Chapter III. EGF and Wnt 3a differentially regulate Tcf/Lef transcription with implications for tumor development

Abstract

Tcf/Lef-mediated transcription plays a prominent role in development and oncogenesis. While Wnt ligands are the classical agonists of Tcf/Lef signaling, epidermal growth factor (EGF) also stimulates Tcf/Lef transcriptional activity with potential implications for breast cancer development. However, the mechanisms by which EGF stimulates Tcf/Lef signaling and the potential crosstalk between EGF and Wnt are poorly understood. Here, we demonstrate that EGF and Wnt 3a tap distinct sub-pools of β-catenin to stimulate Tcf/Lef transcriptional activity, even though both factors rely on some common intermediate signals (Erk and PKC). While Wnt 3a operates by the canonical β-catenin stabilization pathway, EGF utilizes a siRNA-resistant sub-pool of β-catenin in a Src-dependent manner. We further show that these distinct sub-pools of β-catenin provide independent and additive contributions to Tcf/Lef signaling when cells are co-stimulated with EGF and Wnt 3a. These results suggest therapeutic strategies to selectively target EGF and Wnt contributions to Tcf/Lef signaling.

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1. Introduction

The Wnt family of ligands regulates both embryonic and adult development in several tissues, including the gastrointestinal tract, the neural crest and the mammary gland (Hatsell et al., 2003; Ille and Sommer, 2005). Furthermore, hyperactivation of Wnt-mediated signaling plays a key role in the development of cancers of the colon, breast, and prostate (Polakis, 2000). The broad role of Wnt in development and cancer stems from its ability to coax an otherwise structural protein, β-catenin, into inducing the expression of target genes within the nucleus.

β-catenin is a 90 kDa proto-oncogene that mediates intercellular adhesion by bridging transmembrane cadherin proteins to the actin cytoskeleton. Canonical Wnt ligands, including Wnt 3a, induce developmental cues through transcription of gene targets by nuclear β-catenin, which forms a bipartite transcription factor with the Tcf/Lef family of proteins. A key aspect of transcriptional activation by Wnts is the tight regulation of β-catenin stability. In the absence of Wnt ligands, β-catenin is phosphorylated on N-terminal serine and threonine residues by a multiprotein complex consisting of axin, APC, and glycogen synthase kinase 3β (GSK3β). Phosphorylated β-catenin is then ubiquitinated and subsequently degraded by the proteasome. Upon binding to the co-receptor complex of Frizzled (Frz) and LRP 5/6, canonical Wnt ligands inhibit the β-catenin degradation complex, allowing accumulation of cytoplasmic β-catenin. Stabilized β-catenin then translocates to the nucleus and, in partnership with Tcf/Lef transcription factors and a multitude of co-activators, catalyzes transcription of oncogenes such as *cyclin D1* and *c-myc* (He et al., 1998; Tetsu and McCormick, 1999).

Mutations that impair the β-catenin degration complex stabilize β-catenin and permit constitutive transcription of Tcf/Lef gene targets in several cancer types (Polakis, 2000). Interestingly, similar mutations are not found in human breast cancers even though hyperactive β-catenin signaling is observed in approximately 60% of cases (Lin et al., 2000). Thus, the mechanistic basis of hyperactive β-catenin signaling in human breast cancer remains unclear. Recent evidence suggests that epidermal growth factor (EGF) signaling may be involved. We and others have shown that EGF activates Tcf/Lef-mediated transcription in a variety of cell systems (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002). Furthermore, Wnt 1 and Wnt 3 can cooperate with EGFR ligands such as TGFα to initiate neoplastic progression in murine mammary glands, although the mechanism is not known (Schroeder et al., 2000). In addition, Wnt-mediated tumorigenesis in both murine models and humans may require association of β-catenin with EGFR (Schroeder et al., 2002).

These reports suggest the intriguing possibility that Wnt and EGF may co-regulate Tcf/Lef signaling in physiological contexts such as human breast cancer and have raised several important questions. What are the mechanisms by which EGF stimulates Tcf/Lef transcriptional activity? Do EGF and Wnt operate through the same canonical pathway, or do these factors utilize complementary and distinct mechanisms? Are the pathways configured such that EGF and Wnt work together to stimulate Tcf/Lef signaling to a level beyond what each factor promotes by itself? To address these questions, we sought to delineate the intracellular signaling pathways by which EGF

stimulates Tcf/Lef signaling and to quantify the coordinate effects of EGF and Wnt on Tcf/Lef signaling.

2. Results

2.1. EGF activates Tcf/Lef transcriptional activity in 293T-EGFR cells.

To investigate the possibility of EGF/Wnt crosstalk in Tcf/Lef signaling, we used the HEK-293T cell line, in which both EGF (Lu et al., 2003) and canonical Wnts (Chen et al., 2000) induce Tcf/Lef transcriptional activity. Because HEK-293T cells express low levels of endogenous EGFR (Johns et al., 2004), cells were transfected with a plasmid encoding human EGFR or an empty control vector. As expected, EGF treatment of empty vector transfectants (293T-LPCX) yielded minimal tyrosine phosphorylation (Figure III-1A). Meanwhile, EGF treatment of 293T cells expressing exogenous EGFR (293T-EGFR) stimulated robust tyrosine phosphorylation near the molecular weight of EGFR (~180 kDa).

Using the Tcf/Lef-dependent reporter TOPFLASH, which contains consensus Tcf/Lef binding sites upstream of the *luciferase* gene (Korinek et al., 1997), we monitored Tcf/Lef transcription in response to EGF stimulation. In 293T-EGFR cells, EGF stimulated TOPFLASH reporter activity within 6 h of stimulation; by 12 h, the reporter response had increased ~ 3-fold above its initial basal level (Figure III-1B). In contrast, EGF did not activate the TOPFLASH reporter in 293T-LPCX cells, consistent with the inability to generate a measurable phospho-tyrosine response. As a second

negative control, we employed the FOPFLASH reporter, which carries mutated Tcf/Lef binding sites. EGF treatment yielded a significantly weaker signal from the FOPFLASH negative control than the TOPFLASH reporter (Figure III-1C). Furthermore, EGF-mediated Tcf/Lef signaling was dose-dependent, reaching maximal response near a dosage of 40 ng/ml (Figure III-1D).

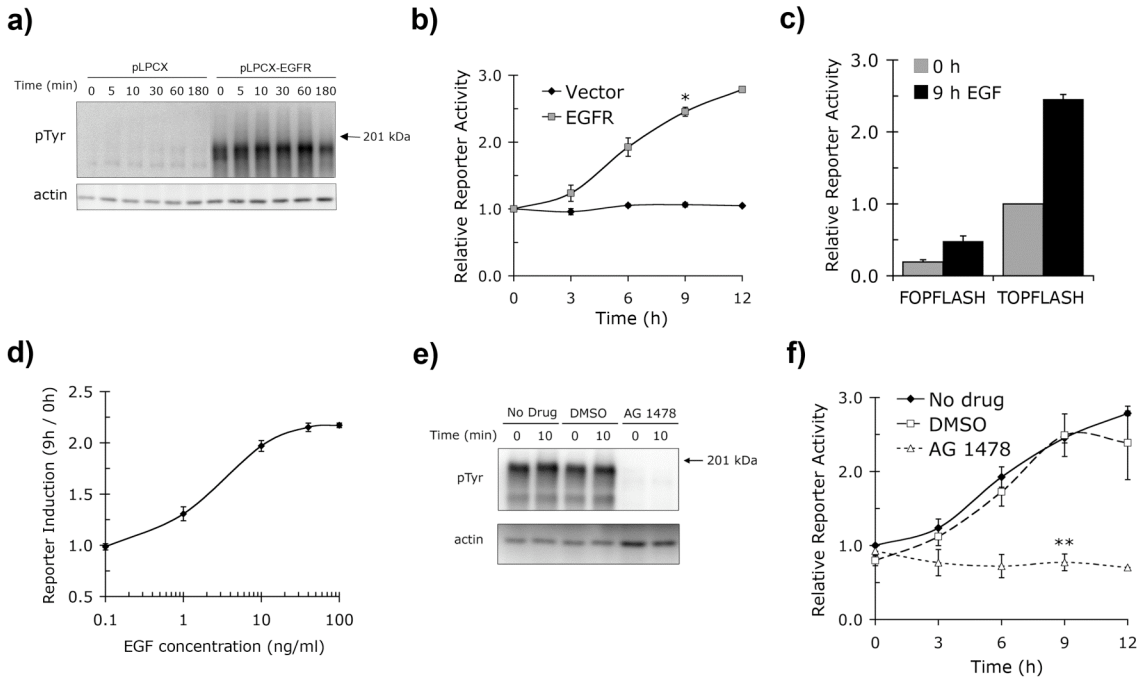

Figure III-1. EGF induces Tcf/Lef transcriptional activity in 293T-EGFR cells.
a) 293T cells were transfected with pLPCX-EGFR or the empty vector pLPCX, serum-starved and then stimulated with EGF (40 ng/ml) before lysis at the indicated times. Whole cell lysates were immunoblotted for phospho-tyrosine and actin.
b) TOPFLASH reporter activity was quantified at the indicated times after stimulation of serum-starved 293T-LPCX or 293T-EGFR cells with EGF. The *asterisk* denotes $P < 0.01$ comparing reporter activity at 0 and 9 h in 293T-EGFR cells.
c) 293T-EGFR were transfected with either TOPFLASH or the negative control FOPFLASH, serum-starved, stimulated with EGF, and the reporter activity was quantified.
d) 293T-EGFR were stimulated with varying concentrations of EGF, lysed at 0 h and 9 h following stimulation, and the extent of TOPFLASH reporter induction (9h / 0h) is reported.
e) After pre-treatment with AG 1478 (5 μM) or the solvent DMSO, serum-starved 293T-EGFR were stimulated with EGF (40 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for phospho-tyrosine and actin.
f) 293T-EGFR pre-treated with AG 1478 or the solvent DMSO were stimulated with EGF before lysis at the indicated times and quantification of TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.001$ comparing reporter activity at 9 h in the absence and presence of AG 1478.

To further demonstrate the specific requirement for EGFR in EGF-induced Tcf/Lef transcriptional activity, we treated cells with AG 1478, a pharmacological inhibitor of EGFR kinase activity, effectively blocking EGFR tyrosine phosphorylation (Figure III-1E). In the presence of AG 1478, the EGF-mediated TOPFLASH response was ablated in 293T-EGFR cells (Figure III-1F). Meanwhile, the solvent control had no effect, demonstrating a strict requirement for EGFR kinase activity in EGF-mediated Tcf/Lef transcription.

2.2. EGF and Wnt 3a additively activate Tcf/Lef transcription.

Having quantified the magnitude and kinetics of EGF-mediated Tcf/Lef transcriptional activity, we sought to compare and contrast signaling by this non-Wnt ligand to the canonical Wnt pathway. Stimulating 293T-EGFR with recombinant mouse Wnt 3a provoked a ~ 2.5-fold increase in TOPFLASH reporter activity within 9 h (Figure III-2A). Notably, the magnitude and kinetics of Wnt 3a-mediated TOPFLASH activity were similar to that of EGF. As negative controls, we verified that Wnt 3a did not activate the FOPFLASH reporter (Figure III-2B) and that recombinant mouse Wnt 5a, a non-canonical Wnt ligand (Weeraratna, 2005), did not induce TOPFLASH reporter activity (Figure III-S1).

Because EGF and Wnt 3a both activate the TOPFLASH reporter with similar magnitude and dynamics, we asked whether these two ligands could cooperate to induce Tcf/Lef reporter activity in an additive or synergistic fashion. Indeed, co-stimulation of 293T-EGFR cells with EGF and Wnt 3a provoked a stronger reporter response than

either ligand alone (Figure III-2C). In fact, adding the reporter response from each individual ligand closely matches the data from co-stimulated cells, demonstrating that EGF and Wnt cooperate in an additive manner. Synergistic activation of the TOPFLASH reporter was never observed, even at sub-saturating doses of EGF and Wnt 3a (data not shown).

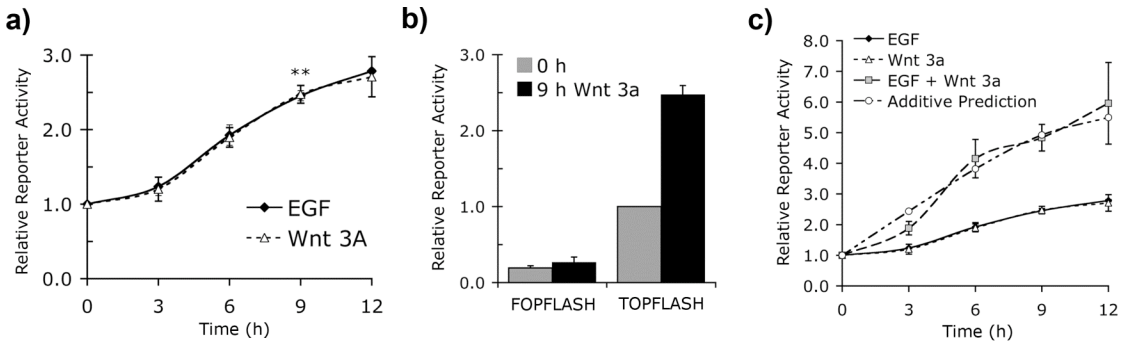

Figure III-2. Wnt 3a and EGF cooperate to activate Tcf/Lef transcriptional activity.
a) 293T-EGFR cells were serum-starved, stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml) and then lysed at the desired times before quantification of TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.001$ in comparing reporter activity between 0 and 9 h for the Wnt 3a-mediated response.
b) 293T-EGFR transfected with either TOPFLASH or the negative control FOPFLASH were serum-starved, stimulated with Wnt 3a, and lysed at the desired times before quantification of the reporter activity.
c) Serum-starved 293T-EGFR were stimulated with either EGF, Wnt 3a, or EGF plus Wnt 3a, and the TOPFLASH reporter activity was quantified at the indicated times. *Additive prediction* denotes the sum of the reporter activities induced by EGF and Wnt 3a alone.

2.3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms.

Because EGF and Wnt 3a additively activate Tcf/Lef transcriptional activity, it raises the possibility that EGF and Wnt 3a function through divergent mechanisms. To test this hypothesis, we determined whether only Wnt 3a, or both Wnt 3a and EGF, induce Tcf/Lef transcriptional activity via the canonical Wnt mechanism. Since serine 9 phosphorylation of GSK3β can inhibit kinase activity towards primed substrates like β-catenin (Shaw and Cohen, 1999), we examined the effect of EGF and Wnt 3a treatment on GSK3β serine phosphorylation. Western blotting demonstrated that neither EGF nor

Wnt 3a increased levels of phospho-serine 9 (Figure III-S2), suggesting EGF and Wnt 3a do not regulate GSK3β activity through inhibitory phosphorylation in 293T-EGFR. However, inferring effects on β-catenin stability based on GSK3β phosphorylation is somewhat tenuous, since Wnt may function independently of GSK3β phosphorylation (Ding et al., 2000; McManus et al., 2005).

To more directly assess β-catenin stability, we measured the effect of Wnt 3a or EGF on the pool of β-catenin that is not associated with the transmembrane glycoprotein E-cadherin, and therefore, available for nuclear translocation. We assayed this "free" pool of β-catenin using an established technique based on pre-clearing of E-cadherin and other glycoproteins from cell lysates using beads coated with concanavalin A (ConA), a lectin that binds glycoproteins with high affinity (Fagotto et al., 1999). Following Wnt 3a stimulation, the level of free β-catenin was substantially increased (Figure III-3A), confirming that Wnt 3a does inhibit β-catenin degradation. As a control for the efficacy of the ConA pre-clearing procedure, E- cadherin was undetectable in pre-cleared lysates (Figure III-S3). In contrast to Wnt 3a, EGF stimulation induced only a small but reproducible increase in the pool of free β-catenin. Thus, although Wnt 3a stabilizes β-catenin, elevating the level of free β-catenin available for nuclear translocation and transactivation, EGF does not, suggesting that EGF activates Tcf/Lef transcription through a non-canonical mechanism.

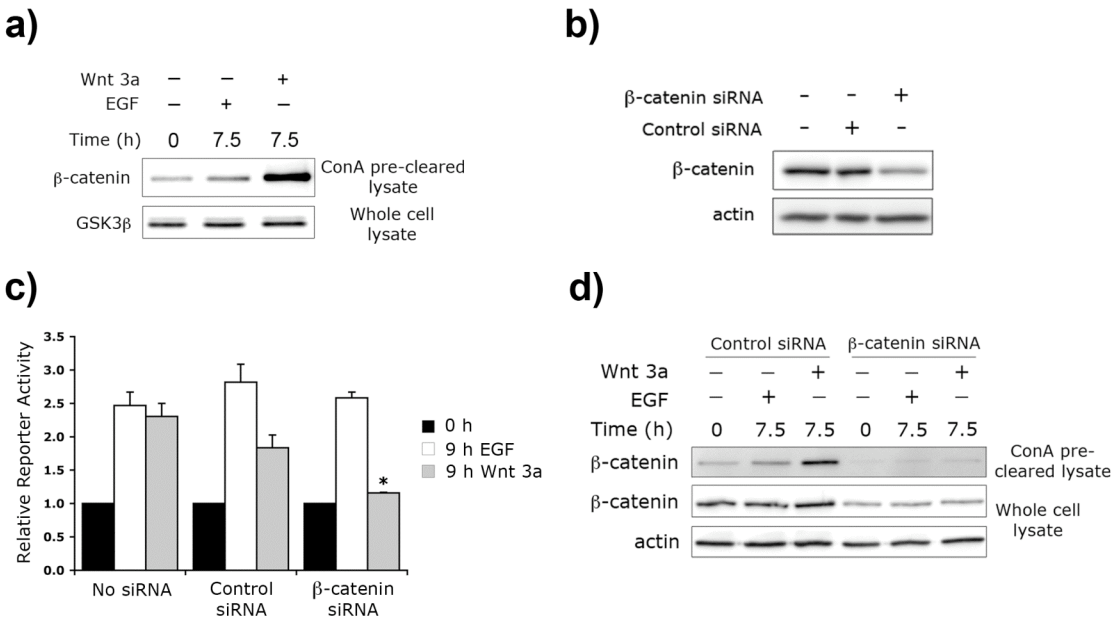

Figure III-3. Wnt 3a and EGF activate Tcf/Lef transcription via different mechanisms.
a) 293T-EGFR were serum-starved, stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml), and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads before immunoblotting for β-catenin. Immunoblotting of whole cell lysate for GSK3β confirmed equal protein loading.
b) 293T-EGFR were transfected with β-catenin or control siRNA (10 nM). Whole cell lysates were immunoblotted for β-catenin and actin.
c) 293T-EGFR were transfected with β-catenin or control siRNA (10 nM) or not transfected with siRNA. After serum starvation, cells were stimulated with either EGF or Wnt 3a and the TOPFLASH reporter activity was quantified at the indicated times. The *asterisk* denotes $P < 0.05$ comparing reporter activity at 9 h between cells transfected with control and β-catenin siRNA. The observed difference in Wnt-mediated reporter activity between control siRNA and no siRNA cells was not statistically significant ($P = 0.12$).
d) 293T-EGFR were transfected with β-catenin or control siRNA (15 nM) or not transfected with siRNA. After serum starvation, cells were stimulated with either EGF or Wnt 3a, and ConA pre-clearing and immunoblotting was performed as in (A).

To explore this possibility, we sought to determine whether β-catenin was required for EGF-mediated activation of the TOPFLASH reporter. Transfection of β-catenin-specific siRNA caused substantial, but not complete, knockdown of total cellular β-catenin, whereas transfection of control siRNA did not affect β-catenin expression (Figure III-3B). In cells transfected with control siRNA, TOPFLASH reporter induction by EGF and Wnt 3a was not significantly different than in cells untreated with siRNA (Figure III-3C). Transfection of β-catenin siRNA, however, resulted in complete

inhibition of Wnt 3a-mediated TOPFLASH activation. In contrast, β-catenin siRNA did not affect the induction of Tcf/Lef transcription by EGF. These results raise two possibilities: either β-catenin is not required for EGF-mediated TOPFLASH reporter activation, or the pool of β-catenin that is resistant to siRNA treatment contributes selectively to EGF-mediated Tcf/Lef transcriptional activity.

To examine the latter possibility, we reasoned that subcellular pools of β-catenin whose turnover is significantly less than that of free β-catenin might be resistant to siRNA treatment. One such highly-stable pool is the fraction of β-catenin that is associated with E-cadherin (Papkoff, 1997). In cells transfected with control siRNA, EGF and Wnt 3a induced accumulation of free β-catenin similar to cells untreated with siRNA (Figure III-3D). However, in cells treated with β-catenin siRNA, the pool of free β-catenin was completely ablated in both EGF- and Wnt 3a-treated cells, even though total cellular β-catenin was only partially reduced. Thus, free β-catenin is highly sensitive to siRNA treatment. In contrast, the glycoprotein-associated fraction of β-catenin, including β-catenin bound to E-cadherin, is at least partially resistant to siRNA, raising the possibility that this siRNA-resistant pool of β-catenin is involved in EGF-mediated Tcf/Lef transcriptional activity.

2.4. ERK is required for EGF- and Wnt 3a-mediated Tcf/Lef signaling.

Since EGF-mediated Tcf/Lef transcriptional activity does not occur via the canonical Wnt pathway, we sought to determine which EGF-mediated signaling pathways might be involved. We first examined the role of the MAP kinase ERK, a

prominent EGF-related signal. Upon treatment with EGF, 293T-EGFR exhibit robust, sustained activation of ERK 1/2 (Figure III-4A). ERK phosphorylation in response to EGF was significantly diminished by treatment with the pharmacological agent PD 98059, which inhibits MEK, the upstream activator of ERK (Figure III-4A). EGF-mediated Tcf/Lef transcription was also completely blocked by inhibition of ERK signaling with PD 98059 (Figures III-4B and III-S4A). Corroborating this observation, a second, structurally distinct MEK inhibitor (U1026) blocked TOPFLASH reporter activity in a dose-dependent manner (Figures III-4B and III-S4B). Additionally, because pharmacological inhibitors can have non-specific effects, we tested TOPFLASH reporter activity in cells transfected with MKK1-K97M, an inactive mutant of MEK (Mansour et al., 1994). Compared to 293T-EGFR cells transfected with an empty vector, EGF-mediated Tcf/Lef transcriptional activity was blocked by dominant-negative MEK in a dose-dependent fashion (Figure III-4C), clearly demonstrating that ERK signaling is required for EGF-mediated Tcf/Lef transcriptional activity.

Because Wnt 3a has also been reported to stimulate ERK activation (Almeida et al., 2005; Yun et al., 2005), we examined whether Wnt 3a can induce ERK activity in 293T-EGFR. Indeed, Wnt 3a treatment transiently activated ERK in a manner sensitive to MEK inhibitors (Figure III-4D), although the magnitude and duration of ERK phosphorylation were less than that induced by EGF. Despite reports that Wnts can stimulate MAPK through transactivation of EGFR (Civenni et al., 2003), Wnt 3a-mediated ERK activity was not affected by pharmacological inhibition of EGFR (data not shown).

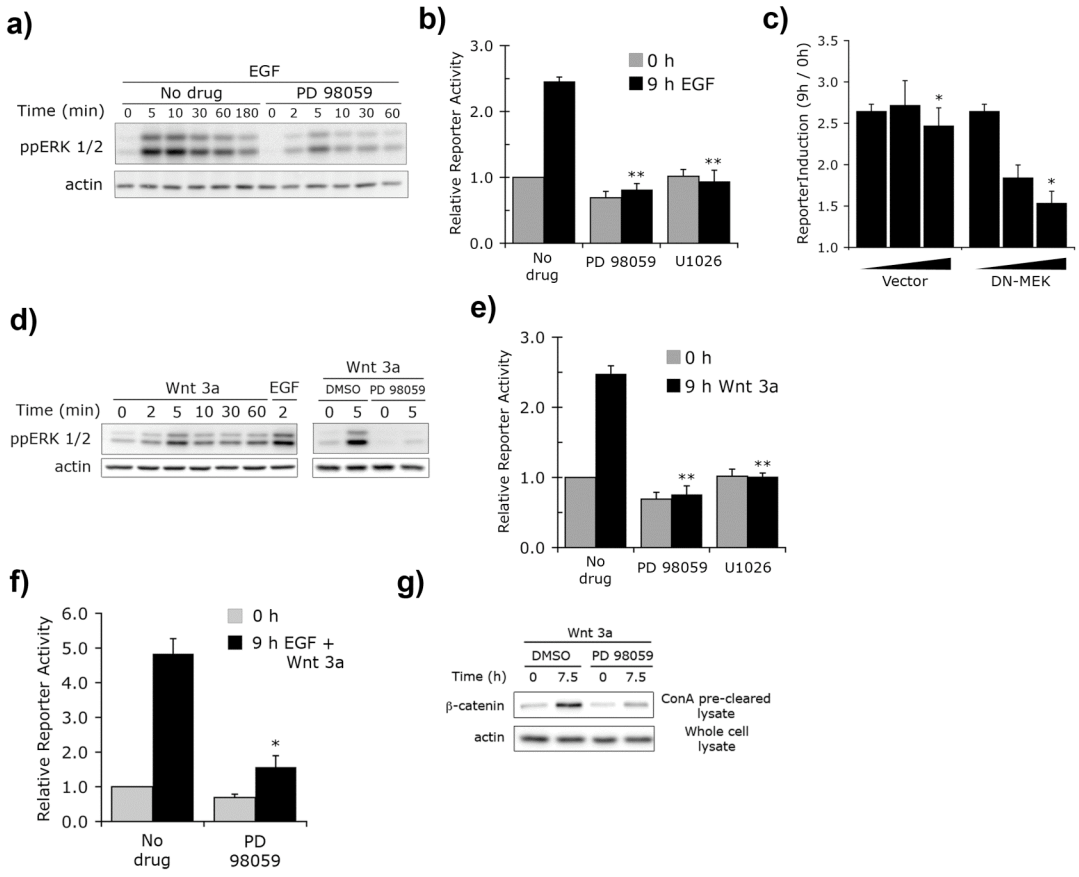

Figure III-4. ERK is required for both EGF- and Wnt 3a-mediated Tcf/Lef signaling.
a) 293T-EGFR were pre-treated with PD 98059 (50 μM), stimulated with EGF (40 ng/ml), and then lysed at the desired times. Whole cell lysates were immunoblotted for dually-phosphorylated ERK 1/2 and actin.
b) After pre-treatment with PD 98059, U1026 (10 μM), or no drug, serum-starved 293T-EGFR were stimulated with EGF, and the TOPFLASH reporter activity was quantified at the indicated times. The *double asterisk* denotes $P < 0.01$ comparing reporter activity at 9 h in the absence and presence of the pharmacological agents.
c) 293T-EGFR were transfected with increasing amounts (0, 5, or 10 μg) of the dominant-negative MEK mutant or the corresponding empty vector. After serum starvation, cells were treated with EGF and the extent of TOPFLASH reporter induction (9h / 0h) is reported. The *asterisk* denotes $P < 0.05$ comparing TOPFLASH reporter activity between the MEK mutant and the empty vector.
d) 293T-EGFR were either stimulated with Wnt 3a (50 ng/ml) or EGF (left panel) or pre-treated with PD 98059 or the solvent DMSO before stimulation with Wnt 3a (right panel). Whole cell lysates were immunoblotted for dually-phosphorylated ERK 1/2 and actin.
e) 293T-EGFR were pre-treated with PD 98059, U1026, or no drug, and then stimulated with Wnt 3a before quantification of the TOPFLASH reporter activity. The *double asterisk* denotes $P < 0.01$ in comparing reporter activity at 9 h in the absence and presence of the indicated pharmacological agents.
f) 293T-EGFR were pre-treated with PD 98059 or no drug, stimulated with Wnt 3a, and the TOPFLASH reporter activity was quantified. The *asterisk* denotes $P < 0.05$ comparing reporter activity at 9 h in the absence and presence of PD 98059.
g) After pre-treatment with PD 98059 or the solvent DMSO, 293T-EGFR were stimulated with Wnt 3a and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads before immunoblotting for β-catenin. Whole cell lysates were also immunoblotted for actin.

To determine whether ERK was involved in Wnt 3a-mediated Tcf/Lef signaling, we again employed the MEK inhibitors PD98059 and U1026. Similar to EGF-mediated signaling, Wnt 3a-induced Tcf/Lef reporter activity was completely ablated by both MEK inhibitors (Figure III-4E). Additionally, PD 98059 substantially reduced TOPFLASH induction by co-stimulation with EGF and Wnt 3a (Figure III-4F), demonstrating that EGF- and Wnt-3a-mediated Tcf/Lef transcription, which proceed via distinct mechanisms, have a common requirement for ERK signaling.

We next examined whether ERK signaling is involved in Wnt-mediated stabilization of β-catenin. Although the vehicle control did not affect stabilization of free β-catenin by Wnt 3a, PD 98059 treatment of 293T-EGFR diminished β-catenin stabilization (Figure III-4G), corroborating observations that ERK signaling is partially required for Wnt-mediated stabilization of cytoplasmic β-catenin (Almeida et al., 2005).

2.5. PKC, but not PKA, is required for EGF- and Wnt 3a-mediated Tcf/Lef transcription.

To further parse the divergent mechanisms by which EGF and Wnt 3a activate Tcf/Lef transcription, we examined the role of protein kinase C (PKC), which can mediate Tcf/Lef transcription in several contexts (Chen et al., 2000; Yang et al., 2006). We first probed EGF-mediated TOPFLASH activity with calphostin C, a pharmacological inhibitor of all PKC isoforms. In 293T-EGFR cells, low doses of calphostin C (0.1 and 0.5 μM) did not block EGF-mediated TOPFLASH activity (Figure III-S5). At a dose of 1 μM, however, calphostin C reduced EGF-mediated Tcf/Lef transcriptional activity by ~60 % (Figure III-5A).

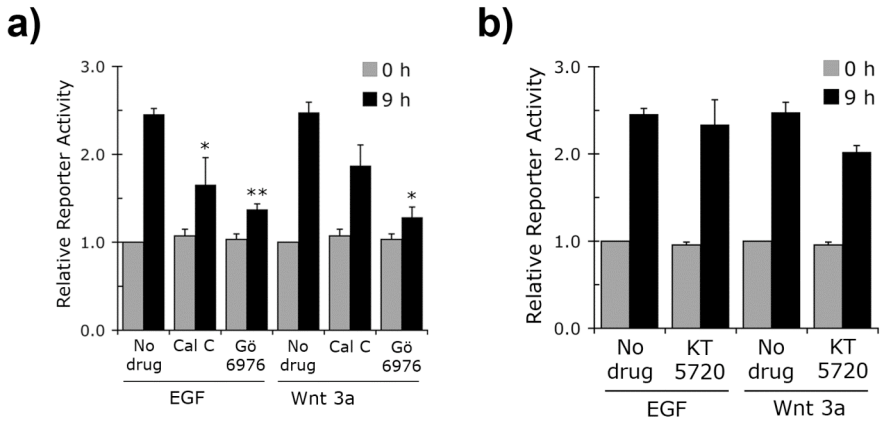

Figure III-5. PKC, but not PKA, is required for EGF- and Wnt 3a-mediated Tcf/Lef transcriptional activity.
a) After pre-treatment with either calphostin C (1 μM) or Gö 6976 (0.5 μM), 293T-EGFR were stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml), and the TOPFLASH reporter activity was quantified. The *asterisks* and the *double asterisk* denote $P < 0.05$ and $P < 0.01$, respectively, comparing the reporter activity at 9 h in the absence and presence of the indicated pharmacological inhibitor.
b) 293T-EGFR were pre-treated with KT 5720 (1 μM), stimulated with either EGF or Wnt 3a, and the TOPFLASH reporter activity was quantified. The observed differences in reporter activity were not statistically significant ($P = 0.63$ and 0.12 for EGF and Wnt 3a, respectively).

Since calphostin C only partially blocked EGF-mediated TOPFLASH reporter activity, we sought to confirm the effect of PKCs in the induction of Tcf/Lef transcription by EGF. Using a second pharmacological inhibitor, Gö 6976, which selectively inhibits the α/β1 isoforms of PKC, confirmed that PKC is functionally required for EGF-mediated Tcf/Lef signaling (Figure III-5A). Some reports have suggested that PKC lies upstream of ERK activation (Schonwasser et al., 1998); however, treatment with Gö 6976 did not reduce ERK phosphorylation in response to EGF (Figure III-S6). Thus, PKC and ERK are distinct, parallel requirements for EGF-mediated Tcf/Lef transcription.

Similar to EGF, Wnt-3a-mediated induction of Tcf/Lef transcriptional activity was also blocked by the PKC inhibitors calphostin C and Gö 6976 (Figure III-5A), confirming that activation of the TOPFLASH reporter by canonical Wnts is PKC-

dependent (Chen et al., 2000). Thus, similar to ERK, PKC α/β1 is not a unique requirement of EGF-mediated Tcf/Lef signaling. However, in contrast to ERK, the PKC α/β1 inhibitor Gö 6976 did not affect Wnt 3a-mediated stabilization of β-catenin (Figure III-S7), indicating that PKC is essential for Wnt signaling at a step downstream of β-catenin stabilization.

Because several agonists of protein kinase A (PKA) reportedly induce Tcf/Lef transcriptional activity (Hino et al., 2005; Taurin et al., 2006), we next tested the role of PKA in EGF- and Wnt 3a-mediated Tcf/Lef transcription. Inhibiting PKA with the drug KT 5720, which does not affect PKC, did not affect TOPFLASH reporter activity in response to stimulation with EGF or Wnt 3a (Figure III-5B).

2.6. Src family kinase activity is required for EGF-, but not Wnt 3a-, mediated Tcf/Lef transcription.

The Src family of kinases have also been implicated in Tcf/Lef transcription (Coluccia et al., 2006; Haraguchi et al., 2004; Ress and Moelling, 2006). To parse the role of Src in EGF- and Wnt-3a-mediated β-catenin signaling, we used the drug PP2, which inhibits all Src family members. Treating 293T-EGFR with PP2 blocked EGF-mediated induction of TOPFLASH by ~ 70 % (Figure III-6A), suggesting that Src kinase activity is required for induction of EGF-mediated Tcf/Lef transcriptional activity. Because Src may lie upstream of ERK activation (Daulhac et al., 1999), we tested whether the PP2 drug affected EGF-mediated phosphorylation of ERK. At 10 min after EGF stimulation, levels of dually-phosphorylated ERK were not changed in PP2-treated

cells; however, at 30 min, ERK phosphorylation was slightly reduced (Figure III-S6). Thus, Src family kinases may partially mediate ERK signaling, as opposed to being fully distinct, parallel requirements.

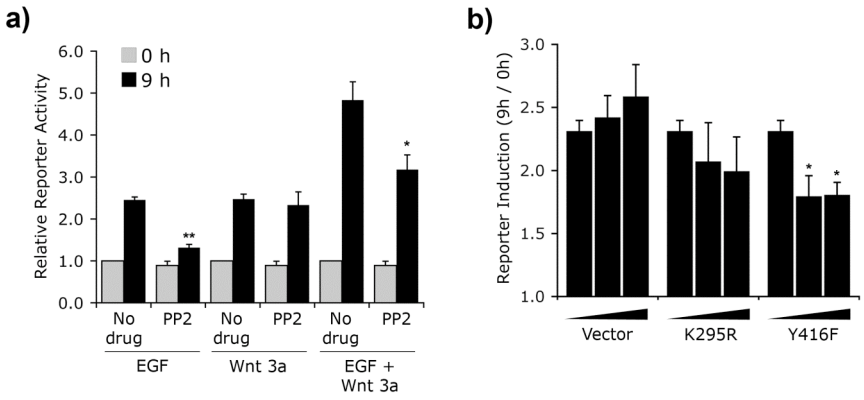

Figure III-6. EGF, but not Wnt 3a, requires Src family kinase activity to activate Tcf/Lef transcriptional activity.
a) Serum-starved 293T-EGFR were pre-treated with PP2 (10 μM), stimulated with EGF (40 ng/ml), Wnt 3a (50 ng/ml), or EGF plus Wnt 3a, and the TOPFLASH reporter activity was quantified. The *asterisk* and *double asterisk* denote $P < 0.05$ and $P < 0.001$, respectively, comparing reporter activity in the absence and presence of PP2.
b) 293T-EGFR were transfected with increasing amounts (0, 5, 10 μg) of the indicated dominant-negative Src mutants or the corresponding empty vector. After serum starvation, cells were treated with EGF, and the extent of TOPFLASH reporter induction (9h / 0h) is reported. The *asterisks* denote $P < 0.05$ comparing reporter induction of empty vector-transfected cells to Src-Y416F-transfected cells.

In contrast to EGF, Wnt-3a-mediated TOPFLASH reporter activity was unaffected by Src inhibition (Figure III-6A). Consistent with its inability to block Wnt-3a-mediated Tcf/Lef signaling, the Src inhibitor PP2 did not block stabilization of free β-catenin by Wnt 3a (Figure III-S7). As such, it appears that the kinase activity of Src family members is required for activation of Tcf/Lef transcription by EGF, but not Wnt 3a. In support of this conclusion, 293T-EGFR co-stimulated with EGF and Wnt 3a in the presence of PP2 activate the TOPFLASH reporter to the same magnitude as Wnt 3a alone (Figure III-6A), demonstrating that Src inhibitors can selectively ablate the EGF-mediated contribution to Tcf/Lef transcription in co-stimulated cells.

To verify the requirement of the Src family kinases in EGF-mediated Tcf/Lef signaling, we tested two dominant-negative mutants of Src, one deficient in ATP binding (K297R) (Kamps and Sefton, 1986) and one lacking a key phosphorylation residue (Y416F) (Piwnica-Worms et al., 1987). Compared to the empty vector, both inactive Src mutants reduced EGF-mediated TOPFLASH reporter activity (Figure III-6B), corroborating results from pharmacological inhibition of Src.

3. Discussion

A central question in biology is how cells make appropriate response decisions in the presence of numerous environmental cues. Part of the answer lies in uncovering the topology of the molecular signaling networks that couple different environmental signals. In this study, we investigated crosstalk between two extracellular factors – EGF and Wnt – that play prominent roles in both normal and pathological physiology. We demonstrate that EGF and Wnt 3a utilize distinct but partially overlapping pathways that converge on Tcf/Lef transcriptional activity (Figure III-7). Because Tcf/Lef signaling regulates the expression of several prominent gene switches, including the oncogenes *cyclin d1* and *c-myc*, the intricate connectivity between EGF and Wnt signaling pathways raises the intriguing possibility that crosstalk between these two factors may synergistically regulate development and tumorigenesis.

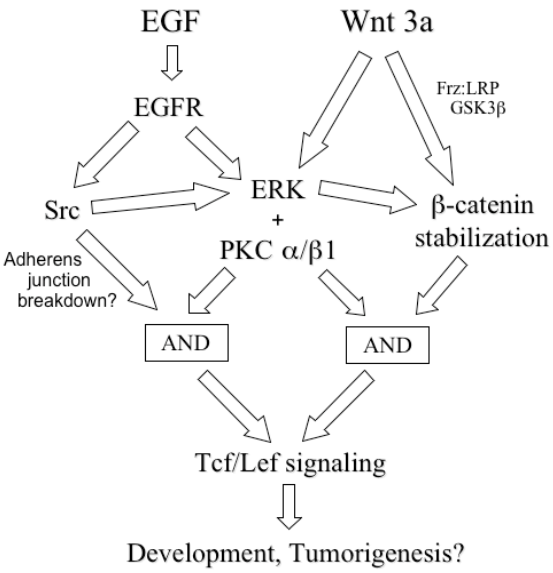

Figure III-7. EGF and Wnt 3a activate Tcf/Lef signaling via a distinct but partially overlapping network.
Wnt 3a signals via the canonical Wnt mechanism involving stabilization of cytoplasmic β-catenin, presumably through the Frz:LRP receptor complex and inhibition of GSK3β. In contrast, EGF induces Tcf/Lef signaling without affecting β-catenin stability. Both ligands require activation of ERK and PKC, although only ERK is involved in β-catenin stabilization. In addition to ERK and PKC, EGF also requires Src kinase activity, presumably because Src promotes adherens junction dissociation and β-catenin nuclear activity through tyrosine phosphorylation. This strongly-coupled signaling network indicates that crosstalk between EGF and Wnt may regulate Tcf/Lef-dependent phenomena such as development and oncogenesis.

Our data show that EGF and Wnt 3a stimulate Tcf/Lef transcriptional activity by regulating β-catenin in distinct ways. While Wnt 3a functions through the canonical mechanism involving β-catenin stabilization, EGF targets an siRNA-resistant, more stable sub-pool of β-catenin. The net quantitative effect is that EGF and Wnt 3a co-stimulate Tcf/Lef-mediated transcription in an additive manner. This additive convergence on Tcf/Lef transcriptional activity masks an intricate signaling network in which EGF and Wnt 3a utilize both distinct (Src) and common (Erk and PKC) signaling molecules to regulate Tcf/Lef-mediated transcription (Figure III-7).

To our knowledge, this is the first report to demonstrate the coordinate effect of EGF and Wnt 3a on Tcf/Lef-mediated transcription and to elucidate the signaling network underlying this effect. Previous reports have suggested that specific signaling pathways downstream of EGF and Wnt might crosstalk in some cellular contexts. In 293 cells, for example, the GSK3β inhibitor LiCl cooperates with EGF to induce greater Tcf/Lef transcriptional activity than either stimulus alone (Chen et al., 2000). Additionally, co-expression of dominant-negative GSK3β and constitutively-active Ras in hepatocytes synergistically activates the TOPFLASH reporter (Desbois-Mouthon et al., 2001). However, the inhibition of GSK3β and activation of Ras are only partial surrogates for the full signaling capacity of Wnt 3a and EGF ligands, respectively. Moreover, constitutive activation or inhibition of signaling pathways is clearly different from the induction of these intracellular signals by soluble ligands. Thus, it was unclear whether EGF and Wnt 3a could co-regulate Tcf/Lef transcriptional activity, and if so, through what signaling network. Our work addresses this question and reveals a model (Figure III-7) where several EGF- and Wnt 3a-mediated signaling pathways coordinate to stimulate Tcf/Lef transcriptional activity.

3.1. Physiological implications of EGF and Wnt co-regulation of Tcf/Lef transcription

Because co-stimulation with EGF and Wnt 3a provokes quantitatively greater Tcf/Lef signaling than either ligand alone (Figure III-2C), embryonic and adult developmental processes that are concomitantly regulated by these two factors may be shaped by their crosstalk. In the adult mammary gland, multiple developmental stages require signals from both EGF and Wnt ligands, including ductal elongation during

adolescence and alveolar morphogenesis during pregnancy (Brennan and Brown, 2004; Troyer and Lee, 2001). Even in simpler organisms such as *C. elegans*, EGF and Wnt receptors (LET-23 and LIN-17/LIN-18, respectively) operate concomitantly to guide vulval development in the adult hermaphrodite (Inoue et al., 2004; Sternberg and Horvitz, 1989). Our findings raise the intriguing hypothesis that developmental fates may be shaped by cooperative regulation of Tcf/Lef by EGF and Wnt in these contexts.

Crosstalk between EGF- and Wnt-mediated Tcf/Lef signaling may also play a role in the development of human breast cancers. Mutations that deactivate the cytoplasmic degradation machinery of β-catenin, causing abnormal stabilization and nuclear localization of β-catenin, are pervasive in many human cancers, especially those of the colon (Polakis, 2000). As in other cancer types, upregulation of β-catenin signaling is prominent in human breast cancers. One study found that 60% of patient samples tested positive for cytoplasmic and nuclear β-catenin staining (Lin et al., 2000). Furthermore, suggestive of its biological significance, positive staining for β-catenin correlated with cyclin D1 upregulation and poor prognosis.

While there is strong evidence of a role for hyperactivated β-catenin signaling in human breast cancer, the underlying molecular causes are less clear. Mutations that stabilize β-catenin have been found in numerous cancer types, but not in human breast cancer (Hatsell et al., 2003). Autocrine production of Wnt ligands may play a role in breast cancer, since mammary-tissue-specific overexpression of Wnt-1 induces adenocarcinomas in mouse models (Tsukamoto et al., 1988) and some human breast

cancers exhibit autocrine Wnt signaling (Bafico et al., 2004). Our results raise the possibility that hyperactive EGF signaling may independently, or in collaboration with autocrine Wnt production, stimulate Tcf/Lef signaling during breast cancer development. In support of a role for EGF/Wnt synergism in breast cancer development, the EGFR ligand TGFα has been reported to cooperate with Wnt 1 and Wnt 3 to initiate neoplastic progression in murine mammary glands, although the mechanism is not known (Schroeder et al., 2000). Furthermore, there is evidence for interactions between ErbB2/Neu and β-catenin in metastatic human breast cancer (Schroeder et al., 2002).

Uncovering the precise role of EGF in regulating β-catenin signaling during human breast cancer development will be an important future direction. In the meantime, our results suggest that specific therapeutic strategies may provide selective control over EGF- versus Wnt-mediated Tcf/Lef transcription. RNAi-based therapeutic strategies may prove ineffectual in antagonizing Tcf/Lef signaling arising from hyperactive EGF signaling, since EGF utilizes a highly stable subpool of β-catenin. Instead, siRNA targeting the Src family of kinases should selectively inhibit EGF-mediated Tcf/Lef transcriptional activity.

3.2. Mechanisms underlying EGF/Wnt crosstalk in regulating Tcf/Lef transcriptional activity

Although Wnt ligands are the classical agonists of β-catenin:Tcf/Lef transcriptional activity, it is becoming clear that ligands which activate receptor tyrosine kinases (RTKs) also induce Tcf/Lef transcriptional activity. RTK-activating soluble

factors that have been linked to activation of Tcf/Lef transcriptional activity include EGF (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002), hepatocyte growth factor (Monga et al., 2002), insulin-like growth factors (Desbois-Mouthon et al., 2001), fibroblast growth factor 2 (Holnthoner et al., 2002), platelet-derived growth factor (Yang et al., 2006), and the Gas6 growth factor (Goruppi et al., 2001). Most of these non-Wnt ligands have been suggested to activate Tcf/Lef transcription via Wnt-like mechanisms that stabilize cytoplasmic β-catenin, often through the inhibition of GSK3β.

In contrast, our results demonstrate that EGF-mediated Tcf/Lef transcriptional activity proceeds via a non-canonical mechanism and does not involve an increase in the level of free β-catenin (Figure III-3A). In fact, we observed that a β-catenin-targeting siRNA completely downregulates free β-catenin, yet has no effect on EGF-mediated Tcf/Lef transcription. Meanwhile, siRNA treatment only partially downregulated total cellular β-catenin, suggesting that EGF activates Tcf/Lef transcription using a highly-stable, siRNA-resistant sub-cellular pool of β-catenin, such as the fraction bound to E-cadherin (Papkoff, 1997). Indeed, in 293T-EGFR cells, depletion of glycoproteins from whole cell lysates demonstrated that the residual pool of β-catenin in siRNA-treated cells includes E-cadherin-bound β-catenin (Figure III-3D). As such, EGF might induce Tcf/Lef transcriptional activity by shifting this adhesive fraction of β-catenin into a transcriptionally-competent state, without substantially increasing the cytoplasmic pool of β-catenin. Such a mechanism would be consistent with the current paradigm that β-catenin may be primed to selectively function in either adhesion or transcription (Gottardi and Gumbiner, 2004).

In fact, in cancer cells that overexpress EGFR and undergo epithelial-to-mesenchymal transition upon EGF treatment, a stabilization-independent mechanism of β-catenin transcriptional activity has been proposed (Lu et al., 2003). In these cell types, EGF treatment induces caveolin-mediated endocytosis of E-cadherin, causing breakdown of adherens junctions and releasing β-catenin for transcriptional activation. This mechanism would also be consistent with our observation that Src is required for EGF-mediated Tcf/Lef transcriptional activity, since Src-mediated tyrosine phosphorylation has been shown to disrupt adherens junctions both *in vitro* (Piedra et al., 2003; Roura et al., 1999) and *in vivo* (Behrens et al., 1993; Owens et al., 2000). Notably, if Src does disrupt adherens junctions in 293T-EGFR cells, it does not concomitantly induce significant accumulation of cytoplasmic β-catenin (Figure III-3A).

In addition to regulating the adhesive properties of β-catenin, tyrosine phosphorylation by Src kinases may also promote β-catenin transcriptional activity by modifying the affinity of β-catenin for nuclear binding partners such as BCL9-2 (Brembeck et al., 2004). Src-mediated phosphorylation of intracellular proteins other than β-catenin may also facilitate Tcf/Lef transcriptional activity, since phosphorylation of the BCR kinase by Src induces dissociation of BCR from Tcf1, thereby promoting Tcf/Lef transcription (Ress and Moelling, 2006). As such, Src may play multiple roles in priming β-catenin for Tcf/Lef transcription in EGF-treated cells, including dissociation from adherens junctions, nuclear localization, and association with Tcf/Lef.

In addition to Src, our results suggest EGF-mediated Tcf/Lef transcriptional activity is completely ERK-dependent (Figure III-4). Recently, EGFR has been shown to relieve Groucho-mediated repression of the Notch signaling pathway via ERK (Hasson et al., 2005). Since the Groucho family of repressors also regulate β-catenin-mediated transcription (Brantjes et al., 2001), it raises the intriguing possibility that de-repression of Groucho via EGFR signaling is involved in EGF-mediated Tcf/Lef signaling. Precisely how ERK- and Src-mediated control of Groucho and tyrosine phosphorylation, respectively, contribute to EGF-mediated Tcf/Lef transcriptional activity is currently under investigation.

Notably, in some cell systems, EGF treatment is not sufficient to induce Tcf/Lef transcriptional activity (Mizushima et al., 2002). For example, A431 cancer cells, which overexpress EGFR, do not exhibit TOPFLASH reporter induction upon stimulation with EGF (Yan et al., 2006). However, in this same system, Tcf/Lef transcriptional activity does become responsive to EGF when levels of the phosphatase PCP-2 are downregulated by siRNA. Thus, the induction of Tcf/Lef transcription by EGF, which has been reported in a variety of cell types (Graham and Asthagiri, 2004; Lu et al., 2003; Muller et al., 2002), may critically depend on the balance between phosphatase and kinase activity.

Although the mechanisms controlling Tcf/Lef transcription by EGF are still under investigation, canonical Wnt signaling has been studied in great detail. While the key event in Wnt signaling is stabilization of cytoplasmic β-catenin, our results also

demonstrate a requirement for other intracellular pathways including ERK and PKC for full activation of Tcf/Lef transcription by Wnt 3a. This observation is underscored by the fact that PKC inhibitors block Wnt-mediated Tcf/Lef transcription even without affecting stabilization of β-catenin (Figure III-S7). Precisely how PKC is involved Wnt signaling is unknown at this time.

In contrast to PKC, ERK is required for both Wnt-mediated β-catenin stabilization and Tcf/Lef transcriptional activity. Previous reports have implicated ERK in the phosphorylation and inactivation of GSK3β (Almeida et al., 2005; Ding et al., 2005); however, since we did not observe GSK3β phosphorylation in response to Wnt 3a (Figure III-S2), this mechanism does not appear to be relevant in 293T-EGFR. Interestingly, siRNA targeting β-catenin preferentially ablated the free pool of β-catenin utilized in Wnt 3a-mediated Tcf/Lef transcription (Figure III-3D). This result suggests that the transcriptionally-active pool of β-catenin in Wnt-3a-treated cells may consist primarily of newly-synthesized β-catenin. In fact, since ERK has been reported to control β-catenin levels by regulating the activity of eukaryotic translation iniation factor 4E (Karni et al., 2005), Wnt 3a may cause accumulation of β-catenin via ERK-dependent regulation of β-catenin synthesis.

In summary, our results reveal an intricate coupling between EGF and Wnt 3a in activation of Tcf/Lef transcriptional activity. Parsing the topology of the signaling networks that couple these prominent extracellular factors reveals that EGF and Wnt stimulate distinct but partially overlapping pathways which converge on Tcf/Lef

transcription. Because the transcriptional targets of Tcf/Lef include several prominent gene switches, including the oncogenes *cyclin d1* and *c-myc*, the connectivity between EGF and Wnt signaling pathways may synergistically regulate development and tumorigenesis. Probing the specific mechanistic roles of Src, ERK, and PKC in this signaling network will provide valuable insight into the role of EGF/Wnt crosstalk in development and cancer, as well as how cells mediate appropriate responses in the presence of numerous environmental cues.

4. Experimental Procedures

4.1. Antibodies and Reagents

Recombinant mouse Wnt 3a and Wnt 5a were purchased from R&D Systems. Recombinant human EGF was obtained from Peprotech. The pharmacological inhibitors AG 1478, calphostin C, Gö 6976, KT 5720, PD 98059, PP2, and U1026 were purchased from Calbiochem and reconstituted in DMSO. The following antibodies were used in this study: anti-actin (Santa Cruz Biotechnology), monoclonal anti-β-catenin (BD Transduction Laboratories), anti-GSK3β (BD Transduction Laboratories), anti-phospho-Ser9-GSK3β (Biosource), monoclonal anti-E-cadherin (BD Transduction Laboratories), anti-phospho-ERK1/2 (Thr202/Tyr204) (Cell Signaling Technology), anti-phosphotyrosine (Santa Cruz Biotechnology).

4.2. Cell Culture

293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), and 1%

(v/v) penicillin/streptomycin (Invitrogen). For serum starvation, the cells were washed once in PBS and then cultured in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma) for 24 h. For studies involving pharmacological inhibitors, cells were starved for 23 h and then pre-treated with the drug in serum-free medium for 1 h prior to stimulation in the continued presence of the drug.

4.3. Plasmid Constructs

pLPCX, pLPCX-EGFR, pLNCX, pLNCX-Src-K295R, and pLNCX-Src-Y416F were generously provided by J. Brugge (Harvard Medical School). The luciferase-based reporters pTOPFLASH and pFOPFLASH were purchased from Upstate Biotechnology, Inc. pRL-TK was purchased from Promega. pMCL -MKK1-K97M and the parental vector pCEP4L were kind donations of N. Ahn (University of Colorado at Boulder)

4.4. Cell Lysis

Cells were washed once in ice-cold PBS and scraped into cold lysis buffer. After incubation on ice for 15 min, the cell lysates were clarified by centrifugation, and the supernatant was collected as whole cell lysate. Protein concentrations were determined using BCA reagents (Sigma). For immunoblotting, samples were lysed in modified RIPA buffer, as described elsewhere (Graham and Asthagiri, 2004), except for the ConA pre-clearing assays, where cells were lysed in Triton-only lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, β-glycerophosphate (pH 7.3) 10 mM NaPP, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). For luciferase reporter measurements, cell lysis was

performed in 1X passive lysis buffer provided by the manufacturer (Promega).

4.5. ConA fractionation

Separation of the glycoprotein-bound and un-bound fractions of β-catenin was performed as described elsewhere (Fagotto et al., 1999). Briefly, cells were plated at a subconfluent density (10^5 cells/35-mm dish) and then transfected with 1 μg pLPCX or pLPCX-EGFR before serum starvation, stimulation with EGF or Wnt 3a in serum-free medium, and lysis. 50 μg of whole cell lysate were then incubated with 100 μl of ConA-sepharose 4B beads (Sigma) in 500 μl Triton-only lysis buffer for 90 min at 4 ˚C. The beads were then spun down and the supernatant (pre-cleared lysate) was collected for immunoblotting.

4.6. Reporter Assays

293T cells were plated at a subconfluent density (10^5 cells/35-mm dish) and co-transfected with 1 μg of the reporter plasmid (pTOPFLASH or pFOPFLASH), 0.1 μg of pRL-TK, and 1 μg of pLPCX-EGFR or the empty vector pLPCX using FuGENE 6 (Roche Applied Science). 24 h after transfection, cells were serum-starved, stimulated and then lysed at desired times. Reporter activity was measured using the dual luciferase assay (Promega), and luciferase activity was normalized to *Renilla* luciferase activity. To pool data from multiple trials, the luciferase:*Renilla* luciferase ratio from all experiments was normalized to serum-starved cells in the absence of pharmacological agents. Error bars represent the sample standard error of at least three independent experiments, and two-tailed Student's *t*-tests were performed to P values. For studies involving siRNA-mediated knockdown of β-catenin, siRNA were transfected approximately 12 h after transfection of the reporter plasmids and 12 h before serum-starvation.

4.7. Immunoblotting

Whole cell lysates or pre-cleared lysates were resolved by SDS-PAGE on 10% gels and blotted onto nitrocellulose membrane (Bio-Rad). The membranes were blocked overnight and then incubated sequentially with primary and corresponding horseradish peroxidase-conjugated secondary antibody. The blots were treated with SuperSignal West Femto Substrate (Pierce) and imaged on the VersaDoc 3000 (Bio-Rad) using Quantity One software (Bio-Rad). All immunoblots presented are representative of at least two independent experiments.

4.8. siRNA knockdown of β-catenin

siRNA directed against β-catenin, as well as non-specific, negative control siRNA, were purchased from Ambion. siRNAs were transfected in antibiotic-free medium using Lipofectamine RNAiMAX (Invitrogen).

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6. Supplemental Data

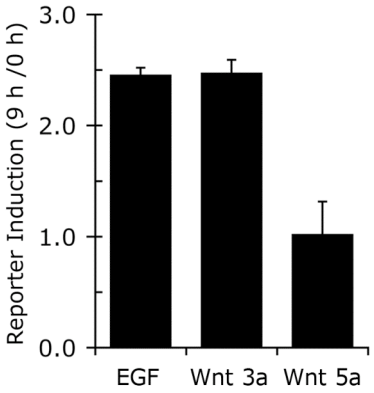

Figure III-S1. Wnt 5a does not activate Tcf/Lef signaling.
Serum-starved 293T-EGFR cells were stimulated with EGF (40 ng/ml), Wnt 3a (50 ng/ml), or Wnt 5a (50 ng/ml). Induction of the Tcf/Lef-dependent reporter TOPFLASH (9h / 0h) was quantified.

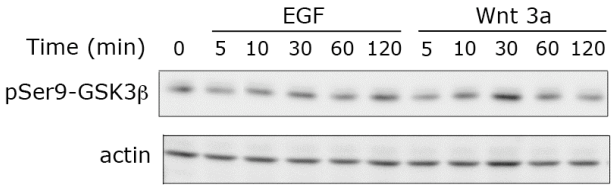

Figure III-S2. Neither Wnt 3a nor EGF induces phosphorylation of GSK3β on serine 9.
Serum-starved 293T-LPCX and 293T-EGFR cells were stimulated with either EGF (40ng/ml) or Wnt 3a (50 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for phospho-serine 9 GSK3β and actin.

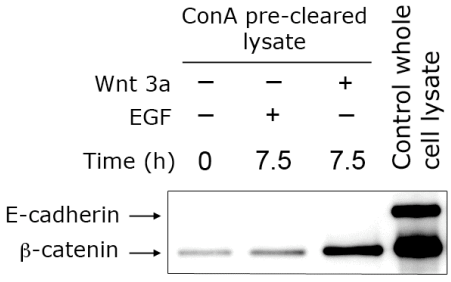

Figure III-S3. ConA pre-clearing of whole cell lysate depletes E-cadherin.
293T-EGFR were serum-starved and then stimulated with either EGF (40 ng/ml) or Wnt 3a (50 ng/ml) for the indicated times. Whole cell lysates were pre-cleared with ConA beads, and the pre-cleared lysates were immunoblotted for E-cadherin and β-catenin. Non-pre-cleared whole cell lysate was included as a positive control for E-cadherin immunoblotting.

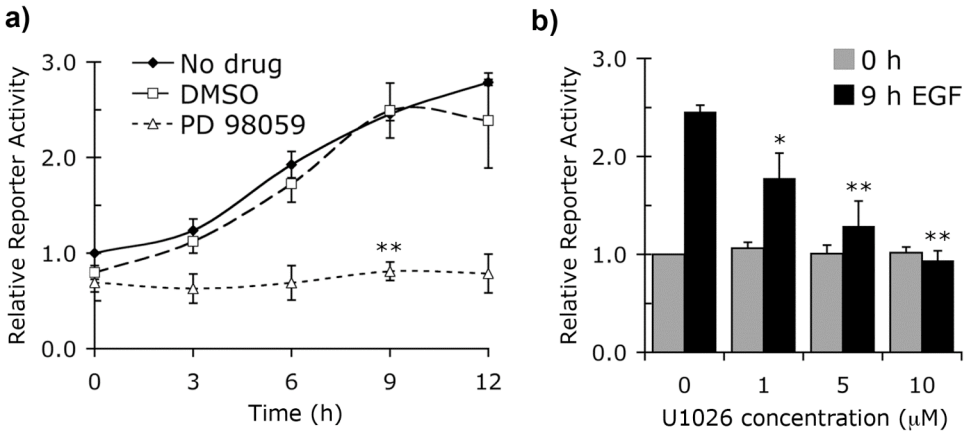

Figure III-S4. ERK signaling is required for EGF-mediated Tcf/Lef signaling
a) Serum-starved 293T-EGFR cells were pre-treated with PD 98059 (50 μM) or DMSO, stimulated with EGF (40 ng/ml), and the TOPFLASH reporter activity was quantified at the indicated times. The *double asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing EGF-induced TOPFLASH reporter activity in the absence and presence of PD 98059 at 9 h.
b) Serum-starved 293T-EGFR cells were pre-treated with the indicated range of concentrations of U1026, stimulated EGF, and the TOPFLASH reporter activity was quantified at the indicated times. The *asterisk* denotes $P < 0.05$ (Student's *t*-test) and the *double asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing EGF-induced TOPFLASH reporter activity in the absence and presence of U1026 at 9 h.

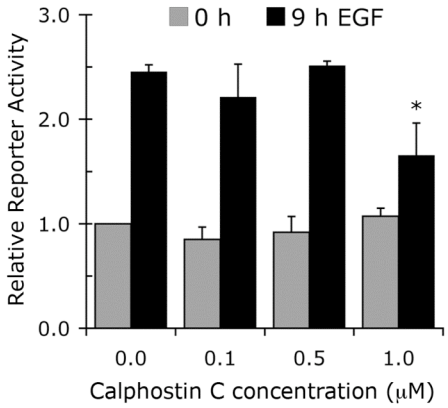

Figure III-S5. The PKC inhibitor calphostin C inhibits EGF-mediated Tcf/Lef signaling only at high concentrations.
Serum-starved 293T-EGFR cells were pre-treated with calphostin C at the indicated concentrations, stimulated with EGF (40 ng/ml), and the TOPFLASH reporter activity was measured at the indicated times. The *asterisk* denotes $P < 0.01$ (Student's *t*-test) in comparing TOPFLASH reporter activity at 9 h in the absence and presence of calphostin C.

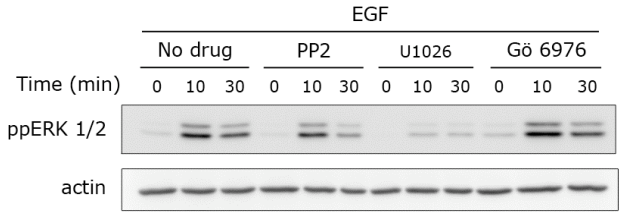

Figure III-S6. PKC does not lie upstream of EGF-mediated ERK activation, but Src possibly does. Serum-starved 293T-EGFR cells were pre-treated with either the solvent DMSO, the Src family kinase inhibitor PP2 (10 μM), the MEK inhibitor U1026 (10 μM), or the PKC α/β1 inhibitor Gö 6976 (0.5 μM), then stimulated with EGF (40 ng/ml) and lysed at the indicated times. Whole cell lysates were immunoblotted for dually-phosphorylated ERK and the equal loading control actin.

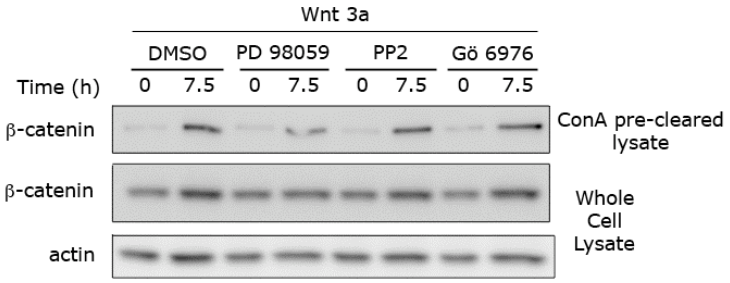

Figure III-S7. PKC and Src are not required for Wnt 3a-mediated stabilization of β-catenin. Serum-starved 293T-EGFR were pre-treated with either the solvent DMSO, the MEK inhibitor PD 98059 (50 μM), the Src family kinase inhibitor PP2 (10 μM), or the PKC α/β1 inhibitor Gö 6976 (0.5 μM), stimulated with Wnt 3a (50 ng/ml) and lysed at the indicated times. Whole cell lysates were pre-cleared with ConA beads, and the pre-cleared lysates were immunoblotted for β-catenin. Whole cell lysates were immunoblotted for total cellular β-catenin and the equal loading control actin.

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