_{\text{betw-level-collab}}$ between any two levels indicates a higher propensity between the regulators from those levels to coregulate their target genes.

Interesting patterns are observed for all five species and for all regulatory networks. First, we find that the highest degree of collaboration is between regulators from the middle level (Fig. 6*B*). In other words, it is most frequent for regulators in the middle level to pair up to regulate their common target genes. Second, the next two highest degrees of collaborations exist between middle and top level, and between regulators both from the top level. Finally, regulators that are both from the bottom level have the lowest tendency to coregulate. It is reasonable that middle-level regulators have a high degree of collaboration because of the large amount of cross talk in this level. Interactions between middle level regulators represent the "computational core" of the regulatory network. Bottom-level regulators, on the other hand, tend to activate terminal differentiation cascades, so lack of coregulation between bottom-level regulators is also reasonable. Each bottom-level regulator acts upon a different cellular process, and we expect minimal interaction between them. Similar observations are also obtained for an alternative approach of building the hierarchy (see *Robustness of Methodology and Incompleteness of Data*).

All these above findings are also readily seen in nonbiological hierarchies such as a corporate hierarchy. It has been shown that in a corporate setting, middle managers play a very important role and interact the most for organizational success (18–22). Middle managers play a critical role in linking the vision of top managers to the day-to-day realities of frontline managers (23, 24). Moreover, it is the junior managers that need least interaction with their peers; they only look after their own division and carry out the jobs assigned to them, which are mostly stand-alone (20).

As coregulation partnership between two regulators instantiates a MIM, we are essentially studying these motifs in a hierarchical context. Our results propose that certain kinds of MIMs are more commonly found in the regulatory networks of the five species than others. Fig. 7*A* lists the three kinds of most significantly present MIMs in the networks. In particular, in all networks, MIMs where both regulators come from the middle level are more frequent than the ones where one regulator is from the middle level and the top level each or the ones where both regulators are from the top level. We also examined the distribution of another kind of motif: the feed-forward loop (FFL) where a regulator regulates another regulator and they both regulate another common target (Fig. 7*B*). We find that the most common kind of FFL occurs between a top-level regulator and a middle-level

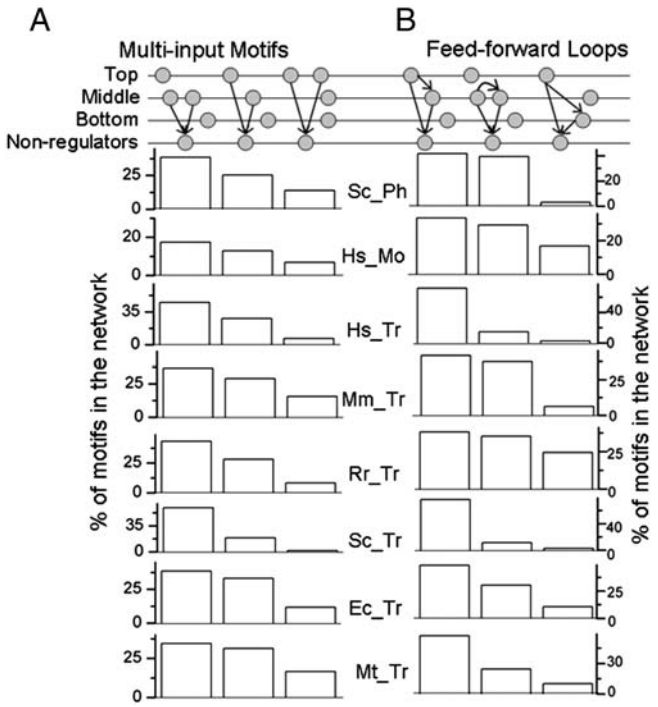

Fig. 7. Three kinds of most significantly present MIMs and FFLs in the decreasing order of their frequency. Values indicate the fraction of that particular kind of motif from all the occurrences of that class of motif (MIM). Network names indicated in the middle follow the same notation as Fig. 2.

regulator with a common target and the next most common FFL is found between two middle-level regulators. These frequencies of different kinds of MIM and FFL motifs are consistent with our observation above that middle-level regulators are most collaborative and with other previous studies (11).

Robustness of Methodology and Incompleteness of Data. One of the issues with studies dealing with the regulatory data that use different methodologies is the robustness of the results to the used definitions; it is often difficult to determine optimum values and definitions. To address this issue, we adapted different definitions and methodologies and repeated the analysis. In addition to above approach, we used another technique to construct hierarchies using both incoming and outgoing edges. First, all regulators were sorted in the increasing order of the incoming edges and decreasing order of outgoing edges. From this sorted list, we assigned top 30% to the top level, the middle 40% to the middle level, and the lowest 30% to the bottom level assigning the regulators with the most number of outgoing edges (and least number of incoming edges) to the top level and the ones with the most number of incoming edges to the bottom level. This addresses the issue of the top level diminishing in light of more data (when they have more incoming edges). We then calculated degree of collaboration between different pairs of levels and obtained similar results as above (Fig. S6) for specific types of networks (Fig. S7 and Fig. S8).

Discussion

In any given genome, the genes are regulated by regulators (which are fairly few in number) that control their expression (hence their amount) or their activity in the cell via combinatorial control where two or more regulators jointly regulate the target gene forming a coregulation networks. We have analyzed three kinds of coregulation networks for their hierarchical organization for five diverse species. These hierarchies had chains of commands going top down (or going horizontally in the middle level). We have uncovered some interesting coregulatory patterns between hierarchical levels common between networks from different species, e.g., the most frequent coregulatory interactions are formed between two regulators from the middle level whereas the least frequent ones are observed between those from the bottom level. Because an instance of coregulation by two regulators essentially represents a MIM motif, we have placed it in a hierarchical context and shown that certain MIMs are more frequent than others. We have also shown that target genes of the middle level are coregulated the most, and their most frequent partners are the other middle level regulators. The observations reported above also seen readily in a typical social setting where middle managers interact the most with their peers to manage those below them (18–20, 25). Similarly, managers at the lowest level supervise their specific department without much co-control over the workers under other managers (20, 25). Our results are also shown to be robust to the adopted methodologies and parameters that can be user-subjective. Interestingly, the above findings are seen more or less consistent across all five organisms suggesting that the above properties are inherent to the regulatory and coregulatory networks of all living species. However, one of the differences between these species is the relative magnitudes of collaborative nature of regulators: Regulators in more complex species demonstrate a higher collaborative nature. We believe that these are due to the vast differences between the size and complexity of these genomes. For example, in yeast, the estimated number of regulators is 250 that regulate 6,000 targets bringing the average number of targets to approximately 25 where as for human the number is about 10 (2,000 regulators regulating 20,000 genes).

In spite of the above similarities between social and biological comanagement hierarchies, some differences between the two should also be noted. First, there are fewer comanagement interactions in corporate settings than in biological hierarchies—the reason being that corporate hierarchies are more modularized by geography or department, e.g., the middle manager of one region does not control the staff from other regions. Second, there is less direct control between levels that are two or more levels apart (e.g., top and bottom levels) in social hierarchies than in biological ones (e.g., the chief executive officer rarely gives direct orders to the janitor), although, indirect control certainly exists in social hierarchies. In biological hierarchies, such controls are more prevalent (e.g., multi-input motif with one node from the top level and one from the bottom level). Finally, whereas in cellular regulatory machinery, most regulators only either activate or inhibit their target, most social settings exhibit simultaneously positive and negative regulation: A boss may task an employee in certain instances and may also prohibit the same employee in some others. Exceptions are regulatory agencies such as the Food and Drug Administration or a police body that only inhibits. It would be interesting to incorporate this kind of dual (positive or negative) regulation in our model.

Nevertheless, our above observations are readily understandable through analogies to social settings. Such studies comparing biological networks and hierarchies to social ones aid to our intuition about the organization of the internal machinery of the cell and give insight into the nonrandom architecture of the biological networks.

Materials and Methods

Dataset. Various data sources were used for different species: the largest collection of regulatory data obtained from published microarray data for *M. tuberculosis* (9), *regulonDB* version 6.2 for *E. coli* (26), results of genetic and biochemical experiments as used in previous studies for yeast (2, 14, 15, 27–31), and Transcriptional Regulatory Element Database database for rat, mouse, and human (as of June 2008) (32). The protein modification network was obtained from the Human Protein Reference Database database (33). Phosphorylation data for yeast was obtained from two large scale experimental studies (34, 35).

Network Transformation. To transform the regulatory network into the coregulatory network, we placed an edge between two TFs if they regulate the same target gene and generated 1,000 control networks with the same degree distribution (in- and out-degree of each node) as the original regulatory network. As in previous studies, the aim was to keep only those coregulation edges that are more probable than random (14, 15). For every pair, the ratio of the number of target genes regulated in real network and the average number of target genes regulated in random networks was calculated. Only edges with the ratio >1 were retained to keep only those coregulatory collaborations that are more frequent than random ones.

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