Genomic analysis of the hierarchical structure of regulatory networks

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Abstract

A fundamental question in biology is how the cell uses transcription factors (TFs) to coordinate the expression of thousands of genes in response to internal and external stimuli. The relationships between TFs and their target genes can be modeled in terms of directed regulatory networks. These, in turn, can be readily compared to commonplace “chain of command” structures in social networks, which have a characteristic hierarchical layout. Here, we develop an algorithmic approach for identifying generalized hierarchies (allowing for various regulatory loops) and use this to show that clear pyramid-shaped hierarchical structures exist in the regulatory networks of representative prokaryotes and eukaryotes (i.e., *E. coli* and *S. cerevisiae*), with most TFs at the bottom levels and only a few master TFs on the top. These masters receive most of the input signals of the whole network through interactions with other proteins. They are situated near the center the protein-protein interaction network -- a different type of network from the regulatory one. The master TFs have maximal influence over other genes. However, surprisingly TFs at the bottom of the regulatory hierarchy are more essential to the viability of the cell. Moreover, one might think that master TFs achieve their wide influence through directly regulating many targets, but actually TFs with most direct targets are in the middle of the hierarchy. We find, in fact, that these middle-level TFs act as bottlenecks of the hierarchy. This large amount of control for "middle managers" has parallels in structures found to be efficient in various corporate and governmental settings.

Introduction

Many biological processes can be modeled as networks, such as protein interaction networks, gene expression networks and transcriptional regulatory networks (1-4). Networks have been used as a universal framework to model many complex systems, such as social interactions, the internet, and ecological food webs (5-7). Individual networks have been globally characterized by a variety of graph-theoretic statistics, such as degree distribution, clustering coefficient (C), characteristic path length (L) and diameter (D) (3, 5-12). Recently, Barabási and colleagues proposed a "scale-free" model in which most of the nodes have very few links, with only a few of them (hubs) being highly connected (7, 8). Concurrently, Watts & Strogatz found that many networks can also be described as having a "small-world" property (12), i.e., they are highly clustered and have small characteristic path lengths. Complex networks can be further divided into two broad categories: directed and undirected. The edges of the directed networks have a defined direction.

Previously, people have compared protein-protein interaction networks to social communication networks and found that protein networks share some common characteristics with them, such as scale-free and small-world properties (3, 9). However, people have yet to do this with regulatory networks. Of all biological networks, regulatory networks are of particular interest, because to some degree they act as the master control system for the cell, tightly coordinating the expression of all genes (13-15). From a graph-theoretical point of view, regulatory networks are different from interaction networks in that they are directed. Both these facts suggest that we should compare regulatory networks to a different type of social networks, such as governmental and corporate organizations that are more oriented toward control than communication. These are known to have hierarchical layouts, with different levels – the stereotypical example would be a corporation with managers who supervise workers (16) (see Figure 1).

Social hierarchical networks are often very complicated, containing many network motifs. Motifs are defined as over-represented local network patterns (1). Four common ones in social hierarchies are shown in Figure 1 and described below:

(1) Single-Input Motifs (SIM), where a group of nodes (i.e., workers) are only regulated by a single node (i.e., manager).

(2) Multi-Input Motifs (MIM), where a group of nodes together regulate another group of nodes.

(3) Feed-Forward Loop (FFL), where a node regulates another; then, the two together regulate a third one.

(4) Feed-Back Loop (also known as Multi-Component Loop; MCL), where an upstream node is regulated by a downstream one.

What makes a hierarchical structure special is that there are central control points at the top. Whether such a hierarchical structure exists in biological regulatory networks is not currently obvious. Here, we have examined regulatory networks in both eukaryotes (*S. cerevisiae*) and prokaryotes (*E. coli*). We show that regulatory networks do, indeed, have a pyramid-shaped hierarchical structure that relates to their social counterparts. By doing so, we have also identified central transcription factors (TFs) in both organisms that are on the top of the hierarchies.

Results

Building generalized hierarchies using breadth-first search

A simple hierarchy in a strict mathematical sense requires that the network contain no loops (i.e., it is "tree-like") (17). However, even though the concept of a simple hierarchy originally came from social studies, it is rather difficult to apply this notion to real social and biological networks, because both these types of networks do indeed have prominent loops (Figure 1A). In a more general sense, a hierarchy just refers to a pyramidal layered or ranked structure organized as those in social networks with few people at the top (managers) and most people at the bottom (workers). Consequently, for this study we want to create a precise construction of "*generalized hierarchies*" that matches our social intuition and allows for loops. In essence, we assign a level number to each TF in the regulatory network to determine which TFs are at the top and which are at the bottom.

We call this construction method "BFS-level" (Breadth-First Search to define Level). As described in Figure 1B, it is based on a straightforward application of breadth-first search: we first identified all TFs at the bottom level (i.e., level 1). A TF is at the bottom level if and only if it does not regulate other TFs. TFs that only regulate themselves (i.e., auto-regulation) are also placed at the bottom. Starting from each bottom TF, we then performed a breadth-first search to convert the whole network into a "breadth-first tree" (18) (see Figure 2A and Table 1A). In other words, we define the level of a non-bottom TF in the hierarchy as its shortest distance from a bottom one. Here, the construction procedure is only focused on inter-regulation between TFs (or officials in social networks). A top TF could directly regulate non-TF target genes (or a higher-ranked official could have an assistant with no managerial responsibility), but this will not affect the constructed hierarchy. If the resulted layered structure has a pyramidal shape (i.e., few nodes at the top and most nodes at the bottom), we, then, considered it as a generalized hierarchy.

Note a few features about this construction:

> (1) It is mathematically precise. There is only one unique solution for a given network and a node is unambiguously placed at a single level.
>
> (2) It subsumes simple hierarchies. If a network does not contain loops, the BFS-level method would assign levels to nodes according to the perfect simple hierarchy of the network.

(3) It does not change the network topology or connections (i.e., it does not "amputate" the network). In particular, it preserves all loops and takes into account all connections in assigning level.

(4) It makes biological and social sense in that it builds from the ground up. One could imagine doing a similar breadth-first search from the top down (see supplementary materials). However, we believe that this does not match our social intuition (e.g., putting the owner of a small business at the same level of hierarchy as the president of a country).

(5) It is not trivial to construct a hierarchy for any given directed network. There are a number of possible variations as discussed below and in the supplementary materials.

Pyramidal regulatory hierarchies and their non-monotonic out-degree distributions

Figure 2A and Table 1A clearly show that the yeast regulatory network has a four-layer pyramid-shaped hierarchical structure: i.e., the number of TFs on each level is smaller than that of the previous level. A similar pyramidal hierarchy was also observed in *E. coli* (see Figure 2C and Table 1B).

This hierarchical structure is actually very similar to that in social networks. Figure 2B shows a representative social hierarchy – the Macao government. (This was chosen because, although it is realistic, it is sufficiently simple to represent on a single page.) In Figure 2B, there is only one chief executive (i.e., the president). Five secretaries are at the level immediately below the chief executive. There is a clear inverse relationship between the level in the hierarchy and the number of people at each level.

Intuitively, one might expect that the out-degree distribution at each level should parallel the pyramidal structure of hierarchy. For instance, it could increase uniformly as one goes from the bottom to the top, since, as you go up, there is more to regulate. However, this is not the case for social hierarchies. It has been shown that a typical organization scheme for companies is that middle managers supervise the most people, not those at the bottom or top of the hierarchies (16), as illustrated by Figure 2B.

We then examined the average number of targets for TFs at different levels of the regulatory hierarchies for both *S. cerevisiae* and *E. coli*. We found the same relationship, i.e., TFs at the second level have the most targets, while those at the bottom and higher levels all have fewer targets, by and large (see Figure 2A and C).

We also tested the robustness of our results by adding, deleting or rearranging 20% of the regulatory interactions at random. All results remain the same, suggesting that the global conclusions from our calculations would be largely unaffected by noise in the datasets (see supplementary materials). It is also noteworthy that there might be hidden

organizational structures since there are many within-level regulations, which is one of the possible directions for future analysis.

Bottlenecks of the hierarchies lie in the middle

Figures 2A and 2C clearly show that the regulatory information in the hierarchies is passed from the top to the bottom. A path in the regulatory network represents a specific regulation (activation or inhibition) of a downstream TF by an upstream one. If any intermediate TF along this path is disabled, the regulation is broken. If we consider each path as a unique flow of regulatory information, the number of paths through each node is therefore how much flow it controls. In graph theory, "betweenness" is an important topological parameter that describes precisely this concept. The betweenness of a node is defined as the number of shortest paths going through this node. If there are more than one shortest path between a pair of nodes, each path is given equal weight so that the overall weight of all paths is unity (10, 19). We call nodes with the highest betweenness "bottlenecks", in analogy to heavily-used intersections leading to major highways or bridges in social transportation systems. Since the TFs in the middle of the hierarchy not only pass the information directly to their targets but also carry the information flows from the top TFs to the bottom ones, it is quite intuitive to see that these TFs should be the bottlenecks that control the most information flows.

We calculated the average betweenness of all TFs at each level in the hierarchy. Our results agree well with our expectation (see Figure 2D): the TFs in levels two and three have significantly higher betweenness than those at the top or bottom of the hierarchy. Similar results were also observed in the *E. coli* hierarchy (see Supplementary Figure 10). To some degree, these results also validate the way we constructed hierarchies using our BFS-level method. Because the calculation of betweenness is only based on the connectivity of the network, completely independent of how we placed the nodes into layers within the hierarchy, the fact that the calculated results agree with our expectation confirms the plausibility of our method. Please note that one should not take the betweenness calculation as a definitive measure of the information flow, because it does not take into account some other possible contributing factors (e.g., gene expression and protein abundance).

Regulatory hierarchies are well organized

Next, we investigated random networks to see whether a similar hierarchical organization can be achieved by chance. We randomly rewired the edges between TFs and their targets within the whole yeast regulatory network (see materials and methods). Figure 2E clearly shows that the pyramid-shaped hierarchical structure does not exist in random networks, whose layered structures consist of many more levels (on average 7.2 levels) than real hierarchies (P-value < 0.001). Furthermore, the average out-degree is almost constant between different levels of random networks. Similar results were also found for randomly rewiring the *E. coli* hierarchy (see Supplementary Figure 9).

In a social context, it has been shown that flatter hierarchies give managers at each level more freedom (20). Moreover, the number of levels in a hierarchy is determined by the degree of standardization of the work processes. In a corporation where workers perform similar tasks (e.g., in an auto assembly plant), hierarchies tend to be flatter (21). In a similar fashion, different types of genes are known to cooperate together to carry out a certain function. Therefore, it is quite reasonable for the regulatory hierarchies to be flatter than random expectation.

It has also been found that the number of people supervised by each manager is determined by the nature of the job (21). In a situation where workers under the same manager perform different tasks and need more mutual accommodation (e.g., in a law firm), the average number of people supervised by a single manager is very small (22-24). A similar situation exists in the cell. At the top of the regulatory hierarchies, interplay between top-level and downstream TFs is needed to initiate a process. Furthermore, notice that top-level TFs tend to regulate TFs associated with many different pathways and functions (see below). Therefore, it is quite reasonable that the average out-degree at the topmost level is small. After commitment, however, the middle-level TFs can turn on massive expression of many genes in response to stimuli, reflecting their larger average out-degree. At the bottom of the hierarchy, TFs regulate only few specific target genes.

Decision-making schemes in regulatory hierarchies

We further analyzed the regulatory hierarchies in *S. cerevisiae* and *E. coli*. We were able to observe two distinct types of regulatory processes in them. These are readily understandable as different decision-making schemes, given that we know gene expression is regulated in response to various internal and external stimuli.

1. “Reflex” processes

A non-negligible number of TFs (52 in *S. cerevisiae*; 30 in *E. coli*) do not regulate other TFs, nor are they regulated by other TFs. They respond to specific stimuli turning on (or shutting down) the expression of their targets. We call this type of decision-making a “reflex” process. The regulation of the *trp* operon in *E. coli* is a perfect example. The *trp* operon encodes genes for the synthesis of tryptophan. TrpR is a repressor that, when activated by tryptophan binding, represses the expression of the *trp* operon. TrpR is not regulated by any other TFs (25). In *S. cerevisiae*, a similar example is Arg81, a TF involved in arginine metabolism (26). Upon the presence of arginine, Arg81 shuts down the expression of many enzymes involved in arginine biosynthesis, such as *ARG*1, *ARG*3, and *ARG*8 (27). (Note that some of the reflex assignments to TFs may result from incompleteness of the known regulatory datasets).

2. “Cogitation” processes

The majority of TFs in both regulatory networks are regulated by other TFs. Most of these regulate other TFs, as well. Thus, TFs at the top become the global modulators for all down-stream ones. The decision is amplified and executed while being passed down. We call this a “cogitation” process.

Cogitation processes have some nice parallels to the overall description of decision making in apoptosis. Apoptosis consists of three phases: decision, commitment and execution (28-30). In the first phase, the cell senses pro-apoptotic signals and determines whether it should die. This is reversible. In the commitment phase, however, the cell makes an unstoppable decision to die, which leads to the execution phase, where the actual destructive process is carried out (28-30). Such a multi-step decision-making scheme has two advantages: (1) it can work as a signal amplifier to rapidly increase the magnitude of the response just like the cAMP-cascade in glycogen metabolism (31); (2) it can act like a noise filter to convert continuous inputs into all-or-none switch-like outputs (32).

We can see clear examples of cogitation processes in the yeast regulatory hierarchy. In particular, the expression of *MOT*3, a top-level TF involved in aerobic growth, is activated by heme and oxygen (33, 34) (see Figure 3), representing the decision phase. Mot3 in turn activates the expression of *NOT*5 and *GCN*4 (1, 35), which are both mid-level TF hubs with a large number of targets. Once their expression is turned on, the cell is committed. Finally, in execution, Gcn4 activates two specific bottom-level TFs, Put3 and Uga3, which trigger the expression of enzymes in proline and nitrogen utilization, respectively (36, 37).

Please note that the distinction between the cogitation and reflex processes is purely based on the topology of the regulatory network. It is of course the case that even a reflex process could be very slow if the non-transcriptional events that underlie it are exceptionally time-consuming. However, transcriptional processes are normally much slower than non-transcriptional ones (e.g., phosphorylation). So it is quite reasonable to believe that our conclusions based on network topology reflect the actual timing of the processes.

Top-level TFs receive signals through protein-protein interactions

Through our analyses, we have shown that the regulation of gene expression in the cell normally happens in a multi-step fashion starting from the top TFs. Because (1) the cell regulates the expression of its thousands of genes in response to internal and external stimuli; and (2) TFs receive these signals through interactions with other molecules, mainly other proteins, since they usually function within the nucleus, we hypothesized that the TFs at the top of the hierarchy would receive most of the stimulating signals, and thus should have more interaction partners.

Figure 4A clearly shows that the top-level TFs on average interact with more proteins than the others, confirming our hypothesis. Furthermore, we examined another important topological quantity – closeness, defined as the inverse of the sum of the distances from a certain node to all other nodes (19). Figure 4B shows that the top TFs, by and large, have significantly higher closeness in the interaction network than all other TFs, indicating that these TFs are at the center of the interaction network (i.e., close to all proteins) (19). This further confirms our hypothesis that these TFs receive signals through protein-protein

interactions. The signals are then processed and passed onto lower-level TFs along the hierarchy. Finally, we analyzed the functional composition of the interaction partners of the TFs at each level of the hierarchy using the MIPS functional classification schemes (38). As shown in Figure 4C, we found that three functional categories are significantly enriched within the interaction partners of the top TFs compared with those of the bottom ones ($P < 0.05$). They are:

(1) Cellular organization. Most of the proteins in this category are localized to different organelles within the cell to keep their integrity.

(2) Metabolism. The cell utilizes these proteins to respond to the nutrition changes in the environment, such as during the diauxic shift when the yeast cell switches from using glucose to using ethonal as a carbon source (39).

(3) Cell defense and rescue. Obviously, most proteins in this category carry out defenses against various types of stress that the cell may sustain.

A good example is the protein Ire1 (see Figure 5), belonging to all of the three categories. It is a trans-membrane protein on the endoplasmic reticulum (ER) membrane, with serine-threonine kinase and endoribonuclease activities (40, 41). It is one of the main factors involved in the unfolded protein response and myo-inositol metabolism (40, 41). Upon the presence of unfolded proteins, Ire1 activates the SAGA complex (comprising Ada2, Gcn5, Hfi1, Ngg1, Spt20, Spt3, and Spt7) through directly interacting with Ada2 to enhance transcriptional induction of ER stress-responsive genes (42). In the available regulatory network, one possible path is that Ada2 successively turns on the expression of three TFs: Rtg3, Hmra1, and Ime4. Ime4 then induces the expression of 18 other genes. For example, Egd2 is a subunit of the heteromeric nascent polypeptide-associated complex that binds unfolded proteins in the ER to help them form secondary structures (43); Vik1 is involved in ER organization and biogenesis (44); and Zwf1 is required for oxidative stress response and fatty acid metabolism (45, 46).

One might think that most top-level TFs are involved in chromatin-remodeling complexes, because these complexes affect a large number of transcriptional events and their components have high degrees in the interaction network. However, this is, in fact, not the case (Please refer to Supplementary Table 1 for detailed descriptions on functions of top-level TFs). Even though there is no strong functional pattern for the top-level TFs. most of them seem to be global modulators that respond to various cellular stresses (e.g. anomalous levels of nitrogen or glucose).

The paradox of influence and essentiality

1. Higher-level TFs are more influential

We next examined the influence of each TF using the Rosetta knock-out experiments (47). Figure 6A shows that deletions of genes at higher levels of the hierarchy affect more genes than deletions of those at the bottom: i.e., higher-level TFs are more influential.

(Note that because the Rosetta knock-out experiments were only performed on 276 genes, no genes at level 3 were tested in the experiments.)

Furthermore, we investigated the influence of TFs in terms of the ability of their human homologs to initiate disease, especially cancer. We calculated the fraction of TFs at different layers that have cancer-related homologs in human. Our calculations show that human homologs of TFs at higher levels have a higher tendency to be cancer related (see Figure 6B), further confirming the influence of high-level TFs in the hierarchy.

2. Lower-level TFs are more essential

Since we have shown that TFs at higher levels are more influential, it is reasonable to assume that these TFs should also be more essential (i.e. lethal) (48). However, based on our calculations in yeast, we found that TFs at the lower levels of the network have much higher tendency to be essential (see Figure 6C). A similar result can also be obtained in *E. coli* (see Figure 6D). One possible explanation for the separation of the influence from essentiality may be that TFs at the top of the hierarchy act more like modulators coordinating gene expression across different pathways (e.g., Mot3); therefore, all pathways remain functional upon deletion of these TFs, even though the precise expression between most pathways will not be well organized. On the other hand, TFs at the bottom are in charge of specific pathways (e.g., Put3 and Uga3). Upon their deletion, certain pathways will cease operating, causing the cell to die.

Discussion

In general, our results show that there is a pyramid-shaped hierarchical structure in regulatory networks, which is well organized in a clearly non-random manner. The major decision-making scheme in this hierarchy is a "cogitation" like multi-step process, where the TFs at the top receive signals from internal and external stimuli through protein-protein interactions. These TFs strongly influence those below (in terms of the overall fraction of cellular genes affected). However, surprisingly, the TFs at the bottom are more essential to the viability of the cell.

Because bottom TFs are relatively easy to define in regulatory networks, our BFS-level method is a reasonable way to turn the network into a tree in graph theory (18). However, as mentioned above, it is not trivial to construct a hierarchy for any given directed network – there are a variety of possible variations that readily come to mind. In particular, our method essentially assigns the lowest possible level of each TF as its level in the hierarchy because it is shortest-path based. Alternatively, one could calculate the longest path from a TF to a bottom node and assign this number as its level. For simple hierarchies, both methods will produce exactly the same results. For networks containing loops, the constructed hierarchies will be slightly different. Our BFS-level method has problems solving feed-forward type of situations; while the longest-path method has problems solving feed-back type of situations. It is hard to argue which method is better. In the supplementary materials, we described implementing this variant and other related

ones. Our results show that, in fact, most variations have similar global trends, confirming the validity of our conclusions.

Furthermore, as shown in Figure 2E, our BFS-level method could assign a level number to every node in any directed network, even one randomly generated. However, the key aspect of a generalized hierarchy is its pyramidal shape. As we showed in Figure 2, regulatory hierarchies have a similar pyramidal shape as social ones. We are also able to show that the topological features of the regulatory hierarchy correspond well to aspects associated with efficiency in its social counterparts. As discussed in detail above, these features are completely different from those in random networks, suggesting their functional implications.

Moreover, previous studies have examined the relationships between the essentiality of a TF and its number of descendants (i.e., out-degree). It has been shown that TFs regulating more targets tend to be more essential (49).

Materials and methods

Regulatory networks. We constructed the *S. cerevisiae* regulatory network by combining the results of various genetic, biochemical and ChIp-chip experiments in yeast (1, 2, 50-54). To ensure the quality of the network, we manually examined the network and removed all questionable ORFs and DNA-binding enzymes (e.g., PolIII). The final network contains 8,371 regulatory interactions involving 286 TFs and 3,369 targets. The *E. coli* regulatory network was constructed in a similar manner, which consists of 2370 regulatory interactions between 145 TFs and 1063 genes (55, 56).

Yeast interaction network. The interaction network was created by combining various databases and large-scale experiments (38, 49, 57-63). Because large-scale experiments are known to be error-prone (64, 65), we only considered protein pairs with multiple sources of support (using the likelihood ratio $\geq$ 300 criteria from Jansen et al (66)). The final network contains 23,294 interactions involving 4,743 proteins.

Generation of random networks. We first generated random networks by randomly connecting TFs with target genes, while keeping the total numbers of TFs (286), target genes (3369) and edges (8371) as constant. Then, we ran BFS-level method to build the layered structure from the randomized network and repeated all calculations. This procedure was repeated 1000 times. The results were averaged and showed in Figure 2E. We also performed similar calculations for the *E. coli* regulatory network and found similar results (see Supplementary Figure 9).

Figure captions

Figure 1.

(Part A). Four common network motifs in social networks. Different colors represent different motifs. All four schematics came from real social networks shown in Supplementary Figure 11. (I) SIM. For example, node 1 is a professor or a director, and nodes 2 and 3 are his/her students or assistants, respectively. In the yeast regulatory network, node 1 is *NDD1*; nodes 2 and 3 are *STB5* and *MCM21*, whose only regulator is *NDD1*. (II) MIM. Nodes 1 and 2 can be professors. Nodes 3 and 4 can be two students that they co-advise. In Supplementary Figure 11B, nodes 1 and 2 are Senior Director and Executive Director; nodes 3 and 4 are different departments that they co-supervise. In the yeast regulatory network, nodes 1 and 2 are *FKH1* and *FKH2*. Together, they regulate node 3 (*DBF2*) and node 4 (*HDR1*). (III) FFL. For example, node 1 is the chairman of a department. Node 2 is a professor in the department. And node 3 is a shared secretary. In yeast regulatory network, node 1 (*MBP1*) regulates node 2 (*SWI4*). Then, they collectively regulate node 3 (*SPT21*). (IV) MCL. In Supplementary Figure 11D, node 1 is a chairman; node 2 is a director; node 3 is a coordinator; and Node 4 is a scientist. Then, some of the scientists form an advisory committee that oversees the chairman. In yeast regulatory network, node 1 is *REB1*; node 2 is *SIN3*; node 3 is *UME6*; and node 4 is *HSF1*.

(Part B). Illustration on how to determine a generalized hierarchy using our BFS-level method. (I) A toy example with all four motifs mentioned in part A. Each color represents a motif. The color coding is the same as that in part A. (II) Finding all the bottom nodes in the network. A TF is a bottom node if and only if it does not regulate other transcription factors. TFs that only regulate themselves (i.e., auto-regulation) are also considered as bottom nodes. All bottom nodes in the network are colored red. (III) Finding mid-level nodes. One does a one-level deep BFS search starting at each of the bottom nodes to find what regulates them. Direct regulators of all bottom nodes are considered as level-2 nodes, which are colored green. (IV) Finding top-most nodes. The procedure in the previous step (III) is repeated until all levels are determined. We call this overall process BFS-level. In this toy example, there are only three levels. The node at the top level is colored blue. But in the yeast regulatory network, there are 4.

Figure 2. Common characteristics of the hierarchical structures between regulatory networks and the Macao governmental organization. A. Illustration of the yeast regulatory hierarchy. The light blue arc arrows indicate the regulations between TFs at the same level. Many of these regulations are involved in loop structures (FFLs and MCLs). B. Illustration of the Macao governmental hierarchy. The bottom layer consists of people who do not manage anyone based on the available information, which are similar to the non-TFs in yeast. Therefore, level 1 of the hierarchy consists of peoples managing those at the bottom. C. Illustration of the regulatory hierarchy in *E. coli*. Average out-degree and total number of nodes at different levels are shown parallel to the hierarchies. P values in panels A and C were calculated using the student T tests to

compare the average out-degree of level-1 TFs with that of the TFs at other levels. D. Average betweenness at each level of the year hierarchy. P values are calculated using the student T tests to compare the average betweenness of the top and bottom TFs with that of the middle-level TFs, respectively. E. Comparison between yeast regulatory network and randomized networks.

Figure 3. A biological example to illustrate the multi-step cogitation processes in the regulatory hierarchy: aerobic growth mediated by Mot3. We divided the whole figure into two parts: nucleus and cytoplasm, because TFs only function in the nucleus whereas other proteins (such as the enzymes Put1, Put2, Uga1, Uga2, and Uga3) normally function in the cytoplasm.

Figure 4. (A). Average number of interaction partners for the TFs at each level. (B) Average closeness for the TFs at each level. P values in panels A and B were calculated using the student T tests to compare the top bar with the sum of the test bars. (C) Enrichment of functional categories relative to level 1. For each functional category in the MIPS functional classification schemes, we calculated the percentage of the interaction partners of the TFs that have this function. The percentage of a certain category was then normalized against the corresponding one at level 1. Therefore, all bars at level 1 have a value of 1. Because we are analyzing the transcriptional regulatory networks, we ignored the functional category "transcription". P values were calculated using cumulative binomial distributions to compare the statistical significance of the enrichment at level 4 to that of the sum of the other levels (see supplementary materials).

Figure 5. A biological example to illustrate that the top-level TFs receive internal and external signals through protein-protein interaction: unfolded protein response mediated by Ire1.

Figure 6. A. Deletion of TFs at higher levels disrupts the expression of more genes. A gene is defined as disrupted if it has a P-value smaller than 0.05 determined by Rosetta knock-out experiments (47). Because the knock-out experiments were only performed on 41 TFs, T tests cannot be performed to examine the statistical significance of the differences between the average numbers of affected genes across different levels. Therefore, we performed a χ^2 test and found that deletion of TFs at higher levels disrupts the expression of more genes, which is statistically significant when compared with random expectation ($P < 10^{-45}$; see supplementary materials). B. TFs at higher levels in the hierarchy have a strong tendency to have human homologs associated with cancer. P values measure the statistical significance between the fractions of human cancer gene homologs among TFs at a certain level with that at level 4. C. TFs at the bottom of the yeast hierarchy have a strong tendency to be essential genes. P values measure the statistical significance between the fractions of essential genes among TFs at a certain level with that at level 2. P values were calculated using cumulative binomial distributions (see supplementary materials). D. TFs at the bottom of the *E. coli* hierarchy have a strong tendency to be essential genes. All calculations are similar to those in panel C.

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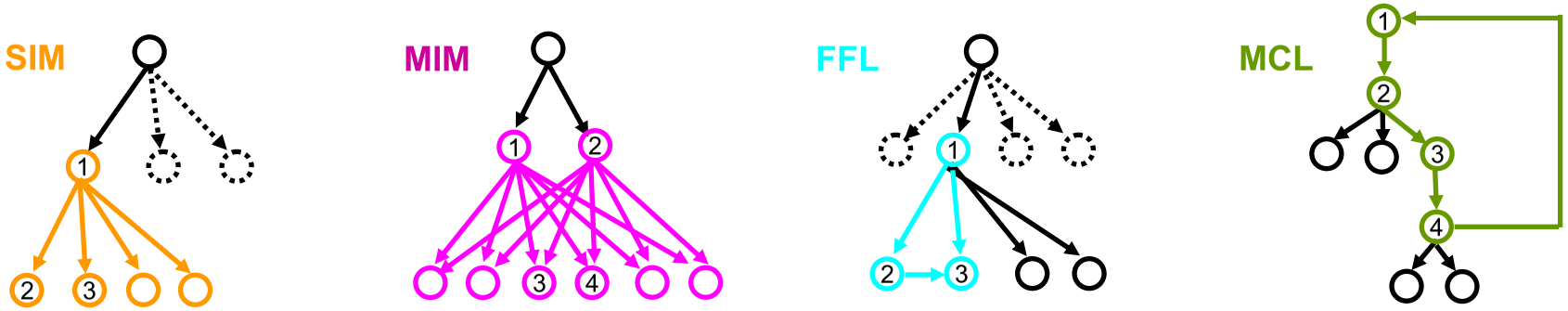
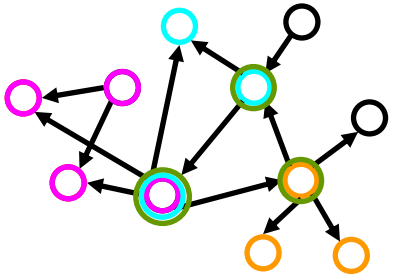
A. Motifs in Social and Biological Networks

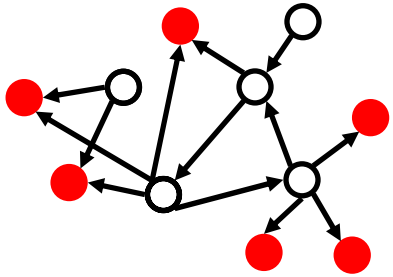


B. Determining Generalized Hierarchy with BFS-level

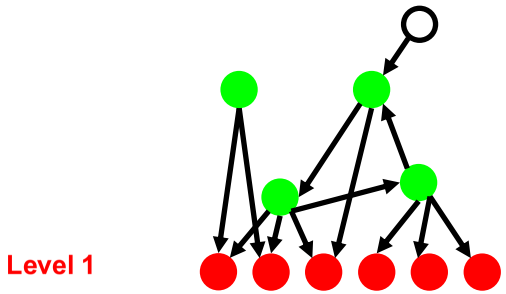
I. Example network with all 4 motifs



II. Finding terminal nodes (Red)



III. Finding mid-level nodes (Green)



IV. Finding top-most nodes (Blue)

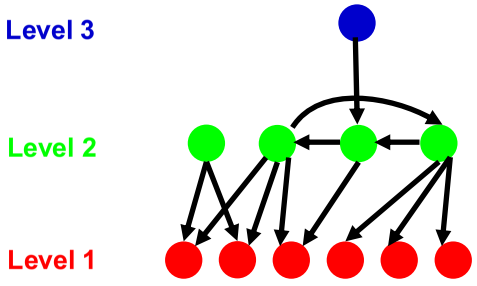
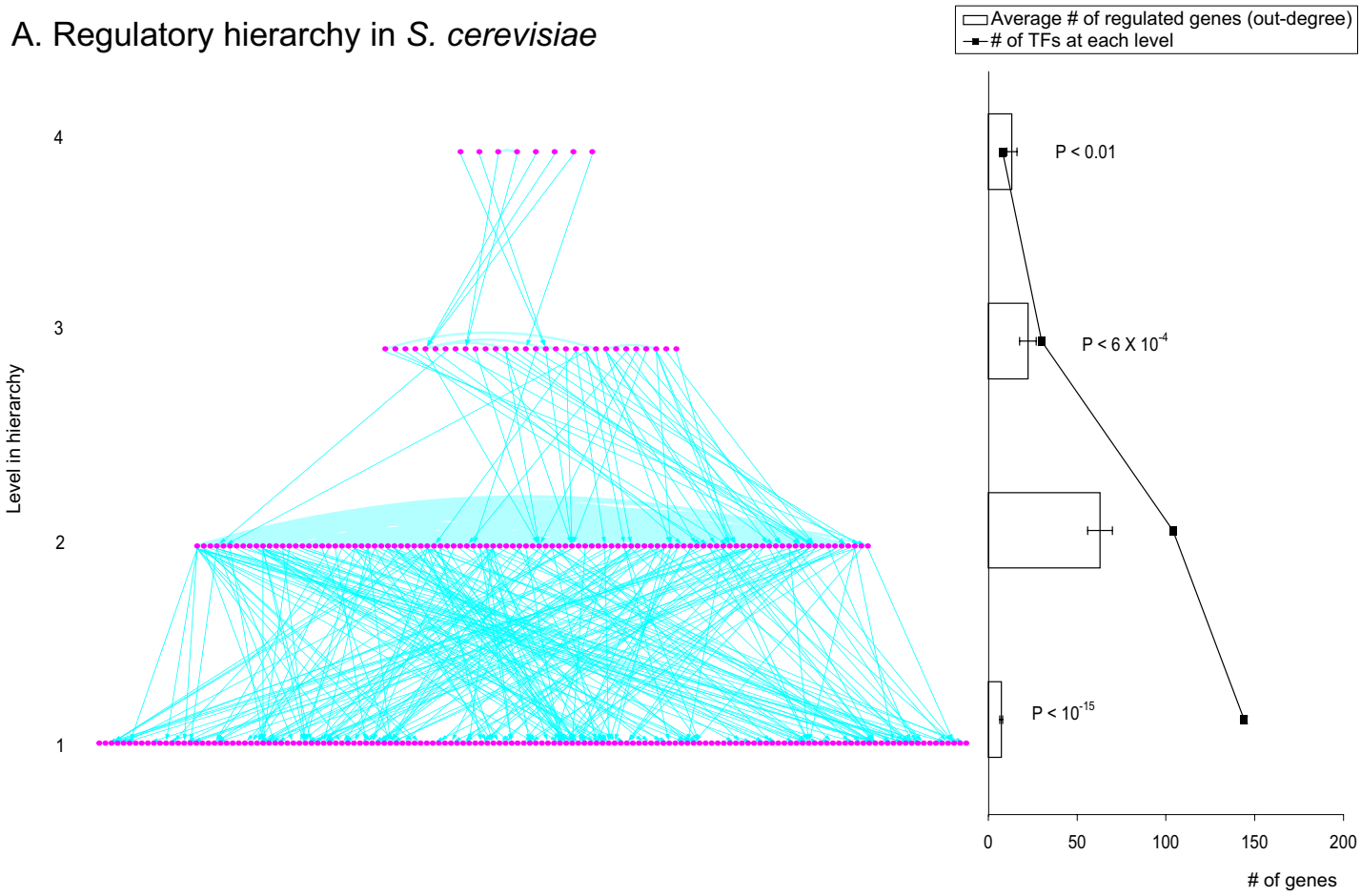


Figure 1

A. Regulatory hierarchy in *S. cerevisiae*



B. Governmental hierarchy of a representive city (Macao)

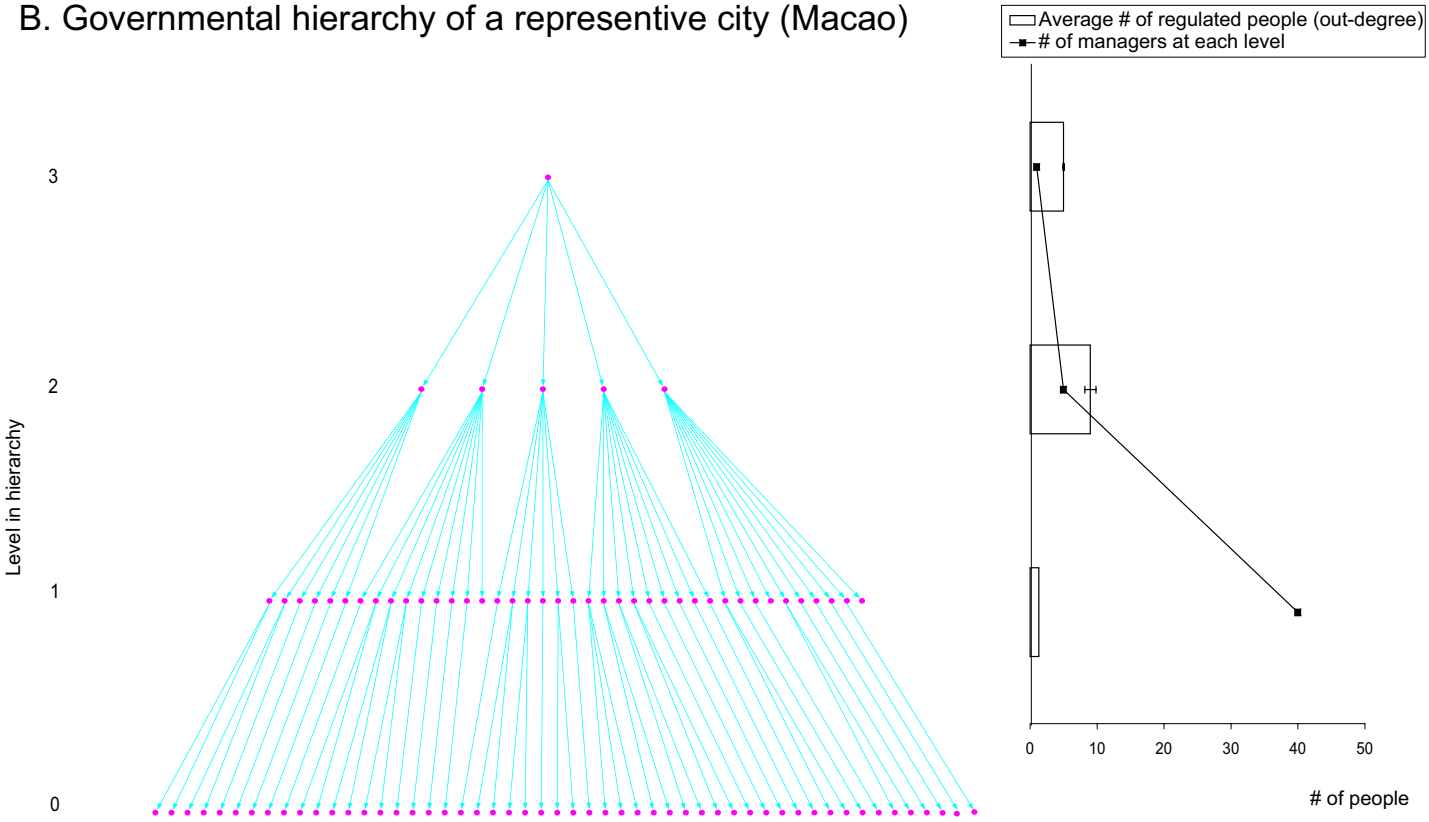
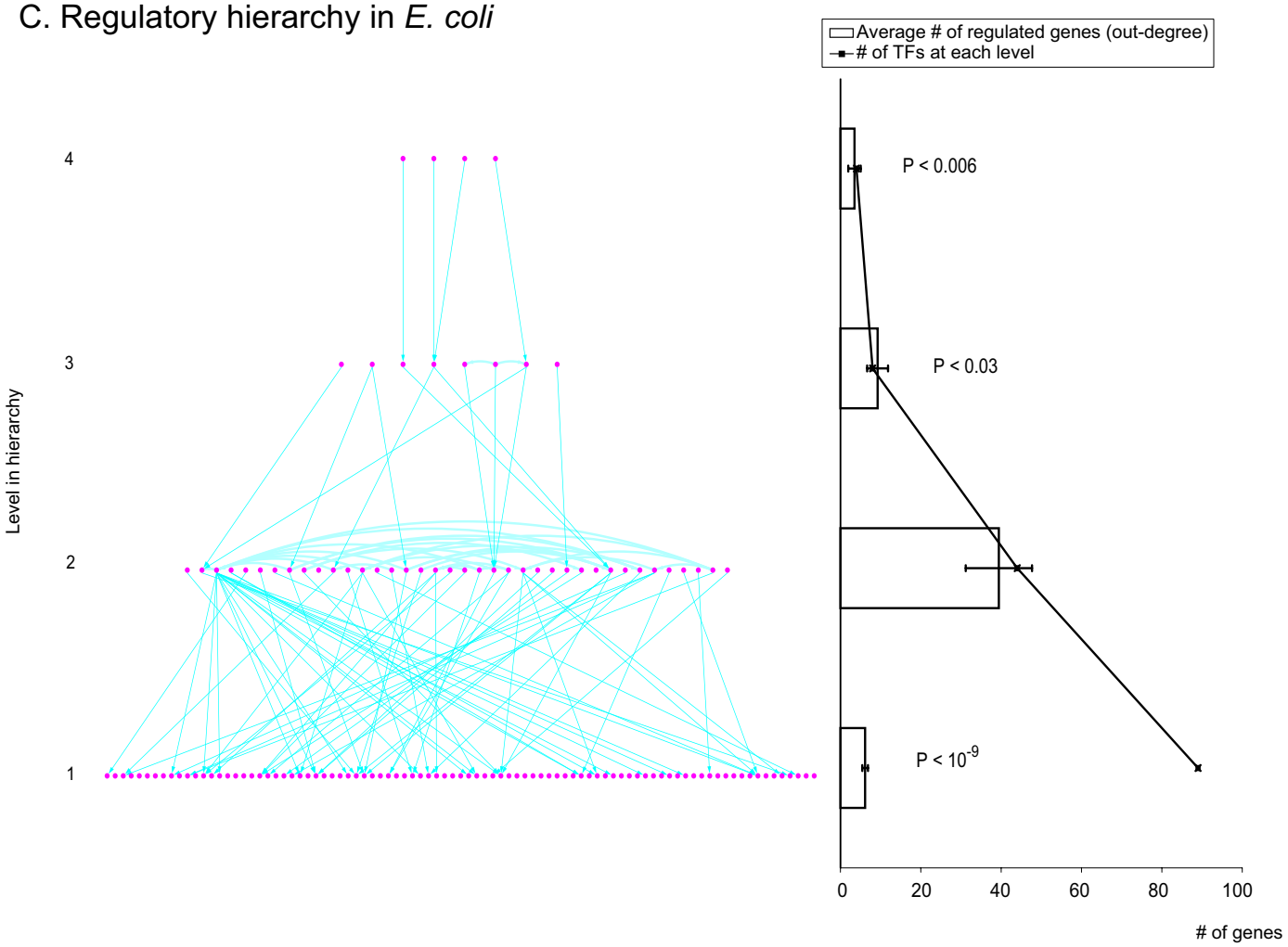
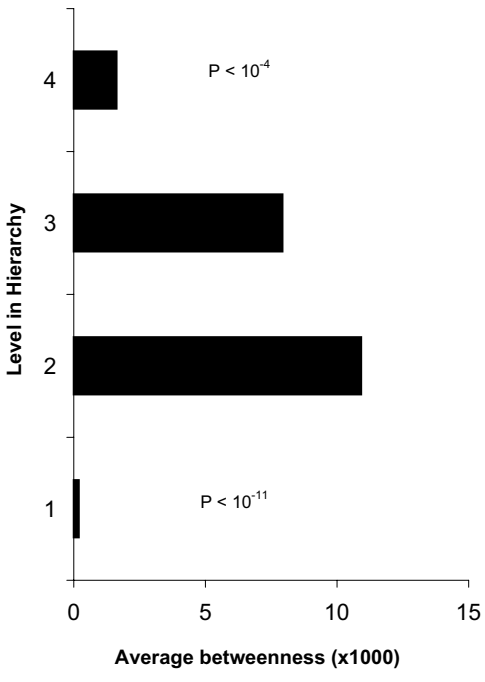


Figure 2AB

C. Regulatory hierarchy in *E. coli*



D. Average betweenness at each level



E. Comparison with random expectation

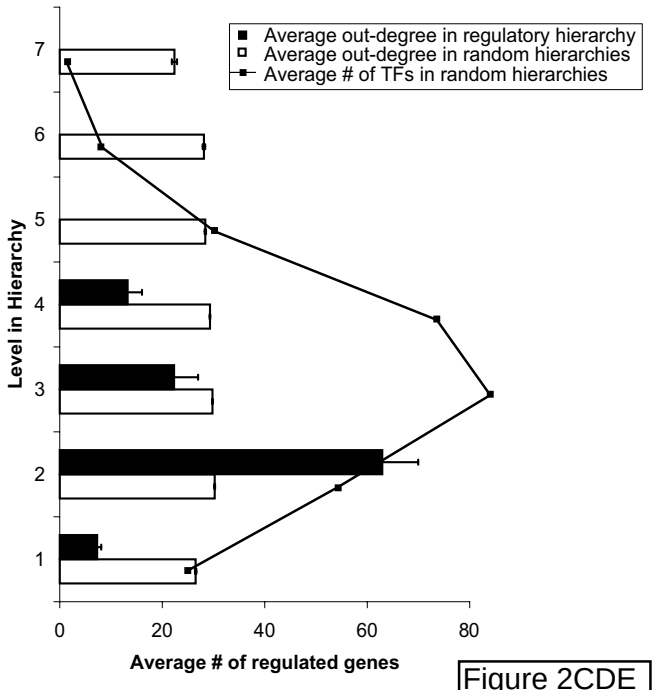


Figure 2CDE

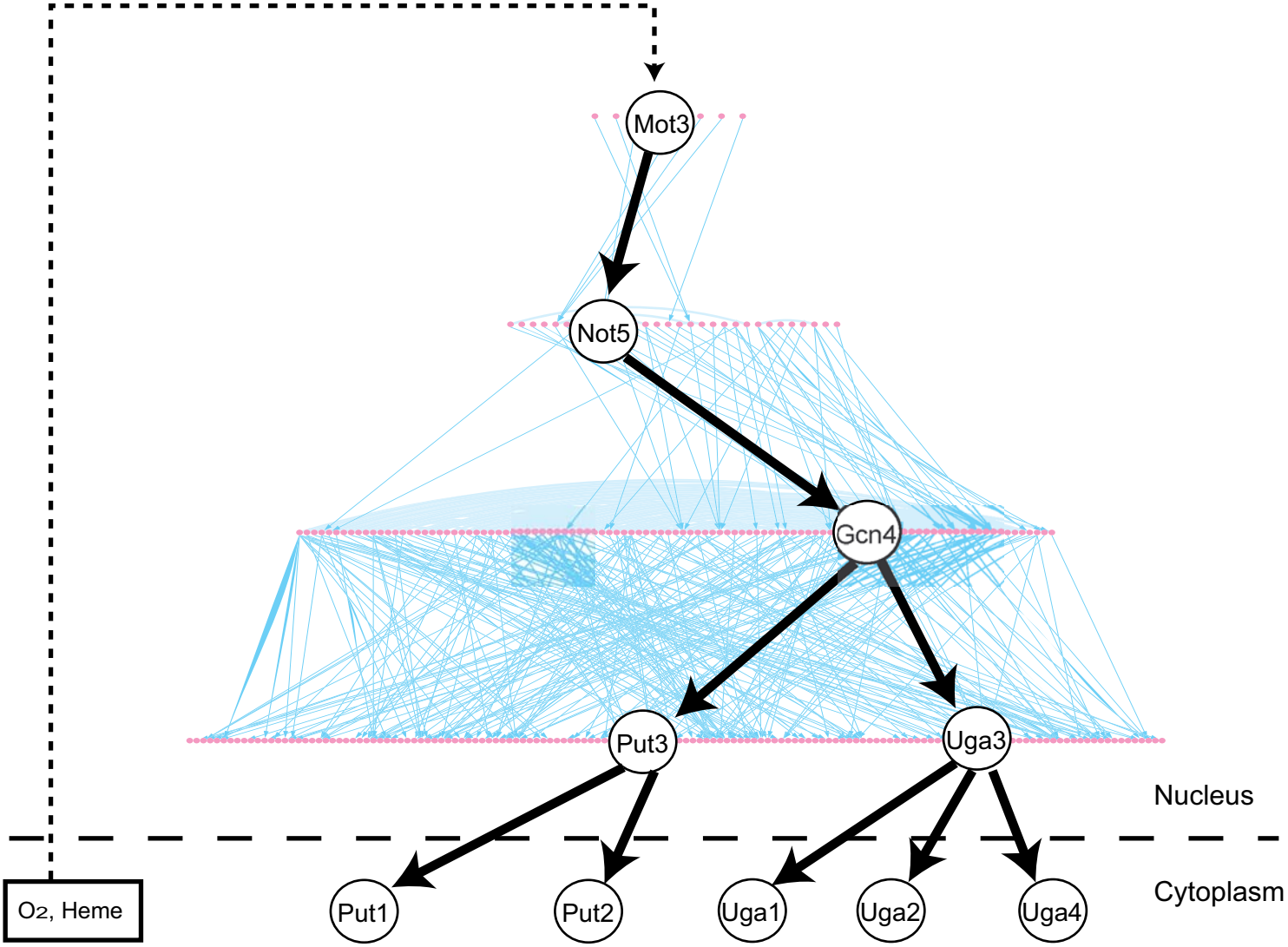

Mot3
Not5
Gcn4
Put3
Uga3
Nucleus
Cytoplasm
O2, Heme
Put1
Put2
Uga1
Uga2
Uga4

Figure 3

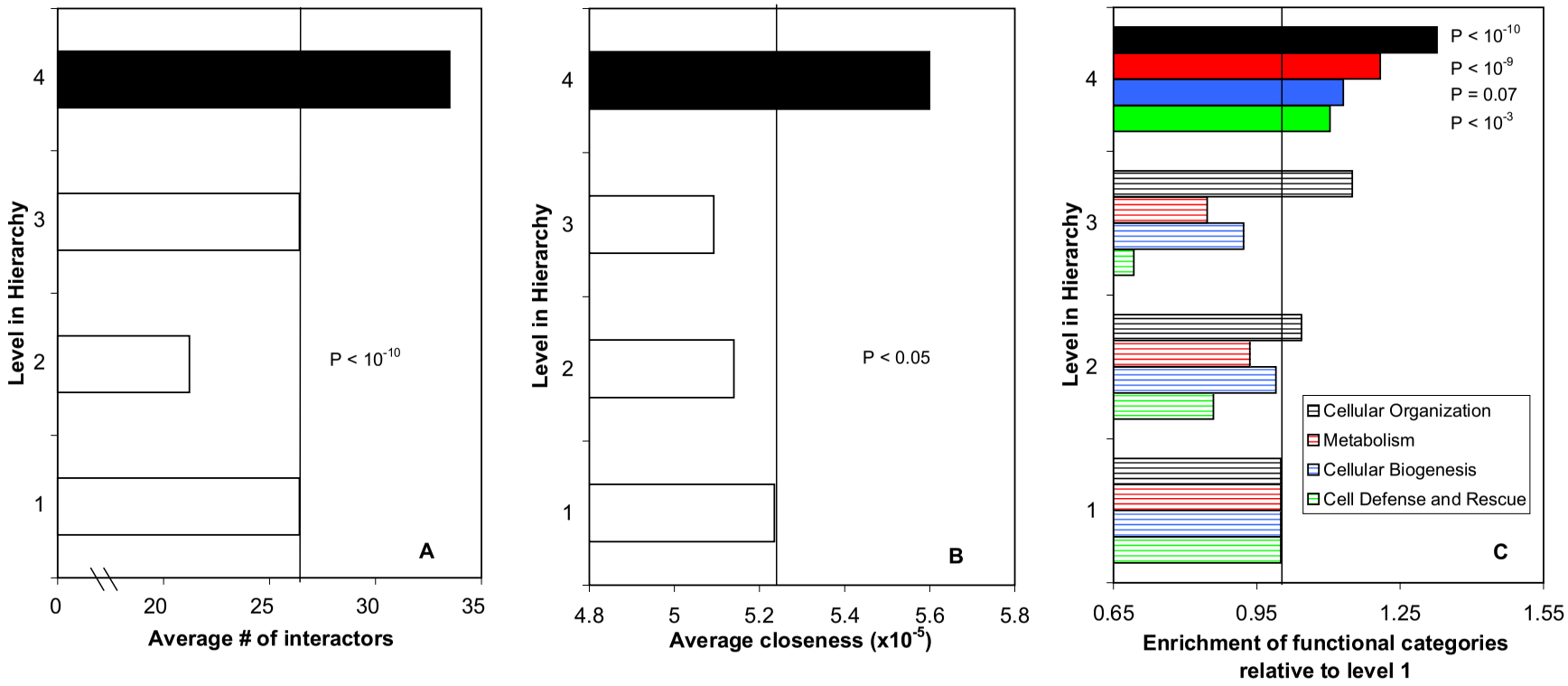

Level in Hierarchy
4
3
2
1
P < 10-10
A
0
20
25
30
35
Average # of interactors
P < 0.05
B
4.8
5
5.2
5.4
5.6
5.8
Average closeness (x10-5)
P < 10-10
P < 10-9
P = 0.07
P < 10-3
Cellular Organization
Metabolism
Cellular Biogenesis
Cell Defense and Rescue
C
0.65
0.95
1.25
1.55
Enrichment of functional categories relative to level 1

Figure 4

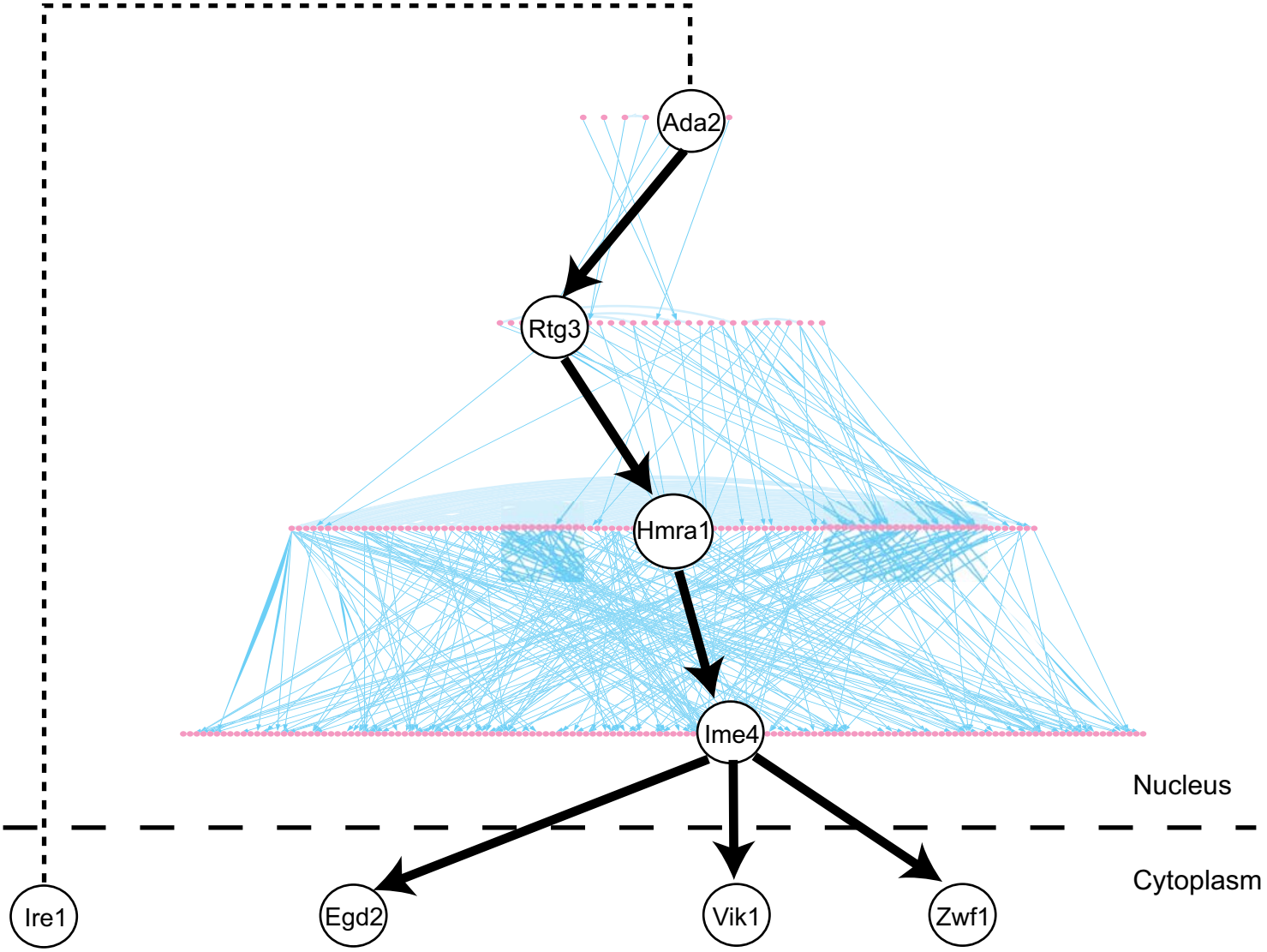


Figure 5

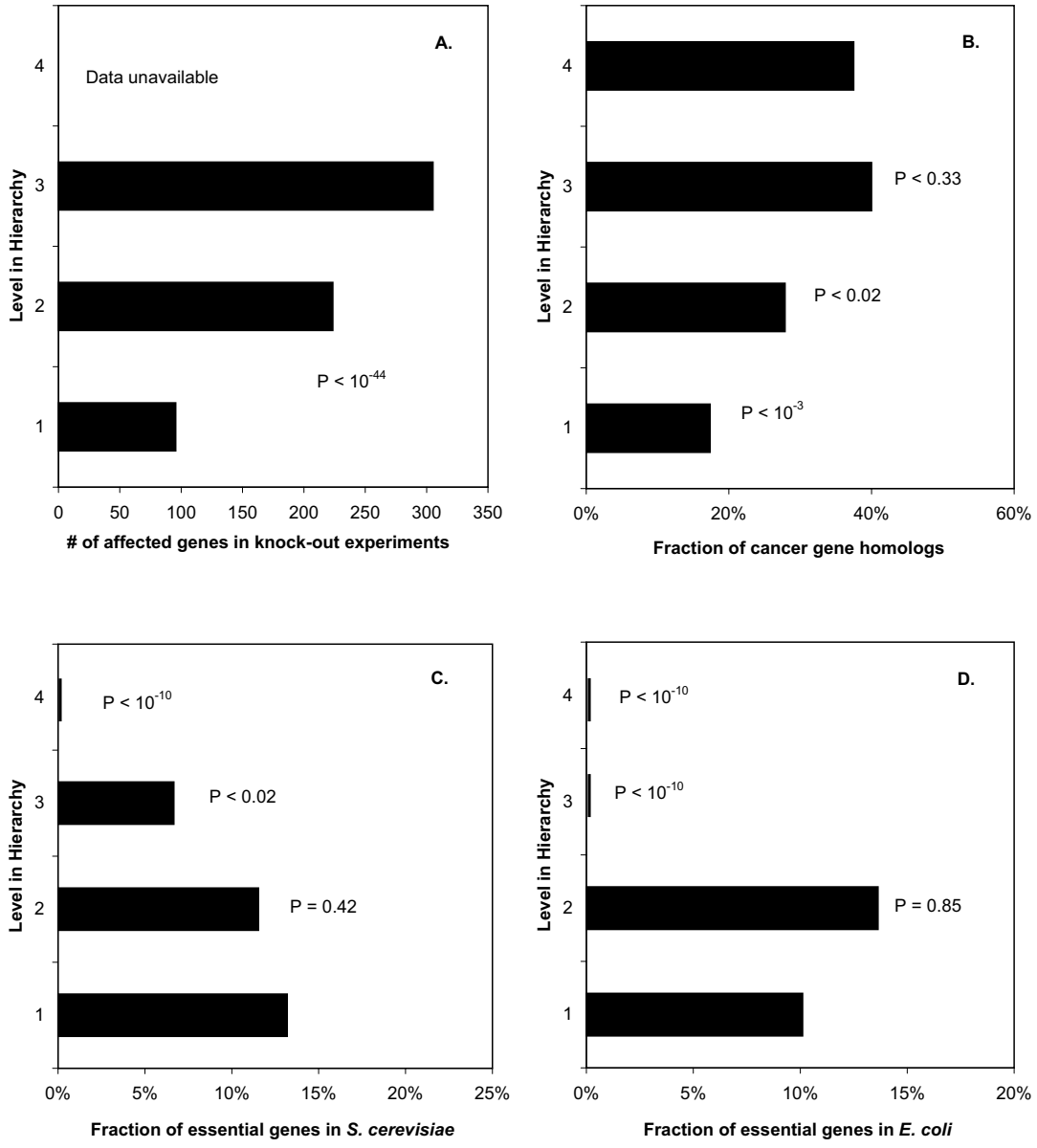

A.
Data unavailable
Level in Hierarchy
4
3
2
1
P < 10-44
0
50
100
150
200
250
300
350
# of affected genes in knock-out experiments
B.
P < 0.33
P < 0.02
P < 10-3
0%
20%
40%
60%
Fraction of cancer gene homologs
C.
P < 10-10
P < 0.02
P = 0.42
0%
5%
10%
15%
20%
25%
Fraction of essential genes in S. cerevisiae
D.
P < 10-10
P < 10-10
P = 0.85
0%
5%
10%
15%
20%
Fraction of essential genes in E. coli

Figure 6

Table 1.

A. Hierarchy of *S. cerevisiae* regulatory network

Level	Genes									
4	*SPT23*	*HIR3*	*ADA2*	*GAT1*	*NGG1*	*DAT1*	*MOT3*	*GZF3*		
3	*MIG2*	*ZMS1*	*SWI3*	*SET2*	*IMP2'*	*MIG1*	*HFI1*	*XBP1*	*RTG3*	*ZAP1*
	SIR2	*SIR4*	*HAP1*	*DAL80*	*CYC8*	*ARO80*	*PHO80*	*SUI2*	*PHO2*	*SPT20*
	GAT3	*BDF1*	*NOT5*	*RIM101*	*SIN3*	*OPI1*	*CDC47*	*MSN4*	*HPR1*	*HMRA2*
2	*SMP1*	*INO2*	*CLN3*	*SIR3*	*SUT1*	*HAC1*	*SNF5*	*IME1*	*SKN7*	*RGT1*
	CUP9	*RFX1*	*YOX1*	*TUP1*	*YAP6*	*CIN5*	*HIR2*	*YFL044C*	*YML081W*	
	HSF1	*HAP3*	*HCM1*	*PHO4*	*NDD1*	*FKH1*	*CLN1*	*UME6*	*CAD1*	*REB1*
	MET4	*ASK10*	*FAR1*	*TOS4*	*CRZ1*	*SPT16*	*STP2*	*SUM1*	*DOT6*	*LEU3*
	GAL4	*MATA1*	*HAP4*	*GCN4*	*RAP1*	*RLM1*	*KTI11*	*FKH2*	*IXR1*	*YHP1*
	YAP1	*MBP1*	*TYE7*	*FZF1*	*POG1*	*NRG1*	*MET32*	*HMLALPHA1*		*STE12*
	ASH1	*HMLALPHA2*		*SPT5*	*NHP6A*	*GAL11*	*OAF1*	*HAP5*	*SWI5*	*DIG1*
	HMS2	*SET1*	*SOK2*	*BCK2*	*SNT2*	*PDR3*	*PDR1*	*PHD1*	*ACE2*	*ADR1*
	CBF1	*RTG1*	*CAT8*	*CSE2*	*MCM1*	*ROX1*	*SWI6*	*PAF1*	*KSS1*	*SWI1*
	RME1	*ABF1*	*ATS1*	*TEC1*	*SFP1*	*MAC1*	*ALPHA1*	*GLN3*	*AZF1*	*FHL1*
	SWI4	*MET31*	*HAL9*	*STB1*	*TOS8*	*NAB3*	*YAP5*			
1	*HAA1*	*ARG81*	*RSC3*	*UPC2*	*THI3*	*SSN2*	*RDR1*	*DST1*	*MED8*	*PDC2*
	DAL82	*CHA4*	*EAF3*	*RGA1*	*CDC36*	*SNF1*	*YAP3*	*PPR1*	*ARG80*	*NOT3*
	MAF1	*ARR1*	*YJL206C*	*IWS1*	*YDR520C*	*GCR2*	*RCO1*	*FLO8*	*TOA1*	*NDT80*
	AFT2	*SDS3*	*SNF6*	*CTI6*	*CDC73*	*GIS1*	*PGD1*	*SRB7*	*MED2*	*MGA2*
	CAF4	*SPT3*	*THI2*	*SPT4*	*SKO1*	*SSU72*	*SPT7*	*RSF1*	*LYS14*	*YPL230W*
		CAF16	*HAP2*	*TPO1*	*WAR1*	*SSN8*	*STB4*	*ITC1*	*ROX3*	*NUT2*
	MBF1	*MSS11*	*NUT1*	*RAD9*	*STE5*	*MIG3*	*RFA1*	*ACA1*	*RSC2*	*RDS3*
	MET28	*MAL13*	*STB5*	*SMK1*	*CDC39*	*CAF130*	*YRR1*	*TFA2*	*MSN1*	*PIP2*
	HST1	*BAS1*	*CAF40*	*PUT3*	*YKU70*	*NRD1*	*RDS1*	*CDC50*	*MGA1*	*CST6*
	KAR4	*RFA2*	*RAD50*	*MF(ALPHA)2*		*GTS1*	*RPH1*	*GCR1*	*CLN2*	*RAD18*
	STP1	*NRG2*	*MSN2*	*RCS1*	*YDR026C*	*SFL1*	*HIR1*	*RPI1*	*TOA2*	*RLR1*
	NHP6B	*RIM4*	*WHI2*	*HMS1*	*PHO23*	*MF(ALPHA)1*		*IME4*	*PLM2*	*SIP4*
	MAL33	*RPN4*	*WTM1*	*RDS2*	*STP4*	*STO1*	*MET18*	*RSC1*	*TFA1*	*TIS11*
	CUP2	*ECM22*	*STB2*	*UME1*	*RGM1*	*MOT2*	*SPT8*	*SRB4*	*SRD1*	*SPT21*
	HOG1	*SPT2*	*UGA3*	*DAL81*	*SET3*	*HTZ1*	*STD1*			

B. Hierarchy of *E. coli* regulatory network

Level	Genes									
4	yhiW	gntR	soxR	cspE						
3	oxyR	lrhA	cspA	yhiX	rob	marR	soxS	exuR		
2	yhiE	fur	crp	lrp	metJ	cytR	tdcR	flhC	rhaR	gutM
	narL	himA	rpoS	feaB	cysB	fis	B2087	cpxR	flhD	rcsB
	rpoN	fruR	fhlA	glnG	marA	nac	fnr	srlR	dnaA	rpoE
	uxuR	modE	himD	hns	ompR	galR	arcA	mlc	feaR	lysR
	rhaS	phoP	pdhR	fadR						
1	metR	appY	trpR	tyrR	argR	glcC	xylR	purR	rpiR	gals
	lldR	mtlR	malT	atoC	malI	hydG	emrR	hycA	cadC	asnC
	yeiL	idnR	ilvY	hupB	betI	uidR	lexA	rpoH	gcvA	fucR
	hcaR	B2531	ada	melR	yiaJ	glpR	rcsA	fliA	cynR	putA
	cbl	dsdC	treR	arsR	nagC	csgD	tdcA	rtcR	farR	phoB
	araC	hupA	hipB	yhhG	fecI	iclR	B2090	torR	caiF	sdiA
	uhpA	yjdG	xapR	evgA	nadR	adiY	narP	B1399	deoR	gcvR
	acrR	leuO	ygaE	envY	alpA	pspF	ylcA	hyfR	yjbK	ebgR
	kdpE	yhdM	slyA	ygaA	lacI	rbsR	nhaR	mhpR	birA	