Mathematical Model of a Telomerase Transcriptional Regulatory Network Developed by Cell-Based Screening: Analysis of Inhibitor Effects and Telomerase Expression Mechanisms

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Abstract
Cancer cells depend on transcription of telomerase reverse transcriptase (TERT). Many transcription factors affect TERT, though regulation occurs in context of a broader network. Network effects on telomerase regulation have not been investigated, though deeper understanding of TERT transcription requires a systems view. However, control over individual interactions in complex networks is not easily achievable. Mathematical modelling provides an attractive approach for analysis of complex systems and some models may prove useful in systems pharmacology approaches to drug discovery. In this report, we used transfection screening to test interactions among 14 TERT regulatory transcription factors and their respective promoters in ovarian cancer cells. The results were used to generate a network model of TERT transcription and to implement a dynamic Boolean model whose steady states were analysed. Modeled effects of signal transduction inhibitors successfully predicted TERT repression by Src-family inhibitor SU6656 and lack of repression by ERK inhibitor FR18209, results confirmed by RT-QPCR analysis of endogenous TERT expression in treated cells. Modeled effects of GSK3 inhibitor 6-bromoindirubin-3'-oxime (BIO) predicted unstable TERT repression dependent on noise and expression of JUN, corresponding with observations from a previous study. MYC expression is critical in TERT activation in the model, consistent with its well known function in endogenous TERT regulation. Loss of MYC caused complete TERT suppression in our model, substantially rescued only by co-suppression of AR. Interestingly expression was easily rescued under modelled Ets-factor gain of function, as occurs in TERT promoter mutation. RNAi targeting AR, JUN, MXD1, SP3, or TP53, showed that AR suppression does rescue endogenous TERT expression following MYC knockdown in these cells and SP3 or TP53 siRNA also cause partial recovery. The model therefore successfully predicted several aspects of TERT regulation including previously unknown mechanisms. An extrapolation suggests that a dominant stimulatory system may programme TERT for transcriptional stability.

Introduction
Immortalisation is a hallmark of cancer commonly achieved by transcriptional reactivation of the telomerase reverse transcriptase gene TERT [1]. Multiple transcription factors modulate TERT and previous studies have identified many of those which individually contribute to activate or repress telomerase levels in cancer cells, resulting in a highly complex picture of TERT regulation [2]. In cancer cells lacking tight control of chromatin mediated silencing present in normal cells, a few factors such as c-Myc and Sp1 may act as “master regulators”. However, many other factors bind the TERT promoter, co-operating with these and other pathways, and acting together to ensure telomerase expression in a wide variety of cancer cells.

It is increasingly recognised that transcription factors do not behave in isolation, but rather as a complex co-operative network [3] and TERT expression most likely also occurs in this context [4,5]. For example, TERT transcriptional suppression by different TP53 family members is mediated through distinct combinations of binding sites for c-Myc, Sp1 and E2F-family proteins [6], while E2F family members themselves activate or suppress TERT in a cell-specific manner [7]. Furthermore, WT1 dependent TERT repression in renal cancer cells involves upregulated expression of TERT repressors SMAD3 and JUN, as well as down-regulation of activators AP-2 and NF1 [8].

We previously observed that GSK3 inhibition causes widespread TERT promoter remodelling and that GSK3 inhibited ovarian cancer cells show long-term unstable telomerase suppression, correlating with altered protein expression and oscillation of several TERT regulatory factors, particularly c-Jun [4]. Thus, upstream telomerase regulatory interventions are mediated through multiple effects at the promoter but can also cause broader network
Author Summary

Tumour cells acquire the ability to divide and multiply indefinitely whereas normal cells can undergo only a limited number of divisions. The switch to immortalisation of the tumour cell is dependent on maintaining the integrity of telomere DNA which forms chromosome ends and is achieved through activation of the telomerase enzyme by turning on synthesis of the TERT gene, which is usually silenced in normal cells. Suppressing telomerase is toxic to cancer cells and it is widely believed that understanding TERT regulation could lead to potential cancer therapies. Previous studies have identified many of the factors which individually contribute to activate or repress TERT levels in cancer cells. However, transcription factors do not behave in isolation in cells, but rather as a complex co-operative network displaying inter-regulation. Therefore, full understanding of TERT regulation will require a broader view of the transcriptional network. In this paper we take a computational modelling approach to study TERT regulation at the network level. We tested interactions between 14 TERT-regulatory factors in an ovarian cancer cell line using a screening approach and developed a model to analyse which network interventions were able to silence TERT.

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of overexpressing E2F1 is shown separately from the other TERT repressors on a log scale in figure 1C because of its very strong self-regulatory effect (73.5-fold activation). Effects at the TERT promoter (right hand side of each panel) were in the expected direction for all factors except FOS. The reason for this discrepancy is not clear, but may reflect the different construct and cell line [27]. We initially tested several candidate network models assembled by using different criteria for inclusion of an interaction in the network and implementing the BN rules on each network. The threshold rule governing model dynamics is given in materials and methods. Briefly, the rule is activation-dominant: for any node, unless more of its repressors than activators are on at any time step, the node will be active on the next time step.

We tested a range of cutoff values based on fold-change in promoter activity and significance (ANOVA) for inclusion of individual interactions from the transfection screen and implemented the rules on each resulting network. The results of this model-fitting exercise are also detailed in supplemental file Text S1. The best results were obtained when we selected cutoffs of minimum 1.5-fold change in reporter activity with $p \leq 0.01$ (in figure 1, interactions meeting the cutoff are marked **). A single relaxation of these cutoffs was made for the effect of STAT3 at the TERT promoter. Although regulation tended in the expected direction, it was not significant. Other studies have shown that STAT3 does activate TERT expression [28] and we have previously detected STAT3 binding to the TERT promoter by ChIP analysis in A2780 cells [4] and we re-performed this analysis here (supplemental file Text S1). We retained STAT3 as a TERT activator since our model performs better with its inclusion. We discuss this decision and the role of STAT3 in our model in detail in supplemental file Text S1, along with results obtained using a network in which STAT3 is excluded. As detailed in the file, the STAT3-deleted model still captured most behaviour we describe in this report, though the model was more aligned with some experiments when it was included. The selected model comprises 92 total interactions of which 50 are activating and 42 are repressive.

We performed literature searching with inclusion/exclusion criteria detailed in materials and methods for each of these individual interactions. We found 47 previously reported interactions (excluding STAT3 activation of TERT since this was retained according to a modelling decision). The cumulative hypergeometric probability of this overlap was $p(\geq 17) = 1.24 \times 10^{-3}$. 35 interactions were found to be in agreement with our results (table 2) [27,29–63]. 12 previously reported interactions were non-concordant with our results, having different effect directions (table 3) [27,49,55,64–72]. The reasons for these differences may be because of the use of different cell lines or constructs. However, the cumulative binomial probability of this concordance is $p(\geq 35) = 5.44 \times 10^{-4}$. Hence, the overlap between our screen and the literature was highly significant. The remaining 44 interactions we identified are given in table 4. We apologise if we have overlooked the work of any authors who have previously demonstrated these interactions according to our literature search criteria. If we are made aware of any such study, we will endeavour to cite it in any future publications relating to our model. We next analysed the model dynamic behaviour in more detail.

### Analysis of the basal model dynamics

The BN model has 32768 total dynamic states, representing the $2^{15}$ possible combinations of on/off states across all 15 nodes in the model. As noted above, classical BN always converge to either steady states or oscillations. 32766 states are transient states evolving to the 2 steady states of our basal model shown in figure 2A, along with the structure of the network model. TERT is active in both steady states, which are highly similar, differing only in JUN activation (in the figure, red indicates on, whereas green indicates off). Therefore, telomerase is “stably expressed” in the model, in line with the stable telomerase expression and telomere maintenance that we previously observed in these cells over 6 months in culture [4].

For all model variants in this paper we have performed statespace analysis [73]. This involves sequentially treating each of the 32768 total dynamic states as the current model state, then for each node determining how many of its activators and repressors are currently on. The approach is only feasible for relatively small networks, but allows identification of all steady states without the need to run multiple simulations starting from different initial conditions.

Since each individual state transits to exactly one next state, the statespace itself can be represented as a network (figure 2B). Here, each node represents a single model state and the arrows represent transitions between them. The single yellow nodes near the centre of each network are the respective steady states shown in figure 2A. All other nodes are transient states evolving toward these. Note that the 32768-state system is too large to show, so a truncated core structure is shown in figure 2B as described in the legend. Steady state 1 (figure 2A, left) dominates, with 20156 associated states which flow to it. If the model is initialised and run from any of these states, it will evolve to steady state 1. 12612 states are associated to steady state 2 (figure 2A, right).

This analysis provides an overview of global dynamics of the model by considering path lengths through statespace. Since no TERT-off attractors exist, if perturbed to an “outer state”, the model must return to a TERT-on state. Long paths indicate that the model takes longer to reach a steady state. In our model, most system states evolve very rapidly toward the steady states which have TERT active. The longest paths are 9 steps and 8 steps for steady states 1 and 2, respectively. Hence, the model exhibits a high degree of global stability with respect to TERT activation. Specifically, if the model is transiently perturbed to turn off TERT,

| Gene | Gene ID |
|------|---------|
| AR   | 367     |
| JUN  | 3725    |
| TPS3 | 7157    |
| SP1  | 6667    |
| E2F1 | 1869    |
| MYCN | 4613    |
| MXD1 | 4084    |
| RELA | 5970    |
| MYC  | 4609    |
| FOS  | 2353    |
| HIF1A| 3091    |
| SP3  | 6670    |
| STAT3| 6774    |
| NTRF2| 7026    |
| TERT | 7015    |

**Table 1.** Accession numbers of all human genes from the model.

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Figure 1. Definition of the TERT transcriptional neighbourhood in A2780 cells by transfection screening. (A), overexpression of TERT activators. A2780 cells were transfected with the luciferase reporters shown on the vertical axis. Each reporter was co-transfected alongside vector control or transcription factor expression plasmid shown in the right hand boxes. Each bar type represents a different expression vector relative to control. 48 h post-transfection, promoter activities were analysed by luciferase assay. (B), overexpression of TERT repressors, transfected as in (A). (C), overexpression of E2F1 against the promoter panel, transfected as above. Because of the very strong self-regulatory effect on its own promoter, E2F1 is shown on a different scale and separately from the other TERT repressors. Mean ± SEM of 3 experiments (ns: not significant; *: p<0.5; **: p<0.01).

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Table 2. Common, concordant interactions in literature and data-derived models.

| From → To | Literature Effect | Reference |
|-----------|-------------------|-----------|
| AR→TPS3 | Inhibition | Rokhlin et al (2005) [53] |
| AR→TERT | Inhibition | Moehren et al (2008) [48] |
| JUN→JUN | Activation | Angel et al (1988) [31] |
| JUN→TERT | Inhibition | Takakura et al (2005) [27] |
| TPS3→MYC | Inhibition | Ho et al (2005) [39] |
| TPS3→E2F1 | Inhibition | Oookawa et al (2001) [52] |
| TPS3→SP1 | Inhibition | Tapias et al (2008) [56] |
| TPS3→FOS | Inhibition | Kley et al (1992) [45] |
| TPS3→TERT | Inhibition | Kanaya et al (2000) [44] |
| SP1→JUN | Activation | Chen et al (1994) [33] |
| SP1→MYCN | Activation | Hossain et al (2012) [40] |
| SP1→FOS | Activation | Duan et al (1998) [36] |
| SP1→TERT | Activation | Kyo et al (2000) [46] |
| E2F1→E2F1 | Activation | Johnson et al (1994) [43] |
| E2F1→HIF1A | Activation | Sengupta et al (2011) [54] |
| E2F1→AR | Inhibition | Davis et al (2006) [35] |
| E2F1→MYC | Activation | Thalamé et al (1989) [57] |
| E2F1→TERT | Inhibition | Crowe et al (2001) [34] |
| MYCN→TPS3 | Activation | Chen et al (2010) [32] |
| MYCN→E2F1 | Activation | Oliver et al (2003) [51] |
| MYCN→TERT | Activation | Mac et al (2000) [47] |
| MXD1→TERT | Inhibition | Oh et al (2000) [50] |
| RELA→JUN | Activation | Xing et al (2013) [61] |
| RELA→TPS3 | Activation | Hsu and Lee (2011) [41] |
| RELA→FOS | Activation | Anest et al (2004) [30] |
| RELA→TERT | Activation | Gizard et al (2011) [38] |
| MYC→JUN | Inhibition | Zeller et al (2006) [63] |
| MYC→TERT | Activation | Wu et al (1999) [60] |
| HIF1A→TERT | Activation | Anderson et al (2006) [29] |
| SP3→SP1 | Inhibition | Nicolas et al (2003) [49] |
| SP3→TERT | Inhibition | Wooten-Blanks et al (2007) [59] |
| STAT3→JUN | Activation | Durant et al (2010) [37] |
| STAT3→FOS | Activation | Yang et al (2003) [82] |
| STAT3→MXD1 | Activation | Jiang et al (2008) [42] |
| NR2F2→TERT | Inhibition | Wang et al (2004) [58] |

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Table 3. Common, non-concordant interactions in literature and data-derived models.

| From → To | Literature Effect | Reference |
|-----------|-------------------|-----------|
| JUN→AR | Inhibition | Yuan et al (2004) [72] |
| JUN→FOS | Inhibition | Schöntahl et al (1989) [70] |
| TP53→NR2F2 | Activation | Neelsen et al (2011) [68] |
| TP53→STAT3 | Activation | Kim et al (2009) [67] |
| E2F1→TP53 | Activation | Choi et al (2002) [66] |
| E2F1→SP1 | Activation | Nicolas et al (2003) [49] |
| E2F1→SP3 | Inhibition | Tapias et al (2008b) [55] |
| MYCN→MYCN | Inhibition | Sivak et al (1997) [71] |
| HIF1A→MXD1 | Activation | Cho et al (2013) [65] |
| STAT3→TP53 | Inhibition | Niu et al (2005) [69] |
| NR2F2→E2F1 | Activation | Chen et al (2012) [64] |
| FOS→TERT | Inhibition | Takakura et al (2005) [27] |

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this will be rapidly reversed. This is an interesting feature of the model, although clearly a qualitative BN model cannot be used to predict the rate of reversal in cells. However, telomere lengths and telomerase expression are stable over greater than 6 months in culture in A2780 cells, suggesting that any noise that does occur must indeed be reversed rapidly enough to facilitate ongoing telomere maintenance [4].

Modelling inhibitor effects on the TERT transcriptional network

It is possible that different states exist in the endogenous TERT transcriptional network some of which are more compatible for TERT silencing by particular approaches than others. It is of interest to evaluate this question in the modelling context and to compare model behaviour with actual regulation of endogenous TERT under signalling interventions. We therefore examined effects of several small molecule kinase inhibitors on the transcription factor promoter panel and incorporated these effects into our model. The compound IUPAC names are given in table 5. A2780 cells were transfected with the promoter panel and 32 h post-transfection were treated for 16 h with either the Src-family inhibitor SU6656 (5 μM), the ERK inhibitor FR180204 (10 μM), or the GSK3 inhibitor 6-hormonidinurin-3’-oxime (BIO, 5 μM). Effects of SU6656 and FR180204 on the promoter panel are shown in the lefmost panels of figures 3A and 3B, respectively.

SU6656 significantly stimulated several promoter constructs. However, only 5 constructs achieved the cutoffs for inclusion in the model (as before, FC>1.5 and p<0.01). These were JUN (FC 2.59; p 0.0048), TP53 (FC 1.99, p 0.0092), MYCN (FC 3.19, p 0.0089), MXD1 (FC 1.84, p 0.0081) and SP3 (FC 2.5, p 0.005). Hence, SU6656 had a strong stimulatory effect on the promoters of several TERT repressors. In contrast, FR180204 significantly inhibited only two promoters, both of which achieved the cutoffs. These were TP53 (FC 0.63, p 0.0083) and FOS (FC 0.6, p 0.0037).

To model these results for SU6656, the updating rules for JUN, TP53, MYCN, MXD1 and SP3 were modified to result in constitutive activity: these nodes were set to the on state independent of any combination of their upstream activators or repressors. Similarly, those of TP53 and FOS were modified for constitutive
repression by SU6656. In the case of FR180204, a single TERT activator and a single repressor are both inhibited. In the new steady state, TERT remains on and differs from basal state 1, above, only in TP53 status. This result could be interpreted either as a prediction of no change or of activation. These alternatives cannot be readily discriminated in this modelling framework.

We next tested the effects of each compound on endogenous TERT expression in repeat treatment schedules. A2780 cells were treated twice weekly with SU6656 (5 μM), or FR180204 (10 μM) for 2 weeks (SU6656), or 3 weeks (FR180204). Compounds were not removed between treatments. In the case of FR180204, both control and treated cells were maintained in continuous log-phase for the 3 week treatment duration. However, 5 μM SU6656 induced a complete and sustained growth arrest and the treated cells could be maintained with ongoing treatment for only 2 weeks in this state (not shown).

RT-QPCR analysis revealed profound suppression of TERT levels in day 14 SU6656-treated A2780 (right panel, figure 3A). Treated cells had TERT levels 8.4% those of control levels. In contrast, in day 21 FR180204 treated cells, no change in TERT was observed (figure 3B, right panel). In treated cells TERT mRNA levels were 110% of control, but this was not significant. The endogenous effect of both compounds was therefore in line with modelling predictions. To our knowledge this is the first report in which a Boolean network model of a transcriptional network has successfully predicted the outcome of a signalling intervention in respect of gene expression.

The effect of treating the transfected promoter panel for 16 h with 5 μM GSK3 inhibitor BIO is shown in the top panel of figure 4A. BIO significantly affected the promoters of E2F1, FOS and STAT3. However, only FOS (FC 0.64, p 0.0088) and STAT3 (FC 2.77, p 0.0017) met the model cutoffs. As above, the rule-tables for these nodes were modified to simulate constitutive repression of FOS and constitutive activation of STAT3.

We have previously reported the effect on TERT levels of very long term culture in the presence of BIO [4]. We found that TERT is initially suppressed, reaching minimal levels at 3 weeks treatment. Expression subsequently recovers slowly over a period of months, though levels remain low enough to achieve telomere shortening. However, TERT expression eventually recovers sufficiently for brief telomere re-elongation, before its levels and telomere length are again suppressed. During this treatment period, levels of JUN protein are found to oscillate over a very wide range.

Analysis of the model under BIO modified rules reveals two steady states (figure 4A, heat map labelled “model”). In the dominant BIO state 1 (associated with 26002 system states), TERT is off. In BIO state 2 (associated with 6766 system states), TERT is on. Therefore, modelling of the effect of GSK3 inhibition on the transcription factor network panel predicts that two conflicting network states are possible under BIO treatment: TERT suppressed or not. As with the steady states of the basal model, the key difference between these states is in activity of JUN.

### Noise influences TERT repression under simulated GSK3 inhibition

To explore the modelling result for BIO in more detail, we examined the impact of simulating transient noise in either the basal model or under the BIO rule modifications. We adopted the “bit-flip” approach for noise simulation: the objective is to model impact of a transient change in each node on the stability of a steady state of interest. The system is initialised with a single state change at one node relative to the steady state of interest (for example, JUN on→off relative to steady state 1 of either basal or

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**Table 4. Candidate novel interactions included from the screen.**

| From  | To    | Effect |
|-------|-------|--------|
| AR    | RELA  | Inhibition |
| AR    | HIF1A | Inhibition |
| JUN   | STAT3 | Activation |
| TP53  | JUN   | Inhibition |
| TP53  | MXD1  | Inhibition |
| TP53  | RELA  | Inhibition |
| TP53  | HIF1A | Inhibition |
| TP53  | SP3   | Inhibition |
| SP1   | STAT3 | Activation |
| E2F1  | MXD1  | Inhibition |
| E2F1  | RELA  | Inhibition |
| E2F1  | JUN   | Inhibition |
| E2F1  | STAT3 | Inhibition |
| MYCN  | AR    | Activation |
| MYCN  | JUN   | Activation |
| MYCN  | SP1   | Activation |
| MYCN  | MYC   | Activation |
| MYCN  | HIF1A | Activation |
| MYCN  | FOS   | Activation |
| MYCN  | SP3   | Activation |
| MYCN  | STAT3 | Activation |
| MXD1  | MYCN  | Activation |
| RELA  | NR2F2 | Inhibition |
| MYC   | SP1   | Inhibition |
| MYC   | MXD1  | Inhibition |
| MYC   | RELA  | Inhibition |
| MYC   | FOS   | Inhibition |
| MYC   | SP3   | Inhibition |
| MYC   | NR2F2 | Inhibition |
| HIF1A | MYC   | Activation |
| HIF1A | NR2F2 | Activation |
| HIF1A | AR    | Activation |
| FOS   | AR    | Activation |
| FOS   | MXD1  | Activation |
| FOS   | RELA  | Activation |
| FOS   | STAT3 | Activation |
| FOS   | NR2F2 | Activation |
| SP3   | NR2F2 | Inhibition |
| STAT3 | AR    | Activation |
| STAT3 | RELA  | Activation |
| NR2F2 | JUN   | Inhibition |
| NR2F2 | NR2F2 | Inhibition |

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The model is then run to steady state and it is determined whether the transiently modified state returns to the original steady state or not. This is performed sequentially for each individual node and each steady state.

As shown by the arrows representing steady state shifts induced by each node in figure 4B (top), the basal model is readily influenced by simulated noise. State 2 (top right) can be reached from State 1 (top left) through a transient change on 8/14 nodes in the network. However, the reverse transition can be achieved through noise at only 5/14 factors. Therefore, although state 1 associates with the larger fraction of statespace, under noise a switch to steady state 2 is likely. In contrast, the main GSK3 inhibited state 1 (bottom left) is highly resistant to noise with only 2/14 factors able to affect a shift to state 2 (bottom right). Interestingly, the reverse transition to the TERT-off state (BIO state 1) is readily achieved through transient changes at 7/14 factors. Importantly, noise at JUN is able to promote any shift.

We also examined the effect of a rule-set shift (basal→BIO, or the reverse) in determining which steady states are visited (vertical and diagonal arrows between top and bottom panels). This is intended to model effects of BIO treatment and effect wear-off applied to the different network states. When initialised in basal state 1, application of the BIO rule-set results in evolution to BIO state 1 (TERT repressed). However, from basal state 2, the BIO

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**Table 5.** IUPAC names and CAS numbers of the compounds used in the study.

| Compound    | IUPAC name                                                                 | CAS          |
|-------------|----------------------------------------------------------------------------|--------------|
| SU6656      | (3Z)-N,N-Dimethyl-2-oxo-3-[(4,5,6,7-tetrahydro-1H-indol-2-ylmethylidene)-2,3-dihydro-1H-indole-5-sulfonamide] | 330161-87-0  |
| BIO         | (3Z)-6-bromo-3-[3-(hydroxyamino)indol-2-ylidene]-1H-indol-2-one            | 667463-62-9  |
| FR180204    | 5-[2-(Phenyl-pyrazolo[1,5-a]pyridin-3-yl)-1H-pyrazolo[3,4-c]pyridazin-3-ylamine] | 865362-74-9  |

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rule modification causes evolution to BIO state 2 (TERT active). In the reverse rule shift, both BIO simulated states evolve to basal state 1. Extrapolating to the biological case, we interpret these results as a model prediction that a significant subset of cells may be at least transiently resistant to TERT suppression under BIO treatment (in the model, entering BIO state 2 from basal state 2). However, over time, noise could promote a network state compatible with TERT suppression. Therefore, TERT levels should initially decrease over time [4]. The impact of JUN in mediating the effects of BIO may be central. The model predicts that network noise select for JUN over-expression under BIO treatment (as more cells move to BIO state 1). We did observe progressive up-regulation of JUN under long term BIO treatment up to 4 months treatment, after which an oscillation is observed [4]. Up-regulation of JUN was also previously found to be important in mediating mesenchymal stem cell differentiation through GSK3β inhibition [74]. However, very high levels of JUN may be poorly tolerated. If mechanisms exist to reduce JUN expression under such conditions, we suggest that unstable TERT expression will result.

Subsystems in the TERT transcriptional network

One key reason to model complex systems is to interrogate potential functional roles of underlying structures in overall system behaviour. In the context of TERT it is of interest to attempt to define critical hubs for interventions to stably repress telomerase, and to identify how these cooperate to affect stability. We have previously proposed that stable activation or repression of TERT expression using pathway specific inhibitors which affect expression and activity of diverse transcription factors may depend partly on the structure and states of the transcriptional network and, in particular, on the relative effects on feedforward subsystems [4]. We next used our model to further investigate this hypothesis.
Figure 5A shows 4 types of feedforward loop (FFL) motif widely present in transcriptional networks (FFL type I–IV). In each case, two transcription factors (labelled X and Y in the figure) control expression of a third gene, Z. In addition, X also transcriptionally regulates Y. Each interaction may be activating (solid lines) or inhibiting (dashed lines). Of the 8 possible configurations of this motif, type I–IV are designated “coherent”, since the overall effect on Z resulting from the path X→Y→Z is the same as the direct path X→Z in terms of activation or repression. In particular, types I and IV are activating at Z, whereas types II and III are repressive. These have previously been characterised in a kinetic modelling context as delay elements which may reduce noisy expression of Z [75].

It is a reasonable expectation that the expression of a regulated gene Z, such as TERT, may be affected by the extent to which multiple FFL, centred on its regulation interact and overlap to form coherent activation or repression “modules”, and by the relative dominance of one or other of these subsystems. Specifically, in the current context, we define the “activation module” to be the set of overlapping FFL types I and IV focused on activation of TERT and the corresponding repression module to be the set of overlapping FFL types II and III focused on its repression.

We extracted these subsystems from our network model of TERT transcription (figure 5B). The method of extraction is detailed in materials and methods and supplemental file Text S1. The activation module comprises 12 nodes including TERT, and 31 interactions, of which 22 are stimulatory and 9 inhibitory. MYC plays a key role in blocking TERT repression in this system through several interactions which antagonistic TERT repressors. Most other interactions involve TERT activators which support each other’s expression. The repression module comprises 13 nodes and 27 interactions (9 activating, 18 inhibiting). Therefore, the 2 systems seem reasonably balanced at first glance.

However, further analysis suggests the activation module is structurally more co-operative. Longer paths are present, suggesting more scope for positively reinforcing delays: activation module network diameter (length of the longest of all shortest paths between any 2 nodes in the network) is 3 compared with 2 for the repression module. We also quantified relative participation of each factor in pathways in either subsystem using the betweenness centrality and flow centrality metrics [76,77]. Betweenness centrality of a node n is the fraction of all shortest paths between all other pairs of nodes in which n participates. By this analysis, MYC, MYCN, HIF1A, STAT3 and FOS all participate strongly as intermediates on shortest paths in the activation module, whereas only AR plays this role in the repression module (table 6). Flow centrality is a related measure which considers all paths, not only geodesics. As can be seen from table 6, this analysis provided similar results to the betweenness centrality analysis. Therefore, the TERT activator subsystem is more co-operatively connected in our model.

Robust MYC dependent TERT repression

To determine the contribution of each factor to overall activation of TERT in the model, we investigated the effect of simulating constitutive activation or suppression of each node individually. As above, appropriate changes were made in the rule-tables of each node and steady states were investigated. We quantified, in each case, the total proportion of model states which evolve to steady states with TERT-on. The weak components of
Boolean Network Model of hTERT Transcription

A. Boolean Network Model of hTERT Transcription

B. Network Diagram of Gene Interactions

C. Bar Chart of % state-space TERT on

D. Relative TERT expression

E. Western Blot Analysis of MYC, MXD1, TP53

** and ns indicate statistical significance.
statespace (basins of attraction) were obtained by depth-first search and the sizes of these were quantified. The total proportion of statespace contributed by each basin of attraction harboring a TERT-on steady state was quantified (figure 5C). In BN models, attractors are usually taken to be distinct "phenotypes" [78]. Hence, the steady states should be interpreted as different network states, each capable of supporting TERT expression. The fraction of statespace associated with them is a rough measure of the probability that any deviation from those steady states (through noise or intervention) will be reversed and maintain TERT-on rather than leading to a steady state transition to stable repression.

**Table 6.** Node betweenness centrality values for the activation and repression modules.

| Subnetwork | Node | Betweenness | Flow Betweenness |
|------------|------|-------------|-----------------|
| AM         | JUN  | 0.0000      | 0.200           |
|            | SP1  | 0.0000      | 0.833           |
|            | MYCN | 0.0545      | 6.917           |
|            | MXD1 | 0.0000      | 0.533           |
|            | RELA | 0.0000      | 1.667           |
|            | MYC  | 0.0909      | 10.500          |
|            | HIF1A| 0.0091      | 1.167           |
|            | FOS  | 0.0091      | 3.750           |
|            | SP3  | 0.0000      | 0.200           |
|            | STAT3| 0.0091      | 3.750           |
|            | NR2F2| 0.0000      | 0.200           |
|            | TERT | 0.0000      | 0.000           |
| RM         | AR   | 0.0151      | 2.500           |
|            | JUN  | 0.0000      | 0.000           |
|            | TP53 | 0.0000      | 0.000           |
|            | SP1  | 0.0000      | 0.825           |
|            | E2F1 | 0.0000      | 0.000           |
|            | MYCN | 0.0000      | 0.125           |
|            | RELA | 0.0000      | 0.658           |
|            | MYC  | 0.0000      | 0.125           |
|            | HIF1A| 0.0000      | 0.458           |
|            | FOS  | 0.0000      | 0.125           |
|            | SP3  | 0.0000      | 0.700           |
|            | STAT3| 0.0000      | 0.325           |
|            | TERT | 0.0000      | 0.000           |

Most factors had little effect individually on the stable expression of TERT in the model. However, constitutive repression of both MYC and FOS fully ablated all stable on-states (black bars for basal model, absent for MYC- and FOS-off). Therefore, the frequently observed critical role of MYC in TERT expression was reproduced [2,79]. Suppression of the MYC and HIF1A nodes also had a substantial impact on TERT. MYCN suppression produced 2 stable off-states, associated with 63.5% and 5.7% of model states, in addition to a small oscillator (5%). The remaining 25.7% of system states associate to the single remaining on-state. HIF1A suppression produced a single large off-state associated to 78.9% of statespace and a single smaller on-state. It is noteworthy that these factors all score as important in the betweenness analysis of the activation module, above.

Among the responders, constitutive activation of MXD1 produced the largest off-state (61.6% of statespace). Activation of either NR2F2 or TP53 had a mild effect in producing small off-states. Despite its key topological role in the repression module, activation of AR did not suppress TERT. Instead, constitutive AR suppression resulted in an off-state, though its size was negligible (0.7%). Overall, we make 2 conclusions from these results. Firstly, the model reproduces, from a network perspective, well known findings that both MYC and MXD1 are centrally important in TERT expression [80,81]. Secondly, the impact on TERT expression of targeting individual activators in the model seems to depend closely on their structural contribution in the activation module.

Because of the known importance of MYC in TERT regulation in the cellular setting, we next investigated the resistance to reversal of MYC dependent TERT repression observed in our modelling context. Starting from the MYC constitutively suppressed rule set, we again modified the rules for each of the other transcription factor nodes to simulate co-activation or co-repression alongside MYC inhibition. Steady states were analyzed and the ability of each condition to restore TERT activity relative to the MYC ablated state was scored (figure 5C, grey bars – presence of a grey bar indicates recovery of TERT expression). Most secondary ruleset mutations had no effect. However, AR suppression resulted in complete TERT recovery, consistent with its central topological role in the repression module. SP3 suppression was also able to promote partial recovery, producing a single small on state (8.9% of statespace). Therefore, MYC dependent TERT repression appears substantially robust in the model, but is still not entirely resistant to reversal.

To determine whether this prediction holds experimentally, we conducted RNAi double knockdown experiments in A2780 cells to simultaneously reduce expression of MYC alongside individual TERT repressors. We examined co-suppression of MXD1, TP53, AR, SP3, or JUN. Western blotting of each RNAi target following...
transfection of 100 nM non-specific or specific siRNA in figure 5E demonstrates that each individual siRNA successfully resulted in target knockdown, though the efficiencies varied between siRNA. Control blots for ERK were also performed. In most cases, RNAi had no effect on ERK, although SP3 knockdown did slightly reduce ERK levels, suggesting positive regulation of ERK by SP3 in these cells. However, knockdown of SP3 itself was clearly substantially greater.

We next quantified the effect of the double knockdowns on endogenous TERT expression by RT-QPCR in A2780 cells. As expected from its well known role in endogenous TERT regulation, MYC knockdown alone substantially reduced TERT mRNA levels to 40.6% of the non-specific control transfectants. Interestingly, as predicted by the model, AR co-suppression substantially restored TERT expression relative to MYC knockdown alone. SP3 knockdown had a very mild effect, as predicted, restoring TERT expression to 57% of control levels. Additionally, TP53 knockdown was found to have slightly stronger effect than SP3, restoring levels to 62.9% of control, although this was not predicted by the model. Neither JUN nor MXD1 knockdown had a significant effect in our hands. Therefore, our model successfully predicted that MYC inhibition produces TERT suppression which is largely but not completely resistant to targeted attempts at reversal. Additionally, the model identified the only factor, AR, which was able to reverse MYC dependent TERT suppression by RNAi in these experiments. Therefore, the model can be used to provide previously unknown mechanistic insights into TERT regulation.

Modelling Ets-factor gain of function at the TERT gene

During preparation of this manuscript, several papers emerged reporting identification of somatic and germline mutations in the TERT promoter in a range of tumour types and cell lines including melanoma, glioma, hepatocellular carcinoma and others [23–25]. Among those reported to occur at high frequency, particularly in melanoma, are the C228T and C250T mutations which occur close to the transcriptional start site and introduce gain of function binding sites for Ets family transcription factors resulting in increased TERT promoter activity. Since we have not considered Ets-factors in our model, we were interested to determine the effect of adding an Ets-factor into the basal model.

We therefore acquired a promoter reporter and expression vector for ETS2 and tested these as before for the effect of ETS2 overexpression in our promoter panel and for the effect of our expression vector panel on the ETS2 promoter (figure 6A and 6B). The ETS2 promoter was suppressed by overexpression of TP53 and activated by MYCN and STAT3 in addition to its own overexpression (figure 6B). Overexpressed ETS2 up-regulated its own promoter and that of AR, and repressed those of JUN, HIF1A, FOS, SP3, STAT3 and N2F2 with fold change >1.5 and p<0.01 which satisfied our cut-offs described earlier. We did not detect a significant effect of ETS2 overexpression alone on our TERT promoter construct. However, we were also interested in whether there may be interplay between MYC and ETS2. We therefore co-transfected cells with MYC siRNA and ETS2 expression vectors (figure 6C). MYC siRNA strongly repressed promoter activity to 18% of control levels in vector co-transfected cells. However, in ETS2 co-transfected cells, promoter activity was only reduced to 30% of control levels. Hence, ETS2 did appear to stimulate the TERT promoter under conditions of MYC inhibition. It is possible that c-Myc protein at the TERT promoter participates in complexes that occlude Ets2 binding. Since ETS2 expression did stimulate the promoter in these conditions and our objective is to model a gain of function, we included ETS2 as an activator of TERT. These interactions were incorporated into the model to generate a new 16 node network. The included ETS2 subnetwork is shown in figure 6D.

We re-performed the analysis of the effect of constitutive activation or suppression of each node under the new basal 16-node ETS2-added condition (figure 6D, black bars). In this analysis, TERT repression was still achieved by MYC inhibition. However, this was the only modification able to substantially ablate TERT on-states in the new model. Loss of MYCN or activation of TP53 were the only other modifications to have any effect, though these effects were very mild. In contrast to the results in figure 5C, when we repeated the analysis under the condition of MYC inhibition in the ETS2-added model (figure 6D, grey bars), TERT repression was found to be fragile and easily reversible, rather than robust. This is in line with our finding that ETS2 overexpression stimulated the TERT promoter only when MYC was knocked down, although the model did not report that ETS2 itself recovered TERT expression. Rather, not only loss of AR, but also of repressors TP53 and E2F1 or activation of HIF1A caused complete or substantial recovery. Therefore, ETS2 gain of function may enhance TERT expression stability. Surprisingly, recovery was also observed when activators FOS and STAT3 were suppressed, or when repressor NR2F2 was activated. These effects presumably result from reprogramming of the network balance through introduction of ETS2 interactions at a variety of nodes (figure 6D).

Topological control of TERT on state multiplicity in the model

Our results so far suggest that the model can predict at least some experimentally verifiable aspects of TERT regulation. To explore in more detail the role of the transcriptional neighbour- hood topology in TERT regulation, we focused on the roles of the activation and repression modules (AM/RM) in the model by specifically targeting their connections.

One problem in analysis of the role of topology in complex networks is that reagents are unavailable to efficiently target multiple individual network interactions in a selective manner. However, panels, or ensembles, of random Boolean networks have previously been used effectively as a statistical approach to probe how topological features or interventions regulate system dynamics [82,83]. We have adopted this approach to study to study the roles of AM/RM. We are unaware of any BN study that has previously investigated roles of these feedforward systems in network dynamics. AM/RM edges were deleted from the model in a series of random attacks of increasing severity targeting either the AM alone or both AM/RM. Probability for each edge to be deleted was varied between 0.1 and 0.7 in increments of 0.05. This produced a series of re-wired networks based on the original model but with differing contributions from AM/RM. All edges connecting directly to TERT were left unmodified in each case.

We extracted AM/RM from each resulting modified network and quantified the number of edges remaining in the re-wired subnetworks in each case as a measure of overall prevalence of either system. In the case of the “wild-type” basal model, AM is slightly dominant with an edge ratio AM/RM of 1.15 although, as noted, the AM also has greater betweenness. We also calculated the statespace of each model variant and quantified the number of TERT stable on-states. The relation between these metrics is shown in figure 7A. It is clear that AM dominance associates with emergence of multiple on-states. The median edge ratios for 0, 1, 2, or 3 more on-states were 0.78, 1.04, 1.14 and 1.36, respectively. These differences were all highly significant (p<0.01, by Wilcoxon Rank Sum).

Lastly we investigated the roles of AM/RM in the more general case of semi-random networks. We generated 300 (15 node)
Figure 6. Simulation of Ets family transcription factor gain of function at the TERT promoter. (A), overexpression of ETS2 against the promoter panel. A2780 cells were transfected with the luciferase reporters shown. Each reporter was co-transfected alongside vector control or pCMV-ETS2. 48 h post-transfection, promoter activities were analysed by luciferase assay. Mean ± SEM of three experiments (ns: not significant; *: p<0.05; **: p<0.01). (B), regulation of the ETS2 promoter by the transcription factor panel. A2780 cells were co-transfected with ETS2-luciferase reporter alongside vector control or expression vectors shown. 48 h post-transfection, promoter activities were analysed by luciferase assay. Mean ± SEM of three experiments (ns: not significant; *: p<0.05; **: p<0.01). (C), effect of ETS2 expression on the TERT promoter under MYC inhibition. A2780 cells were co-transfected with the TERT-luciferase reporter and with pCMV control or pCMV-ETS2 with non-targeting or MYC-specific siRNA. 48 h post-transfection, promoter activities were analysed by luciferase assay. Mean ± SEM of three experiments (D), interactions in the ETS2 subnetwork added into the model with cutoffs FC>1.5, p<0.01 from the transfection data. The subnetwork was visualised in Cytoscape [105]. Arrows indicate activation, T-shape indicates repression. (E), Effect of single- and double-node targeting on TERT on-states in the ETS2 modified model. Rule-sets for each node were modified in turn individually (black bars) to simulate constitutive repression or activation. For each rule-set change, statespace was derived and the proportion of system states evolving to attractor states with TERT stably on was quantified. The analysis was repeated in the background of the MYC suppressed rule-set (grey bars). doi:10.1371/journal.pcbi.1003448.g006
networks of increasing edge seeding density (100 networks each with each possible edge included with probability 0.1, 0.15, or 0.2). The probability for any included edge to be activating or inhibitory was 0.5. We imposed the constraint that the 15th node was downstream of all other nodes as in the case of TERT on-state multiplicity. Topology of the model was altered by a series of 600 random attacks deleting activation and repression module interactions with increasing probability. Direct interactions with TERT were left unaltered in all attacks. The remaining sub-networks were extracted from each model variant as described in materials and methods and the number of edges in each were counted to determine the edge ratio AM/RM. The statespace of each model was calculated and the number of stable on states present for the TERT node was quantified and plotted against the calculated AM/RM edge ratio for each variant network. Significance of edge ratio population differences was tested in Matlab by Wilcoxon rank-sum test (**: p<0.01). (B), influence of AM dominance in random networks. A series of 300 (15 node) networks was generated with semi-random edge seeding and increasing edge density. All networks were constrained to have one regulated node which was connected downstream of all others. The number of activators and repressors of the node was allowed to vary randomly. Statespace and AM/RM edge ratios were calculated for each network and compared as in (A), calculating number of stable on-states for the fully connected node. Significance of edge ratio population differences was tested in Matlab by Wilcoxon rank-sum test (**: p<0.01).

Discussion

Previous studies have taken mathematical modelling approaches to investigate cellular senescence responses to telomere homeostasis and oxidative stress [84,85]. The current report is the first investigation of TERT transcription using modelling to probe its regulation at the systems level. Telomerase regulation has been widely studied at the level of identification of individual transcription factor regulators of TERT. However, like most complex biochemical systems, a clear view of their co-operative activities is lacking. Multiple pathways regulate TERT and some such as MYC may be more critical than others globally or context-independently, whereas some may play essential context-dependent roles [2,4,6,23,29,79-81,86]. From the point of view of therapeutics based around TERT transcription, including signalling inhibitors, better understanding of system behaviour would be advantageous. Clearly, endogenous gene transcription involves multiple complex events which are mechanical in nature as well as dynamical, and which are not easily captured in simplistic models. These include chromatin effects, complex formations, promoter melting, abortive initiation, promoter escape and elongation [87]. Classical Boolean networks offer only a qualitative modelling solution and suffer from several major disadvantages. For example, it is necessary to define a static network structure by literature-curation or, as in the model reported here, by experiment. This “snap-shot” does not take account of the possibility that network structure also changes in a dynamic way. This is potentially an extremely interesting route for refinement in future models by combining time-dependent network inference with advanced dynamic features such as memory [88,89]. However, as an entry point to systems level study of TERT, even simple models such as that presented here could be useful. In this study, as a direct consequence of application of the model, a previously unknown role for AR in stabilising MYC dependent TERT repression was revealed. Furthermore, our model predicted the effects of several signal transduction inhibitors, including the powerful TERT repressor effect of SU6656 in cells. The model also suggests that Ets-factors may functionally co-operate with MYC expression to enhance TERT transcription stability.

It is noteworthy that the experiments required in order to show phenotypic effects of telomerase inhibition are usually very long term and their outcome is rarely certain at the start of an experiment. In our previous work, BIO was effective in suppressing TERT expression but not in long term treatments [4]. Our model suggests this may be the result of susceptibility to noise, which is in line with our previous observations. Therefore, the possibility that small reporter screens as performed for SU6656, coupled with application of models, could be used as a filter to increase confidence in the potential for the long term effects of a compound is intriguing. Modelling might also assist in clarifying which network interventions are fragile or reversible in targeting telomerase, potentially allowing better approaches to be designed. To develop our network model we applied a screening approach. To our knowledge this is the first report of a BN model of transcription generated in this way. We initially tested a range of literature-curated models which did not perform as well as the data derived model. This may be because the interactions in these networks are curated from a range of experiments performed in different contexts. The overlap between our screening results and the literature was highly significant, although some interactions were found to be non-concordant. These may reflect different effects in different cell systems or the use of different constructs. Regulation of transcriptional network interactions is likely to differ substantially among different cell lines. In contrast, excellent models of core signal transduction pathways can be literature curated since specific cell models and relatively standard reagents are often widely employed in studying their kinetics. That the model was able to make several de novo predictions that could be verified in cells suggests that this might be a useful general approach to develop BN models of transcriptional systems.
Because of the nature of the threshold rule, which is a balance between activating and repressive interactions, in model development we felt that a network having an evenly balanced number of TERT activators and repressors would likely give better results. In preference to reducing the model size to achieve that balance, we therefore retained \(\text{STAT3}\) as an activator throughout model selection. We previously found that \(\text{STAT3}\) protein binds the core TERT promoter and we repeated this analysis here. As discussed in supplemental file Text S1, a model without \(\text{STAT3}\) still had most of the behaviour of the complete model.

One limitation of the Boolean framework analysis is in modelling constitutive repression or over-expression by mutating the rules for a node to be “always off” or “always on”. This does not reflect the biological setting, where a relative decrease or increase in concentrations would more likely be observed. One possibility to refine the existing model would be to make use of the thresholds and weights for nodes and interactions to more accurately reflect this scenario. However, our approach did capture behaviours that we demonstrated experimentally. Thus, the current qualitative model does seem able to reproduce some essential aspects of endogenous TERT regulation.

Although \(\text{MYC}\) has been well studied in the context of TERT regulation in cancer cells, the stability of TERT suppression by \(\text{MYC}\) inhibition is not well understood. Our modelling results suggested this state is substantially but not completely resistant to reversal. Our structural analysis of the model suggests that \(\text{MYC}\) may be important not only in its direct effects at the TERT promoter but also by its “brokerage” role throughout the transcription network.

We examined the role of network topology in promoting stable TERT expression in the modelling context. We identified feedforward systems in the network whose structure suggests coordinated functional regulation of transcriptional interactions. The activation module sub-system was topologically dominant over the repression module sub-system in the “wild-type” model. Targeted edge attacks showed the activation module contributes to TERT on state multiplicity in the model. If this model prediction is similar to the endogenous scenario, then network organisation would indeed play a significant role in TERT transcriptional stability.

Clearly, these results are an untested extrapolation, but interesting nevertheless since experimental confirmation that individual interactions give rise to emergent behaviour would require reagents capable of ablating individual endogenous interactions. No current reagents are able to efficiently produce this effect since reagents such as siRNA can only target gene products and not their interactions. On the other hand, BN panels have previously been used to infer relations between topology and network dynamics [82,83]. To our knowledge, the roles of the feedforward systems we describe have not been directly tested before and our results suggest they may play important general roles in network dynamics. In our model of TERT regulation, the activation module was more co-operative than the repression module by the measures of betweenness centrality and flow centrality (table 6).

In the endogenous context, TERT expression clearly is essential in most cancer cells and it is likely that a variety of backup mechanisms exist which are capable of subverting TERT suppression as we previously observed [4]. A highly co-operative AM could promote stable expression dynamics of TERT by providing for long signal delays and substantial redundancy among activators, thereby allowing the transcriptional neighbourhood to adopt divergent states while maintaining expression of TERT and promoting stability.

In respect of the therapeutic scenario, multiple approaches have been suggested to target telomerase including transcriptional suppression through intervention with its regulatory pathways [86,90,91]. If this is indeed a viable approach, and if the true regulatory network does indeed share the features we describe, then it seems one should aim to hit the pathways as broadly as is possible while retaining acceptable toxicity. Suppression of TERT through \(\text{MYC}\) knockdown was substantially robust but, as we have shown, also fully reversible under appropriate conditions. Hence, it is probably insufficient to consider targeting TERT transcription in terms of individual factors since targeting any single transcription factor may not guarantee repression in the long term. Advanced therapeutic approaches based on TERT suppression may need to explicitly target the systems complexity of telomerase regulation by, for example, polypharmacology strategies. In general, broad inhibition of the activation module or broad activation of the repression module may be preferable.

SU6656 appears to engage the latter mechanism and to cause both profound TERT suppression in cells and a powerful cell cycle arrest. Given the functions of several repression module factors in cell cycle arrest, DNA damage and senescence, it may be that its co-ordinate activation selectively promotes cell cycle arrest and/or premature senescence rather than a classic telomerase inhibitor phenotype. Similar results might also be obtained with other compounds which engage senescence pathways [92]. If senescence and immortalisation are considered to be opposing processes, regulated by opposing mechanisms, then just as the activation module appears evolved for efficient telomerase expression in immortal cancer cells, it may be that the opposing repression module is evolved for efficiency of arrest. Highly co-ordinated regulation of telomerase expression appears hard-wired in both processes.

**Materials and Methods**

**Cell lines, plasmids, siRNA and inhibitors**

The cells used were A2780 ovarian adenocarcinoma cells. Reporter pGL3-TERT contains the TERT promoter region – 585/–9, relative to the translational start site. All other reporters were obtained from Switchgear Genomics (Menlo Park, CA). Constructs were \(\text{AR}(\text{S7149912}), \text{JUN}(\text{S721598}), \text{TP53}(\text{S721662}), \text{SPI}(\text{S722903}), \text{E2F1}(\text{S719961}), \text{MYCN}(\text{S719321}), \text{MXD1}(\text{S708003}), \text{p65}(\text{S720207}), \text{MYC}(\text{S719565}), \text{HIF1A}(\text{S721637}), \text{FOS}(\text{S721638}), \text{SP3}(\text{S711215}), \text{STAT3}(\text{S706087}), \text{NR2F2}(\text{S708324}), \text{ETS2}(\text{S719772}), \text{pCMV} \text{expression vectors for MYCN, FOS, NR2F2, STAT3 and MXD1 were obtained from Cambridge Bioscience (Cambridge, UK). Expression vector for TP53 was obtained from Clontech Laboratories (Mountain View, CA). We previously reported the expression vectors for SPI, SP3 and HIF1A [29,93,94]. Expression vectors for E2F1 and RELA [95,96] were kind gifts from Professor Kevin Ryan (Beatson Institute for Cancer Research, Glasgow, UK). Avian \(\text{MYC}\) and v-Jun vectors [97,98] were kind gifts from Professor David Gillespie (Beatson Institute for Cancer Research, Glasgow, UK). \(\text{AR}\) knockdown was substantially robust but, as we have shown, also fully reversible under appropriate conditions. Hence, it is probably insufficient to consider targeting TERT transcription in terms of individual factors since targeting any single transcription factor may not guarantee repression in the long term. Advanced therapeutic approaches based on TERT suppression may need to explicitly target the systems complexity of telomerase regulation by, for example, polypharmacology strategies. In general, broad inhibition of the activation module or broad activation of the repression module may be preferable.

**Transfections and luciferase assay**

All transfections were performed in triplicate (3 technical replicates) using superfect reagent according to the manufacturer’s
instructions using a 2.5:1 ratio reagent:DNA (Qiagen, Crawley, UK). 250 ng reporter plasmid and expression vector per well were transfected in 96-well luminometer plates (ThermoFisher Scientific UK, Leicestershire, UK). 48 h post-transfection, luciferase activities were determined using luciferase assay reagents according to the manufacturer’s instructions (Promega Ltd, Madison, WI). All experiments were repeated at least 3 times (3 biological replicates).

### Western blotting

Protein extracts (1 technical replicate per experiment) were prepared in passive lysis buffer (Promega Ltd, Madison, WI). Protein concentrations were estimated at ODS95 using the BioRad protein assay (BioRad Laboratories Ltd, Hemel Hempstead, UK). 20 μg protein were separated by SDS-PAGE, blotted onto PVDF filter (Millipore, Watford, UK) and blocked overnight in PBS-T containing 5% non-fat dried milk. Antibodies used were Androgen Receptor (ab9474) and TP53 (ab7757), obtained from Abcam (Cambridge, UK), MXD1 (sc-222), SP3 (sc-644), MYC (sc-764), obtained from Santa Cruz Biotechnology Inc (Heidelberg, Germany) and JUN (9165), obtained from New England Biolabs UK (Hitchin, UK). Primary antibodies were detected with HRP-conjugated secondary. HRP was detected using ECL HRP detection reagents (Amersham Pharmacia, Buckinghamshire, UK). All experiments were performed at least twice (2 biological replicates).

### Quantitative RT PCR

Q-PCR was performed in triplicate (3 technical replicates) using Genetic Research Instrumentation (Essex, UK) Opticon monitor equipment and software. Sybr green was used as fluorophore. The primers used were: RPS15, 5′-TTCCGCAATTTACCTACC and 5′-CCGGCCGCGCATGCTTACC; TERT 5′-CTGCTGCGCAGCTTGGAA and 5′-GGACACCTGGCGGAAGG. Optical read temperatures were optimised to exclude primer dimers. All treatments were repeated three times (3 biological replicates) and Q-PCR was performed twice for each assay.

### Boolean networks modelling framework and topology analysis

BN comprise a set of N nodes and the set of their activating or inhibitory interactions. Each node takes one of 2 states: 1 (on) or 0 (off). Hence, there are 2^N possible combinations of node states. The system is initialised at time 0 under some combination of on/off states and the dynamics of each node during simulation of further time steps are determined entirely by a rule-table specifying the next state of each node given each possible state combination of its regulators [100]. In the classical BN all nodes are synchronously updated. We use the “threshold” rule,

\[ \sigma_j(t+1) = \begin{cases} k_j \sum_{i=1}^{k_j} a_{ij} \sigma_i(t) + \phi \geq 0 \\ 1, \text{otherwise} \end{cases} \]

Here, \( \sigma_j(t+1) \) is the next state of node \( j \), \( \sigma_j(t) \) defines the current state of the \( t \)-th node \( j \), \( a_{ij} \) is the weight of the interaction from node \( i \) to node \( j \) (1 if the interaction is activating, -1 if repressive), and \( k_j \) is the input-degree of the regulated node \( j \). The threshold variable \( \phi \) is zero in all rule-sets in this study. Hence, a node will be turned off at time \( t+1 \) only in the case that more of its repressors than activators are on at time \( t \). Topology of the basal model was defined using data from the transfection screen. Cut-offs for assignment of an interaction were fold-change in reporter activity, FC>1.5 (up- or down-regulation), and p-value (ANOVA)<0.01. Each \( a_{ij} = 1 \) if promoter \( j \) was activated by transcription factor \( i \), or \( a_{ij} = -1 \) in the case of repression.

In at most 2^N+1 time steps during simulation of classical BN, a previous state must be revisited as must all subsequent steps. The system is then locked in an attractor. Commonly, far fewer steps are required. Therefore, BN rapidly converge either to point attractors or limit cycles [101], though we use the informal terminology steady states or oscillations here. Characterisation of these is a principal method for exploring BN models. In this paper we have performed statespace analysis of all models [73,102]. Basins of attraction were identified as the weak components of statespace and the corresponding attractors as their input degree core. The states of the TERT node were analysed in each attractor. Limit cycles were considered to have stable TERT activity if the node was on in all sub-states. During the study, we developed a Windows console application which implements the statespace analysis and other functions on Pajek net files (http://sourceforge.net/projects/statespaceminer).

### Literature search

We apologise to any authors if we have overlooked your study which meets our inclusion/exclusion criteria in the preparation of table 4. Please contact us and the relevant references will be included in any future publications concerning this model or refined versions. To determine the literature in this area, we analysed references within the MetaCore database [4], the Transcriptional Regulatory Element Database [106], in addition to performing PubMed searches. In our PubMed searches, we included a range of search terms to find regulator → target interactions including “regulator” AND “target promoter”, “regulator” AND “target mRNA”, “regulator bind” AND
Inclusion criteria on the searches:

1. Demonstration in human cells and any or all of:
   - Selective modulation of regulator causes regulation of target mRNA.
   - Selective modulation of regulator causes regulation of target promoter activity.
   - Regulator binds to target promoter in vitro or in vivo with effect on target mRNA or protein.

Exclusion criteria:

1. Only found in non-human system.
2. Regulator binds target promoter but without validated expression change.
3. Genomic-scale studies without specific validation of effect on target.

References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646–674.
2. Daniel M, Peek GW, Tollefson TO (2012) Regulation of the human catalytic subunit of telomerase (hTERT). Gene 498: 135–146.
3. Tang B, Hsu PY, Huang TH, Jin VX (2012) Cancer omics: From regulatory networks to clinical outcomes. Cancer Lett 304(2):277–283.
4. Bilsland AE, Hoare S, Stevenson K, Plumb J, Gomez-Roman N, et al. (2009) Dynamic telomerase gene suppression via network effects of GSK3 inhibition. PLoS One 4: e6459.
5. Lafferty-Whyte K, Cairney CJ, Will MB, Serakinci N, Daidone MG, et al. (2004) Tyrosine kinase inhibition. Cell Signal 23: 407–416.
6. Yao Y, Bellon M, Shelton SN, Nicot C (2012) Tumor suppressors p53, p63TAalpha, p63TAy, p73alpha, and p73beta use distinct pathways to repress telomerase expression. J Biol Chem 287: 20737–20747.
7. Won J, Yim J, Kim TK (2002) Opposing regulatory roles of E2F in human telomerase reverse transcriptase (hTERT) gene expression in human tumor and normal somatic cells. J Biol Chem 277: 1949–1954.
8. Saratur AM, Degerman S, Ljungberg B, Andersson E, Oji Y, et al. (2010) Wilms’ tumour 1 can suppress hTERT gene expression and telomerase activity in clear cell renal cell carcinoma via multiple pathways. Br J Cancer 103: 1255–1262.
9. Nelson DE, See V, Nelson G, White MR (2004) Oscillations in transcription factor dynamics: a new way to control gene expression. Biochem Soc Trans 32: 1090–1092.
10. Bilsland AE, Revie J, Keith W (2013) MicroRNA and Senescence: The Senectome, Integration and Distributed Control. Crit Rev Oncog 18: 373–390.
11. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, et al. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 34: 267–273.
12. Weibel G, Chomel F, Primig M (2005) goCluster integrates statistical analysis and functional interpretation of microarray expression data. Bioinformatics 21: 3575–3577.
13. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545–15550.
14. Geard N, Willadsen K (2009) Dynamical approaches to modeling developmental gene regulatory networks. Birth Defects Res C Embryo Today. pp. 131–142.
15. von Kriegsheim A, Baiocchi D, Bittwistle M, Sumpton D, Bienvenut W, et al. (2009) Cell fate decisions are specified by the dynamic ERK interaction. Nat Cell Biol 11: 1458–1464.
16. Golovin A, Faratian D, Laughon SF, Bown J, Goryain O, et al. (2011) Compensatory effects in the PI3K/PTEN/akt signaling network following receptor tyrosine kinase inhibition. Cell Signal 23: 407–416.
17. Benson N, Matsuura T, Smirnov S, Demin O, Jones HM, et al. (2013) Systems pharmacology of the nerve growth factor pathway: use of a systems biology model for the identification of key drug targets using sensitivity analysis and the integration of physiology and pharmacology. Interface Focus 3: 20130071.
18. Greffet J, Kim S, Kauffman S (2006) An analysis of the class of gene regulatory functions implied by a biochemical model. Biosystems 84: 81–90.
19. Kauffman S, Peterson C, Samelson L, Troein C (2003) Random Boolean network models and the yeast transcriptional network. Proc Natl Acad Sci U S A 100: 14796–14799.
20. Akman OE, Watterson S, Parton A, Binnis N, Miller AJ, et al. (2012) Digital clocks: simple biological models can quantitatively describe circadian systems. J R Soc Interface 9(74): 2865–2872.
21. Lee WB, Huang JY (2009) Robustness and topology of the yeast cell cycle Boolean network. FEBS Lett 583: 927–932.
22. Schlagter R, Smich K, Avalos Vazcarra I, Schuurich P, Sauter T, et al. (2009) ON/OFF and beyond a novel model of apoptosis. PLoS Comput Biol 5: e1000595.
23. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, et al. (2013) TERT promoter mutations in familial and sporadic melanoma. Science 339: 959–961.
24. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, et al. (2013) Highly recurrent TERT promoter mutations in human melanoma. Science 339: 957–959.
25. Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, et al. (2013) TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. Proc Natl Acad Sci U S A 110: 6021–6026.
26. Bilsland AE, Fletcher-Monaghan A, Keith WN (2005) Properties of a telomerase-specific Cre/Lox switch for transcriptionally targeted cancer gene therapy. Neoplasia 7: 1020–1029.
27. Takamura M, Kyo S, Inoue M, Wright WE, Shay JW (2005) Function of AP-1 transcription in the telomerase reverse transcriptase gene (TERT) in human and mouse cells. Mol Cell Biol 25: 8037–8043.
28. Konnokova L, Simeone MC, Kruger MM, Kotek C, Cochran BH (2005) Signal transducer and activator of transcription 3 (STAT3) regulates human telomerase reverse transcriptase (hTERT) expression in human cancer and primary cells. Cancer Res 65: 6516–6520.
29. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN (2006) Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. Oncogene 25: 61–69.
30. Anest V, Cogswell PC, Baldwin AS Jr. (2004) I kappa B alpha kinase alpha and p65/RelA contribute to optimal epidermal growth factor-induced c-fos gene expression independent of Ikappa B alpha degradation. J Biol Chem 279: 31183–31189.
31. Angel P, Hansel K, Smral T, Karin M (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55: 837–845.
32. Chen I, Inaci N, Gherardi S, Gamble LD, Wood KM, et al. (2010) p53 is a direct transcriptional target of MYCN in neuroblastoma. Cancer Res 70: 5177–5180.
33. Chen LI, Nishinaka T, Kwan K, Kitabayashi I, Yokoyama K, et al. (1994) The TERT promoter is a target for integration of retrovirus in human cancer and TERT promoter mutations in familial and sporadic melanoma. Science 339: 959–961.
34. Nelson DE, See V, Nelson G, White MR (2004) Oscillations in transcription factor dynamics: a new way to control gene expression. Biochem Soc Trans 32: 1090–1092.
35. Takamura M, Kyo S, Inoue M, Wright WE, Shay JW (2005) Function of AP-1 transcription in the telomerase reverse transcriptase gene (TERT) in human and mouse cells. Mol Cell Biol 25: 8037–8043.
36. Konnokova L, Simeone MC, Kruger MM, Kotek C, Cochran BH (2005) Signal transducer and activator of transcription 3 (STAT3) regulates human telomerase reverse transcriptase (hTERT) expression in human cancer and primary cells. Cancer Res 65: 6516–6520.
37. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN (2006) Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. Oncogene 25: 61–69.
38. Angel P, Hansel K, Smral T, Karin M (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55: 837–845.
39. Chen I, Inaci N, Gherardi S, Gamble LD, Wood KM, et al. (2010) p53 is a direct transcriptional target of MYCN in neuroblastoma. Cancer Res 70: 5177–5180.
40. Chen LI, Nishinaka T, Kwan K, Kitabayashi I, Yokoyama K, et al. (1994) The TERT promoter is a target for integration of retrovirus in human cancer and TERT promoter mutations in familial and sporadic melanoma. Science 339: 959–961.
41. Nelson DE, See V, Nelson G, White MR (2004) Oscillations in transcription factor dynamics: a new way to control gene expression. Biochem Soc Trans 32: 1090–1092.
59. Wooten-Blanks LG, Song P, Senkal CE, Ogretmen B (2007) Mechanisms of human orphan receptor E2F-1. In: Enzyme-linked immunoassay. Methods Enzymol 393: 189–190.

60. Liao H, Zeng Q, Wang Q, Li X, Hou L, Zhang B (2004) The evidences of human orphan receptor E2F-1. In: Enzyme-linked immunoassay. Methods Enzymol 393: 189–190.

61. Yuan H, Pan Y, Young CY (2003) Overexpression of the human TERT and TERT promoter during differentiation of HL60 cells. J Biol Chem 278: 10653–10657.

62. Yang E, Lerner L, Beser D, Darnell JE, Jr. (2003) Independent and cooperative activation of chromosomal c-fos promoter by STAT3. J Biol Chem 278: 15794–15799.

63. Zeller KL, Zhao X, Lee CW, Chiup KP, Yao F, et al. (2006) Global mapping of c-fos binding sites and transcription factors in human B cells. Proc Natl Acad Sci U S A 103: 17834–17839.

64. Chen X, Qin J, Cheng CM, Tsai MJ, Tsai SY (2012) COUP-TFI is a major regulator of cell cycle and Notch signaling pathways. Endocrinol 26: 1247–1257.

65. Cho K, Shin HW, Kim YJ, Cho CH, Chun YS, et al. (2013) Med1 mediates hypoxia-induced doxorubicin resistance in colon cancer cells by inhibiting mitochondrial function. Free Radic Biol Med 60: 201–210.

66. Chen L, Lee H, Rho HMK, Faivre S, Yu J, Jones KL, et al. (2011) Telomerase activation in attherosclerosis and induction of telomerase reverse transcriptase expression by inflammatory stimuli in macrophages. Arterioscler Thromb Vasc Biol 31: 245–252.

67. Ho JS, Ma W, Mao DY, Benchimol S (2005) p53-dependent transcriptional repression of c-myf is required for G1 cell cycle arrest. Mol Cell Biol 25: 7425–7431.

68. Hossain S, Takatori A, Nakamura Y, Suenaga Y, Kamiyo T, et al. (2012) NLRK enhances EGF-mediated MYC/ING in neuroblastoma and accelerates tumor growth in vivo. Cancer Res 72: 7331–7336.

69. Hsu SP, Lee WS (2011) Progesterone receptor activation of extranuclear signaling pathways in regulating p53 expression in vascular endothelial cells. Mol Endocrinol 25: 421–432.

70. Jiang K, Hein N, Eckert K, Laucher-Fridal J, Lusher B (2008) Regulation of the MADI promoter by G-CSF. Nucleic Acids Res 36: 1517–1531.

71. Johnson DG, Ohtani K, Nevins JR (1994) Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 8: 1514–1523.

72. Yuan H, Pan Y, Young CY (2004) Overexpression of c-JUN induced by quercetin and resveratrol inhibits expression and function of the androgen receptor in human prostate cancer cells. Cancer Lett 213: 153–163.

73. Willadens K, Wiley J (2007) Robustness and state-space structure of Boolean gene regulatory models. J Theor Biol 249: 749–765.

74. Cho J, Rameshwar P, Sadoshima J (2009) Distinct roles of glycogen synthase kinase (GSK)-3alpha and GSK-3beta in mediating cardiomyocyte differentiation in murine bone marrow-derived mesenchymal stem cells. J Biol Chem 284: 36647–36650.

75. Mangan S, Alon U (2003) Structure and function of the feed-forward loop motif. Proc Natl Acad Sci U S A 100: 11980–11985.

76. White DR, Bregatti SP (1994) Betweenness centrality measures for directed graphs. Social Networks 16: 335–346.

77. Freeman LC, Borgatti SP, White DR (1991) Centrality in valued graphs: a measure of betweenness based on network flow. Social Networks 13: 141–154.

78. Huang S, Ernberg I, Kauffman S (2009) Cancer attractors: a systems view of gene regulatory networks. PLoS Comput Biol 13: e1002669.

79. Mangan S, Alon U (2003) Structure and function of the feed-forward loop motif. Proc Natl Acad Sci U S A 100: 11980–11985.

80. Schonthal A, Buscher M, Angel P, Rahmsdorf HJ, Ponta H, et al. (1989) The Fos and Jun/AP-1 proteins are involved in the downregulation of Fos transcription. Oncogene 4: 629–636.

81. Frick LS, Tai F, Smith SM, Dillon PA, Broder GM, et al. (1997) Autoregulation of the human Myc oncogene is disrupted in amplifed but not single-copy neuroblastoma cell lines. Oncogene 15: 1937–1946.

82. Huang H, Pan Y, Young CY (2004) Overexpression of c-JUN induced by quercetin and resveratrol inhibits expression and function of the androgen receptor in human prostate cancer cells. Cancer Lett 213: 153–163.

83. Torres-Sosa C, Huang S, Alon U (2012) Criticality is an emergent property of gene networks that exhibit evolvability. PLoS Comput Biol 8: e1002660.

84. Arkus N (2005) A mathematical model of cellular apoptosis and senescence through the dynamics of telomere loss. J Theor Biol 235: 13–32.

85. Cho J, Rameshwar P, White DR (1991) Centrality in valued graphs: a measure of betweenness based on network flow. Social Networks 13: 141–154.

86. Huang S, Ernberg I, Kauffman S (2009) Cancer attractors: a systems view of tumors from a gene network dynamics and developmental perspective. Semin Cell Dev Biol 20: 869–876.

87. Lafferty-Whyte K, Bilsland AE (2008) Targeting telomerase: Therapeutic options for cancer treatment. Telomeres and Telomerase in Ageing, Disease, and Cancer: Springer-Verlag Berlin. pp. 247–295.

88. Graudenzi A, Serra R, Villani M, Damiani C, Colacci A, et al. (2011) Mad1 mediates hypoxia-induced doxorubicin resistance in colon cancer cells by inhibiting mitochondrial function. Free Radic Biol Med 60: 201–210.

89. Matsuura Y, Lee H, Rho HMK, Faivre S, Yu J, Jones KL, et al. (2011) Telomerase activation in attherosclerosis and induction of telomerase reverse transcriptase expression by inflammatory stimuli in macrophages. Arterioscler Thromb Vasc Biol 31: 245–252.

90. Agrawal A, Dang S, Gabrani R (2012) Recent patents on anti-telomerase agents. Curr Drug Targets 13: 1257–1270.

91. Keith WN, Bilsland AE (2008) Targeting telomerase: Therapeutic options for cancer treatment. Telomeres and Telomerase in Ageing, Disease, and Cancer: Springer-Verlag Berlin. pp. 247–295.

92. Shreevatsa K, Sreenivasan C, Pan T, Anantharaman V, Ganguly S, et al. (2011) Telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proc Natl Acad Sci U S A 108: 11980–11985.

93. White DR, Bregatti SP (1994) Betweenness centrality measures for directed graphs. Social Networks 16: 335–346.

94. Freeman LC, Borgatti SP, White DR (1991) Centrality in valued graphs: a measure of betweenness based on network flow. Social Networks 13: 141–154.

95. Mangan S, Alon U (2003) Structure and function of the feed-forward loop motif. Proc Natl Acad Sci U S A 100: 11980–11985.
92. Lafferty-Whyte K, Bihland A, Cairney CJ, Hanley L, Jamieson NB, et al. (2010) Scoring of senescence signalling in multiple human tumour gene expression datasets, identification of a correlation between senescence score and drug toxicity in the NCI60 panel and a pro-inflammatory signature correlating with survival advantage in peritoneal mesothelioma. BMC Genomics 11: 532.

93. Bihland AE, Stevenson K, Atkinson S, Kolch W, Keith WN (2006) Transcriptional repression of telomerase RNA gene expression by cJun-NH2-kinase and Sp1/Sp3. Cancer Res 66: 1363–1370.

94. Zhao J, Bihland A, Jackson K, Keith WN (2005) MDM2 negatively regulates the human telomerase RNA gene promoter. BMC Cancer 5: 6.

95. Bell LA, O’Prey J, Ryan KM (2006) DNA-binding independent cell death from a minimal proapoptotic region of E2F-1. Oncogene 25: 5606–5613.

96. Ryan KM, Ernst MK, Rice NR, Vousden KH (2000) Role of NF-kappaB in p53-mediated programmed cell death. Nature 404: 892–897.

97. Black EJ, Walker M, Clark W, MacLaren A, Gillespie DA (2002) Cell transformation by v-Jun deactivates ERK MAP kinase signalling. Oncogene 21: 6540–6548.

98. Crouch DH, Fisher F, La Rocca NA, Goding CR, Gillespie DA (2005) Viral mutations enhance the Max binding properties of the vMyc b-HLH-LZ domain. Nucleic Acids Res 33: 5235–5243.

99. Gnanapragasam VJ, Robson CN, Neal DE, Leung HY (2002) Regulation of FGF8 expression by the androgen receptor in human prostate cancer. Oncogene 21: 5069–5080.

100. Machado D, Costa RS, Ferreira EC, Tidor B, et al. (2011) Modeling formalisms in Systems Biology. AMB Express 1: 45.

101. Xiao Y (2009) A tutorial on analysis and simulation of boolean gene regulatory network models. Curr Genomics 10: 511–525.

102. Wouters A (1998) Genomic regulation modeled as a network with basins of attraction. Pac Symp Biocomput: 89–102.

103. Batagelj V, Mrvar A (1998) Pajek: a program for large network analysis. Connections 21: 47–58.

104. Borgatti SP, Everett MG, Freeman LC (2002) Ucinet for Windows: software for social network analysis. Harvard, MA: Analytic Technologies.

105. Kohl M, Wiese S, Warscheid B (2011) Cytoscape: software for visualization and analysis of biological networks. Methods Mol Biol 696: 291–303.

106. Jiang C, Xuan Z, Zhao F, Zhang MQ (2007) TRED: a transcriptional regulatory element database, new entries and other development. Nucleic Acids Res 35: D157–160.