decision-making and contribute to the management of disease with biological drugs. The need to generate clinically relevant immunogenicity information should be recognized early in drug development so that appropriate ADA sampling and characterization strategies can be built into clinical trial design and protocols. However, the all-encompassing and idealized example presented here may not be feasible or necessary for all biologic drugs; the extent of immunogenicity evaluation for any biologic drug, and subsequent labeling, should be driven by the drug-specific immunogenicity risk assessment and consultations with pertinent regulatory authorities.

COMPETING FINANCIAL INTERESTS
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DISCLAIMER
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Silence on the relevant literature and errors in implementation

To the Editor:
In the August 2013 issue of this journal, Barzel & Barabási reported a method for reconstructing network topologies[1]. Here we show that the Barzel & Barabási method is a variant of a previously published method, modular response analysis (MRA)[2]. We also demonstrate that the implementation of their algorithm using statistical similarity measures as a proxy for global network responses to perturbations is erroneous and its performance is overestimated.

The reconstruction of network connections from data remains a fundamental problem in biology. It is not immediately obvious how to capture direct links between individual network nodes from experimental data because a perturbation to a component propagates through a network, causing widespread (global) changes, thereby masking direct (local) connections between nodes. This question has been previously studied in >100 publications, collectively representing MRA (reviewed in refs. 3–7). MRA quantifies direct interactions between network nodes (*i* and *j*) using the local response coefficients (also known as connection coefficients), which describe direct effects of a small change in node *j* on node *i*, while keeping the remaining nodes unchanged to prevent the spread of the perturbation. The local responses cannot be directly assessed, whereas the global responses can be measured; when following a perturbation to node *j*, the entire network relaxes to a new steady state. In this new state, nodes that are directly affected by node *j* have changed, but other indirectly affected nodes also change because the initial perturbation has propagated through the network. MRA has solved the problem of finding local, direct links between components through the global responses for networks of any size and complexity[2]. The development and application of methods that are conceptually similar to MRA (e.g., regulatory strength analysis[8] and maximum likelihood-based MRA[9,10]) has reinforced the validity of using MRA-type methods to reconstruct network connections[11–16].

The Barzel & Barabási study[1] uses the same concept and strikingly similar terminologies to reconstruct networks by deriving the local connection coefficients from the global response coefficients. Key equations (3) and (4) in their silencing method[1] express the local coefficients in terms of the global coefficients and are a subset of the published MRA equations[2,9,10,17–19] with a formal replacement of the diagonal elements of the local response matrix by zeros instead of minus ones (**Supplementary Note 1**). Another formal difference is that the variant of the global response matrix used by Barzel & Barabási[1] considers the global change in each node that results

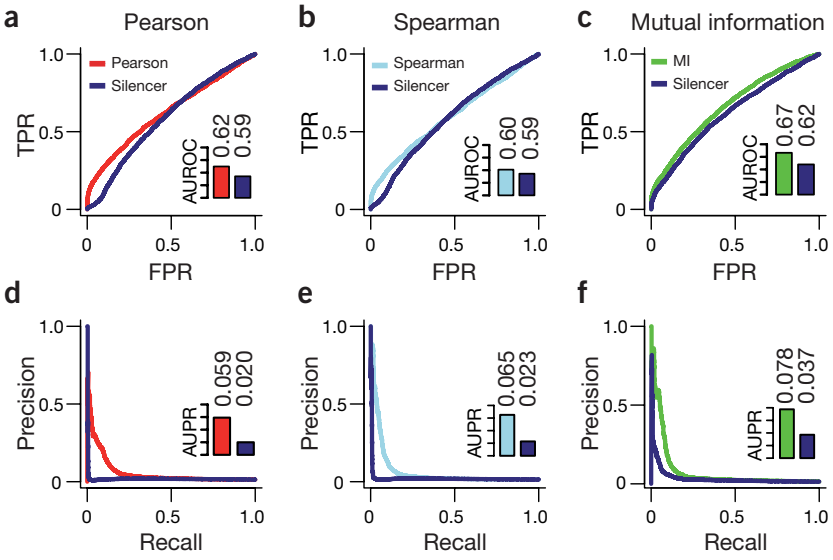

Figure 1 The DREAM5 challenge performances of the Barzel & Barabási 'silencer' method (dark blue) and the raw statistical similarity measures, the Pearson (red) and Spearman (light blue) correlations and mutual information (MI, green). As in the original DREAM5 challenge, the performance was estimated using two scores: (**a**–**c**) AUROC. (**d**–**f**) AUPR. TPR, true-positive rate; FPR, false-positive rate; 'Precision' indicates the fraction of correctly inferred true interactions; 'Recall' equals TPR. Insets show the AUROC and AUPR scores for the Barzel & Barabási algorithm, the Pearson and Spearman correlations, and mutual information. In all three cases, the performance of the Barzel & Barabási algorithm was lower than the performance of the raw similarity measures alone (note that in Fig. 3 of the original publication[1], the Barzel & Barabási algorithm was inappropriately applied and scored).

from the change in every other node of the network, whereas MRA more generally considers the global changes in network nodes that result from changes in any parameter that might affect several nodes simultaneously[18,19]. Barzel & Barabási claim as one of the main outcomes of their study an approximate solution to equations (3) and (4) (equation (5))[1], whereas MRA offers an exact solution[2,18,19]. Both the approximate[1] and the exact MRA[2] solutions require the inversion of the global response matrix. Consequently, Barzel & Barabási's approximation[1] does not decrease the computational complexity of the exact solution[2,18,19]. In fact, the proposed approximate iterative method (equations S12 and S13)[1] needs repetitive matrix calculations, making it slower than the existing MRA algorithms that provide the exact solution.

The Barzel & Barabási approximate solution relies on the assumption that "typically, perturbations decay rapidly as they propagate through the network, so that the response observed between two nodes is dominated by the shortest path between them"[1]. This assumption disregards well-documented biological evidence, such as the sensitivity amplification in signaling cascades[20,21] and the existence of positive feedback loops in biological networks, which amplify initial signals as they propagate through a network[22]. The common occurrence of positive or double-negative feedback loops invalidates the Barzel & Barabási assumption for many known signaling pathways, including the restriction point pathway[23], cell cycle signaling[24–26], mitogen-activated protein kinase (MAPK) cascades[27,28] that are evolutionary conserved from yeast to mammals, as well as transcription regulation networks[29]. In these regulatory networks, global responses of the neighboring nodes outside of positive feedback loops are typically smaller than the response of a node that lies inside a positive feedback loop to an upstream node outside the loop (**Supplementary Note 1**, **Supplementary Note 2** and **Supplementary Fig. 1**). Sensing and processing of stimuli is the normal function of most, if not all, regulatory biological pathways, whose common feature is to increase the response of a 'target' node to a 'source' node with the distance between them[30]. For example, in an experimental study, Bastiaens and colleagues[31] used MRA to unravel the direct linkage topology and strengths of connections in the MAPK cascade in PC12 cells that were stimulated with epidermal growth factor (EGF) versus nerve growth factor (NGF). They calculated the local response coefficients from experimentally measured global responses and found that EGF elicits negative feedback, whereas NGF induces positive feedback, imposed on the backbone of the same MAPK pathway that propagates signals from both growth factors. The experimentally measured response between immediate neighbors, such as the global response of MEK to small interfering RNA (siRNA) against RAF, was much smaller than the response of the more distant neighbor ERK to RAF siRNA under both EGF and NGF stimulation, regardless of the growth factor–specific difference in network wiring[31]. In contrast to Barzel & Barabási's assumption, real biological networks do not feature a rapid decay of specific perturbations because these pathways have evolved to sense and respond to external cues by processing, amplifying and integrating the signals. Thus, reconstruction of these pathways using the 'average perturbation decay' hypothesis misses key functional features of biological pathways.

Network reconstruction methods that exploit the global network responses require systematic perturbation measurements. Clearly, many high-throughput approaches do not provide such perturbation data, whereas statistical similarity measures can be calculated from omics data. In the absence of perturbation data, Barzel & Barabási reconstructed the network by substitution of the global response coefficients with statistical similarity measures, such as the Pearson and Spearman correlation coefficients, and mutual information[1]. However, this substitution yields a symmetric correlation matrix with very different mathematical properties from the global response matrix. Inference based on these symmetric measures (using equation (5) and equations S11–S13 of ref. 1) results in networks where the local response matrix is always symmetrizable (**Supplementary Note 3**). In these inferred networks, the absence of a direct connection from node j to node i ($S_{ij} = 0$) inevitably implies that there is no direct connection in the reverse direction (node i to node j, $S_{ji} = 0$). Thus, the inferred networks cannot have a one-way connection between any two nodes. This approach violates the reality of cellular networks where one-way connections dominate. For example, ubiquitous post-translational protein modifications are typically one-way connections. When a kinase phosphorylates a substrate, the substrate usually does not phosphorylate the kinase. Equally important, the symmetrizable local response matrix implies that the overall signal amplification or attenuation along a circular path (for instance, formed by a feedback loop) is exactly the same as in the reverse direction along this path, which is also biologically unrealistic. Therefore, the local response coefficients inferred from the correlation or mutual information matrices[1] instead of the global response matrices do not represent real cellular networks and predict erroneous network connections (**Supplementary Note 3**).

There are many established methods that use statistical similarity measures to identify connections between network nodes, including lasso regression-based methods[32–34], the partial correlation method[35,36], Gaussian graphical models[37] and elastic nets[38], which rank the predicted edges on the basis of correlations between experimental observations or regression coefficients, rather than incorrectly using MRA equations to express the local connections through the correlation or mutual information matrices.

Barzel & Barabási claim that their method is robust against noise[1]. However, neither the Barzel & Barabási algorithm nor the standard MRA[2] take explicit precautions against extrinsic or intrinsic noise in the data (**Supplementary Note 4**). Several statistical reformulations of MRA have been developed to allow robust inference of network topology in the presence of noise. For example, the statistical adaptations of MRA based on the Monte Carlo[31] and maximum likelihood[9,10] methods were successfully applied to infer signaling pathway topologies from noisy perturbation data in mammalian cells. A recent Bayesian MRA reformulation is also capable of inferring networks from noisy and even incomplete data sets[39].

We are also unconvinced by the claim that the Barzel & Barabási method "improves upon the top-performing inference methods"[1] in the DREAM5 network inference challenge (Network 3, http://wiki.c2b2.columbia.edu/dream/data/scripts/DREAM5/files/DREAM5_NetworkInference_Evaluation.zip). The DREAM5 data set contains the expression levels of 4,511 *Escherichia coli* genes, including 334 known transcription factors, and contestants were asked to rank the likelihood of interactions between transcription factors and target genes. Performances of the contenders were

then estimated against a gold standard network, which involved only 141 out of 334 transcription factors. After we were unable to reproduce the results of Barzel & Barabási, the authors provided us with the source code for their algorithm. We found three issues in their analysis that rendered the comparison of their algorithm performance with the performances of the DREAM5 contestants invalid (**Supplementary Note 5**). First, although the identity of the 141 transcription factors of the gold standard network on which the performance was evaluated was unknown to the contestants, Barzel & Barabási exploited this information to zero out the correlations involving the other 193 transcription factors in their correlation matrix (used as a proxy for matrix G). Second, Barzel & Barabási incorrectly calculated the receiver operating characteristics (ROC) curves that estimated the performance of their algorithm (**Supplementary Note 5**). The DREAM5 challenge gold standard network contains only 141 transcription factors and 1,080 out of 4,511 potential target genes, whereas the interactions involving the remaining 3,431 genes are neither included in the gold standard network nor considered for the performance evaluation in the DREAM5 challenge (http://wiki.c2b2.columbia.edu/dream/data/scripts/DREAM5/files/DREAM5_NetworkInference_Evaluation.zip). Even so, Barzel & Barabási erroneously count these omitted interactions (between the 141 transcription factors and the remaining 3,431 genes) as true negatives, resulting in the inflated area under the ROC curve (AUROC) estimate (**Supplementary Note 5**). Finally, to evaluate performance of their algorithm, Barzel & Barabási disregarded the precision recall score (AUPR) and used only the AUROC score, which is known to be insufficient and can be misleading when the numbers of true positive (the interactions present in the gold standard network) and true negatives (the interactions absent in the gold standard network) differ significantly[40]. In the *E. coli* network, true negatives are about 100-fold more abundant than true positives. Consequently in the DREAM challenge, the performances were estimated using scores that combined both AUROC and AUPR. After we corrected these errors (**Supplementary Note 5**) and properly recalculated AUROC and AUPR using the evaluation script of the DREAM5 challenge (http://wiki.c2b2.columbia.edu/dream/data/scripts/DREAM5/files/DREAM5_NetworkInference_Evaluation.zip), the Barzel & Barabási inference algorithm performed poorly compared with the best performers in the DREAM5 competition (using either AUROC or AUPR as criteria; **Supplementary Note 5**; supplementary materials are also available on GitHub and figshare: http://figshare.com/articles/NBT_correspondence/1356170 GitHub: https://github.com/SBIUCD/NBT_correspondence.git). Specifically, the performance of the Barzel & Barabási algorithm estimated by AUPR ranks between 20th and 28th, and using AUROC it ranks between 3rd and 14th of the 29 participants, depending on whether the algorithm was applied to the Spearman correlation, Pearson correlation or Mutual Information. Predictions, in which we used merely 'raw' statistical similarity measures as substitutes of the local connections, ranked better (AUPR ranked between 7th and 13th, and AUROC ranks between 2nd and 10th depending on the statistical similarity measure used) than their algorithm (**Fig. 1**).

In summary, the concerns raised in this Correspondence cast doubt both on the level of conceptual advance and the practical usefulness of the silencing method proposed in the Barzel & Barabási study[1]. Ironically, many of the practical issues could have been remedied by consulting the extensive published literature describing MRA and MRA-based methods, which Barzel & Barabási unfortunately seem to have overlooked.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3185).

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Barzel and Barabási reply:

Bastiaens *et al.*[1] raise several pertinent issues regarding the silencing method we proposed in ref. 2. They argue that the method is conceptually similar to modular response analysis (MRA)[3–5] and that the use of correlation-based predictions as input for silencing generates symmetrizable network predictions, which prevents the inference of directionality. We agree that the principles that underpin the silencing method reported in our manuscript[2] are similar to those used to derive MRA[3–5] methods and regret that we did not cite the relevant literature, which we were unaware of at the time of publication. However, the main contribution in ref. 2 was that silencing, unlike MRA, is designed to improve correlation and mutual information–based predictions. These statistical similarity measures are frequently used in the context of link prediction[6,7], and thus, a method that can enhance their predictive power is a useful contribution towards the mapping of regulatory interactions[8–10].

We find, however, the second criticism of Bastiaens *et al.*[1], regarding the use of symmetrical predictions as inputs for silencing, to be rather unusual, as it does not seem to be directed towards our method, but rather towards the common practice of using the symmetrical correlation and mutual information–based methods for link predictions. Indeed, it has no relevance to our silencing method, which does not advocate the use of such predictions, but rather is designed to improve them. We would like to make it clear that silencing is not a stand-alone method, but instead should be used as a post-processing step for enhancing preexisting predictions. The symmetry that Bastiaens *et al.*[1] criticize originates from the characteristics of the preexisting predictions (e.g., correlations), but has little bearing on the improvement to these predictions offered by our silencing method.

The final criticism of Bastiaens *et al.*[1] is that our evaluation of the performance of our silencing method did not follow the precise DREAM5 protocol that was used by Marbach *et al.*[6]. However, silencing was not designed to compete with the methods reported by Marbach *et al.*[6] in DREAM5; instead, we created our method to improve them. Such improvement is independent of whether one does or does not follow the DREAM5 protocol.

The reservations of Bastiaens *et al.*[1] regarding the applicability of our method to predictions based on correlation and mutual information have prompted us to improve the method's implementation by adding a preprocessing step that broadens the range of suitable input predictions. We present below a substantially expanded validation, reinforcing the conclusions in our original paper[2]. The improved code, now tested using the full DREAM5 evaluation criteria, achieves an average score increase for link prediction of 96% for *Escherichia coli* and several orders of magnitude for *Saccharomyces cerevisiae*. Both the original and improved source codes are also made available in **Supplementary Software 1** and **2** and on figshare (http://dx.doi.org/10.6084/m9.figshare.1348220). In the following text, we respond in detail to the criticisms raised by Bastiaens *et al.*[1].

First, we agree that the principles that we used to derive the silencing method have common roots with the derivation of MRA[3–5], a mapping that, as opposed to our approximation, offers an exact solution to the fundamental equation (4) in our original paper[2]. However, whereas MRA was shown to enhance perturbation experiments, the strength of our silencing method, as reported in the original paper[2], is that it also accounts for correlation-based predictions, namely G_{ij} constructed from statistical similarity measures (see below). This is a crucial complement to MRA, because most current inference efforts rely strongly on correlations and other statistical similarity measures[6]. As we show in Figure 3 from our original paper and discuss in this response, our implementation of the silencing method allows us to enhance the predictive power not only of perturbation-based experiments, for which MRA is designed, but also of correlation-based predictions, thereby offering a broader range of application than MRA.

Second, Bastiaens *et al.*[1] argue that the application of the silencing method to correlation-based G_{ij} results in "symmetrizable" network predictions, which violate the directionality of real biological networks. We find this difficult to reconcile, given that correlation-based matrices are perfectly symmetrical to begin with. It is therefore impossible for any methodology that uses correlation-based matrices as input to recover directionality. The information on the directions of the links is lost in the construction of G_{ij} and cannot be retrieved without exogenous inputs, such as a list of transcription factors, as provided in the DREAM5 challenge[6], which we used to validate our method.

This criticism may have resulted from a misunderstanding of the goal of our original paper in that silencing is not a stand-alone method. Rather, it is designed to take a preexisting G_{ij} as input and enhance its predictive power. Thus, the criticism of Bastiaens *et al.*[1] might be better directed toward the input matrix G_{ij}, on account of its symmetrical structure, and not on the output provided by our method, S_{ij}. Indeed, the use of correlation-based matrices for gene network inference is common practice[6–9], despite the justified reservations of Bastiaens *et al.*[1]. Thus, as imperfect as these inputs are, there is a need to develop methods that improve their performance. The true test is not whether the silenced S_{ij} matrix recovers the network's directionality because that information is already absent from G_{ij}, but rather whether S_{ij} improves on G_{ij}'s predictive power, namely does it predict direct links with higher fidelity. Our results as reported in our original paper clearly document that it does.

We agree with Bastiaens *et al.*[1] that perturbations, the input for which silencing is ultimately designed, have different properties to correlations. However, like many other successful scientific

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Barzel and Barabási reply:

Bastiaens *et al.*[1] raise several pertinent issues regarding the silencing method we proposed in ref. 2. They argue that the method is conceptually similar to modular response analysis (MRA)[3–5] and that the use of correlation-based predictions as input for silencing generates symmetrizable network predictions, which prevents the inference of directionality. We agree that the principles that underpin the silencing method reported in our manuscript[2] are similar to those used to derive MRA[3–5] methods and regret that we did not cite the relevant literature, which we were unaware of at the time of publication. However, the main contribution in ref. 2 was that silencing, unlike MRA, is designed to improve correlation and mutual information–based predictions. These statistical similarity measures are frequently used in the context of link prediction[6,7], and thus, a method that can enhance their predictive power is a useful contribution towards the mapping of regulatory interactions[8–10].

We find, however, the second criticism of Bastiaens *et al.*[1], regarding the use of symmetrical predictions as inputs for silencing, to be rather unusual, as it does not seem to be directed towards our method, but rather towards the common practice of using the symmetrical correlation and mutual information–based methods for link predictions. Indeed, it has no relevance to our silencing method, which does not advocate the use of such predictions, but rather is designed to improve them. We would like to make it clear that silencing is not a stand-alone method, but instead should be used as a post-processing step for enhancing preexisting predictions. The symmetry that Bastiaens *et al.*[1] criticize originates from the characteristics of the preexisting predictions (e.g., correlations), but has little bearing on the improvement to these predictions offered by our silencing method.

The final criticism of Bastiaens *et al.*[1] is that our evaluation of the performance of our silencing method did not follow the precise DREAM5 protocol that was used by Marbach *et al.*[6]. However, silencing was not designed to compete with the methods reported by Marbach *et al.*[6] in DREAM5; instead, we created our method to improve them. Such improvement is independent of whether one does or does not follow the DREAM5 protocol.

The reservations of Bastiaens *et al.*[1] regarding the applicability of our method to predictions based on correlation and mutual information have prompted us to improve the method's implementation by adding a preprocessing step that broadens the range of suitable input predictions. We present below a substantially expanded validation, reinforcing the conclusions in our original paper[2]. The improved code, now tested using the full DREAM5 evaluation criteria, achieves an average score increase for link prediction of 96% for *Escherichia coli* and several orders of magnitude for *Saccharomyces cerevisiae*. Both the original and improved source codes are also made available in **Supplementary Software 1** and **2** and on figshare (http://dx.doi.org/10.6084/m9.figshare.1348220). In the following text, we respond in detail to the criticisms raised by Bastiaens *et al.*[1].

First, we agree that the principles that we used to derive the silencing method have common roots with the derivation of MRA[3–5], a mapping that, as opposed to our approximation, offers an exact solution to the fundamental equation (4) in our original paper[2]. However, whereas MRA was shown to enhance perturbation experiments, the strength of our silencing method, as reported in the original paper[2], is that it also accounts for correlation-based predictions, namely G_{ij} constructed from statistical similarity measures (see below). This is a crucial complement to MRA, because most current inference efforts rely strongly on correlations and other statistical similarity measures[6]. As we show in Figure 3 from our original paper and discuss in this response, our implementation of the silencing method allows us to enhance the predictive power not only of perturbation-based experiments, for which MRA is designed, but also of correlation-based predictions, thereby offering a broader range of application than MRA.

Second, Bastiaens *et al.*[1] argue that the application of the silencing method to correlation-based G_{ij} results in "symmetrizable" network predictions, which violate the directionality of real biological networks. We find this difficult to reconcile, given that correlation-based matrices are perfectly symmetrical to begin with. It is therefore impossible for any methodology that uses correlation-based matrices as input to recover directionality. The information on the directions of the links is lost in the construction of G_{ij} and cannot be retrieved without exogenous inputs, such as a list of transcription factors, as provided in the DREAM5 challenge[6], which we used to validate our method.

This criticism may have resulted from a misunderstanding of the goal of our original paper in that silencing is not a stand-alone method. Rather, it is designed to take a preexisting G_{ij} as input and enhance its predictive power. Thus, the criticism of Bastiaens *et al.*[1] might be better directed toward the input matrix G_{ij}, on account of its symmetrical structure, and not on the output provided by our method, S_{ij}. Indeed, the use of correlation-based matrices for gene network inference is common practice[6–9], despite the justified reservations of Bastiaens *et al.*[1]. Thus, as imperfect as these inputs are, there is a need to develop methods that improve their performance. The true test is not whether the silenced S_{ij} matrix recovers the network's directionality because that information is already absent from G_{ij}, but rather whether S_{ij} improves on G_{ij}'s predictive power, namely does it predict direct links with higher fidelity. Our results as reported in our original paper clearly document that it does.

We agree with Bastiaens *et al.*[1] that perturbations, the input for which silencing is ultimately designed, have different properties to correlations. However, like many other successful scientific

applications, the silencing method is based on specific approximations. In this case, the approximation is that statistical similarity measures

$$G_{ij} = \mathrm{Corr}(x_i, x_j) \quad (1)$$

can be used as substitutes for the terms of the linear response matrix

$$G_{ij} = \frac{dx_i}{dx_j} \quad (2)$$

At no point do we claim that the silencing method is exact when applied to equation (1). Indeed equation (1) and equation (2) represent different measures, with several distinct mathematical properties. However, both are aimed at capturing similar characteristics of the system: quantifying the association between the activities of pairs of nodes *i* and *j*. Loosely speaking, these two quantities are expected to show similar behavior. For instance, a large G_{ij} in equation (2) indicates that x_i exhibits a strong response to changes in x_j. Under most circumstances such dependency will lead to a correspondingly large correlation in equation (1). Indeed, state changes in x_i will follow changes in x_j, which in turn, will lead to strong statistical correlations between them (**Supplementary Note 1**). Thus, applying the method to matrices of the form of equation (1) constitutes an uncontrolled approximation that must be tested either numerically or empirically, as we do in the paper, showing that the approximation of equation (1) is highly beneficial under both tests (Figs. 2 and 3 in ref. 2 and **Fig. 1** below).

To better understand the class of input matrices that represent valid candidates for silencing we return to the original derivation of the silencing method. We show that we can write equation (5) in ref. 2, the silencing transformation, as

$$G = I_D \sum_{n=0}^{\infty} S^n \quad (3)$$

where $I_D = I - \mathcal{D}((G - I)G)$ (**Supplementary Note 2**). Equation (3) is a matrix representation of the derivation based on network paths that we provide in Supplementary Note I.2 in our paper[2]. Indeed, since $S_{ij} \neq 0$ only along direct links, the terms of S^n account for all paths of length *n* that link between *i* and *j*. Thus, equation (3) describes the observed response matrix G_{ij} as a summation over the contribution of all paths leading from the source node *j* to the target node *i*. A similar approach is reported in Feizi *et al.*[10], published in the same issue of *Nature Biotechnology* as our original paper[2], where the authors present an almost identical method to silencing, starting from equation (3) and taking I_D to be the identity matrix, *I*.

This derivation of the method, based on network paths, provides us with the general criterion that an input matrix must satisfy to be silence-able, that is, a good candidate for the silencing method. Consider a perturbation propagating along a path $i \to k \to j$. According to equation (3) the contribution of this single path is given by

$$G_{i \to k \to j} \sim S_{jk} S_{ki} \quad (4)$$

Indeed, summing over all paths of length two between *i* and *j* provides

$$\sum_k G_{i \to k \to j} = \sum_k S_{jk} S_{ki}$$

which, in accordance with equation (3), is nothing but the *j, i* term of S^2. Thus silencing is applicable as long as the propagation along paths follows the multiplicative rule of equation (4). This allows us to relax the stringent criteria for G_{ij}, and expand it from perturbation-based matrices of the form of equation (2) to a broader class of inputs, including other measures that satisfy equation (4). Thus, the application of silencing to correlation-based predictions of the form of equation (1) is valid as long as correlations propagate multiplicatively as in equation (4). Although this is not guaranteed, it is commonly the case that such multiplicative propagation is observed (**Supplementary Note 2**). Such interpretation of the propagation of indirect correlations was previously offered by Wright's path coefficients[11].

To summarize, we agree with Bastiaens *et al.*[1] that correlation-based matrices of the form of equation (1) have different properties to response matrices defined in equation (2). The question is, however, whether the application of the method to correlation-based matrices represents a valid approximation. Our derivation provides the relevant criterion: that the propagation along network paths is governed by a multiplicative rule, as described in equation (4).

Third, Bastiaens *et al.*[1] criticize the empirical validation in our original paper (Fig. 3 from our original paper[2]) for not adhering to the protocol that was used in the DREAM5 challenge[6]. Indeed, our implementation benefited from two advantages that the original participating groups did not have, namely the list of participating transcription factors (141 versus 334) and the number of nodes in the gold standard used for validation (4,511 versus 1,080). Thus Bastiaens *et al.*[1] are correct to point out these differences, which prevented them from successfully reproducing our findings. Our goal, however, was not to compete with the methods in DREAM5, but to improve them. We maintain that the improvement achieved by silencing remains valid, even if our evaluation protocol differed from that used in DREAM5, as long as we consistently used the same criteria both before and after applying silencing. Indeed, our validation rigorously and fairly tested the silencing method against all the other methods reported under exactly the same experimental conditions, albeit those conditions were not the same as those used in the original evaluation of the methods reported in DREAM5. We emphasize that the two reported advantages were invested in the construction of the input matrix, G_{ij}, and not in the method's output matrix, S_{ij}. As explained above, silencing is not a stand-alone method; it is designed to take the prediction of an existing method, for example, Pearson correlations, as input, and improve on it by silencing indirect paths. The challenge is thus to construct the best possible input matrix, benefiting from all a priori knowledge available, and then show that silencing can further improve its predictive power. Indeed, the discrepancies in the deviation from the DREAM5 protocol only improved the baseline performance of the preexisting predictions. Given the fact that the improvement for which our method was tested is measured with respect to that baseline, the reported deviations did not grant any advantage to our method.

Bastiaens *et al.*[1] also claim that the silencing method failed to improve the input when tested using the same protocol as the DREAM5 challenge evaluation. We tested this and found that the original code for silencing presented with our original paper[2] (**Supplementary Software 2**) performed poorly when tested using the DREAM5 evaluation scheme, confirming the concerns of Bastiaens *et al.*[1].

The above finding prompted us to reassess the performance of the silencing method, resulting in an improved implementation that is presented in this response (**Supplementary Software 1**).

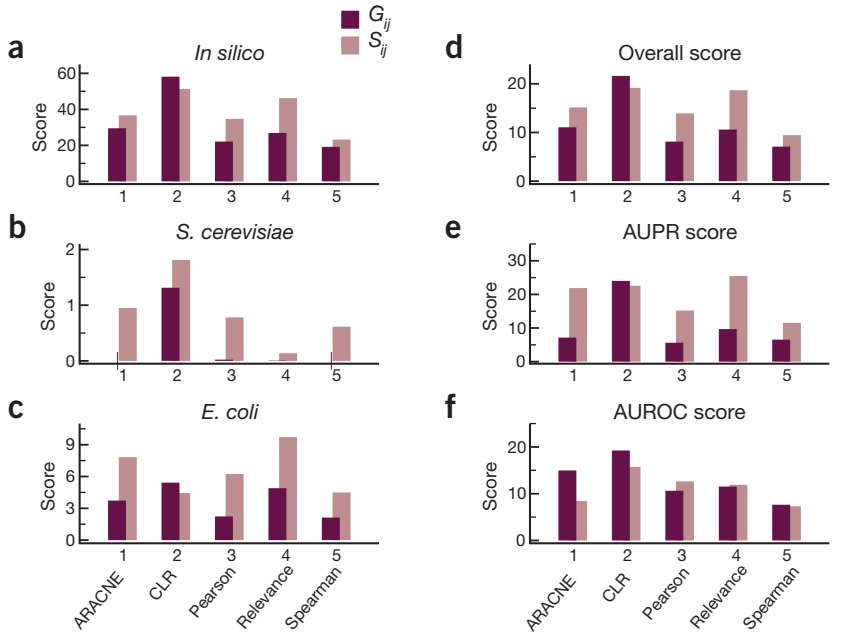

Figure 1 Silencing correlation– and relevance-based predictions. We applied the silencing method to the network predictions obtained from ARACNE (1), CLR (2), Pearson correlations (3), relevance networks (4) and Spearman correlations (5), provided in ref. 10, and used the DREAM5 evaluation criteria[6] to score the predictions before and after silencing (**Supplementary Note 3**, see also ref. 6). (**a**) For the *in silico* model organism silencing led to an average score increase of 32%. (**b**) Although most methods scored very low against the *S. cerevisiae* gold-standard network, silencing significantly improved their quality of prediction, in some cases increasing the score by orders of magnitude. (**c**) For *E. coli* the average gain was 96%. (**d**) The overall score increased by an average of 41%. (**e**) The average AUPR score, gaining 122%, is the main source of the improvement achieved by silencing. (**f**) The AUROC score was not significantly affected, showing a small decrease of 8% on average.

Consider again the path-based derivation of equation (3). As the equation suggests, for silencing to be effective, G_{ij} must be the result of a geometric series, which aggregates the contributions of all paths. If this is indeed the case, the silencing transformation uses self-consistency to expose the kernel of the series S_{ij} from its sum G_{ij}. However, what is implicitly assumed in this analysis is that such a self-consistent solution is available, namely that the geometric series at the right-hand side of equation (3) is convergent. This requires that the spectrum of S_{ij}, λ_S, is between 1 and −1, namely that (**Supplementary Note 2**)

$$\max(|\lambda_S|) < 1 \qquad (5)$$

Thus the full criterion for an input matrix G_{ij} to be silence-able is that there exists a matrix for which both equation (3) and equation (5) are simultaneously satisfied. Satisfying equation (3) is, of course, an intrinsic property of G_{ij}, as we discussed in detail above. Equation (5), however, can always be satisfied by renormalizing the off-diagonal terms of G_{ij}[10].

Below we now offer an improved implementation of the method, where we add a preprocessing step that renormalizes the raw correlation-based matrix, by multiplying all off-diagonal terms by a constant until (5) is satisfied (**Supplementary Note 3**, see also ref. 10, in which a similar approach was introduced). Such renormalization preserves the ranks of all entries in G_{ij}, and thus has no effect on its performance as a link-prediction matrix. This step is not required if G_{ij} is a perturbation-based matrix of the form of equation (2), but crucial with correlation-based matrices, in which the specific values of the terms are arbitrary, and only their ranking plays a role in the prediction. Using this renormalization scheme we re-applied the silencing method, this time following the precise DREAM5 protocol[6]. We used the original data sets and the evaluation scripts provided by the DREAM5 team to make sure that we strictly adhere to the 'rules of the game' (data sets were downloaded from ref. 10 and evaluation scripts from ref. 6). Source code and data reproducing this analysis is available on figshare (http://dx.doi.org/10.6084/m9.figshare.1348220).

Of the methods used in DREAM5, the most relevant are the ones implementing correlation/relevance-based predictions. Thus, we tested the silencing method on Pearson correlations, Spearman correlations, Relevance networks[7], ARACNE[8] and the context likelihood of relatedness (CLR) algorithm[9]. In addition to the results presented in our original paper[2], we now include results for all three organisms that were used to score DREAM5: the *in silico* model organism (**Fig. 1a**), and the empirical data sets for *S. cerevisiae* (**Fig. 1b**) and *E. coli* (**Fig. 1c**). Scoring was done using the evaluation script provided by the DREAM5 team[6] (**Supplementary Note 3**). With the exception of CLR (see below), we find that silencing significantly improves all tested methods, with an average score increase of 54%, excluding CLR, and 41%, including CLR (**Fig. 1d**). We observe the most substantial improvement for the empirical data sets: the average improvement for *E. coli* is 96%, and for *S. cerevisiae* silencing enhances the average score by several orders of magnitude. In DREAM5 most methods performed very poorly on the gold standard constructed for *S. cerevisiae* some scoring no better than a random guess[6], with scores as low as 2×10^{-4} (ARACNE) or 8×10^{-5} (Spearman). The fact that silencing was able to improve these predictions more than 1,000-fold (0.95 and 0.63, respectively) shows that silencing can extract hidden information even from extremely low-quality inputs. The only exception is CLR, for which silencing leads to a marginal decrease in overall performance. This might be because CLR, like silencing, uses global information to prune indirect effects[9]. Thus silencing is perhaps redundant for use with CLR. Note that the overall score in DREAM5, which averages performance of an algorithm over all three organisms, gives little weight to *S. cerevisiae*, whose typical scores are much lower than those of the other two organisms. For instance, the 5,000-fold increase observed for ARACNE in *S. cerevisiae*, from 2×10^{-4} to 0.95, is marginalized by the 110% increase for this method in *E. coli* (3.7 to 7.8) and the 25% increase for *in silico* (29.5 to 36.7) because averaging gives substantially more weight to the latter two organisms. Had we controlled for that, the overall improvement would have been significantly higher, and even CLR would have shown an overall increase in performance (−12%, −18%, and +38% for *in silico*, *E. coli* and *S. cerevisiae*, respectively). In response to a remark of Bastiaens *et al.*[1] that AUPR provides a more relevant measure than AUROC for these systems[12], we tested the specific improvement achieved in the score for AUPR. We find that AUPR is the

main source of improvement, showing an average increase of 122% across all methods and algorithms (Fig. 1e,f). These results substantially reinforce the conclusions from our original paper[2] and show that we can achieve a large improvement on a wide range of methods using our approach.

A last claim of Bastiaens *et al.*[1] is that our approximation based on correlation decay disagrees with biological reality. We concur that in certain cases a local perturbation may increase as it propagates along a network path, rather than decay. However, our application of the silencing method focused on statistical similarity measures, such as correlations, which always decrease along paths, and by definition cannot exceed unity. Moreover, even regarding perturbations, we argue that such amplification is not typical in biological networks. Indeed, if small perturbations were repeatedly amplified during their propagation, the implications on the stability and robustness of living cells would be dramatic; every local disturbance would lead to a macroscopic response and the modular nature of the cell's functionality would be constantly distracted by the cross-talk between distant genes. Thus, it is not surprising that both theoretical and empirical analyses of cellular dynamics indicate, time and again, that the impact of perturbations is, in most cases, strictly local[13]. Studies have shown that perturbations typically feature an exponential decay as they penetrate the network[14–18]. Others have quantified the impact of perturbations by measuring cascade sizes, that is, the number of genes that exhibit a significant response following a perturbation. These reports find that most cascades are tiny and only rarely does a perturbation affect a substantial number of genes[19–21]. This paucity of large cascades further supports the notion that most perturbations do not penetrate deeply into the network.

Finally, the premise of network inference relies on the notion that the magnitude of the terms in the prediction matrix G_{ij} correlates with the likelihood of direct linkage[6–9]. If, as Bastiaens *et al.*[1] suggest, there are cases where the G_{ij} terms systematically increase with the distance between i and j, then in these cases G_{ij} is a poor candidate for network inference in general, with or without silencing, and thus we would not consider it a suitable input for our method.

To summarize, although we disagree with much of the criticism made by Bastiaens *et al.*, we wish to thank them for raising several important issues and igniting a discussion that has ultimately led to the development of the improved silencing algorithm presented here.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3184).

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Opportunities for drug repositioning from phenome-wide association studies

To the Editor:
Results from large-scale phenome-wide association studies (PheWAS) allow association of genetic variants with a wide spectrum of human disorders and have provided considerable insight into disease etiologies[1]. The PheWAS strategy relies on electronically available phenotypic data collected from patient cohorts. PheWAS is similar to a genome-wide association study (GWAS), but whereas a GWAS asks "What genetic variants are associated with a disease?", a PheWAS asks "What diseases are associated with a genetic variant?" In 2013, *Nature Biotechnology* published a study by Denny *et al.*[2] in which they conducted a comprehensive PheWAS on 3,144 single-nucleotide polymorphisms (SNPs) that had been previously associated with a variety of phenotypes by GWAS. This PheWAS, like many other PheWAS published[1,3], used International Classification of Diseases version 9 (ICD9) codes extracted from electronic medical record systems in large patient cohorts to define case-control groups for many phenotypes. In the United States, ICD9 coding is primarily used for billing and can have variable effectiveness in regard to describing discrete phenotypes. Regardless, Denny *et al.*[2] demonstrated that for many of the GWAS SNPs, PheWAS was able to rediscover expected SNP-disease associations while also identifying novel associations[2]. We propose that PheWAS results may also provide new opportunities to identify candidates for drug repositioning.

Drug repositioning is the process of discovering new indications for existing drugs and is becoming an important component of drug development as success rates for novel drugs in clinical trials decrease and costs increase[4]. Critical to drug repositioning is the initial identification of candidate drug-disease relationships. Genetic-based association studies, including GWAS, have proven to be effective for generating hypotheses related to drug repurposing[5,6]. GWAS can identify disease susceptibility genes that are targets for existing drugs used to treat different conditions. For example, a large GWAS implicated flavopiridol, a cyclin-dependent kinase 4 (CDK4) inhibitor and anticancer

Supplementary Information.

Comment on "Network link prediction by global silencing of indirect correlations": entangling the wires again?

Philippe Bastiaens[1], Marc R. Birtwistle[2], Nils Blüthgen[3,4], Frank J. Bruggeman[5], Kwang-Hyun Cho[6], Carlo Cosentino[7], Alberto de la Fuente[8], Jan B. Hoek[9], Anatoly Kiyatkin[10], Steffen Klamt[11], Walter Kolch[12,13,14], Stefan Legewie[15], Pedro Mendes [16,17,18], Takashi Naka[19], Tapesh Santra[12], Eduardo Sontag[20], Hans V. Westerhoff[16,21,22], Boris N. Kholodenko[12,13,14*]

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Supplementary Note 1.

Equations 3 and 4 of the Barzel & Barabási study are a subset of the published MRA equations.

Here we derive a part of MRA equations, which uses the assumption that perturbations affect only single network nodes, and show that this simple MRA equation variant is equivalent to key Equations 3 and 4 of the Barzel & Barabási paper[1]. A more general version of MRA formalism that relaxes this assumption and allows for perturbations which can affect multiple modules is published elsewhere[2-4].

We assume that the network dynamics is described by a set of ordinary differential equations (ODE),

$$dx/dt = F(x, p), \quad x = x_1,...,x_n, \quad p = p_1,...,p_n, \qquad \text{(S1.1)}.$$

where a state variable x_i is assigned to each network node i, representing its concentration or activity level, and the corresponding function F_i describes how the rate of change of x_i depends on all other elements of the network. The parameters $p = p_1,...,p_n$ represent any external or internal condition maintained constant, as, e.g., external concentrations, rate constants, etc. It is assumed that the system has a stable steady state (x^0, p^0),

$$F(x^0, p^0) = 0, \qquad \text{(S1.2)}.$$

where the Jacobian matrix ($\partial F/\partial x$) is non-singular. According to the implicit function theorem, there is a unique vector $x(p)$ solving the set of Eqs. 2 in some neighborhood of a particular value p^0 (further we omit superscript 0 for simplicity of notations).

If the Jacobian element $\partial F_i(x, p)/\partial x_j$, $i,j = 1,\ldots n$, of the matrix F is zero, component x_j has no direct effect on component x_i. In this case, there is no edge from node j to node i in the connection graph associated with the network. For a non-zero element $\partial F_i(x, p)/\partial x_j$, node j connects to node i in the connection graph. MRA quantifies the direct connections between nodes i and j in terms of the fractional changes ($\Delta x_i/x_i$) in the activity of node i brought about by the activity change ($\Delta x_j/x_j$) of node j, provided the activities of all other nodes (x_k, $k \neq i,j$) remain fixed, while node i is allowed to relax to its steady state[5,6]. A mathematical definition requires the changes ($\Delta x/x$) to be infinitesimally small, resulting in log to log derivatives,

$$r_{ij} = \frac{\partial \log x_i}{\partial \log x_j}; \;\; x_k = const \; (k \neq i,j)$$

In MRA, the coefficient r_{ij} is referred to as the *local* response coefficient that precisely quantifies the sign and the strength of direct connection from node j to node i. Although the use of the dimensionless response coefficients given by the logarithmic derivatives r_{ij} is preferable due to many reasons, hereafter we use non-normalized derivatives to show that key Equations 3 and 4 of the Barzel & Barabási paper are a subset of MRA equations. The non-normalized local response coefficients will be denoted by S_{ij} similarly as in the Barzel & Barabási paper[1],

$$S_{ij} = \frac{\partial x_i}{\partial x_j}; \quad x_k = const \; (k \neq i,j) \qquad \text{(S1.3)}.$$

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The local response ($\partial x_i/\partial x_j$) can be expressed in terms of the Jacobian elements of the ODE system (Eq. S1.3) using the steady-state condition for node *i*, considered at constant values of x_k for $k \neq i,j$.

$$F_i(x_1,\ldots,x_i,\ldots,x_j,\ldots,x_n,p) = 0,$$

The differentiation with respect to x_j at constant values of x_k, $k \neq i,j$ gives[4,6],

$$S_{ij} = \frac{\partial x_i}{\partial x_j} = -\frac{\partial F_i}{\partial x_j} \Big/ \frac{\partial F_i}{\partial x_i} \; ; \; j \neq i, \; x_k = const \; (k \neq i,j) \qquad \text{(S1.4)}.$$

Note that in this MRA expression, $S_{ii} = -1$.

The global response of node *i* occurs when following a parameter perturbation that affects only node *j*, an entire network is allowed to relax to the new steady state. Since for simplicity, we consider perturbations that affect only single nodes, we assume that each parameter (p_i) affects only single node (*i*) of the network, implying that,

$$\partial F_i(x,p)/\partial p_k \neq 0 \quad only\ if\ i = k. \qquad \text{(S1.5)}.$$

By differentiating Eqn, S1.2 with respect to a parameter p_k ($k \neq i$), we obtain

$$\sum_{j=1}^{n} \frac{\partial F_i}{\partial x_j} \cdot \frac{dx_j}{dp_k} + \frac{\partial F_i}{\partial p_k} = 0, \; i = 1,2,\ldots n, \; k \neq i$$

Here $\frac{dx_j}{dp_k} = R_{jk}$ are the global response coefficients (R_{ik}) for each node *j* to a perturbation (the parameter p_k), which directly affects only node *k*. Dividing this equation by $\frac{\partial F_i}{\partial x_i}$ and taking into account Eqs. S1.4 and S1.5, we arrive at,

$$\sum_{j=1}^{n} S_{ij}R_{jk} = 0 \qquad i = 1,2,\ldots n, \; k \neq i \qquad \text{(S1.6)}.$$

For each node *i*, $i = 1, \ldots, n$, Eq. S1.6 can be rewritten as a linear equation system to determine *n*-1 unknown local response coefficients S_{ij} of node *i* to all other nodes *j* from measured global responses R_{jk} to *n*-1 perturbations that affect each node *k* except node *i* ($k \neq i$),

$$\sum_{j=1,j\neq i}^{n} S_{ij}R_{jk} = R_{ik} \qquad i = 1,2,\ldots n, \; k \neq i \qquad \text{(S1.7)}.$$

In addition to Eqs. S1.6 and S1.7, MRA also provides a concise matrix expression to calculate all local response coefficients from the global response coefficients[6]. This expression requires only a single inversion of the global response matrix (R_{ik}) and does not require repetitive matrix calculations, as the Barzel & Barabási iterative method[1]. Key Equations 3 and 4 of the Barzel & Barabási paper are immediately obtained from Eqs. S1.7 by (*i*) formally selecting $p_k = x_k$ (considering the change in the activity x_k of node *k* instead of considering the change in the parameter p_k that directly affects only node *k*), (*ii*) renaming R_{jk} as $G_{jk} = \frac{dx_j}{dx_k}$, and (*iii*) replacing the diagonal elements of the local response matrix by zeros instead of minus ones, $S_{ii} = 0$ (that helps to derive the matrix Equation 4 from Equation 3).

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We also note that a large body of previously published methodologies, completely ignored by Barzel & Barabási, provided similar mathematical approaches that describe how to capture direct links between individual network nodes from experimental data on the global perturbation responses of the network[2,7-14]. These methods differ in terms of the mathematical formalism, whereas the key equations of Barzel & Barabási study are equivalent to the published equations of the deterministic part of MRA.

Supplementary Note 2.In signaling networks the global responses are often larger for distant nodes than for neighboring nodes.

As their main result, Barzel & Barabási claim Equation 5, an approximate solution to Equations 3 and 4[1]. Importantly, this approximate solution "uses the fact that, typically, perturbations decay rapidly as they propagate through the network, so that the response observed between two nodes is dominated by the shortest path between them"[1]. This is a rather naive assumption that disregards well documented biological realities, such as the sensitivity amplification that occurs in signaling cascades and positive feedback loops, which are part of network motifs designed to enhance initial signals as they are processed through a network[5,15,16]. Sensing and processing of stimuli is the normal function of most if not all biological pathways. The common occurrence of positive or double negative feedback loops invalidates the Barzel & Barabási assumption for most known regulatory pathways, including the restriction point pathway[17], cell cycle signaling [18-20] and mitogen-activated protein kinase (MAPK) cascades[21,22], which are evolutionary conserved from yeast to mammals. In these real networks, global responses of the neighboring nodes outside of positive feedback loops are typically smaller than the response of a node within a positive feedback loop to a distant node (Eq. S2.4). Below, we illustrate this general phenomenon, using an example of a protein modification cascade[5]. We show that the response of a "target" node to a "source" node increases with the distance between these nodes.

Signaling networks usually include cascades of protein modification cycles, where each cycle consists of two or more interconvertable forms of a signaling intermediate (e.g. a phosphorylated and dephosphorylated protein), and one (or more) of these forms affects the interconversion of forms at the next level down the cascade. As an illustration, we consider a linear chain of protein modification cycles with two forms of protein, an active (x_i) and inactive (x_i^I) at each level (i). An active protein form x_i at each level $i = 1, 2, \ldots, N$ catalyzes the transformation of an inactive form x_{i+1}^I at the next level (i+1) into an active form x_{i+1}. The signal S activates the protein at the first level. To compare the responses, it is convenient using the dimensionless response coefficients given by the logarithmic derivatives describing the corresponding fractional changes. The local response of node k to the immediately preceding node k - 1 and the global response of node k to node j are expressed as follows,

$$r_{k,k-1} = \frac{\partial \log x_k}{\partial \log x_{k-1}}; x_j = const\ (j \neq k-1, k), \quad R_{kj} = \frac{d \log x_k}{d \log x_j} \qquad \text{(S2.1)}$$

where x_i is the steady state activity (or concentration) of node i. The well-known feature of protein modification cycles is "ultrasensitivity", which implies that the local responses $r_{k,k-1}$ exceed 1 [5,16,23]. For a linear cascade without feedback, the global response of node k to node j ($k > j$) is the product of the local responses along the path from node j to node k [5],

$$R_{kj} = r_{j+1,j} r_{j+2,j+1} \ldots r_{k,k-1} = \prod(path\ j \rightarrow k) \qquad \text{(S2.2)}$$

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Note that the expression for non-fractional response coefficients is similar to Eq. S2.2, and the global response G_{kj} is the product of the local responses, $S_{j+1,j}S_{j+2,j+1}\ldots S_{k,k-1}$. Thus, G_{kj} can be expressed using Eq. S2.2, by multiplying the right hand side by the ratio of the steady state activities, $\frac{x_k}{x_j} = \frac{x_{j+1}}{x_j} \cdot \frac{x_{j+2}}{x_{j+1}} \cdot \ldots \cdot \frac{x_k}{x_{k-1}}$.

Owing to typically observed ultrasensitivity of phosphorylation cycles in real biological pathways[20,21,23,24] the sensitivity to the signal at each level is more than 1 ($r_{i,i-1} > 1$). Thus, Eqn. (S2.2) implies that the response of a "target" node to a "source" node increases with the distance between these nodes along the cascade, which is a well-known property of signaling cascades[24,25], invalidating the assumption by Barzel & Barabási[1]. Often, merely having more levels greatly increases the sensitivity of the target to the signal change, enabling the cascade to operate as a switch. For example, if each of three levels amplifies the signal 10 fold then the whole cascade amplifies the signal 1000 fold.

Positive feedback loops.

Positive feedback loops are frequently present in biological networks, controlling the cell physiology[17-22]. For instance, positive feedback between Akt and mTOR acts as a perpetual signal booster in a growing skeletal muscle[26], whereas positive feedback between p42 and cdc2 in Xenopus oocytes creates a 'memory module' that governs cell fate decisions[27]. Here we show that the global responses between neighbouring nodes outside of a positive feedback loop are typically much smaller than the global response of a node within this loop to a distant node (when feedback is sufficiently strong), which further invalidates the assumption by Barzel & Barabási[1]. As an illustrative example, we again consider a linear signaling pathway that consist of protein modification cycles, but assume that there is positive feedback from the active protein (x_N) at the last layer to an upstream protein (x_m). Owing to this feedback, the transition of an inactive form x_m^I into an active form x_m (which is catalysed by the protein x_{m-1}) is activated by x_N. For any particular mechanism of activation, this positive feedback strength is described in terms of the local response coefficient, $r_{m,N}$, as follows[5],

$$r_{mN} = \frac{\partial \log x_m}{\partial \log x_N};\ x_j = const\ (j \neq m, N) \qquad \text{(S2.3)}$$

The global response of any node within a feedback loop, for instance node N to any distant node outside of the feedback loop, for instance node k ($k < m$) is amplified by the positive feedback (even if local response coefficients $r_{i,j}$ are less than 1), as follows [5],

$$R_{Nk} = \frac{\prod(path\ k \to m-1)\prod(Loop)}{1 - \frac{r_{mN}}{r_{m,m-1}}\prod(Loop)},\ \prod(Loop) = r_{m,m-1}r_{m+1,m}\ldots r_{N,N-1} \qquad \text{(S2.4)}$$

Thus, while the global responses of the neighbouring nodes outside of a positive feedback loop, for instance node m-1 to node m-2 is given by $r_{m-1,m-2}$, the global response of the distant node N to the same node m-2 is amplified by the feedback by a factor of $\prod(Loop)$ / $(1 - \frac{r_{mN}}{r_{m,m-1}}\prod(Loop))$, which is typically much large than 1 for a sufficient strength of positive feedback. Numerical simulations in

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Fig. S1 illustrate this point, showing the failure of the assumption by Barzel & Barabási[1] in pathways with positive feedback loops.

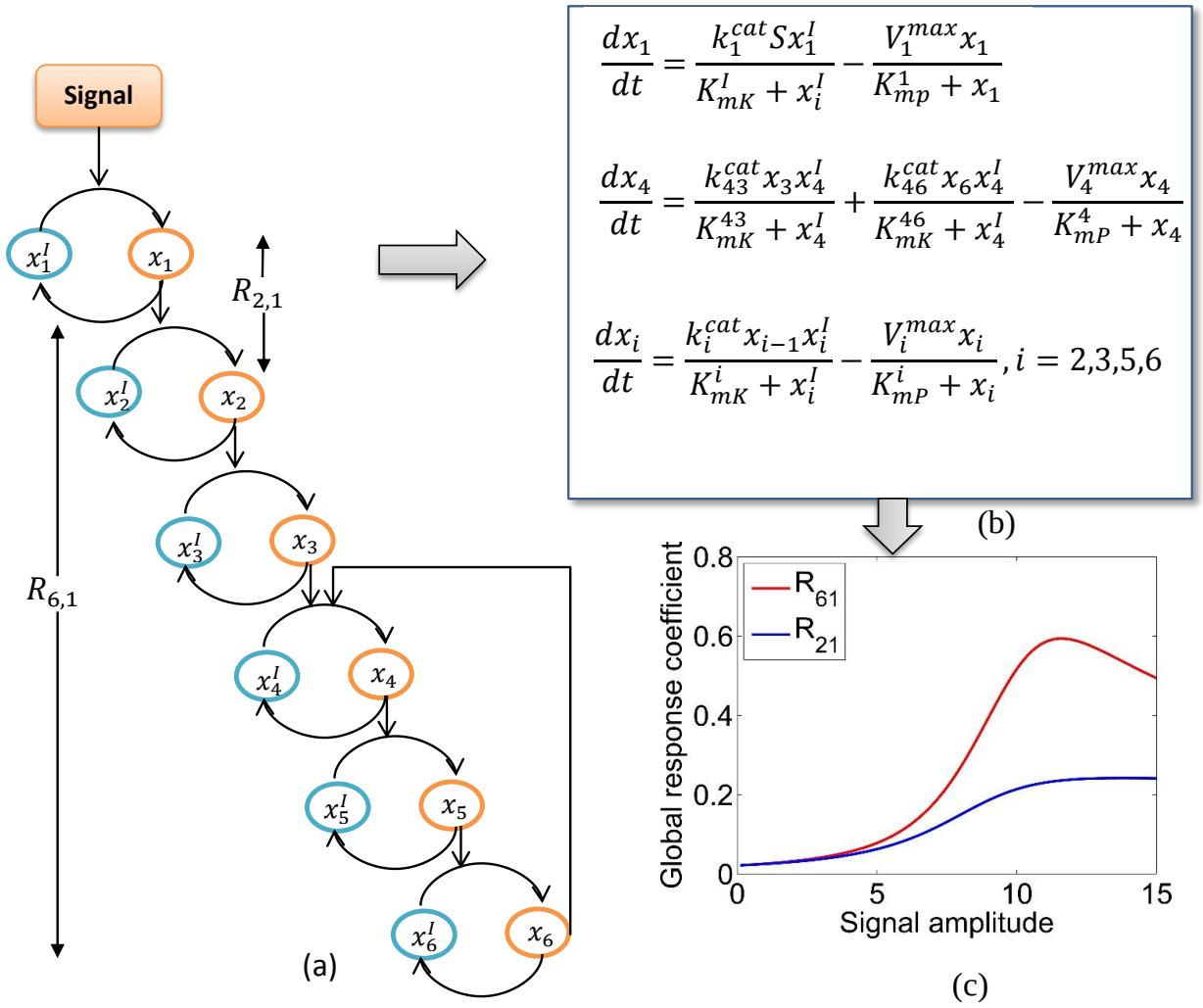

Figure S1. Local and global responses in a six tier signaling cascade with positive feedback. (a) Kinetic scheme of a six level cascade with positive feedback from level 6 to level 4. (**b**) Kinetic equations. The parameter values and initial concentrations for this model are $x_1^T = x_1^I + x_1 = 7, x_i^T = x_i^I + x_i = 10, i = 2,3, x_4^T = 20, x_5^T = 20, x_6^T = 20, x_i(0) = 0, k_1^{cat} = k_{43}^{cat} = 0.8, k_{46}^{cat} = 4, K_{mk}^1 = K_{mk}^{43} = K_{mk}^{46} = 1, V_1^{max} = 10,\ K_{mp}^1 = K_{mp}^4 = 4, k_2^{cat} = k_5^{cat} = 2, K_{mk}^2 = K_{mk}^5 = 10, V_4^{max} = 10, V_2^{max} = V_5^{max} = 10, K_{mp}^2 = K_{mp}^5 = 3, k_3^{cat} = k_6^{cat} = 2, K_{mk}^3 = K_{mk}^6 = 1, V_{max}^3 = V_{max}^6 = 7, K_{mp}^3 = K_{mp}^6 = 2$. (**c**) Steady-state responses of the active protein forms to changes in the signal amplitude (S). The global response coefficients $R_{2,1}$ (between neighbouring levels 2 and 1) are smaller than the global response $R_{6,1}$ (between distant levels 6 and 1) in a range signal amplitudes.

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Another shortcoming of the Barzel & Barabási study is the neglect of moiety conservations [1], whereas MRA explicitly considers this general biochemical feature[3,6,15]. In biological systems most moieties exist in many forms, e.g. a typical tyrosine kinase receptor can exist in more than a thousand different phosphorylation states that together add to the total receptor moiety, which can be conserved during the time of a typical experiment[28]. The dynamic system framework treats these forms as independent variables, which will inevitably collapse the Barzel & Barabási approximate solution into the abyss of complexity. Classic MRA avoids this problem by considering network modules constrained by moiety conservations as single nodes, letting all internal intermediates relax to a steady state and analyzing only the composite change in the module's output[3,6,15]. As an example, we again consider the MAPK/ERK cascade, where at each level there are several differentially phosphorylated forms of kinases, RAF, MEK and ERK. Since only the forms, which are phosphorylated on activating residues and dephosphorylated at inhibiting residues can phosphorylate and activate the targets, these forms are the module outputs. When the signal comes to a module, all its kinase intermediates are allowed to relax to a new steady state, and only the change in the fully active form is analyzed. This formalism is generalized in MRA to allow for modules having multiple outputs [6].

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Supplementary note 3.

Local response coefficients inferred from correlation coefficients instead of global responses do not represent the biological reality.

Statistical correlations and mutual information have fundamentally different mathematical properties compared to global response coefficients. Both Spearman's and Pearson's correlation coefficients, as well as mutual information are symmetric measures. For instance, the correlation coefficient (C_{ij}) between node i and node j is the same as the correlation (C_{ji}) between node j and node i. However, global response coefficients are asymmetric measures, i.e. the global response ($G_{ij} = \frac{dx_i}{dx_j}$) of node i to a perturbation to node j generally differs from the global response ($G_{ji} = \frac{dx_j}{dx_i}$) of node j to a perturbation to node i. Here we show that if as suggested by Barzel & Barabási[1], the global response matrix ($\boldsymbol{G}$) is replaced by a correlation matrix ($\boldsymbol{C}$), the inferred local response matrix ($\boldsymbol{S}$) is a 'symmetrizable matrix', which results in erroneous inference of direct connections between nodes for the majority of biological signal transduction and gene networks.

We start with the mathematical definitions of symmetrizable matrices. Using these definitions, we prove that if global response coefficients are replaced by correlation coefficients, then the inferred local response matrix is always symmetrizable. Finally, using the properties of symmetrizable matrices, we demonstrate that the replacement of global response coefficients by correlation coefficients makes the calculated local response matrix biologically unrealistic.

Definition S3.1. A matrix M is symmetrizable is there exists a positive definite matrix P such that MP is Hermitian[29].

Since we consider only real matrices, a Hermitian matrix is a symmetric matrix.

Theorem S3.1: If $\boldsymbol{C}$ is a correlation matrix, then the local response matrix $\boldsymbol{S} = \left(\boldsymbol{C} - \boldsymbol{I} + D\left((\boldsymbol{C} - \boldsymbol{I})\boldsymbol{C}\right)\right)\boldsymbol{C^{-1}}$ is a symmetrizable matrix.

Here D is the diagonalization operator such that for any matrix $\boldsymbol{X}$, $D(X)_{ii} = X_{ii},\ D\ (X)_{ij} = 0$.

Proof: Since $\boldsymbol{C}$ is a correlation matrix, it is positive semi-definite. Additionally, in order for $\boldsymbol{S}$ to exist, $\boldsymbol{C}$ must be invertible, and therefore it must be positive definite. Multiplying $\boldsymbol{S}$ by $\boldsymbol{C}$ yields,

$$\boldsymbol{SC} = (\boldsymbol{C} - \boldsymbol{I} + D((\boldsymbol{C} - \boldsymbol{I})\boldsymbol{C})) \qquad \text{(S3.1)}$$

Since the matrices $\boldsymbol{C}$, $\boldsymbol{I}$ (identity matrix) and $D((\boldsymbol{C} - \boldsymbol{I})\boldsymbol{C})$ are symmetric matrices, the right hand side of Eq. S3.1 is symmetric and therefore Hermitian. By definition, if $\boldsymbol{SC}$ is Hermitian then $\boldsymbol{S}$ is symmetrizable.

Theorem S3.2: If $\boldsymbol{C}$ is a correlation matrix, then the local response matrix obtained by Barzel & Barabási's iterative method[1], $\boldsymbol{S^{(n+1)}} = \left(\boldsymbol{C} - \boldsymbol{I} + D\left(\boldsymbol{S^{(n)}C}\right)\right)\boldsymbol{C^{-1}}$ is a symmetrizable matrix for any $n > 1$ (where n is the iteration number).

Proof: The proof of this theorem is similar to the proof of theorem S3.1.

Properties of symmetrizable matrices.

Theorem S3.3: If $\boldsymbol{S}$ is symmetrizable matrix, then the two following properties hold:

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i. S_{ij}=0 implies that S_{ji}=0.

ii. for any sequence of indexes $i_1, i_2, \ldots i_n$, $S_{i_1 i_2} * S_{i_2 i_3} * \ldots S_{i_n i_1} = S_{i_2 i_1} * S_{i_3 i_2} * \ldots S_{i_1 i_n}$.

Proof. Although this theorem can be proved using Definition S3.1 above, the proof is easier, if the following definition of symmetrizable matrices is used:

Definition S3.2. A matrix M is symmetrizable if it can be expressed as the product of an invertible diagonal matrix ($\boldsymbol{U}$) and a symmetric matrix $\boldsymbol{T}$ (for detail see, e.g., Ref {Hazewinkel, 2009 #35, pp. 514),

$$\boldsymbol{M} = \boldsymbol{UT} \qquad \text{(S3.2)}$$

It can readily be shown that the local response matrix $\boldsymbol{S}$ can be expressed in the above form, since the equations (Equations 5 and S11-S13{Barzel, 2013 #20}) for $\boldsymbol{S}$ can be be rewritten as follows:

$$\boldsymbol{S} = \left(\boldsymbol{C} - \boldsymbol{I} + D\left((\boldsymbol{C} - \boldsymbol{I})\boldsymbol{C}\right)\right)\boldsymbol{C}^{-1} = (\boldsymbol{I} - \boldsymbol{U_s}\boldsymbol{C}^{-1}) = \boldsymbol{U_s}(\boldsymbol{U_s}^{-1} - \boldsymbol{C}^{-1}) = \boldsymbol{U_s}\boldsymbol{T_s}$$

$$\boldsymbol{S}^{(n+1)} = \left(\boldsymbol{C} - \boldsymbol{I} + D\left(\boldsymbol{S}^{(n)}\boldsymbol{C}\right)\right)\boldsymbol{C}^{-1} = \left(\boldsymbol{I} - \boldsymbol{U}_{\boldsymbol{s}}^{(n)}\boldsymbol{C}^{-1}\right) = \boldsymbol{U}_{\boldsymbol{s}}^{(n)}\left(\boldsymbol{U}_{\boldsymbol{s}}^{(n)^{-1}} - \boldsymbol{C}^{-1}\right) = \boldsymbol{U}_{\boldsymbol{s}}^{(n)}\boldsymbol{T}_{\boldsymbol{s}}^{(n)} \qquad \text{(S3.3)}$$

Here $\boldsymbol{U_s} = (\boldsymbol{I} - D((\boldsymbol{C} - \boldsymbol{I})\boldsymbol{C}))$, $\boldsymbol{U}_{\boldsymbol{s}}^{(n)} = (\boldsymbol{I} - D(\boldsymbol{S}^{(n)}\boldsymbol{C}))$ are diagonal invertible matrices, and $\boldsymbol{T_s} = (\boldsymbol{U_s}^{-1} - \boldsymbol{C}^{-1})$, $\boldsymbol{T}_{\boldsymbol{s}}^{(n)} = \left(\boldsymbol{U}_{\boldsymbol{s}}^{(n)^{-1}} - \boldsymbol{C}^{-1}\right)$ are symmetric matrices. The above reformulation leads to the following equations:

$$S_{ij} = U_{s_{ii}} T_{s_{ij}}, \qquad S_{ji} = U_{s_{jj}} T_{s_{ji}}$$

$$S_{ij}^{(n)} = U_{s_{ii}}^{(n)} T_{s_{ij}}^{(n)}, \quad S_{ji}^{(n)} = U_{s_{jj}}^{(n)} T_{s_{ji}}^{(n)} \qquad \text{(S3.4)}$$

Since, U_s and $U_s^{(n)}$ are invertible matrices, $U_{s_{ii}}, U_{s_{ii}}^{(n)} \neq 0$ for all i. Therefore, $S_{ij} = U_{s_{ii}} T_{s_{ij}} = 0 \ \ and \ S_{ij}^{(n)} = U_{s_{ii}}^{(n)} T_{s_{ij}}^{(n)} = 0$ implies that $T_{s_{ij}} = 0, T_s^n{}_{ij} = 0$. Since, both $\boldsymbol{T_s}$ and $\boldsymbol{T}_{\boldsymbol{s}}^{(\boldsymbol{n})}$ are symmetric matrices, $T_{s_{ij}} = 0, T_s^{(n)}{}_{ij} = 0$, implies that $T_{s_{ji}} = 0, T_s^{(n)}{}_{ji} = 0$. Therefore, $S_{ij} = U_{s_{ii}} T_{s_{ij}} = 0 = U_{s_{jj}} T_{ji} = S_{ji}$, and $S_{ij}^n = U_{s_{ii}}^{(n)} T_{s_{ij}}^{(n)} = 0 = U_{s_{jj}}^{(n)} T_{s_{ji}}^{(n)} = S_{ji}^{(n)}$ which proves the property S3.3(i).

Property S3.3(ii) can be proved using the decomposition S3.3 of the local response matrices. Replacing S_{ij} by $U_{ii}T_{ij}$ in $S_{i_1 i_2} * S_{i_2 i_3} * \ldots S_{i_n i_1}$ and $S_{i_1 i_n} * S_{i_n i_{n_1}} * \ldots S_{i_2 i_1}$ yields,

$$S_{i_1 i_2} * S_{i_2 i_3} * \ldots S_{i_n i_1} = U_{s_{i_1 i_1}} T_{s_{i_1 i_2}} * U_{s_{i_2 i_2}} T_{s_{i_2 i_3}} *..* U_{s_{i_n i_n}} T_{s_{i_n i_1}} = \left(\prod_{i=i_1}^{i_n} U_{s_{ii}}\right).\left(T_{s_{i_1 i_2}} T_{s_{i_2 i_3}} .. T_{s_{i_n i_1}}\right)$$

$$\boldsymbol{S_{i_1 i_n}} * \boldsymbol{S_{i_n i_{n_1}}} * \ldots \boldsymbol{S_{i_2 i_1}} = \boldsymbol{U_{s_{i_1 i_1}}} \boldsymbol{T_{s_{s_{i_1 i_n}}}} * \boldsymbol{U_{s_{i_n i_{n-1}}}} \boldsymbol{T_{s_{i_n i_{n-1}}}} *..* \boldsymbol{U_{s_{i_2 i_2}}} \boldsymbol{T_{s_{i_2 i_1}}} = \left(\prod_{\boldsymbol{i=i_1}}^{\boldsymbol{i_n}} \boldsymbol{U_{s_{ii}}}\right).\left(\boldsymbol{T_{s_{i_1 i_n}}} \boldsymbol{T_{s_{i_n i_{n-1}}}} .. \boldsymbol{T_{s_{i_2 i_1}}}\right) \qquad \text{(S3.5)}$$

Since $\boldsymbol{T_s}$ is a symmetric matrix $\left(T_{s_{i_1 i_2}} T_{s_{i_2 i_3}} .. T_{s_{i_n i_1}}\right) = \left(T_{s_{i_1 i_n}} T_{s_{i_n i_{n-1}}} .. T_{s_{i_2 i_1}}\right)$. Therefore, $S_{i_1 i_2} * S_{i_2 i_3} * \ldots S_{i_n i_1} = S_{i_1 i_n} * S_{i_n i_{n_1}} * \ldots S_{i_2 i_1}$ which proves S3.3 (ii) for the local response matrix $\boldsymbol{S}$. This

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property can also be proved for $\boldsymbol{S^{(n)}}$ (the iterative solution for the local response matrix in Barzel & Barabási's paper[1]) in the same manner as shown above (see also http://en.wikipedia.org/wiki/Symmetric_matrix).

Symmetrizable local response matrices provide erroneous network connections for the majority of cellular networks.

The first property (S3.3(i)) of symmetrizable matrices (for details see Ref. [30]) implies that the absence of a network connection from node *j* to node *i* guarantees the absence of a network connection in the reverse direction from node *i* to node *j*. Thus, if the local response matrix is symmetrizable, the corresponding network does not have any one way (unidirectional) connections. In contrast, in biology unidirectional connections are ubiquitous, including posttranslational protein modifications, such as phosphorylation. For instance, if a kinase phosphorylates a substrate, the substrate often does not modify its kinase, and thus the corresponding connection is unidirectional. The second property (S3.3(ii)) of symmetrizable matrices[30] implies that the overall signal amplification or attenuation along a circular path (which usually includes a feedback loop) is exactly the same as that in the opposite direction along that path. This property does not hold for almost every known signalling or gene network. Therefore, the local response coefficients, which are obtained by using the correlation method suggested by Barzel & Barabási, do not represent real biochemical networks in cells[1].

Networks inferred from the correlation matrices by Barzel & Barabási's method can be erroneous.

We first demonstrate that correlation and mutual information matrices are quantitatively and qualitatively different from global response matrices. Applying GeneNetWeaver[31] (used by the DREAM consortium for generating benchmark datasets), we simulated the perturbation responses of a 100 gene sub-network of E. coli gene regulatory network (GRN) (see Suppl. Data. 1). The global response matrix was calculated by downregulating one gene at a time by 50% (termed as knockdown in GeneNetWeaver [31]). After each perturbation, the network was allowed to relax to new steady state, and then the fractional changes in the gene concentrations were calculated. The Pearson,Spearman correlation and mutual information matrices were also calculated. The resulting global response matrix, the Person and Spearman correlation and mutual information matrices are visualized as heat maps in Fig. S2 (a), (b), (c), (d) respectively. It can be seen from Fig. S2 that the asymmetric global response matrix (Fig.S2a) has little in common with the symmetric Pearson (Fig.S2b) ,Spearman (Fig.S2c) correlation and mutual information (Fig 2d) matrices of the simulated *E.coli* GRN.

Next we show that the networks inferred from the correlation matrices using Barzel & Barabási's method can be erroneous. Using Equation 5 proposed by Barzel & Barabási[1], we inferred the local response coefficients from the Pearson,Spearman correlation and mutual information matrices. The inferred networks were compared with the true network using the area under the Receiver Operating Characteristics (*AUROC*) and the Precision Recall (*AUPR*) curves[32]. The *AUROC* and *AUPR* scores can take values between 0 and 1 ($0 \leq AUROC, AUPR \leq 1$), where zero indicates no resemblance with the original network and one indicates that the inferred network is 100% accurate and identical to the original network (*AUROC*=0.5 indicates that the inferred network is only as

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accurate as a random network)[32, Davis, 2006 #40]. The networks inferred from the Pearson, Spearman correlation and mutual information matrices by Barzel & Barabási's algorithm had the *AUROC* values 0.68, 0.55 and 0.51, and the *AUPR* values 0.050,0.054 and 0.020, respectively. Thus, these networks are only marginally better than random networks in predicting the true topology of the E.coli GRN. At the same time, the network calculated from the global response matrix by standard MRA method[33] had *AUROC*=0.98 and *AUPR*=0.91 inferring the E.coli GRN much more accurately.

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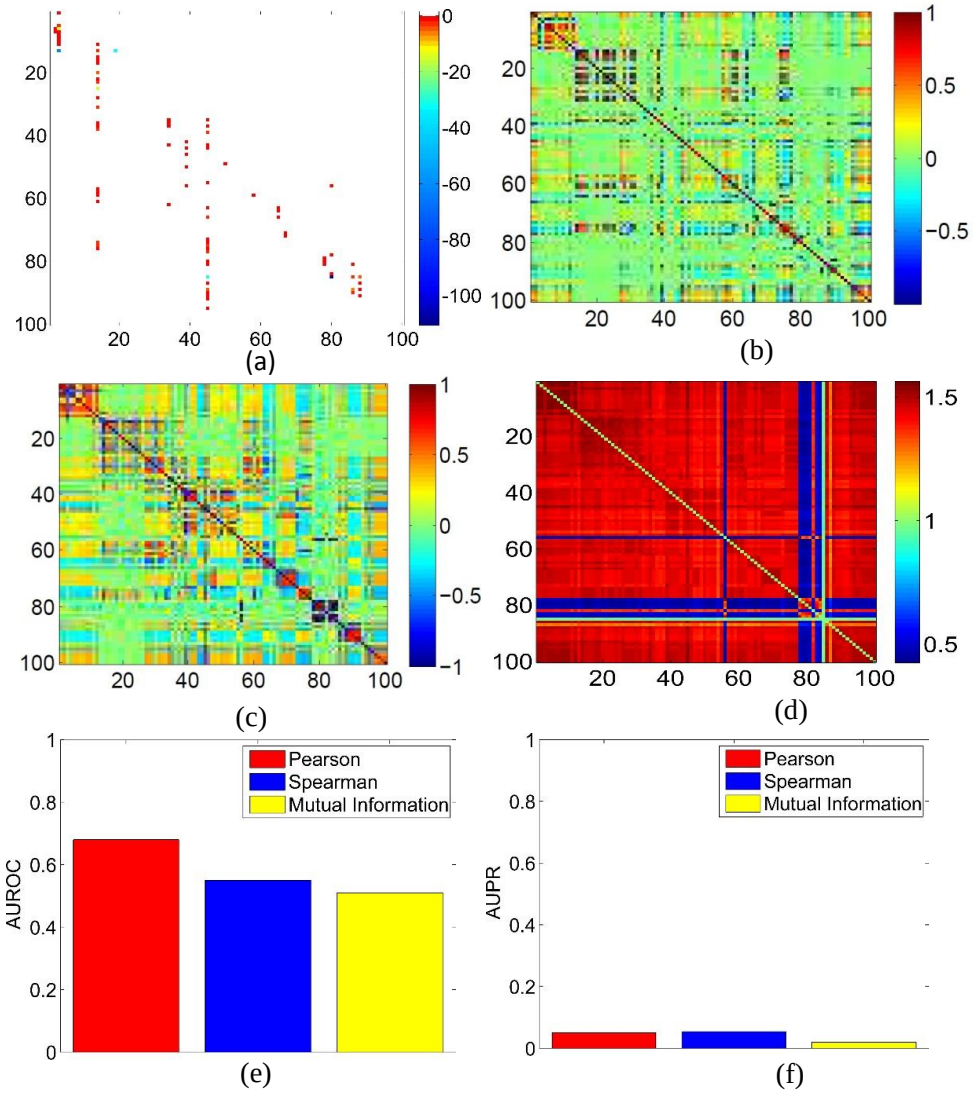

Figure S2. The global response and correlation matrices of a 100 gene subnetwork of E.coli and the performance of Barzel and Barabási's correlation coefficient method. (**a**) Heatmap representation of the global response matrix. (**b**), (**c**) & (**d**) Heatmap representations of the Pearson, Spearman correlation & mutual information matrices, respectively. (**e**) and (**f**) Performance of Barzel & Barabási's algorithm applied to the Pearson, Spearman correlation & mutual information matrices.

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Supplementary Note 4. Robustness against noise.

To evaluate the robustness of Barzel & Barabási's algorithm against noise, we generated a 100 node scale-free network (Suppl. Data 2) and simulated perturbation responses, as described above. We then calculated the Pearson and Spearman correlation matrices and inferred the corresponding local response matrices using Barzel & Barabási's method for this relatively small network[1]. The inferred networks were compared with the true networks using the area under the Receiver Operating Characteristics (*AUROC*) and the Precision Recall (*AUPR*) curves. The *AUROC* and *AUPR* scores can take values between 0 and 1 ($0 \leq AUROC, AUPR \leq 1$), where zero indicates no resemblance with the original network and one indicates that the inferred network is 100% accurate and identical to the original network (*AUROC*=0.5 indicates that the inferred network is only as accurate as a random network)[32,34]. The comparison of the inferred networks using Barzel & Barabási's method with the true network resulted in $AUROC \approx 0.8$ and $AUPR \approx 0.22$. Then we added small Gaussian noise to the perturbation responses and calculated the corresponding correlation matrices. Denoting by *y* the perturbed steady state value, the noise was characterized by $\mu = 0, \sigma = 1\%$ of *y*. Again Barzel & Barabási's formulation (Equation 5) was used to calculate the local response coefficients, which were then compared with the true network by calculating AUROC and AUPR values. For 1% noise, we obtained $AUROC = 0.42, 0.53$ and $AUPR = 0.025, 0.024$, when using the Pearson and Spearman correlation matrices, respectively (Fig. S3). When we gradually increased the noise level ($\sigma = 2\%, 3\%, .4\%, \ldots, 20\%$) and repeated the above procedure, we found that the performance of Barzel & Barabási's algorithm did not significantly improve. This suggests that that Barzel and Barabási's algorithm is highly sensitive to noise in contrast with their statement[1].

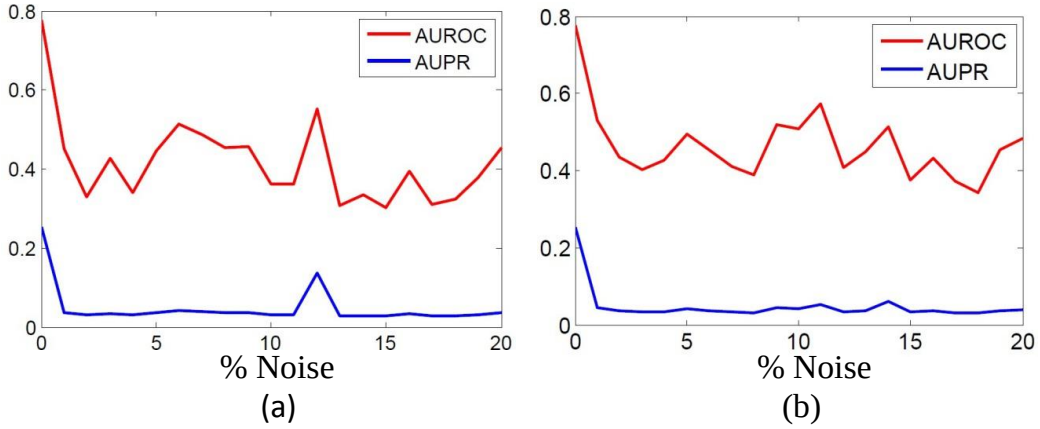

Figure S3. Robustness of Barzel & Barabásis's method against noise. The AUROC and AUPR values (Y-axis) for the networks inferred using Barzel & Barabási's method are plotted against noise variance (***σ***, X-axis), which is expressed as percentage of the signal amplitude. Panels (**a**) and (**b**) show the method performances for the Pearson and Spearman correlation matrices, respectively.

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Supplementary Note 5.

True performance of Barzel & Barabási's inference algorithm for *E. Coli* interaction network in the DREAM5 challenge is much worse than reported and is inferior compared to the top-performing methods.

Barzel & Barabási claim that their algorithm "improves upon the top-performing inference methods"[1] in the DREAM5 network inference challenge (Network 3, [35]). We found three issues in Barzel & Barabási's evaluation of their algorithm performance, which render their claim unsubstantiated.

First, they use *a posteriori* knowledge of which transcription factors (TFs) to exclude from their inference model. The DREAM challenge provides a dataset containing the expression levels of 4511 E.coli genes, including 334 known TFs. Performance in the challenge was estimated against a gold standard network (GSN), which involves only 141 out of 334 known TFs. While the actual contestants in the DREAM5 challenge performed inference on a dataset with all 334 known TFs, Barzel & Barabási used information unavailable to the participants allowing them to restrict their inference model to the 141 TFs appearing in the GSN and to zero out the correlations involving the other 193 TFs in their G-matrix. Their results therefore are not directly comparable to the performance of actual DREAM5 contenders.

A second reason we are unconvinced about the performance of their method is that Barzel & Barabási only used the area under the Receiver Operating Characteristics (*AUROC*) and did not consider the Precision Recall (*AUPR*) score to evaluate their algorithm performance, which is considered insufficient and even misleading when the number of true positive (interactions present in GSN) and true negatives (interactions absent in GSN) differ strongly [34]. In the E. coli network, true negatives are about 100 fold more abundant than true positives. Consequently in the DREAM challenge, the performances were estimated using scores that combined *AUROC* and *AUPR* neglected by Barzel & Barabási.

Finally, Barzel & Barabási incorrectly estimated the performance of their algorithm, using the ROC curves. When scoring their method, they erroneously gave themselves credit for true-negatives that did not appear in the actual GSN used in DREAM5. The ROC curves are calculated by comparing the predicted network with the actual GSN and calculating the proportions (rates) of false-positive (FPR) and true positive (TPR) interactions. The GSN provided by the DREAM5 challenge for the evaluation purposes contains only 1080 potential target genes and $141 \times 1080 = 152\,280$ interactions out of $141 \times 4511 = 636\,051$ possible interactions between 141 TFs and 4511 genes. Instead of comparing their predicted network with this actual DREAM5 GSN, Barzel & Barabási made a new GSN where they erroneously scored the interactions, for which no information was provided by the DREAM5 GSN, as the absence of interaction (true negatives). As a result, the total number of true negatives in Barzel & Barabási's calculations was almost four times higher than in the original GSN. Therefore the FPR (= false positive interactions/(false positive interactions + true negative interactions)) that was calculated by Barzel & Barabási is almost four times smaller than it would be if it were calculated correctly. This incorrect reduction of the false positive rate inflated the area under the ROC curve, making the calculated *AUROC* score invalid.

We corrected these mistakes by (1) incorporating all 334 TFs in the model, (2) including AUPR as a performance measure, and (3) recalculating the *AUROC* and *AUPR* scores using the

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performance evaluation script of the DREAM5 challenge (which was also used to evaluate the performance of the contestants of the DREAM5 competition (http://wiki.c2b2.columbia.edu/dream/data/scripts/DREAM5/files/DREAM5_NetworkInference_Evaluation.zip). The results shown in Fig. S4 demonstrate that Barzel & Barabási's inference algorithm performed far worse than the best performer of the DREAM5 competition (either when the *AUROC* score or *AUPR* score were considered). The "raw" statistical association measures, such as correlations or mutual information alone performed better than their algorithm.

It was previously shown (see, e.g. Refs.[36,37]) that partial correlations can be employed to disentangle direct from indirect correlations. The partial correlation is defined as, $p_{ij} = -\omega_{ij}/\sqrt{\omega_{ii}\omega_{jj}}$ with ω_{ij} being the elements of the inverse of the Pearson correlation matrix[38]. The results shown in Fig. S4 suggest that the partial correlation also outperforms the method proposed by Barzel & Barabási.

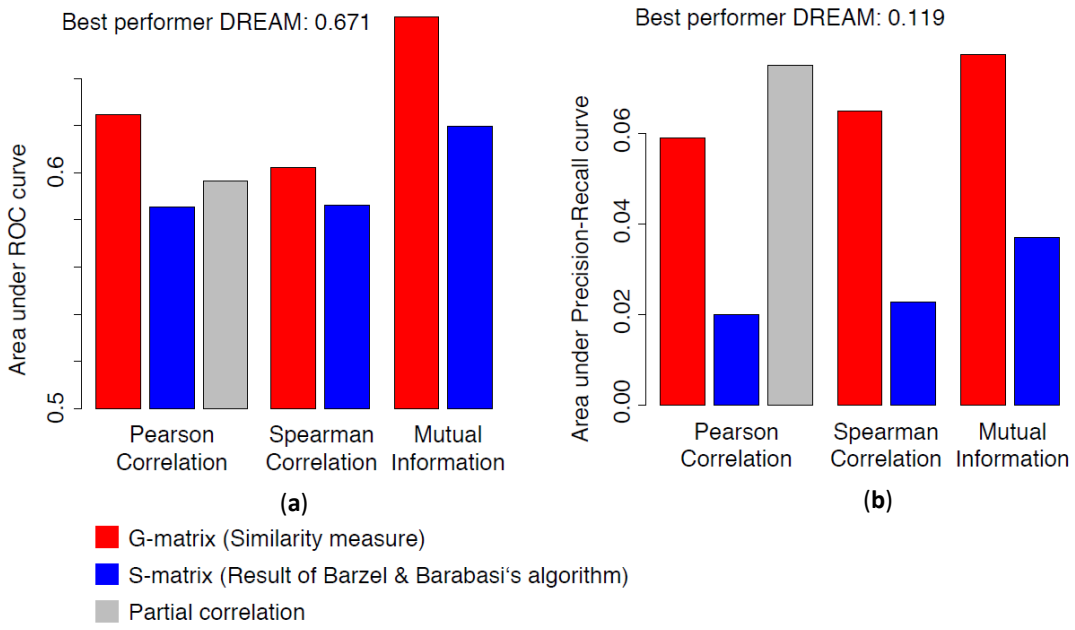

Figure S4. Unbiased evaluation of the performance of Barzel & Barabási's algorithm on DREAM 5 data. (**a**) The *AUROC* scores calculated (red, matrix G) - using pure similarity measures, (blue) - using the local response coefficients given by matrices S that were calculated from the G-matrices using Barzel & Barabási's algorithm, and (grey) - using partial correlations obtained from the Pearson correlation matrix. (**b**) The *AUPR* scores calculated for each of these methods.

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Supplementary Item for Fig. 1.

The data underlying the graphical representations used in Fig. 1 are freely downloaded using the following link: http://wiki.c2b2.columbia.edu/dream/index.php/D5c4