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SNAIL driven by a feed forward loop motif promotes TGFβ induced epithelial to mesenchymal transition

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Abstract

Epithelial to Mesenchymal Transition (EMT) plays an important role in tissue regeneration, embryonic development, and cancer metastasis. Several signaling pathways are known to regulate EMT, among which the modulation of TGFβ (Transforming Growth Factor-β) induced EMT is crucial in several cancer types. Several mathematical models were built to explore the role of core regulatory circuit of ZEB/miR-200, SNAIL/miR-34 double negative feedback loops in modulating TGFβ induced EMT. Different emergent behavior including tristability, irreversible switching, existence of hybrid EMT states were inferred though these models. Some studies have explored the role of TGFβ receptor activation, SMADs nucleocytoplasmic shuttling and complex formation. Recent experiments have revealed that MDM2 along with SMAD complex regulates SNAIL expression driven EMT. Encouraged by this, in the present study we developed a mathematical model for p53/MDM2 dependent TGFβ induced EMT regulation. Inclusion of p53 brings in an additional mechanistic perspective in exploring the EM transition. The network formulated comprises a C1FFL moderating SNAIL expression involving MDM2 and SMAD complex, which functions as a noise filter and persistent detector. The C1FFL was also observed to operate as a coincidence detector driving the SNAIL dependent downstream signaling into phenotypic switching decision. Systems modelling and analysis of the devised network, displayed interesting dynamic behavior, systems response to various inputs stimulus, providing a better understanding of p53/MDM2 dependent TGF-β induced Epithelial to Mesenchymal Transition.

Introduction

The art of communication among cells performing various tasks in response to limitless signals around them is phenomenal. These signals play a crucial role in cell cycle control and regulation (Tyson and Novak 2014). Failure in this signal transduction to elicit appropriate biological response results in a wide array of diseases including cancer (Hanahan and Weinberg 2011; Sever and Brugge 2015). Evidence suggests that the process of carcinogenesis is a multistep process that occurs due to alterations in these signaling pathways (Hanahan and Weinberg 2000).

One of the widely observed characteristics of carcinomas is Epithelial-Mesenchymal Transition (EMT) (Thiery 2002; Thiery and Sleeman 2006; Thiery *et al* 2009; Scheel and Weinberg 2012; Nieto *et al* 2016; Lambert *et al* 2017; Moustakas and de Herreros 2017). EMT (figure 1) is a process where epithelial cell loses its cell polarity, adhesive properties and subsequently gains migratory, invasive phenotype known as mesenchymal cells (Kang and Massague 2004; Onder *et al* 2008; Lamouille *et al* 2014; Prislei *et al* 2015; Savagner 2015). Literature has illustrated the involvement of several important molecules, transcription factors and their associated pathways in cancer. Signaling pathways including EGF, EGFR, TKs, ERK,

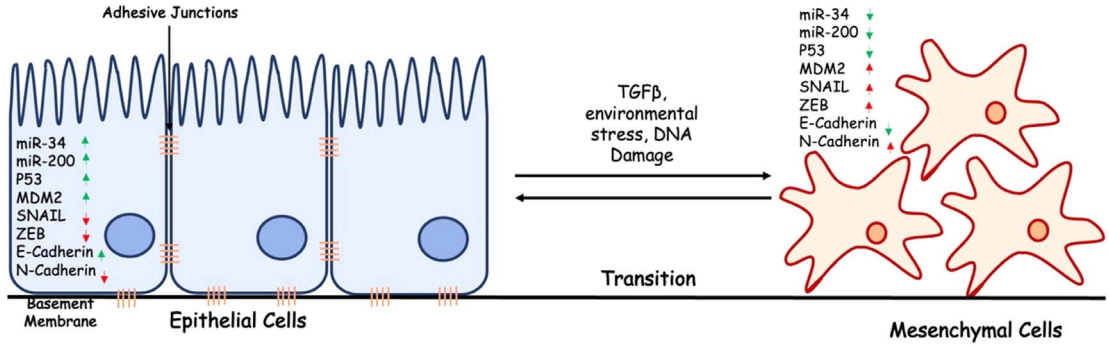

Figure 1. Physiology of Epithelial to Mesenchymal Transition. In the Prescence of the external stress (TGFβ, environmental stress) or internal dysregulation such as DNA Damage. The epithelial cell transforms into mesenchymal cell by losing its adhesive properties. This process alters the cell phenotype by upregulating the mesenchymal markers (Red Arrow) and downregulating the epithelial markers (Green Arrow) thus attaining phenotypic plasticity and migratory behaviour.

Ras, MAPK, PI3K, mTOR, CREB, Rho-Rock, Wnt, Notch delta, Shh, TGFβ are attributed to cause the mammalian cells to undergo EMT. (Kalluri and Weinberg 2009; De Craene and Berx 2013)

Among the above-mentioned pathways, the family of TGFβ is well known for its dual role (as suppressor and promoter of tumour) and plays a remarkable part in modulating EMT (Dickson *et al* 1987; Miyazono 2000; Derynck and Zhang 2003; Bierie and Moses 2006; Xu *et al* 2009; Ceppi *et al* 2010; Ikushima and Miyazono 2010; Gregory *et al* 2011; Bedi *et al* 2012; Bryant *et al* 2012; Reichl *et al* 2012; Xu *et al* 2012; Giannelli *et al* 2014). TGFβ (Transforming Growth Factor-β) and its family of cytokines are involved in countless physiological/biological processes responsible for regulating cellular differentiation, proliferation, migration, and apoptosis by activation of their serine/threonine transmembrane receptors (Attisano and Wrana 1996, 1998; Massague 1998; Attisano and Wrana 2000; Attisano and Wrana 2002; Zavadil and Bottinger 2005; Wu and Hill 2009). Activation of these receptors by phosphorylation results in a complex cascade of signaling in both SMAD dependent (Canonical) and SMAD-independent manner (Non-Canonical) (Heldin *et al* 1997; Attisano and Wrana 1998; Attisano and Wrana 2000; Johnsen *et al* 2002; Derynck and Zhang 2003; Massague *et al* 2005; Xu *et al* 2009; Lamouille *et al* 2012; Massague 2012).

SMAD dependent activation of TGFβ results in the activation of crucial transcription factors including SNAI (SNAIL/SLUG), ZEB (ZEB1, ZEB2) family of regulators which are known to directly repress the epithelial marker E-Cadherin and upregulate the Mesenchymal marker N-cadherin (Thiery *et al* 2009; Lamouille *et al* 2013). Several miRNAs are found to be involved in this process among which families of miR-34a and miR-200 are well studied (Gregory *et al* 2008; Park *et al* 2008; Paterson *et al* 2008; Ceppi *et al* 2010; Gregory *et al* 2011; Siemens *et al* 2011). These miRNA families are well known for their double negative feedback loops with ZEB and SNAI transcription factor families. (Bracken *et al* 2008; Burk *et al* 2008; Wellner *et al* 2009; Gregory *et al* 2011; Siemens *et al* 2011; Tian *et al* 2013). Several mathematical models of EMT are built around these regulators to understand the dynamics of EMT.

SNAIL is a zinc finger transcription factor family comprising of SNAIL1, SNAIL2, and SNAIL3. Overexpression of SNAIL family of regulators were observed to be associated with cancer and metastasis (Hemavathy *et al* 2000; Nieto 2002; Vega *et al* 2004; Yang *et al* 2009; Franco *et al* 2010; Li *et al* 2011; Fan *et al* 2012; Zhang *et al* 2013; Yang *et al* 2017; Cai *et al* 2019). SNAIL was observed to recruit cofactors like HDAC1, CBP300, HDAC2, SIN3A, ctBP, etc to form a repressor complex to repress epithelial gene expression (Peinado *et al* 2004; Peinado *et al* 2007). It is a highly unstable protein in cytoplasm and needs to translocate into nucleus to exert its function. PAK1 is known to phosphorylate SNAIL at S246 that favours its nuclear localization (Yang *et al* 2005). The process of EMT and cancer resistance are closely linked with each other. Snail expression was also found to be associated with chemo- and radio-resistance increasing cell stemness (Smith and Bhowmick 2016). SNAIL was observed to show resistance to the drugs that have been developed promoting cancer progression and metastases. They include 5- Fluorouracil, Cisplatin, Doxorubicin in targeting Breast, HNSCC (Head and Neck Squamous Cell Carcinoma) and ovarian cancer (Hsu *et al* 2010; Li and Zhou 2011; Haslehurst *et al* 2012; Shen *et al* 2012; Zhang *et al* 2012; Mariano *et al* 2015). Considering the numerous studies that have been developed to understand the signaling molecules that drive EMT and their correlation with chemoresistance, SNAIL has become an attractive target to explore cancer resistance.

In addition to SNAI and ZEB families, recent experimental studies have shown the evidence of MDM2 in regulating SNAIL expression by promoting TGFβ induced EMT. Studies have shown that the activation of oncogene MDM2 through TGFβ induced SMAD complex (Araki *et al* 2010). MDM2 enhances its own activity through a positive feedback loop

upregulating R-SMAD phosphorylation and thus promotes activation of SMAD complex (Araki *et al* 2010; Lu *et al* 2016; Chen *et al* 2017; Tang *et al* 2019). Previous studies also have shown complementary role of MDM2 in degrading SNAIL by ubiquitination, when in complex with wild type p53 thus inhibiting EMT (Wang *et al* 2009; Lim *et al* 2010). Wild type p53 is a well-known tumour suppressor gene notable for its regulatory functions in cell cycle (Rivlin *et al* 2011; Kim *et al* 2019). In many cancer types p53 is mutated, resulting in upregulation of MDM2 that transcriptionally activates SNAIL along with suppression of p53 (Lu *et al* 2016). p53 is also known to regulate the expressions of epithelial markers miR-34a, miR-200 which are part of the double negative feedback loops ZEB/ miR200 and SNAIL/miR34 promoting EMT (Hunziker *et al* 2010; Chang *et al* 2011; Kim *et al* 2011; Coradini *et al* 2012; Ren *et al* 2013).

Several mathematical models were built to understand the dynamics of TGFβ signaling and their significance in inducing Epithelial to Mesenchymal transition (Miettinen *et al* 1994; Heldin *et al* 1997; Vilar *et al* 2006; Zi and Klipp 2007; Turner and Kohandel 2010; Vilar and Saiz 2011; Malfettone *et al* 2017; Tripathi *et al* 2020). Amongst which various models were built around the core regulatory network of TGFβ induced EMT. The network mainly comprises of double negative feedback loops of miR-200/ZEB and miR-34a/SNAIL to understand the dynamics of transformation from epithelial to mesenchymal phenotype (Lu *et al* 2013; Tian *et al* 2013; Zhang *et al* 2014; Jolly Jia *et al* 2016; Jolly Tripathi *et al* 2016). Studies show the prominence of double negative feedback loops in modulating the reversible and irreversible bistable switching from epithelial to mesenchymal phenotypes along with existence of a hybrid EMT/ MET phenotypes. It was also observed that release of miR-200-mediated inhibition of TGFβ is required to maintain a stable mesenchymal phenotype (Gregory *et al* 2011; Tian *et al* 2013). A model developed by Lu *et al* (2013), depicts the significance of mRNA: miRNA coupling in the process of EMT. Autoregulation of the transcription factor SNAIL was observed to act as a noise integrator in the process of EMT (Lu *et al* 2013). However, many of these models found in literature review are focused on (i) the upstream SMAD signaling and its nucleocytoplasmic shuttling, (ii) the downstream double negative feedback loops controlling the E/M, M/E (ir)reversible switch. There is no dynamic ordinary differential equation based modelling framework that explores the role of p53, MDM2 dependent alterations to the SNAIL and SNAIL dependent miRNA regulations of EMT. Therefore, motivated by the role of p53-MDM2 in TGFβ induced EMT and their involvement in regulating miRNAs, a mathematical model was developed coupling SMAD regulation along with its downstream core regulatory network as shown in figure 2.

Methods

Network that regulates epithelial to mesenchymal transition

TGFβ signaling pathways were observed to be the major players in the development of epithelial to mesenchymal phenotypic switch and thus migratory behavior in several cancer cells. Therefore, with the objective to explore the role of various key regulators involved in this regulatory process, a pathways network was assembled. An extensive literature survey was performed to obtain the signaling flow following the recent experiments that has revealed the regulatory roles of SMAD, p53, MDM2 in regulating SNAIL expression (Lu *et al* 2016; Chen *et al* 2017; Tang *et al* 2019). Hence, a gene regulatory network depicting TGFβ induced EMT was assembled (figure 2) for a cancerous cell which includes 29 nodes (genes, receptors, miRNAs, Phenotype) connected through 45 edges (activation, repression, nucleocytoplasmic shuttling). The network consists of cancer marker genes p53, MDM2 in negative feedback loop activated through DNA Damage. ZEB, SNAIL mesenchymal markers regulated through MDM2, SMAD complex that initiates and facilitates the process of EMT through inhibition of E-Cadherin, activation of N-Cadherin, activation of latent TGFβ (represented as enTGFb). Also epithelial markers miR-34 and miR-200 that are in double negative feedback loops with mesenchymal markers were considered. SNAIL activated through upstream regulatory molecules (MDM2, SMAD complex) subsequently activates the latent TGFβ and its receptors TGFβR (Represented as enTGFb and TGFbR in figure 2) through a positive feedback dependent regulation, initiating the process of EMT. The network construct also considers the SMADs complex association, dissociation and nucleocytoplasmic shutting. The focus of the study was primarily on the MDM2/SNAIL axis that triggers the TGF-β/SMAD/SNAIL signaling. The network assembled thus is show in figure 2. The logical relationships among nodes was tabulated in supplementary Table S1(b). The network was further analysed and characterised using both discreet (Boolean logical modelling) and continuous dynamic modelling using ordinary differential equations.

Discrete dynamic modelling

The network constructed (figure 2) was translated into the dynamic model to explore EMT/MET and the role of various regulators in the process of transition. Discrete dynamic modelling was utilized as first step because of its computational feasibility and capacity to work with qualitative biological data. Specifically Boolean modelling approach was adopted where each network node was represented by only two known qualitative states: ON or OFF. The ON state means a regulator is in an active form or at its maximum

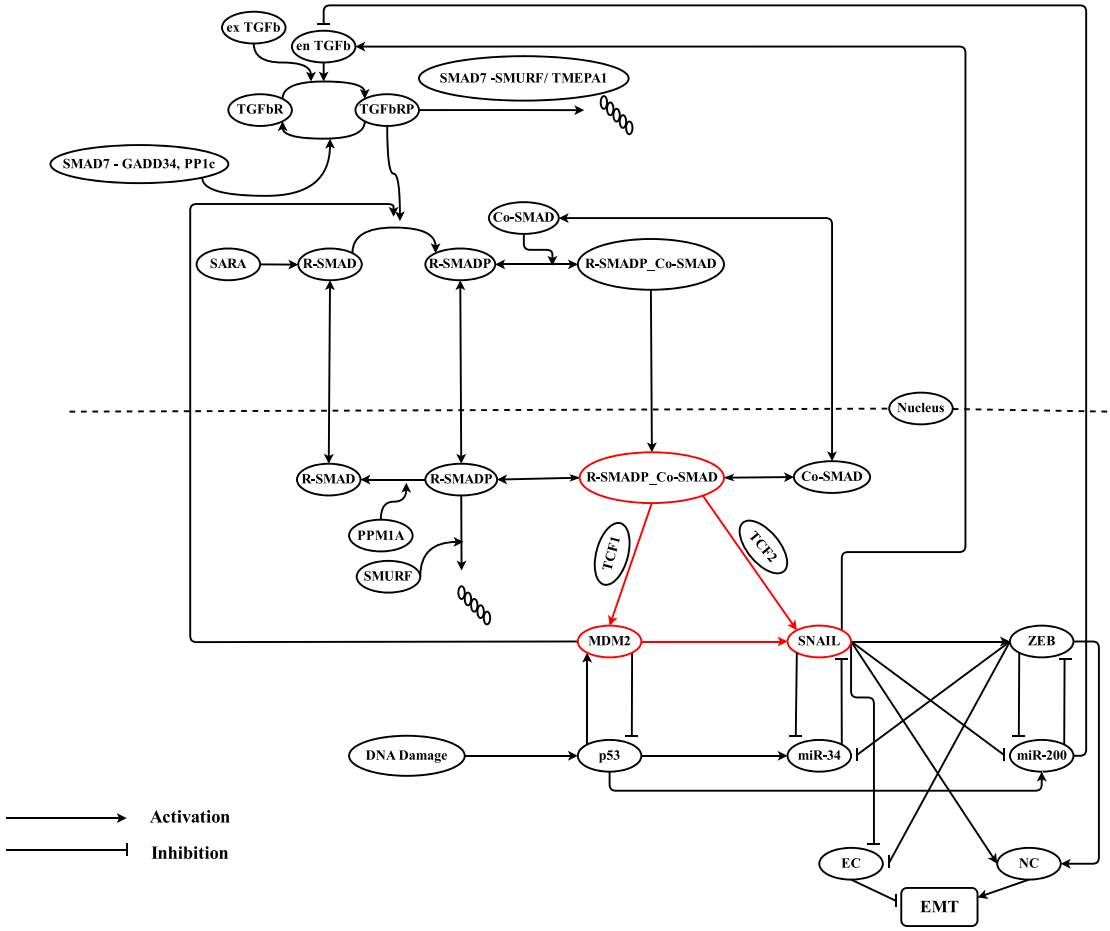

Figure 2. Network devised schematically represents the flow of signal information that moderates the p53 dependent TGFβ induced activation of epithelial to mesenchymal transition (EMT). Network comprises of three layers: (i) Receptor regulation; (ii) SMAD signalling and the p53-MDM2 loop connecting to SNAIL; (iii) SNAIL dependent activators and repressors of EMT. Red highlighted components of the network forms a C1FFL that streams the signal for the EM transition.

Table 1. Various state combinations of TCF1, TCF2 and their resulting phenotype. +/- indicates the presence or absence of hybrid phenotype.

Scenario	TCF1	TCF2	exTGFβ	Hybrid	EMT (Time Steps)
1	0	0	0	+	Hybrid (Oscillations)
2	1	1	0	+	72 Time steps
3	0	1	0	+	113 Time steps
4	1	0	0	+	86 Time steps
5	0	0	1	—	Hybrid (Oscillations)
6	1	1	1	—	10 Steps
7	0	1	1	—	10 Steps
8	1	0	1	—	12 Steps

activity (represented as 1) whereas OFF state means a regulator is inactive form or is at its basal minimum activity (represented as 0). Regulatory functions for each node (Regulator) were formulated depending on their upstream regulators and are known to produce known biological outcomes (Supplementary Table S1 (available online at stacks.iop.org/BPEX/8/045012/mmedia)). The *python* library *Booleannet* downloaded from (https://github.com/ialbert/booleannet) (Albert *et al* 2008) was used to perform the simulations. Python 3.x was used with Ubuntu as working operating system. Considering all the timescales involved in transcriptional and signal transduction events, asynchronous update schema was used. This accounts for different timescales in signaling networks, in which the state of each node was updated according to its own timescale (Garg *et al* 2008; Chatain *et al* 2018).

To evaluate the TGFβ dependent transition of cells from epithelial to mesenchymal transition, epithelial state was assumed to be the initial state of the system responding to the signal DNA Damage. Multiple

simulations were performed with various initial conditions and varying states of certain regulators some of which are tabulated in table 1. To further assess the effect of various regulators on the reversibility of EMT, the initial epithelial conditions was over-raided after 110-time steps in each simulation when the system was assumed to be transitioned completely into mesenchymal state and attained steady state. The simulations were performed for 5000 timesteps and 500 iterations.

Continuous dynamic modelling

To further explore the role of network structure driven dynamic behaviour, continuous dynamic modelling was utilised. ODE modelling and simulations were adopted specifically to deduce the functional role of the SMAD, MDM2 on SNAIL which were observed to be interacting in a coherent type1 feed forward regulatory (C1FFL) interaction (figure 4). Two input functions S_x (TGFβ) , S_y (p53) were considered to be the stimulus that activates SMAD complex and MDM2 which are required for SNAIL transcriptional activation. Various different combinations of continuous, pulsatile inputs were provided in the form of S_x and S_y to observe their influence on SNAIL transcriptional activity. Both AND gate like logic and OR gate like logic were independently explored for S_x and S_y dependent regulation of the regulator SNAIL in C1FFL. These mechanisms for the individual regulators SMAD complex, MDM2 and SNAIL were captured in terms of mass action kinetics and Hill equations-based ODE as shown below.

$$\frac{d[SMAD]}{dt} = a * S_x - Kd1 * [SMAD] \quad (1)$$

$$\frac{d[MDM2]}{dt} = b * S_y + \frac{k1 * [SMAD]^n}{km1^n + [SMAD]^n} - kd2 * [MDM2] \quad (2)$$

$$\frac{d[SNAIL]}{dt} = G(MDM2, k2;\ SMAD, k3) - kd2 * [SNAIL] \quad (3)$$

The regulatory function for AND gate is G_{SNAIL}(MDM2,k2)*G_{SNAIL}(SMAD,k3) follows the hill functions and is given as $\frac{k2 * [MDM2]^n}{km2^n + [MDM2]^n} * \frac{k3 * [SMAD]^n}{km3^n + [SMAD]^n}$. The regulatory function for OR gate is G_{SNAIL}(MDM2, k2)+G_{SNAIL}(SMAD, k3) and is given as $\frac{k2 * [MDM2]^n}{km2^n + [MDM2]^n} + \frac{k3 * [SMAD]^n}{km3^n + [SMAD]^n}$.

The ODE models were simulated using *ode23s* solver of MATLAB R2021a. The Associated parameters and their descriptions were tabulated in supplementary Figure S29 and Table S29.

RESULTS

SNAIL lies at the epicentre of p53 dependent EMT regulation

The network devised (figure 2) comprises of SMADs complex formation, SMADs nucleocytoplasmic shuttling, and the core regulatory network that accounts for the effect of p53-MDM2. We hypothesised that the network gets activated in a layered manner. The first layer comprises the activation of serine/threonine receptors by exogenous or endogenous TGFβ through phosphorylation(Massague 1998; Attisano and Wrana 1996, 2002; Vander Ark *et al* 2018). The second layer comprises of SARA dependent recruitment of R-SMAD to the receptors for activation by phosphorylation. Phosphorylated R-SMAD forms a complex with Co-SMAD that shuttles into the nucleus, and thus promotes transcriptional activation of MDM2, SNAIL in presence of transcription cofactors TCF1, TCF2 (Heldin *et al* 1997; Attisano and Wrana 1998; Johnsen *et al* 2002; Derynck and Zhang 2003). Once shuttled into nucleus the complex disassociates into R-SMADP and Co-SMAD. These disassociated parts further shuttle between the nucleus and cytoplasm or gets degraded depending on the environment (Nakabayashi and Sasaki 2009). In the third layer, DNA damage dependent p53-MDM2 and their associated regulators are considered. MDM2 activates SNAIL, which directly suppresses mir-34, miR-200, E-cadherin and p53 which are required for maintaining epithelial state. By repression of these markers and by activating Zeb, N-cadherin Mesenchymal transition is promoted. These interaction have the double negative feedback loop architecture between SNAIL/miR-34a and ZEB/miR-200 (Ciliberto *et al* 2005; Burk *et al* 2008; Korpal *et al* 2008; Wang *et al* 2009; Hunziker *et al* 2010; Tryndyak *et al* 2010; Kim *et al* 2011; Siemens *et al* 2011; Liu *et al* 2013). MDM2 also enhances its own regulation through phosphorylation of R-SMADs in a positive feedback manner (Chen *et al* 2017; Tang *et al* 2019). In addition to the regulations discussed above, SNAIL also activates the endogenous TGFβ that enhances the TGFβ signaling, and miR-200 negatively inhibits this endogenous TGFβ (Zhang *et al* 2014). We speculate a crucial role for this network in governing the downstream EMT transition. Thus, the network was assembled by reviewing the relevant literature to understand the network influenced behavior that promotes EMT.

Logical modelling and simulation of TGFβ dependent EMT regulation

To understand the dynamics of signaling during the process of epithelial to mesenchymal transition, the core regulatory network shown in figure 2 was translated into Logical and Mathematical Models. Discrete dynamic modelling was used as first step because of its computational feasibility and capacity to work with qualitative biological data. Specifically, Boolean Logical modelling was used, and simulations

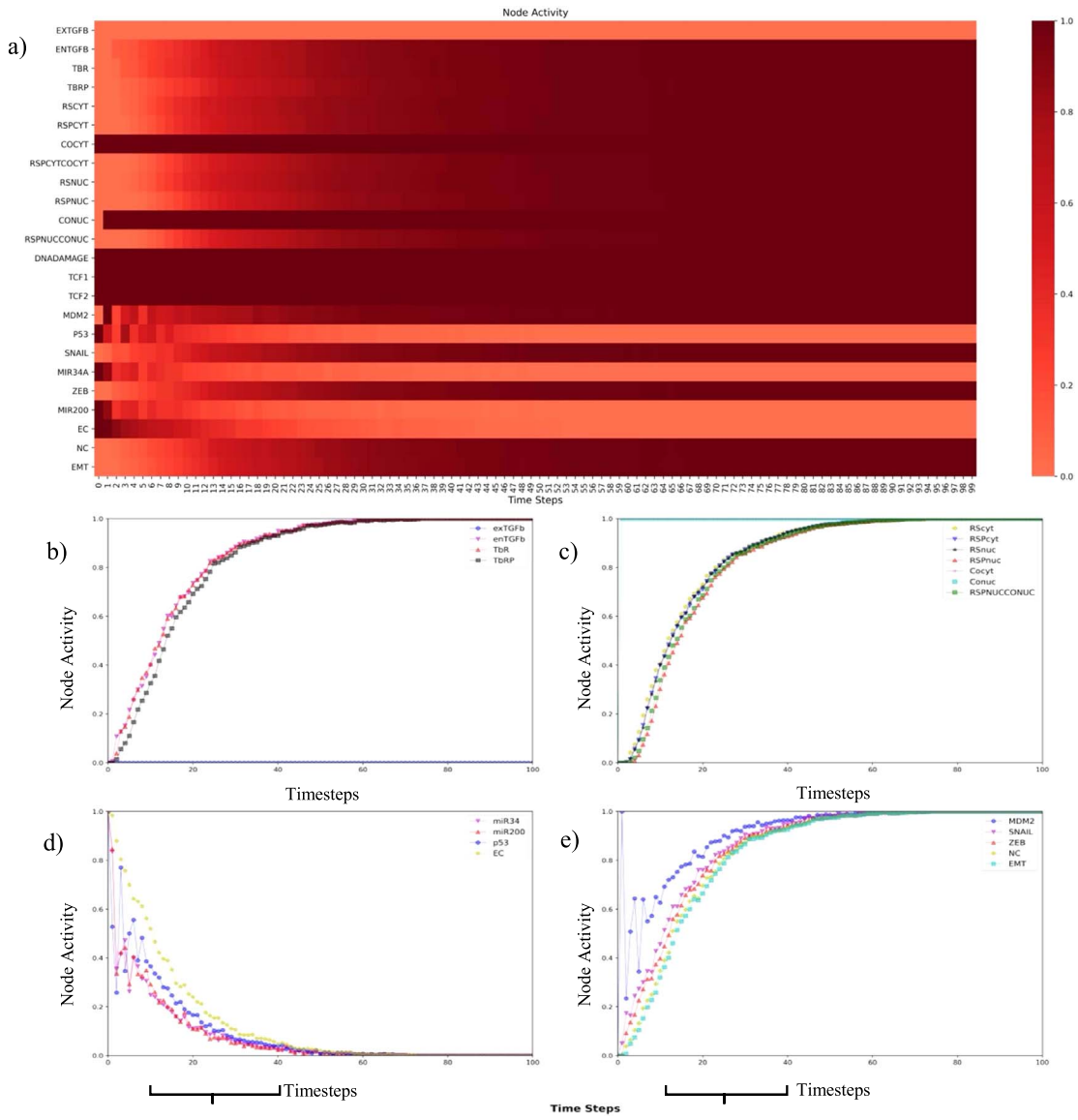

Figure 3. Simulation result of Logical modelling showing Transition from E to M phenotype when DNA Damage = ON (Dysregulation). Simulation was run for 5000 steps, 500 iterations in asynchronous mode. Complete transition was observed with in 100 timesteps. With the increase in total time steps the mesenchymal markers (SNAIL. ZEB, N-Cadherin(NC)) are transcriptionally activated supressing the epithelial markers (p53, miR-34, miR-200, E-Cadherin(EC)). The complete transformation was observed in 72 time steps during the course of which the hybrid phenotype was observed for 30 time steps (Marked) between timesteps 10 to 40.

were conducted using the *python* library *Booleannet* downloaded from (https://github.com/ialbert/booleannet) (Albert *et al* 2008). Logical modelling was based on the components of the regulatory network where each regulatory gene is considered a node and the connection between nodes are considered as edges. Each network node is described by binary states: ON (active) or OFF (inactive) making the model discreet deterministic and parameter free. The regulatory functions in the network were formulated based on the influence from the edges built by logical operators AND, OR, NOT representing biological interactions found in literature (Table S1). While beginning the study we performed simulations for 5000 timesteps, 500 iterations with different input conditions (table 1) to explore the time required for complete EMT transition. Asynchronous update scheme was preferred because it accounts for all the genes at each and every time step during the state transition (Garg *et al* 2008; Chatain *et al* 2018). The simulation captures the complete EMT transition within 100 timesteps (figure 3).

Epithelial to mesenchymal transition

Initially, for logical modelling the nodes were set according to their known epithelial cell states. Specifically, the nodes of epithelial markers p53, miR-200, miR-34a, E-Cadherin were set to ON (1) state and the mesenchymal markers SNAIL, ZEB, N-Cadherin, MDM2, SMADs, exTGF-β, enTGFβ were set to OFF (0) state. The model without any stimulus or dysregulation remains at epithelial state where E-Cadherin is

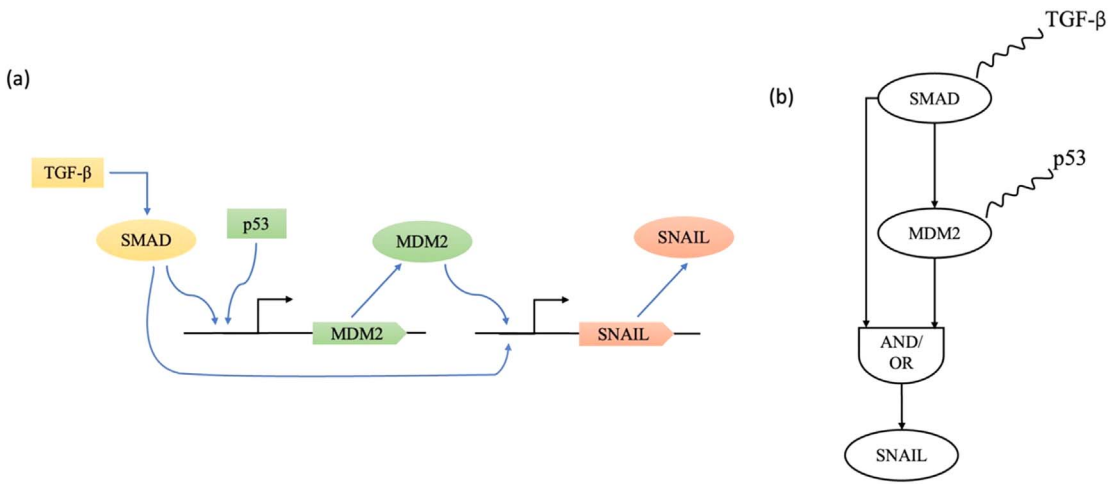

Figure 4. Coherent type I FFL network motif of Snail regulation (a) Gene and transcription signal level regulation of Snail: TGFβ activates SMAD complex (indicated here as SMAD) which binds to the regulatory region of MDM2 and promotes its expression. Together SMAD complex and MDM2 activates the SNAIL transcription by binding to the upstream regulatory region of SNAIL. MDM2 is also transcriptionally regulated by p53. (b) Network representation of SNAIL regulation though MDM2 and SMAD complex. Logical modelling updating rules and truth tables for the same are tabulated in supplementary Figure S26.

at its maximum node activity with N-Cadherin at its minimum node activity as shown in Figure S2. Subsequently, to implement dysregulation in cancer cells, DNA Damage was set to ON state to observe the resulting signaling events (figure 3). When the DNA Damage is set to ON state it was observed that the presence of this dysregulation activates the p53-MDM2 signaling axis further leading to activation of SNAIL. Increased node activity of SNAIL subsequently activates the enTGFβ through positive feedback that regulates SMAD signaling axis thus further enhancing transcriptional activity of MDM2 and SNAIL. This enhanced transcriptional activity of SNAIL subsequently triggers ZEB transcription, which in turn represses the epithelial markers. Turning DNA Damage to ON state ultimately led to the changes in all the nodes particularly EMT hallmarks over the time course of 72 timesteps. All the epithelial markers were observed to be at their minimum activity (0) and mesenchymal markers were observed to be at their maximum activity (1) reflecting TGFβ driven EMT. If a node is reaching steady state with minimum activity (OFF state) to begin with and ends up at maximum activity (ON state) by the end of simulation, it is inferred as network dependent rule driven steady state. Existence of a hybrid EM phenotype, (both epithelial and mesenchymal states) was observed for a period of 40 timesteps before the cell transits completely into mesenchymal phenotype. Prescence of epithelial markers (figure 3d) and mesenchymal markers (figure 3e) were observed from timestep 10 to timestep 40 which was interpreted as EM hybrid state for this study.

To observe the effect of external environmental stress that induces EMT, exTGFβ was set to ON state. It was observed that when exTGFβ is turned ON the steady state is reached within 10 timesteps signifying the role of exTGFβ (any similar stress) in accelerating the EMT switch Figure S3. Further to know the impact of the feedforward (figure 4) relationship amongst SMAD complex, MDM2 in regulating the SNAIL transcriptional activation and their role in EMT, the nodes of their transcription cofactors TCF1 and TCF2 were set to ON & OFF states by keeping (i)exTGFβ at its ON state; (ii) exTGFβ at its OFF state (table 1). When stimulus to MDM2, *i.e.,* TCF1 was set to OFF and exTGFβ was set to OFF, lack of stimulus to MDM2 causes the p53-MDM2 axis to oscillate (Figure S4). However, there is activity of mesenchymal markers that maintains the levels of their counterpart miRNAs as shown in Figure S4. Activation of SNAIL by enTGFβ and by MDM2 enhances its own activity, thus simultaneously suppressing epithelial markers and promoting mesenchymal markers leading to EMT. When the stimulus for SNAIL, *i.e.,* TCF2 along with exTGFβ were set to OFF states, the activation of MDM2 occurs through DNA Damage which in turn activates SNAIL. This further regulates enTGFβ through positive feedback loop which in turn activates MDM2 through SMAD axis thus further enhancing SNAIL expression which promotes EMT (Figure S5). When both the stimulus TCF1, TCF2 were set to OFF along with exTGFβ set to OFF oscillations were observed (Figure S6). These oscillations were observed due to leaky expression of functioning SNAIL (driven by RSMAD-Co-SMAD complex and MDM2) that favors the maintenance of the hybrid phenotype. Table 1 summarizes various state combinations of TCF1, TCF2 and their resulting phenotype. From this it is inferred that the presence of both the signals promotes EMT.

Similar simulations were performed with the background of exTGFβ set to ON state (Figures S7 – S9). EMT was observed to occur swiftly within 10 timesteps. Thus, existence of external environmental

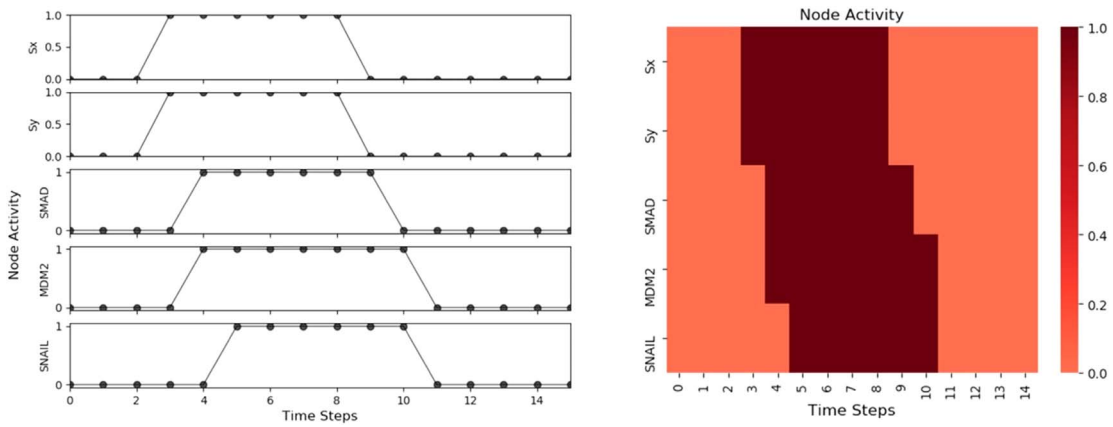

Figure 5. Dynamics of the C1FFL for the AND logic regulation with stimulus Sx = True and Sy = True analysed by logical modelling simulation. When both signals (Sx and Sy) were set to True, SNAIL reaches maximum activity with a delay in one time step. When both the stimuli were withdrawn SNAIL switches OFF instantly as soon as MDM2 reaches its minimum (0).

stress speeds up the epithelial to mesenchymal transition. When SNAIL self-repression was introduced to study the effect of SNAIL auto regulation on EMT, it was observed that the modified structure exhibited nearly similar behavior as previous cases but took longer timesteps to achieve EMT Figures S10- S17. However, there was an exception, for the case when TCF2 was turned to OFF and, TCF1 was turned ON a hybrid phenotype was observed (Figure S12). We performed this set of simulations to explore the impact of self-regulatory role on EMT. SNAIL self-regulation was experimentally explored elsewhere (Peiro *et al* 2006).

Blocking SNAIL expression completely reverses EMT to MET

To assess the effects of various regulators on the reversibility of EMT (mesenchymal to epithelial transition), the simulation conditions used to generate figure 3 were updated after 110 timesteps (assuming steady state is reached after EMT transition at this time point), and a set of systematic analysis were performed. When the stimulus for MDM2 i.e., TCF1 was turned OFF mesenchymal phenotype persists because SNAIL reaches to its maximum activity (Figure S18). Similar results were observed when TCF2 was turned OFF (Figure S19). Thus, in both scenarios mesenchymal phenotype is maintained because SNAIL activity (memory) due to the initial signal that led to EMT was still persistent. Subsequently, when both TCF1 and TCF2 were turned OFF together (Figure S20) the system reaches a hybrid phenotype. To evaluate the effect of miRNAs on the phenotype switching, miR-34 and, miR-200 were expressed by setting their node to ON state. It was observed that expression of both miRNAs (E promoting) resulted in persistent activity of SNAIL and maintenance of mesenchymal markers. Although epithelial markers were activated persistent activity of SNAIL resulted in continued suppression and absence of E-cadherin thus hybrid phenotype was observed (Figure S25). When the ZEB expression alone was knocked down N-Cadherin is downregulated, but E-cadherin is not expressed (Figure S21). When SNAIL expression alone or in combination with ZEB was knockdown altogether, (Figure S22, S23) there was a complete transition from mesenchymal to epithelial phenotype.

SNAIL driven by feed forward loop moderates epithelial to mesenchymal transition

Presence of Coherent type 1 Feed Forward Network motif architecture among MDM2, RSMAD-CoSMAD complex (referred to as SMAD hereon) and SNAIL was a surprising inference of this work during the network assembly (figures 2, 4). The FFL between the SMAD, MDM2 and SNAIL was observed to be coherent type 1 FFL where signal from TGFβ axis (S_x) activates SMAD which directly regulates MDM2. MDM2 is also activated by signal through p53 when there is DNA Damage (S_y). SMAD (direct edge) AND/OR SMAD->MDM2 (indirect edges) regulates SNAIL in a feed forward manner (figure 4). Simulation, analysis of this C1FFL may assist to further understand the specific role of SMAD and MDM2 in regulating SNAIL transcriptional activation that modulates TGFβ induced EMT switch. Hence the focus was shifted to performing a detailed analysis of the FFL using discrete (Logical) and continuous dynamics modelling approaches.

Logical modelling simulations was performed using *python* library *Booleannet* for 100 timesteps in synchronous update mode. In the first case, AND logic scenario was studied by keeping both the input signals S_x and S_y as ON. S_x activates SMAD, SMAD in turn activates MDM2 which is also activated by S_y. Together this direct and indirect edges activate SNAIL figure 5. To understand the architecture further the input signal S_y was kept as OFF and S_x was retained to be present and set as ON. For this, it was observed that with loss of the input signal (S_y) for MDM2, MDM2 is

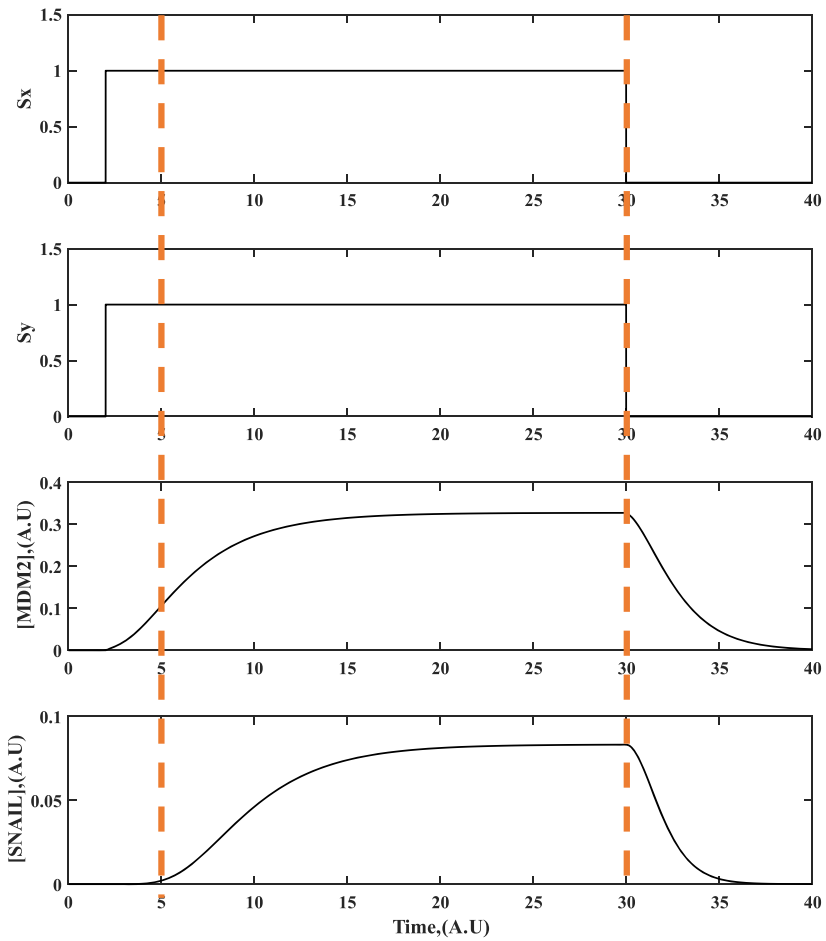

Figure 6. Dynamics of coherent type 1 FFL with respect to AND regulatory logic with the both inputs ON using ODE modelling. A delay in the ON step was observed for SNAIL transcriptional activation. The delay time was observed to be ~ 2.3 timesteps after the ON step. OFF steps after the removal of Sx and Sy were also shown. There was no delay observed following OFF step.

activated only after SMAD gets activated by S_x. SNAIL was observed to be at its ON state, only when both SMAD and MDM2 were at their ON State. Therefore, a delay of 2-time steps was observed in this case as shown in (Figure S27(a)). In the second case OR logic regulation was analyzed (Figure S28).

To understand more about the dynamics of SNAIL based on this C1FFL network motif, Ordinary differential equation (ODE) based dynamic modelling was performed using *MATLAB* solver *ODE23s*. Following activation through input signals (S_x, S_y) in AND logic dependent manner a delayed activation (of ~2.3 timesteps) of SNAIL expression was observed (figure 6). The delay is attributed to the requirement of the necessary thresholds k3 (SMAD on SNAIL) and k2 (Mdm2 on SNAIL) which are simulated in AND logic manner. Different patterns of the input signals reflecting various *in vivo* scenarios were studied (shown in Figures S30). It was observed that SNAIL reaches its maximum threshold only when both the inputs stimulus S_x and S_y were provided to the network motif. Absence of S_x fails to activate SNAIL because AND logic requirement is not fulfilled (Figure S30). However, absence of S_y leads to delayed expression of SNAIL because MDM2 accumulated due to SMAD dependent activation (k1) promotes SNAIL expression (Figure S30).

To further study the effect of input stimulus S_x and S_y, on SNAIL expression, pulse input of S_x was provided to the system in the absence of S_y. A time pulse shorter than the delay time does not lead to SNAIL expression in the C1FFL. This is because SMAD and MDM2 do not have sufficient time to reach the threshold that is essential to activate SNAIL (figure 7). Only persistent pulse longer than the delay time was observed to activate SNAIL expression. Apart from pulse detection, this motif functions like a noise filtering system with respect to S_x. When short consecutive pulses of S_x were provided there was no activation of SNAIL, and these pulses were filtered because of the AND logic like functioning of this motif.

In the subsequent scenario, input pulses of S_x and S_y were given at different intervals of time. When the inputs were provided for shorter span of time MDM2 reaches a threshold level that is sufficient to activate SNAIL figure 8. This indicates that this motif also functions like a coincident detector because of the transient memory storage capacity of MDM2 which is sustained by immediate availability of S_y even after the

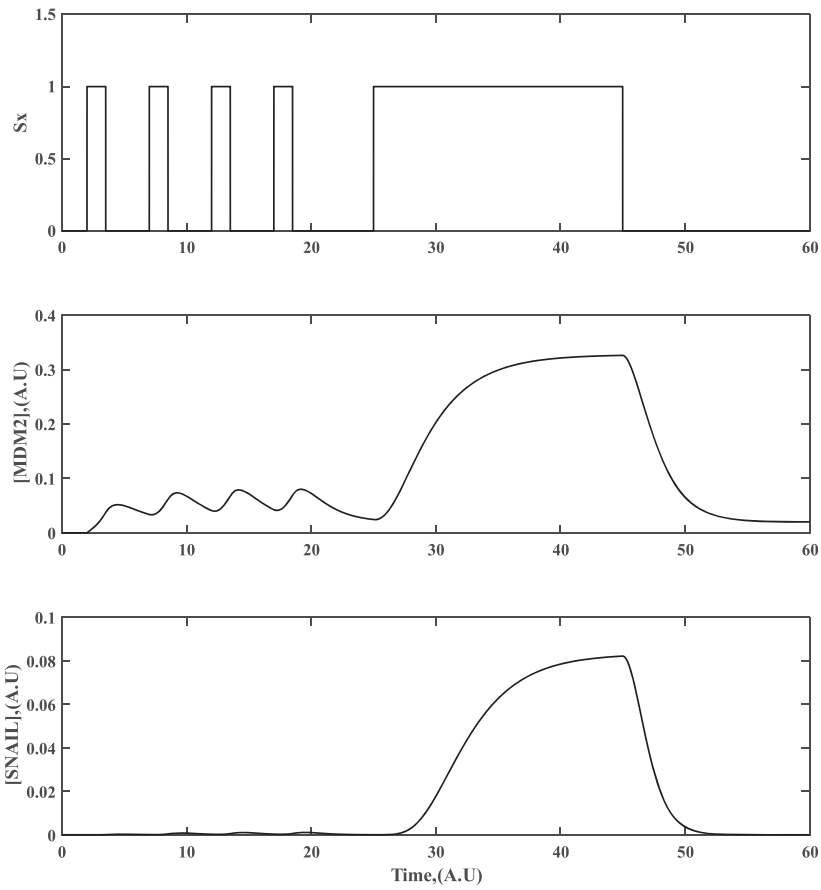

Figure 7. Dynamics of coherent type 1 FFL for AND regulatory logic when stimulus Sx was given as pulsatile stimulus. For low range of input stimulus, the C1FFL was observed to act as a noise filter and when the input stimulus is persistent the C1FFL acts as a persistence detector which is reflected as SNAIL expression. As soon as the stimulus (Sx) is withdrawn both MDM2 and SNAIL expression ceases immediately because of AND logic regulation.

removal of S_x. To assess the OFF-step dynamics for the simulation in figure 6, the stimuli (S_x and S_y) were withdrawn. Immediately after the withdrawal of S_x and Sy SNAIL expression comes to an arrest because of the AND logic dependent activation. Similarly, the SNAIL activation of C1FFL motif was also analysed for OR logic dependent regulation and various difference input stimulus combinations were also explored (Shown in Supplementary file Figure S28, S31).

Discussion

TGFβ induced regulatory processes and network modelling

The regulatory process of the cell is a smooth balance between the cell proliferation and the cell death (Lopez and Tait 2015; Sherekar and Viswanathan 2021). Numerous factors including various environmental conditions result in disturbing this balance causes cancer (Selvaggio *et al* 2020). Numerous mathematical and computational formalisms are developed to analyze these regulatory processes towards understanding the functioning of the cell (Ciliberto *et al* 2005; Kolch *et al* 2005; Zi and Klipp 2007; Albert *et al* 2008; Anderson and Quaranta 2008; Schmierer *et al* 2008; Nakabayashi and Sasaki 2009; Turner and Kohandel 2010; Idikio 2011; Cohen *et al* 2015; Rodriguez *et al* 2015; Jolly and Levine 2017; Khatibi *et al* 2017; Morshed *et al* 2018; Alon 2019; Tripathi *et al* 2020). Among which logical framework proved to be one of the successful one for understanding dynamic features of complex biological signals (Beal *et al* 2018). (Kauffmann *et al* 1973, 1969) was the first to introduce the logical modelling for analysis of generic Boolean network models using synchronous update (Kauffman 1969; Glass and Kauffman 1973). Following which this framework has been used to explore the cellular phenotype/ physiological changes that occur in various cellular processes such as cell cycle, DNA repair, tumorigenesis, cancer pathways etc (Albert and Othmer 2003; Novak and Tyson 2004; Saez-Rodriguez *et al* 2009; Schlatter *et al* 2009; Saadatpour *et al* 2010; Grieco *et al* 2013; Saadatpour and Albert 2013; Tyson and Novak 2014), (Ribba *et al* 2006; Steinway *et al* 2014; Remy *et al* 2015; Steinway *et al* 2015; Rossato *et al* 2019). Such predictive parameter free models are useful to understand the state transitions and their biological implications (Beal *et al* 2018; Eduati *et al* 2020; Beal *et al* 2021; Sherekar and Viswanathan 2021).

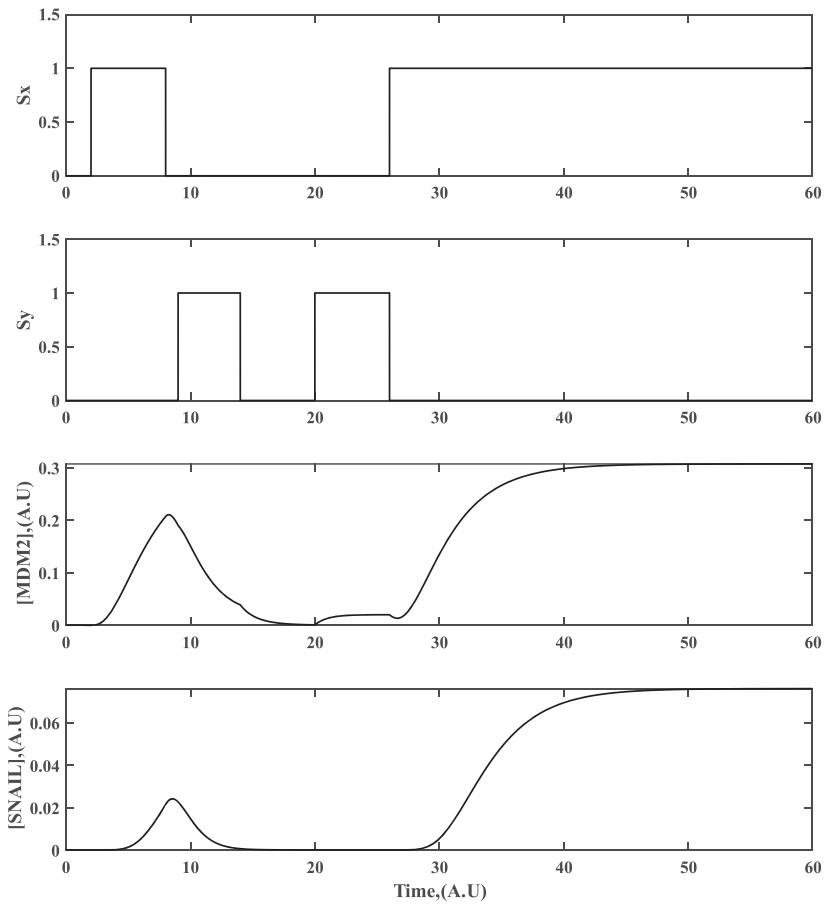

Figure 8. Dynamics of coherent type 1 FFL with respect to AND regulatory logic, when the inputs stimuli Sx and Sy were given simultaneously. The C1FFL acts as a coincidence detector because of availability of MDM2 which we attribute to its transient-memory-storage capacity.

The network assembled (figure 2) comprises of tumour suppressor genes (p53, TGFβ), tumour promoting genes & transcription factors (TGFβ, SNAIL, MDM2, ZEB) and were commonly observed in numerous cancer types including Breast, Ovarian, Hepatocellular, Colorectal, Lung Carcinoma (Bolós *et al* 2016; Lu *et al* 2016; Osorio *et al* 2016; Saitoh *et al* 2016; Yang *et al* 2017; Cai *et al* 2019; Jiang *et al* 2020; Lin *et al* 2020; Tripathi *et al* 2020). Presence of a feed forward network architecture was a surprise observation between the upstream TGFβ activated SMAD complex, MDM2 for the regulation of SNAIL transcriptional activation. Thus, logical modelling framework adopted for this study has demonstrated the EMT transition that promotes metastasis and the role of individual regulators guiding this process.

Implications of logical modelling and analysis on TGFβ induced phenotypic switching

Among the hallmarks of cancer (Hanahan and Weinberg 2000, 2011) EMT is one of the widely explored hallmark of the tumorigenesis. Steinway *et al* (2014) published a TGFβ induced EMT model where the concomitant activation of WNT and SHH signaling was necessary for the TGFβ dependent EMT in Hepatocellular Carcinoma (HCC) (Steinway *et al* 2014). Single and combinatorial perturbation analysis were performed on the logical model developed to observe their effect on TGFβ induced EMT. Single perturbations resulted in a hybrid phenotype and multiple interventions were observed to result in an epithelial phenotype (Steinway *et al* 2015). Analogously, combinatorial perturbations performed both theoretically and experimentally by Watanabe *et al* (2019) by manipulating the essential genes TGFβ and ZEB1 revealed the key functional role of ZEB1 in reciprocity and reversibility of EMT (Watanabe *et al* 2019). Study by rated the importance of microenvironments in controlling the cellular plasticity along EMT and suggests hybrid and mesenchymal phenotype occur via different paths (Selvaggio *et al* 2020). Several other perturbation analysis were also performed by various groups by building logical models to analyze the stability of the cellular phenotype during the transition in different cancer types (Cohen *et al* 2015; Mendez-Lopez *et al* 2017; Selvaggio *et al* 2020). Further logical models were also used to analyze the drug effects and therapeutic strategies (Flobak *et al* 2015; Traynard *et al* 2017; Zanudo *et al* 2018). Thus, logical modelling and simple perturbation analysis can be used to understand the molecular mechanism influencing various key physiological processes such

as EMT in terms of their signaling regulatory components.

The functional principles of EMT/MET was previously described by several mathematical models that revolves around the core regulatory circuit miR-34/SNAIL/miR-200/ZEB in terms of cascade switches and bistable switches (Lu *et al* 2013; Tian *et al* 2013; Jolly and Jia *et al* 2016; Jolly Tripathi *et al* 2016; Jia *et al* 2017; Jolly and Levine 2017; Tripathi *et al* 2020; Tripathi *et al* 2021). Although the response of phenotypic switching is not an all or none response, it is rather a collective response that depends on several input signals and the environment conditions (Gupta *et al* 2011; Huang *et al* 2014; Jolly *et al* 2014; Jolly *et al* 2015; Boareto *et al* 2016; Li and Balazsi 2018).

The phenotypic shift from epithelial to mesenchymal states was observed to be a stepwise process during which existence of hybrid EM state was investigated for its crucial role in tumour progression and organ fibrosis (Li *et al* 2016). Hybrid EMT was noticed to allow collective migration of tumour cells evading immune attack with higher migrating potential (Vinay *et al* 2015; Saxena *et al* 2020). Our model predicts that introducing the negative self-regulation of SNAIL, results in hybrid phenotypes with longer time span before mesenchymal transition. This opens a window for further analysis on hybrid EM phenotypes and its impact on collective migration of cancer cells. Literature shows that knockdown of ZEB (at low levels) and not SNAIL alone (Gregory *et al* 2011) or upregulation of miRNAs miR-34a or miR-200 (thus causing complete downregulation of SNAIL)(Migliore *et al* 2008; Migliore and Giordano 2009; Bader 2012; Wang *et al* 2013; Okada *et al* 2014) reverts the cell to the epithelial phenotype. Our results show that either of them do not revert the epithelial phenotype but downregulation or suppression of SNAIL expression all together can revert the cell to epithelial phenotype. Previous studies have demonstrated the effect of various epigenetic factors / phenotype stability factors, the interconnectedness between these factors with core regulatory circuit and their effect on the stability/resistance on EMT (Padua *et al* 2008; Cieply *et al* 2012; Huang *et al* 2015; Jolly and Tripathi *et al* 2016; Jolly *et al* 2018; Bocci *et al* 2019; Jia *et al* 2019; Jia *et al* 2020; Silveira and Mombach 2020; Silveira *et al* 2020; Subbalakshmi *et al* 2020). The present work investigated the signaling network level regulation, a layer above this core regulatory circuit, which includes an additional p53/MDM2 loop that affects the same phenomenon of EMT. The present work has identified this layer involving SMAD complex, MDM2 and SNAIL to be regulated through a coherent type 1 FFL motif architecture (figure 4). p53 has been well explored for its role as a tumour suppressor, driver gene and p53/mdm2 loop in maintaining the oscillations in the presence of DNA damage (Lev Bar-Or *et al* 2000; Ciliberto *et al* 2005; Cohen *et al* 2015). However, the role of p53/MDM2 in EMT is recently demonstrated experimentally (Wang *et al* 2009; Lim *et al* 2010; Kim *et al* 2011; 2011; Ren *et al* 2013; Wang *et al* 2013; Chen *et al* 2017; Tang *et al* 2019). This is also an attempt to analyse the effect of p53/MDM2 on the core regulatory circuit and phenotypic plasticity. MDM2 is observed to be a part of coherent type 1 FFL motif architecture above the core EMT regulatory circuit.

Our model and model analysis illustrates that for the complete transition from epithelial to mesenchymal phenotype SNAIL requires to be active which occurred within fewer time steps (10 steps). SNAIL activity is required for regulation of its downstream transcription factors. SNAIL attains maximum activity swiftly when there is external environment stress and when both TCF1&TCF2 are set to ON. Absence of any of the input signals or Prescence of negative auto regulation on SNAIL results in longer durations of hybrid phenotype before attaining EMT thus providing the essential time for the system for preparations and final decision making. Knockdown of transcription cofactors alone for both SNAIL and MDM2 or overexpression of miRNAs do not reverse EMT but results in a hybrid phenotype. Whereas complete knockdown of SNAIL expression completely causes a switch from mesenchymal to epithelial phenotype. Therefore, the focus was further shifted to understand the significance of SNAIL regulated by both SMAD complex and MDM2 in a feed forward manner.

Coherent feed forward loop network architecture and guardian role of SNAIL

SNAIL was observed to be one of the prominent molecules affecting the phenotypic plasticity that favours the changes in cell shape (Vega *et al* 2004). Studies by Peiro *et al* (2006) demonstrated the self-regulatory role SNAIL1 family (Peiro *et al* 2006). SNAIL along with SMAD2/3 complex acts as a transcriptional repressor for epithelial markers (Vincent *et al* 2009;). It was also observed that elevated expression of SNAIL forces the cells to attain mesenchymal traits by repressing p53 and p53 induced miRNAs (Siemens *et al* 2011). The role of SNAIL as a noise buffering integrator and its ability to control the fate of hybrid E/M phenotype was demonstrated. (Jolly *et al et al* 2014; Lu *et al* 2013). Our results were observed to corroborate along with these roles discussed. SNAIL functions like noise filtering system in the C1FFL motif structure analysed and it propagates the filtered signal to the downstream core EMT regulators. SNAIL is known to drive cell proliferation in many cancer types including hepatocellular, ovarian, breast, colorectal, lung cancer and tumour progression. We postulate that perhaps the process of tumorigenesis and EMT transformation may involve the C1FFL dependent regulation characterised in this study. This proposed hypothesis can be verified *in vivo* in cancer cell lines by single cell level microscopic studies and through cell culture studies. Further,

'Build it to understand' synthetic biology strategies can be utilised to further refine the C1FFL dependent molecular regulation that lies above the core EMT regulatory switch. Such synthetic biology study may possibly provide a diagnostic tool for early assessment of E-M transitions.

FFLs involving transcription factors and their various subtypes have been analysed in the past (Schleif 2000; Shen-Orr *et al* 2002; Mangan and Alon 2003; Kalir *et al* 2005; Alon 2007; Iyengar *et al* 2017; Zhang *et al* 2018; Alon 2019; Guo and Murray 2019). It has been demonstrated that specific network motifs such as auto-negative regulation or C1FFL can filter or dampen external noise associated fluctuations (Ghosh *et al* 2005; Osella *et al* 2011; Alon 2019). Gill *et al* (2018) experimentally characterise a positive FFL that enforces YAP/TAZ engaging hippo pathway in promoting tumour (Gill *et al* 2018). Reeves *et al* (2009) studied the engineering principles of combining the FFL with a feedback loop (Reeves 2019). Work by Tej *et al* (2019) suggests that the function of small RNA FFL is critically governed by the small RNA not only in generating a speedy and strong response but also in producing a reliable response by regulating the gene expression noise(Tej *et al* 2019). Dynamic analysis of multi-input FFL by Zheng *et al* (2009) in *C. elegans*, illustrates both persistence and coincidence detection among the neuronal cells (Zheng 2009). Similarly, our analysis of the FFL in figure 4 also has demonstrated both persistence and coincidence detection which we postulate may have a crucial role in EM transition. Our study suggests that the FFL governed by two TFs causes a delay in ON(AND) and OFF step (OR) by regulating SNAIL expression. We anticipate that SNAIL functions like a noise filter reminiscent with Lu *et al* (Lu *et al* 2013). Thus, the proposed C1FFL structure through SNAIL guards the downstream miRNA network from getting activated for low range of signals.

In current framework, when considered C1FFL alone, SNAIL activity depends on the input stimulus that comes through the upstream molecules (SMAD complex, Mdm2). However, if the whole regulatory circuit is considered, the C1FFL network architecture functions like a backbone for SNAIL activation and it does not function in isolation. These varying input signals also modulate the p53 dependent regulation of EMT in presence of dysregulation such as DNA Damage, and other environmental conditions. We postulate that this signaling follows a layered manner of activation, driving the activity of elements in the EMT network. Together the logical, ODE modelling and analysis of C1 FFL revealed that both the inputs (S_x, S_y) play a significant role in activating the SMAD and MDM2 which in turn promotes SNAIL which is essential for the downstream signaling to regulate EMT switch. SNAIL do not promote the downstream signaling if there is failure to detect input signals S_x thus playing a very prominent guidance role in this signaling cascade. We also observe that pulsatile input (analogous to noisy signal in terms of S_x) is filtered and EM transition will not be promoted by SNAIL (figure 7). However, simultaneous presence of pulsatile inputs (S_x AND S_y), is detected coincidentally (figure 8) implying the occurrence of EM transition promoted by SNAIL. Thus, SNAIL function as a decision-making element for the downstream EMT signaling regulators.

Conclusion

Diverse range of signaling molecules and pathways were observed to be involved in promoting epithelial to mesenchymal transition. Among which TGFβ and its family of signaling molecules play a prominent role. This study attempted to model and analyse the network that couples the upstream TGFβ signal level regulation of EMT which includes SMADs, SMAD-CoSMAD complexes, p53 and Mdm2.

Logical modelling and analysis revealed that for the transition from epithelial to mesenchymal phenotype to occur completely, SNAIL requires signal from both SMAD complex and MDM2. Blockage of any of the two signals causes the system to stay in hybrid EM phenotype for longer periods of time. Prescence of exogeneous TGFβ accelerates EMT, slimming the chances of hybrid phenotype. Negative autoregulation of SNAIL results in prolonged EM hybrid phenotype whereas complete knockdown results in MET. One of the important observations is the presence of C1FFL motif like regulation of SNAIL involving SMAD complex and MDM2. Dynamic ODE modelling and analysis of the C1FFL displayed a sign sensitive delay and persistence detection of the stimulus S_x. This motif also functioned like a coincidence detector when both stimulus (S_x and S_y) were present. These inferences led us to conclude that this signaling architecture enables SNAIL to filter the noise in terms of its input signals (S_x) to function like a 'Guardian' for the downstream networks performing the phenotypic switching. However, presence of multiple signals (S_x AND S_y), resulted in EM transition promoted by SNAIL expression. Together results from logical and dynamic modelling of the network suggests that SNAIL not only acts as a signal integrator but also plays a significant role in decision making during the phenotypic switching of EMT. We conclude that SNAIL is at the heart of the TGFβ dependent regulation of EMT bridging the C1FFL to the most frequently explored miRNA dependent core regulatory circuit of EMT. Therefore, SNAIL may be targeted for the drugs development and therapeutic interventions.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Declarations:

The authors declare no conflict of interest. The authors declare no competing financial interests.

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