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Article

The hidden switches underlying RORα-mediated circuits that critically regulate uncontrolled cell proliferation

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Prostaglandin E2 (PGE_2) is known to have a key role in the development of colorectal cancer, but previous experiments showed its contrasting (i.e. tumor-promoting or tumor-suppressive) roles depending on experimental conditions. To elucidate the mechanisms underlying such contrasting roles of PGE_2 in tumorigenesis, we investigated all the previous experiments and found a new signal transduction pathway mediated by retinoic acid receptor-related orphan receptor (ROR)α, in which PGE_2/PKCα-dependent phosphorylation of RORα attenuates Wnt target gene expression in colon cancer cells. From mathematical simulations combined with biochemical experimentation, we revealed that RORα induces a biphasic response of Wnt target genes to PGE_2 stimulation through a regulatory switch formed by an incoherent feedforward loop, which provides a mechanistic explanation on the contrasting roles of PGE_2 observed in previous experiments. More interestingly, we found that RORα constitutes another regulatory switch formed by coupled positive and negative feedback loops, which regulates the hysteretic response of Wnt signaling and eventually converts a proliferative cellular state into an anti-proliferative state in a very delicate way. Our results indicate that RORα is the key regulator at the center of these hidden switches that critically regulate cancer cell proliferation and thereby being a promising anti-cancer therapeutic target.

Keywords: colorectal cancer, Wnt signaling, RORα, mathematical modeling, biphasic response, hysteresis, systems biology

Introduction

The development of colon cancer results from a series of pathologic changes, such as APC inactivation, Cox-2 upregulation, K-Ras activation, and p53 inactivation (Janne and Mayer, 2000; Chell et al., 2006a). The genetic mutation of APC enhances colorectal tumorigenesis by constitutively activating Wnt signaling, which triggers a wide range of oncogenes including *Cyclin D1* and *Cox-2* (Tetsu and McCormick, 1999; Araki et al., 2003). Cox-2 is one of the key targets involved in the initial stage of tumor progression and its elevated expression continues during tumor progression. Cox-2 synthesizes prostaglandin E2 (PGE_2) which modulates cancer cell proliferation by regulating its downstream signaling pathways such as PI3K/AKT, Ras/MAPK/ERK, and Wnt/β-catenin pathways (Greenhough et al., 2009). A number of studies provided evidences for the tumor-promoting effects of PGE_2 signaling in various colon cancer cells including HT-29 cells (Qiao et al., 1995; Kawamori et al., 2001; Sheng et al., 2001; Pai et al., 2002; Wang et al., 2004, 2005; Chell et al., 2006b), whereas some other studies reported tumor-suppressive (Parker et al., 1997; Sheng et al., 1998; Wilson and Potten, 2000; Loffler et al., 2008) or both (Qiao et al., 1995; Sheng et al., 1998; Chell et al., 2006b; Loffler et al., 2008) roles of PGE_2 under various experimental conditions (Table 1). Hence, these contrasting reports suggest the necessity of systems analysis to unravel the hidden mechanism underlying the puzzling role of PGE_2 in colorectal tumorigenesis.

The cellular response to PGE_2 depends on the differential expression of four subtypes of prostaglandin E (EP) receptors (EP1-EP4) and their downstream signaling pathways (Hull et al., 2004; Chell et al., 2006a). The recently discovered cross-regulation between the Wnt and Cox-2/PGE_2 pathways indicates that PGE_2 signaling might contribute to colorectal tumorigenesis by activating β-catenin/TCF signaling through the EP2 receptor (Castellone et al., 2005). Interestingly, ectopic expression of Cox-2 was shown to promote its own expression due to the positive feedback loop between the Cox-2/PGE_2 pathway and β-catenin/TCF (Rodriguez et al., 2009). These results support the tumor-promoting roles of

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PGE_2, but the contrasting roles of PGE_2 in colorectal tumorigenesis still cannot be explained, implying that there may exist an additional cross-regulation between PGE_2 and Wnt signaling through the EP1, EP3, or EP4 receptors.

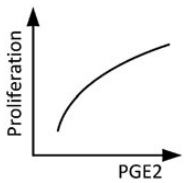
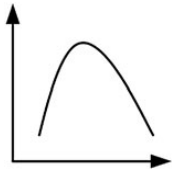
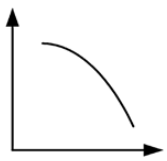
From systems analysis based on the integration of all the previous experiments, we found a new signal transduction pathway mediated by retinoic acid receptor-related orphan receptor (ROR)α, in which PGE_2/protein kinase C (PKC)α-dependent phosphorylation of RORα via the EP1 receptor attenuates Wnt target gene expression in colon cancer cells. By including this signal transduction pathway, we developed a mathematical model of the extended Wnt signaling network and found that a hidden regulatory switch formed by the RORα-mediated incoherent feedforward loop, which induces the biphasic response of Wnt signaling, is responsible for the contrasting roles of PGE_2 in colorectal tumorigenesis. More interestingly, we found that RORα constitutes another regulatory switch formed by coupled positive and negative feedback loops in the extended Wnt signaling network and that this switch critically regulates the hysteretic response of Wnt signaling in a very delicate way. From mathematical simulations combined with biochemical experimentation using colon cancer cells, we revealed that overexpression of RORα can convert a sustained proliferative cellular state under pathological conditions to an anti-proliferative state by reverting the hysteretic response. Together, we conclude that there are hidden switches underlying RORα-mediated circuits that critically regulate uncontrolled cell proliferation and that RORα might be a promising therapeutic target that can abrogate abnormal cell proliferation in colorectal tumorigenesis.

Results

The extended Wnt signaling network and its mathematical modeling

We considered an extended Wnt signaling network with regard to core circuits that are embedded in the network and control Wnt signal flux, by examining the expression of Cyclin D1 in response to Wnt stimulation. The extended Wnt signaling network includes the Wnt, Cox-2/PGE_2, and PI3K/AKT signaling pathways, which have been suggested to play crucial roles in the initiation and early development of colon cancer (Williams et al., 1999; Giles et al., 2003; Brown and DuBois, 2005; Castellone et al., 2005; Greenhough, et al., 2009) (Figure 1A). We also considered a new signal transduction pathway mediated by RORα that we identified in this study; this new pathway receives extracellular signals from PGE_2 and cross-regulates the Wnt signaling. The binding of PGE_2 to EP receptors was shown to promote colorectal tumorigenesis in APC^{min} mice (Watanabe et al., 1999; Mutoh et al., 2002; Amano et al., 2003; Chang et al., 2004) and rats (Kawamori et al., 2003), and PGE_2 was found to enhance cell proliferation in various colon cancer cell lines, whereas tumor-suppressive and anti-proliferative roles of PGE_2 were found in other experimental conditions (Table 1). These results suggest that an unknown interaction may exist between PGE_2 and Wnt signaling. Recently, Lee et al. (2010) found that Wnt5a/PKCα-dependent phosphorylation of RORα results in the inhibitory regulation of Wnt/β-catenin target genes in colon cancer cells. Because the binding of PGE_2 to EP1 receptors results in the activation of PKC (Negishi et al., 1995; Tanaka et al., 2008), we postulated that PGE_2 increases the phosphorylation of RORα via PKCα and thus inhibits Wnt signaling. To examine this possibility, we overexpressed RORα in colon cancer cells (SW620) and found that expression of Wnt/β-catenin target genes, such as *Cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999; Lee et al., 2010), *Cox-2* (Longo et al., 2002; Araki et al., 2003), and *Axin2* (Yan et al., 2001; Jho et al., 2002; Lustig et al., 2002), was suppressed (Figure 1B, left). RORα was phosphorylated on serine 35 by a constitutively activated PKCα, which is consistent with a previous observation (Lee et al., 2010). This activation of RORα leads to significantly reduced polII occupancy at the promoter region of *Cyclin D1*, *Cox-2*, and *Axin2* (Figure 1B, center). In addition, exogenous PGE_2 induced the phosphorylation of PKCα and RORα, which resulted in a significant

Table 1 Contrasting roles of PGE_2 in the proliferation of colon cancer cells.

Response to PGE_2		Cell-line or tissue	References
Proliferative		HT-29	Qiao et al. (1995) and Pai et al. (2002)
		LS174T, HCA-7	Sheng et al. (2001)
		HCA-7, Intestinal adenoma in $APC^{Min/+}$ mice	Wang et al. (2005)
		Colon tumors in rats	Kawamori et al. (2001)
		Colon carcinoma cell line	Chell et al. (2006b)
		Intestinal adenoma in APC^{Min} mice	Wang et al. (2004)
Proliferative or anti-proliferative		SW116	Qiao et al. (1995)
		HCA-7	Sheng et al. (1998)
		HT-29, Caco-2	Loffler et al. (2008)
		Colon adenoma cell line	Chell et al. (2006b)
Anti-proliferative		HCT116	Sheng et al. (1998)
		HT-29, SW480, SW848, Caco-2	Parker et al. (1997)
		SW480	Loffler et al. (2008)
		Intestinal tumors in $APC^{Min/+}$ mice	Wilson and Potten (2000)

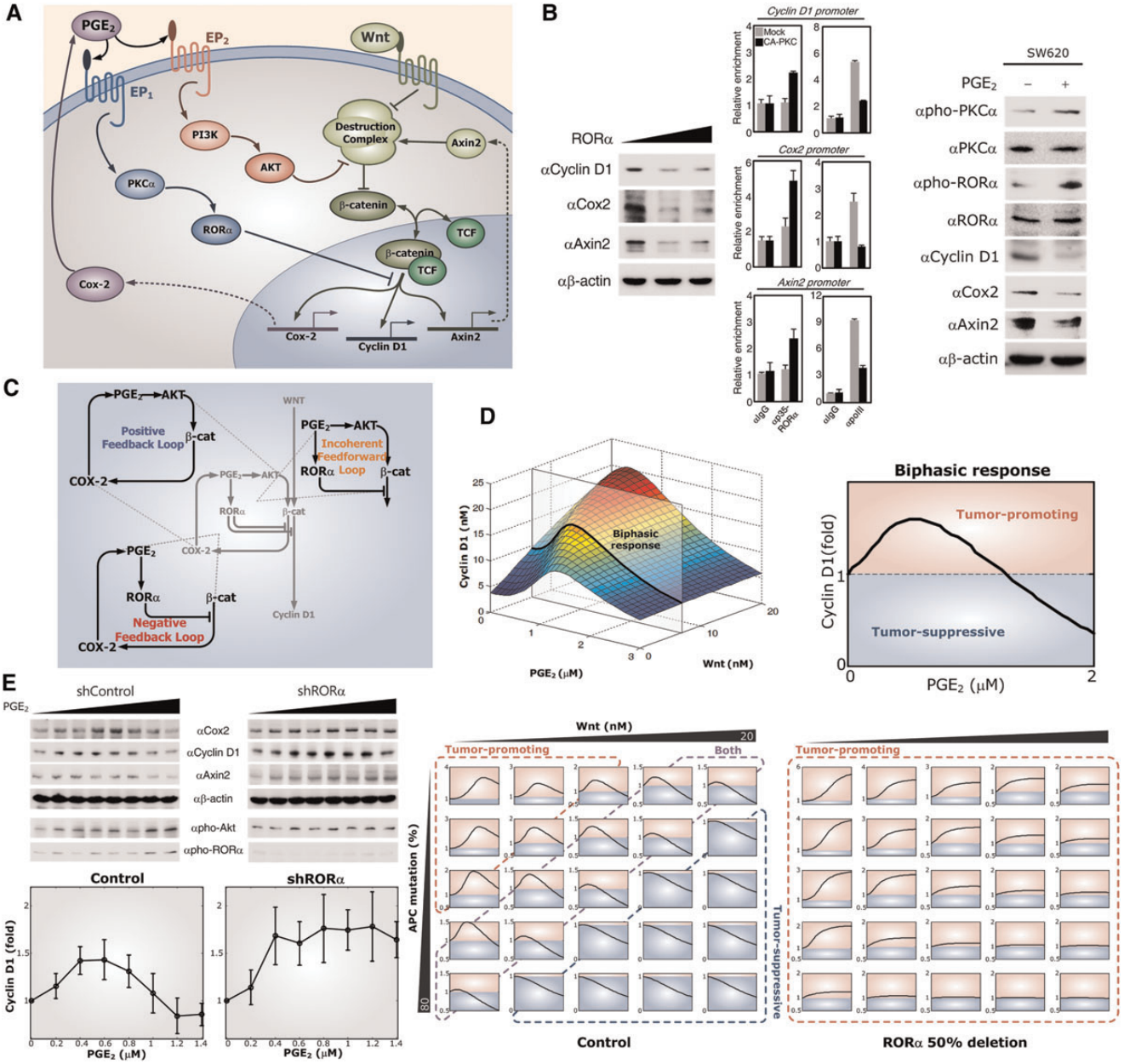


Figure 1 RORα-mediated IFFL in the extended Wnt signaling network induces biphasic responses of Cyclin D1. (**A**) Schematic diagram of the extended Wnt signaling network. (**B**) Left, Suppressed expression of the β-cat/TCF target genes, *Cyclin D1*, *Cox-2*, and *Axin2* arising from the overexpression of RORα. Center, PKCα-dependent phosphorylation of RORα and the subsequent downregulation of *Cyclin D1, Cox-2*, and *Axin2* expression. Constitutively active forms of PKCα (CA-PKC) increased the phosphorylation of RORα on the S35 site and decreased β-cat/TCF target gene expression. Right, Treatment with PGE_2 (1 μM) for 4 h increased the phosphorylation of PKCα and RORα but decreased the expression of Cyclin D1, Cox-2, and Axin2, as assessed by immunoblot analysis. (**C**) The core circuits embedded in the extended Wnt signaling network. (**D**) Top left, Steady state concentration of Cyclin D1 simulated with respect to the Wnt and PGE_2 levels. A cross-sectional view that illustrates the biphasic response to PGE_2 is shown for a fixed value of Wnt stimulation. Top right, A representative biphasic response of Cyclin D1 normalized to the basal Cyclin D1 level before PGE_2 treatment. The changing response profiles of Cyclin D1 to PGE_2 are shown with respect to Wnt stimulation and APC mutation in the control (bottom left) and in the case of a 50% reduction in RORα level (bottom right). APC mutation (%), the quantification of the effect of APC mutation on the destruction complex formation, was implemented in the simulation by changing DC synthesis rate. We further investigated the effect of Axin2-mediated negative feedback on the biphasic response (see Supplementary material for details). (**E**) Immunoblot analysis of Cyclin D1 in the presence (left) or absence (right) of RORα after treatment with elevated concentrations of PGE_2 (from 0 to 1.4 μM) ($n = 3$, error bars represent the SE). Further experiment showed that pho-AKT dominates at an intermediate range of the concentration of PGE_2 whereas pho-RORα dominates at a high range of the concentration of PGE_2 (Supplementary Figure S5).

decrease of Wnt/β-catenin target gene expression (Figure 1B, right). Together, these results confirm that PGE_2 stimulation increases the transrepression activity of RORα through PKCα-mediated phosphorylation and thereby suppresses the expression of Wnt/β-catenin target genes. We further validated the cross-regulation between Wnt and Cox-2/PGE_2 signaling pathways using colon cancer cells. We confirmed that Cox-2 was stabilized by β-catenin/TCF (Supplementary Figure S1) and that Wnt signaling was potentiated by PGE_2 via PI3K/AKT (Supplementary Figure S2). We developed a mathematical model for the extended Wnt signaling network on the basis of these experimental results (see Supplementary material for details). The kinetic parameter values were estimated based on previous results (Hannoush, 2008) as well as our own time course measurements (Supplementary Figures S3 and S4).

A RORα-mediated incoherent feedforward loop functions as a dose-biphasic switch

In this network, we identified two core circuits: an incoherent feedforward loop (IFFL) and coupled positive and negative feedback loops (CPNFL) (Figure 1C). IFFL is subject to positive regulation that is composed of $PGE_2 \rightarrow$ AKT $\rightarrow$ β-catenin and negative regulation that is composed of $PGE_2 \rightarrow$ RORα $\dashv$ β-catenin. CPNFL contain a positive feedback loop that is composed of β-catenin $\rightarrow$ Cox-2 $\rightarrow PGE_2 \rightarrow$ AKT $\rightarrow$ β-catenin and a negative feedback loop that is composed of β-catenin $\rightarrow$ Cox-2 $\rightarrow PGE_2 \rightarrow$ RORα $\dashv$ β-catenin. To determine the role of each circuit in the regulation of Wnt signaling, we investigated the induction of Cyclin D1 when concentrations of PGE_2 and Wnt were varied. We found that the gradual increase of PGE_2 resulted in a biphasic response of Cyclin D1, in which the Cyclin D1 level initially increased and subsequently decreased along with the increase in PGE_2 stimulation (Figure 1D, top left). This biphasic response implies that cell growth would be stimulated at low concentrations of PGE_2 but inhibited at high PGE_2 concentrations because the induction of Cyclin D1 decreases to below its basal level (Figure 1D, top right). Such a switching-off property for high-dose stimulation may change depending on the cellular context, resulting in tumor promotion, tumor suppression, or even both depending on the combinatorial conditions of the Wnt stimulation and the APC mutation (Figure 1D, bottom left). Dose-dependent biphasic responses are often found in biological signaling systems and are known to be induced by incoherent feedforward loops (Kim et al., 2008; Shin et al., 2011). A 50% reduction of RORα, which is the negative regulatory component for IFFL, resulted in the disappearance of biphasic responses and consequently in tumor-promoting effects, which indicates that RORα is responsible for the switching mechanism by which sustained growth at a high level of stimulation can be interrupted (Figure 1D, bottom right). To examine the biphasic response of Cyclin D1 experimentally, we treated SW620 cancer cells with PGE_2 and monitored the induction of Cyclin D1. We observed a clear biphasic pattern in response to PGE_2 (Figure 1E, left) and also found that knock-down of endogenous RORα by shRNA resulted in the disappearance of such biphasic responses (Figure 1E, right), as predicted by the simulation (Figure 1D). The dose-dependent biphasic response is required for biological systems when an output (e.g. cell growth) must respond only to a certain range of input strengths (i.e. switch-on for low doses and switch-off for high doses). The simulations and experiments both demonstrate that IFFL mediated by RORα induces a dose-dependent biphasic response of Cyclin D1, which indicates that RORα-mediated IFFL may play the role of a 'dose-biphasic switch' in cell proliferation. This also elucidates all of the previous contrasting reports on the role of PGE_2 in colorectal tumorigenesis under various circumstances (Table 1). Our results indicate that alterations in biphasic response patterns under different conditions can account for the contrasting roles of PGE_2 in tumorigenesis, depending on the cellular and experimental context.

Bistable switching and hysteresis are induced by positive feedback that is mediated by PI3K and AKT

The extended Wnt signaling network contains CPNFL, where the positive feedback loop is mediated by PI3K and AKT and the negative feedback loop is mediated by RORα. A single positive feedback loop can amplify a signal, create a bistable switch, or enhance the robustness to noise (Brandman and Meyer, 2008; Wang et al., 2010; Kim and Cho, 2012). Additionally, constitutive activation of a positive feedback loop can contribute to the development of tumors by triggering the sustained activation of proliferative signaling (Iliopoulos et al., 2009; Hatziapostolou et al., 2011; Opitz et al., 2011). To explore how PI3K/AKT-mediated positive feedback regulation shapes Wnt signaling and how RORα-mediated negative feedback regulation prevents undesirable activation of Wnt signaling, we measured the induction of Cyclin D1 in response to Wnt stimulation when the following three key components were varied: AKT in the positive feedback loop, RORα in the negative feedback loop, and the DC (destruction complex), which is involved in the APC mutation.

Significant overexpression of AKT was observed in colorectal adenomas and carcinomas, which implies that overexpression of this proto-oncogene may be deeply involved in the initiation of colorectal carcinogenesis (Roy et al., 2002). PI3K/AKT-mediated positive feedback regulation can generate a bistable switching, which allows the system to respond to an input stimulus in an all-or-none manner. When Wnt stimulation increases, the system still resides at the lower branch of the stimulus-response curve, and the activity of Cyclin D1 rises slowly until Wnt stimulation reaches a certain threshold (Figure 2A). Beyond the threshold point, the system state switches to the other branch, where the expression of Cyclin D1 is constitutively active. This bistable switching is accompanied by the hysteretic response to Wnt stimulation along with an increase in the AKT level (Figure 2A). The concentration of Wnt should reach a critical threshold to activate Cyclin D1 from its basal state. However, once activated, Cyclin D1 maintains its active state even if the Wnt concentration falls below the threshold. These results suggest that the PI3K/AKT-mediated positive feedback loop forms an internal 'hysteretic switch' in the regulation of the proliferative signal (see Supplementary material for details).

In addition to the overexpression of AKT, APC mutation is also known as an early event in the initial phase of colorectal

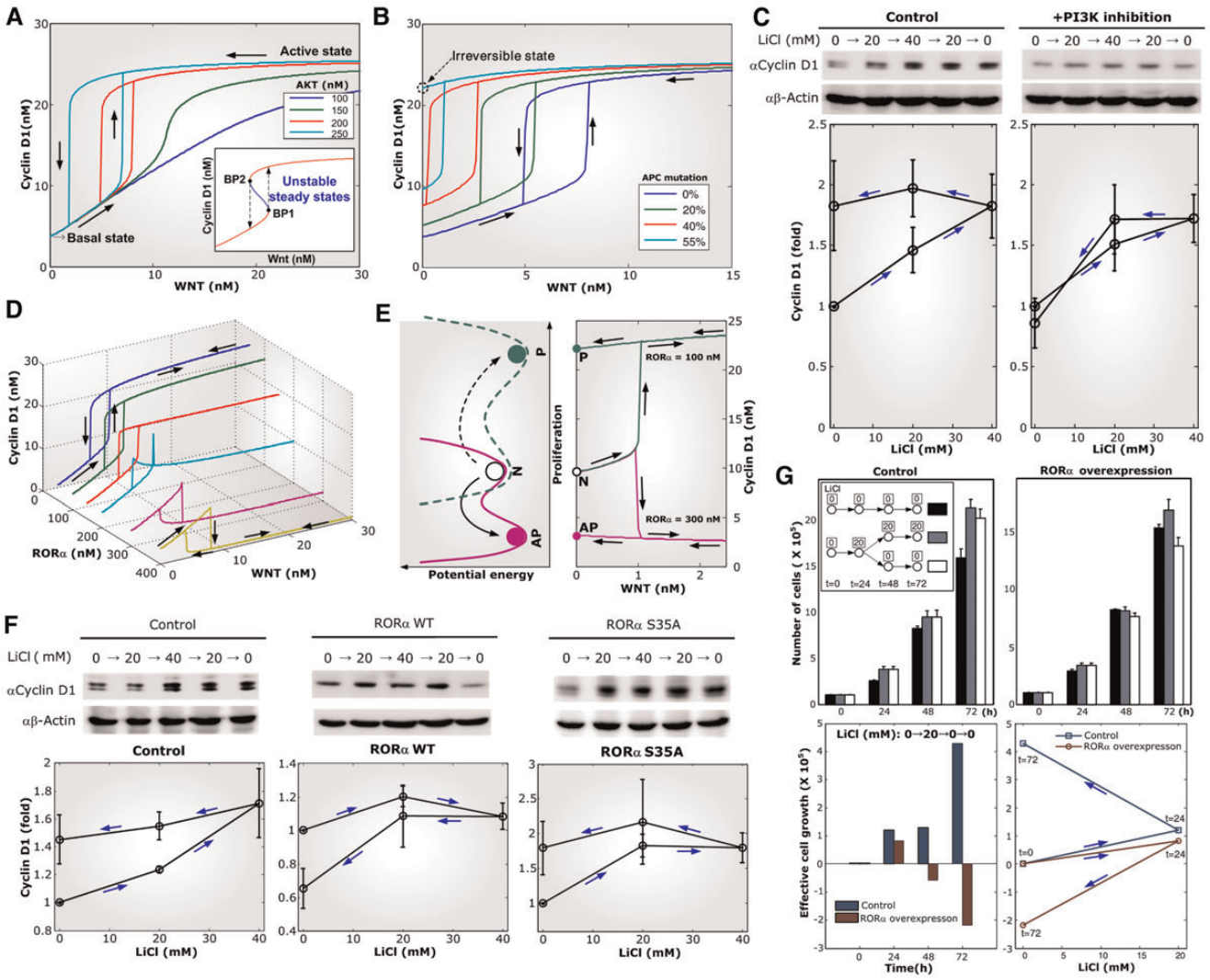


Figure 2 RORα-mediated CPNFL induces the anti-proliferative state transition. (**A**) Hysteretic responses of Cyclin D1 to Wnt stimulation depending on AKT concentration. Saddle-node bifurcation points (BP1 and BP2) and the unstable steady states are indicated in the inset. Dashed lines with vertical arrows represent imaginary response curves (see Supplementary material for bifurcation analysis of the hysteresis as well as a general introduction to the hysteretic phenomena in biological systems). (**B**) The curve for the hysteretic response of Cyclin D1 to Wnt shifts to the left in the presence of an increase in APC mutations, which leads to an irreversible state. (**C**) Irreversible hysteretic behavior of Cyclin D1 in response to LiCl in the control (left) and upon treatment with a PI3K inhibitor (right) ($n = 3$, error bars indicate the SE). (**D**) Various profiles for the hysteretic response of Cyclin D1 to Wnt stimulation concomitant with an increase in RORα. We further confirmed that the inversion of hysteresis by RORα is well conserved under global parameter perturbation (Supplementary Figure S7). (**E**) Comparison of two irreversible hysteretic curves of Cyclin D1 for RORα concentrations of 100 and 300 nM (right). N, P, and AP indicate nominal, proliferative, and anti-proliferative states, respectively. The irreversible hysteretic switching between different proliferative states can be interpreted on the conceptual potential energy landscape (left). (**F**) Comparison of irreversible hysteretic responses of Cyclin D1 in the control (left), in cells transfected with RORα (0.2 μg) (center), and in cells transfected with RORαS35A (1.0 μg) (right) ($n = 3$, error bars represent the SE). (**G**) Cell proliferation with or without LiCl in the control (top left) and in cells transfected with RORα (0.5 μg) (top right). Cells were treated with LiCl (20 mM) initially, followed by washing at 24 h, and were re-incubated for another 48 h with (grey bar) or without (white bar) LiCl (20 mM). The black bar indicates the basal cell growth over 3 days in the absence of LiCl ($n = 3$, error bars represent the SD). The effective cell growth (for the white bar) was obtained by subtracting the basal cell growth (bottom left). This temporal histogram was redrawn as the stimulus-response plot (bottom right) (0, 24, and 72 h were used).

carcinogenesis (Janne and Mayer, 2000; Chell et al., 2006a). To explore the effect of hysteretic switching on cell proliferation under pathological conditions, we examined all of the possible changes in the hysteretic response of Cyclin D1 along with an increase in the APC mutation. The APC mutation promoted the accumulation of nuclear β-catenin, thereby lowering the critical threshold of the Wnt concentration that is required to activate Cyclin D1 (Figure 2B). Intriguingly, we found that a sufficient degree of APC mutation can make the switching process of Cyclin D1 irreversible. Thus, the Cyclin D1 level does not completely

return to its initial state and remains in the active state even if the Wnt stimulus is removed. We further examined these simulation results experimentally by using colon cancer cells (SW620) that contain mutated APC (Garnett et al., 2012). We found that increasing and subsequently decreasing Wnt signaling that was controlled by LiCl (GSK3 inhibitor) led to irreversible hysteretic behavior of Cyclin D1 (Figure 2C, left), which is consistent with our simulation predictions (Figure 2B). To investigate whether such hysteretic behaviors are associated with the PI3K/AKT-mediated positive feedback loop, we treated the SW620 cells with a PI3K inhibitor and observed the disappearance of hysteresis and irreversibility (Figure 2C, right). We further observed the absence of both hysteresis and irreversibility in DLD-1 cells where Cox-2 is not stabilized and thereby the PI3K/AKT-mediated positive feedback loop is not present (Supplementary Figure S6). These results suggest that a synergetic effect of positive feedback and APC mutations may result in the sustained expression of the proto-oncogene, Cyclin D1, by inducing its irreversible hysteretic behavior.

RORα-mediated negative feedback regulation induces hysteresis inversion, which leads to an anti-proliferative state

We investigated the role of RORα-mediated negative feedback in the regulation of irreversible hysteretic switching. We found that augmentation of RORα not only limits the maximal Cyclin D1 level but also weakens the hysteretic behavior of Cyclin D1 by suppressing the positive feedback effect (Figure 2D). Interestingly, we found that the counterclockwise hysteresis loop disappears along with the increase in RORα and a clockwise hysteresis loop then emerges. We also found that this newly emerged hysteresis has a critical effect on the irreversibility of the Cyclin D1 state under pathological conditions. With APC mutations, the Cyclin D1 level decreases to a low inactive state for RORα-mediated strong inhibition (RORα = 300 nM) and is maintained at this inactive state even after withdrawal of the stimulus (Figure 2E, right). This result is quite different from the counterclockwise hysteresis observed for low RORα (RORα = 100 nM). This phenomenon can be explained according to the concept of a potential energy landscape, as illustrated in Figure 2E (left), where the horizontal axis represents the putative potential energy of the molecular interaction network and the vertical axis denotes the proliferation level. For a low level of RORα, the sustained proliferation state (P) is deeper than the nominal state (N), which implies that the cell is more likely to remain at the sustained proliferation state. However, the overexpression of RORα makes the anti-proliferative state (AP) deeper than the nominal state, which prohibits a cellular state transition from the nominal state to the sustained proliferative state. Therefore, the negative feedback regulation mediated by RORα not only decreases the Cyclin D1 level but also inhibits any possible transition from a basal state to the sustained active state under pathological conditions. Our results suggest that RORα-mediated CPNFL may function as a 'hysteresis-inversion switch' in the regulation of cell proliferation because overexpression of RORα inverts the hysteretic behavior of Cyclin D1 and thus induces the switching from the sustained proliferative state to the anti-proliferative state. This hysteresis-inversion switching was observed in our experiments in colon cancer cells (SW620) that were treated with LiCl. We found that the control exhibited the irreversible counterclockwise hysteretic response of Cyclin D1 (Figure 2F, left), whereas the overexpression of RORα resulted in clockwise hysteretic behavior of Cyclin D1 (Figure 2F, center), as predicted by the simulation. Moreover, cells that were transfected with a RORαS35A mutant (Lee et al., 2010), in which the serine residue was mutated to alanine to block the PKCα-induced phosphorylation of RORα, showed counterclockwise hysteresis similar to the control (Figure 2F, right). These results indicate that RORα is the hidden regulator that controls the sustained activation of positive feedback under pathological conditions and thus drives the system toward the anti-proliferative state rather than the sustained proliferative state.

To further confirm the role of RORα as a hysteresis-inversion switch at the cell population level, we investigated the proliferation of cancer cells with or without LiCl by counting the cells. We found that the proliferation of cancer cells after the removal of the LiCl stimulus was not different from the proliferation of cancer cells that were re-incubated with LiCl (20 mM), which indicates that the cells remained in the proliferative state even after the stimulus was removed (Figure 2G, top left). However, the overexpression of RORα was found to result in a significant decrease in proliferation after the withdrawal of LiCl relative to cells that were re-incubated with LiCl, which suggests that the overexpression of RORα inhibits the transition of cells to the sustained proliferative state (Figure 2G, top right). Such a difference is evident if we eliminate the basal growth level of the cancer cells and consider only LiCl-induced cell growth, where the control showed a net increase in proliferation, whereas the overexpression of RORα resulted in a decrease in proliferation when the stimulus was removed (Figure 2G, bottom left). This temporal histogram can be converted to a stimulus-response graph (Figure 2G, bottom right) that shows the same result, as predicted by the simulation (Figure 2E). Thus, we conclude that RORα is the critical regulator that underlies the hysteresis-inversion switch by which the sustained proliferative state under pathological conditions can be converted into the anti-proliferative state.

Phase plane analysis revealing anti-proliferative state transitions

In the foregoing analysis, we found that three major players, i.e. AKT, RORα, and APC, have a crucial role in regulating the expression of Wnt/β-catenin target genes, including *Cyclin D1*, through hysteresis. To quantitatively analyze their roles in determining hysteresis, we defined two measures for Cyclin D1: the irreversibility (ΔCyclin D1) and the mean induction level (<Cyclin D1>). By analyzing these measures with respect to AKT, APC mutation, and RORα, we found that the expression patterns of Cyclin D1 can be categorized into three phases: Phase I, a reversible state with a low Cyclin D1 level; Phase II, an irreversible state with a high Cyclin D1 level; and Phase III, a reversible state with a high Cyclin D1 level (Figure 3A). ΔCyclin D1 = 0 in Phases I and III indicates a reversible state, whereas ΔCyclin D1 > 0 in Phase II denotes an irreversible state because of the counterclockwise hysteresis (Figure 3A, top and Figure 2B). Phases I and III are discriminated if <Cyclin D1> is considered (Figure 3A, bottom). We further investigated the influence of RORα on this phase distribution. As shown previously, overexpression of RORα inhibits Cyclin D1 transcription and also gives

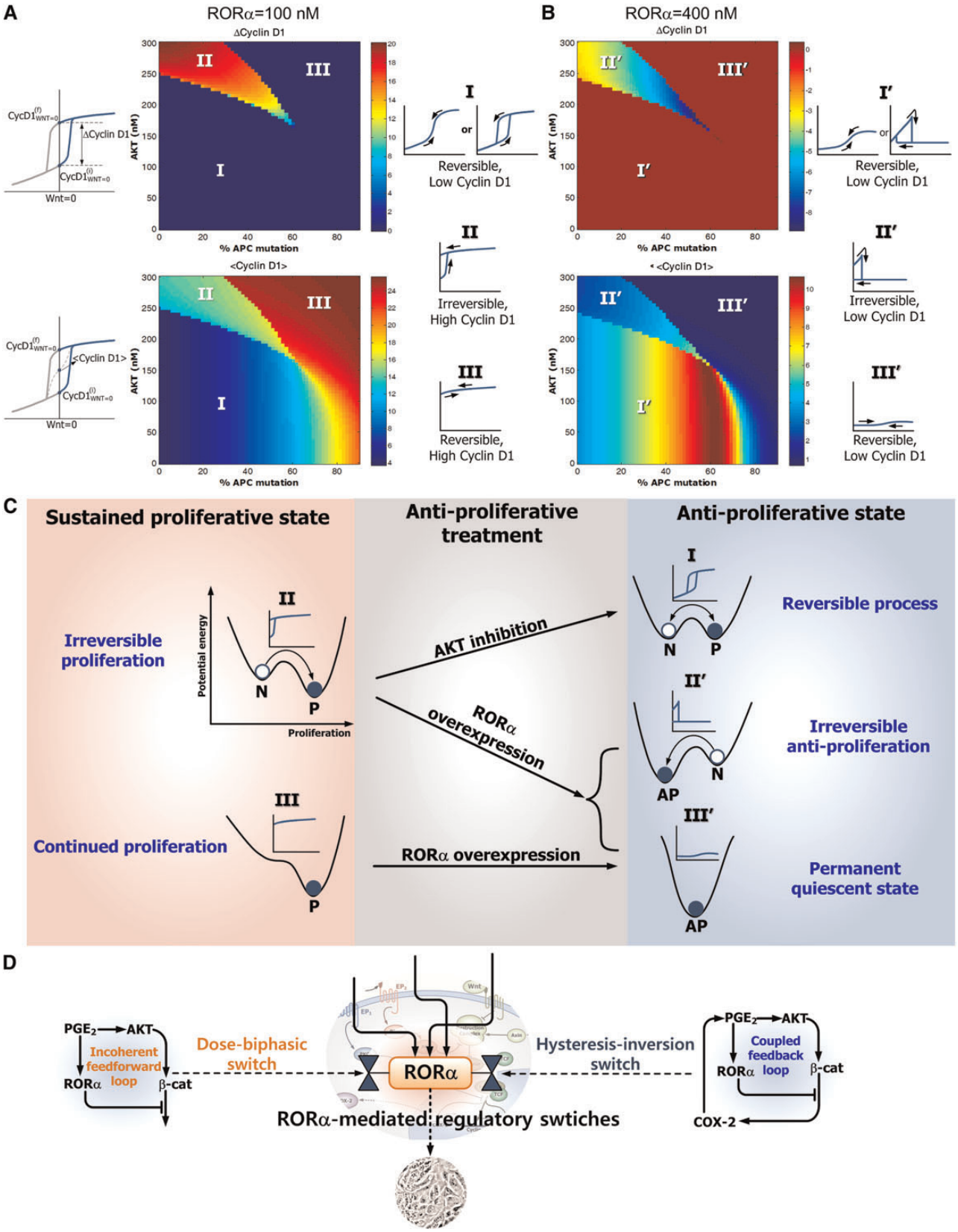


Figure 3 Phase plane analysis reveals all of the plausible state transitions for anti-proliferation with respect to AKT, APC mutation, and RORα. Phase diagrams of Cyclin D1 expression patterns with respect to the AKT level and APC mutations for low (100 nM) (**A**) and high (400 nM) (**B**) concentrations of RORα. ΔCyclin D1 and <Cyclin D1> were defined by $CycD1^{fin}$-$CycD1^{ini}$ and $(CycD1^{fin} + CycD1^{ini})/2$, respectively, when Wnt = 0.

rise to the clockwise hysteretic response of Cyclin D1 to Wnt stimulation. Thus, the Cyclin D1 level was found to be suppressed throughout the whole range of AKT levels and APC mutations in this case (Phase I', II', and III' in Figure 3B). Furthermore, the emergence of clockwise hysteresis leads to a lower final steady state level of Cyclin D1 relative to the initial level (Phase II'), in contrast to Phase II, in which $CycD1^{fin} > CycD1^{ini}$. A cancerous state can be represented by an irreversible proliferative state (Phase II) that is caused by the synergistic effect of positive feedback mediated by Cox-2/PGE_2/AKT signaling and APC mutations or a continued proliferative state (Phase III) under an extremely high level of APC mutation. Using this phase diagram, we can determine efficient anti-cancer therapeutic strategies depending on the pathological conditions represented by molecular states. For example, an irreversible proliferative state in Phase II can be converted to a reversible state in Phase I by using an AKT inhibitor, or it can be converted to either an irreversible anti-proliferative state in Phase II' or a permanent quiescent state in Phase III' by overexpressing RORα (Figure 3C). Although an AKT inhibitor can be used as an anti-proliferative agent, we predict its limited efficacy when the molecular network is at a continued proliferative state in Phase III. Moreover, the phase diagram shows that an AKT inhibitor can only reduce the probability that the cells stay at a proliferative state; in contrast, the overexpression of RORα can further induce a transition toward an anti-proliferative state (Figure 3C). Therefore, RORα might be a promising therapeutic target because it can convert an aberrantly activated state to an anti-proliferative state.

Discussion

In this study, we found that RORα attenuates Wnt target gene expression by PGE_2/PKCα-dependent phosphorylation in colon cancer cells, and that RORα-mediated switches are embedded in the molecular regulatory network of Wnt signaling in the form of the dose-biphasic switch implemented by IFFL combined with the hysteresis-inversion switch by CPNFL (Figure 3D). Our study expounds the contrasting roles of PGE_2 in colorectal tumorigenesis and also provides a conceptual framework that can be used to explore new druggable targets in consideration of the complex molecular interaction networks. The development of targeted therapies for cancer has been accelerated in recent years, but the efficacy of these therapies is still often limited because of the complicated interactions of the target molecule with many other molecules and the resulting unknown dynamic changes of the molecular interaction network. Therefore, a system-level understanding of the regulatory mechanism of oncogenic signaling networks is crucial to identify an optimal target for cancer therapy (Assmus et al., 2006; Won et al., 2012). Our study shows that the regulatory switches formed by RORα-mediated circuits are critical in regulating abnormal cell proliferation in colorectal tumorigenesis and therefore that RORα could be a promising target because it suppresses the Wnt target gene expression and also converts the sustained proliferative state into an anti-proliferative state. This result agrees with recent attempts to develop anti-cancer drugs that target nuclear receptors (Gelmann, 2002; D'Errico and Moschetta, 2008; Jeong et al., 2010; Tang and Gudas, 2011; Wang et al., 2012; Xiong et al., 2012). Our study also suggests that a combined therapy that inhibits PI3K/AKT signaling and increases the phosphorylation of RORα may have a synergetic effect that can provide new therapeutic possibilities. Choi et al. (2012) identified an optimal combined therapy that switches a proliferative state into an apoptotic state by perturbing the molecular interaction network of p53 in breast cancer cells, which also adds another possibility to our network-level targeted approach. As omics data become more available because of the development of high-throughput technologies, network information will accumulate, and the proposed approach will become more widely applicable to further expanded network models.

Materials and methods

Selection of cell lines

To select appropriate cell lines for this study, we screened a number of available colon cancer cell lines, such as COLO201, COLO205, SW480, SW620, LOVO, WIDR, and DLD-1. Among those, RORα-mediated *Cyclin D1* repression was found in SW620 and DLD-1 (Supplementary Figure S8A and B). Cox-2 was stabilized in SW620 cells (Supplementary Figure S1) whereas it was not detected in DLD-1 cells (Supplementary Figure S8C). These results imply that SW620 cells possess all the components of the extended Wnt signaling network that we are going to investigate. So, SW620 cells were chosen for this study (see Supplementary material for further discussion on the selection of cell lines and *in vivo* experiment).

Materials and reagents

The following antibodies were purchased from Santa Cruz Biotechnology: anti-AKT, Cyclin D1, PKCα, phospho-PKCα, GSK3β, Lamin A/C, and RORα. The following commercially available antibodies were used: anti-β-actin (AbFrontier); anti-phospho-AKT, phospho-GSK3β, Axin2, and Cox-2 (Cell Signaling). Anti-phospho-RORαS35 antibody was generated by Abmart. PGE_2 (Prostaglandin E2) and LiCl (Lithium Chloride) were purchased from Sigma-Aldrich.

RNA interference by shRNA or siRNA

The target sequences of siRNA against β-catenin, shRNA against RORα, and nonspecific (NS) shRNA were as follows: siβ-catenin,

Reversibility was characterized by ΔCyclin D1 = 0 (irreversible otherwise). The expression patterns of Cyclin D1 were categorized into three phases, according to ΔCyclin D1 and <Cyclin D1>, and representative expression patterns are shown in the right panel. (**C**) Conceptual potential energy landscapes that illustrate the state transitions mediated by AKT inhibition or overexpression of RORα. There are three types of stable states: a nominal state with a basal Cyclin D1 level (N), a proliferative state with a high Cyclin D1 level (P), and an anti-proliferative state with a low Cyclin D1 level (AP). (**D**) A conceptual diagram of the RORα-mediated regulatory switches that critically regulate cell proliferation (see Supplementary material for further discussion on the coordinated effect of both switches on the biphasic and hysteretic responses of Cyclin D1).

5′-GUCCUGUAUGAGUGGGAAC-3′; shRORα-1, 5′-CGGUGCGCAGAC AGAGCUAUU-3′ (Kim et al., 2005); shRORα-2, 5′-GAGGUAUCUC AGUAACGAAGA-3′; and shNS, 5′-CUGGACUUCCAGAAGAACAUC-3′ (Lee et al., 2010).

Immunoblotting and image analysis

Proteins were isolated by lysing cells in a solution containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors. Extracts were incubated in sodium dodecyl sulfate gel loading buffer containing 5% β-mercaptoethanol at room temperature for 10 min before being loaded on the gel. Images were acquired using a LAS-4000 mini chemiluminescence imager (FUJIFILM), and bands were quantified by densitometry with the Multi Gauge software (FUJIFILM) according to the manufacturer's instructions.

Cell culture and transfections

All cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 25 units/ml penicillin, and 25 units/ml streptomycin. Cells were transfected with the appropriate shRNAs using Lipofectamine Plus reagent (Invitrogen).

ChIP and qPCR

The ChIP assays were conducted as previously described (Baek et al., 2002; Kim et al., 2005). Quantitative PCR (qPCR) was used to measure the enrichment of bound DNA and the value of enrichment was calculated by relative amount to input and ratio to IgG. All reactions were performed in triplicates. The following primers were used: promoter region of *Cyclin D1* forward 5′-GGATGGCTTTTG GGCTCTGC-3′, reverse 5′-ACTCCCCTGTAGTCCGTGTG-3′; promoter region of *Axin2* forward 5′-CTGGAGCCGGCTGCGCTTTGATAA-3′, reverse 5′-CGGCCCCGAAATCCATCGCTCTGA-3′; promoter region of *Cox2* forward 5′-GGGTGAAGGTACGGAGAACAG-3′, reverse 5′-TGAC GACGCTTAATAGGCTGTA-3′.

Hysteresis assay

For hysteresis assay, the protein level of Cyclin D1 was evaluated at sequential time points as follows. The day before preparation of starting point, the same number of cells was seeded in growth media without LiCl. After harvest of starting sample (point 1), rest of cells were cultured in 20 mM of LiCl for 4 h (point 2) and then 40 mM for another 4 h (point 3). After exchange with fresh media, rest of cells were incubated with 20 mM for 4 h (point 4) and then without LiCl for another 4 h (point 5). The average amount of Cyclin D1 was calculated by three independent experiments. We confirmed that β-catenin and CyclinD1 accumulate enough in four hours and maintain their steady state levels until two more hours (Supplementary Figure S9).

Cell growth assay

The proliferation of cells with or without LiCl was evaluated by cell-count using hemocytometer. 1×10^5 cells per well were seeded in 12-well plates and counted at each 12 h for 3 days. For the condition of hysteresis, cells were allowed to grow with LiCl (20 mM) for 24 h followed by wash and re-incubation with or without LiCl for another 48 h. The differences of growth rate were determined by the increased cell numbers compared to control. The number of cells at each time point was obtained through three independent assays.

Mathematical modeling

We developed an extended mathematical model of the Wnt signaling network including four major signaling pathways: Wnt, Cox-2/PGE_2, PI3K/AKT, and PKCα/RORα (Figure 1). All interactions among the signaling molecules were identified by extensive survey of previous experiments as well as our own experiments. When we construct the network model, we did not attempt to describe the exact *in silico* replica of all biochemical species and their interactions, which would be impractical (Borisov et al., 2009). Rather, we reconstructed a minimal essential model of the Wnt signaling network by including only the essential components of the network in view of our modeling purpose (see Supplementary material for further details on the mathematical model and discussion on possible hidden variables in the model).

Parameter estimation

Our extended Wnt signaling network model consists of 11 state variables and 46 kinetic parameters. The kinetic parameter values were estimated based on the previous (Hannoush, 2008) as well as our own time course measurements (Supplementary Figures S3 and S4) by using the genetic algorithm (GA) implemented in Matlab Toolbox™ (see Supplementary material for details).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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