# Chapter I. Introduction

## 1. Introduction

Precise and dynamic control of fundamental cell processes, including proliferation, adhesion and migration, is required for proper organization and homeostasis of mammalian organisms. De-regulation of the mechanisms regulating these behaviors underlies many pathologies, including cancer. For example, proliferation of non-cancerous mammalian cells requires properly-timed mitogenic signals, as well as avoidance of anti-proliferative stimuli, from the cellular microenvironment. However, cancer cells circumvent these requirements, becoming both self-sufficient in mitogenic signals and insensitive to anti-proliferative signals, permitting unchecked cell growth (Hanahan and Weinberg, 2000). With a fundamental understanding of the mechanisms underlying cell behaviors, it may be possible to manipulate these same processes, either for therapeutic benefit or technological applications. A key component of this strategy involves elucidating the topology of the molecular networks that regulate cellular functions.

In this study, we sought to identify mechanisms of crosstalk between soluble factors and cell-cell interactions that regulate the ability of cells to proliferate, migrate, and mediate intercellular adhesion. Probing these mechanisms, we elucidate the architecture of sophisticated molecular circuits that enable biological systems to control cell behaviors and guide multicellular organization. Notably, these studies highlight mechanisms by which pathologies de-regulate biochemical signaling networks in order to

achieve aberrant cellular behaviors. To motivate these studies, it is useful to have some background information on the key molecular players.

## 2. Mechanisms of cell-cell adhesion: Adherens junctions

One of the hallmarks of epithelial tissues is tight intercellular adhesion. Cell-cell contact not only permits epithelial tissues to serve as a physical barrier, but also encodes biochemical signals that regulate cell behaviors such as proliferation. Although there are several adhesive structures present in epithelial cells, including tight junctions, desmosomes, and gap junctions, the structure that is primarily responsible for intercellular adhesion is the adherens junction.

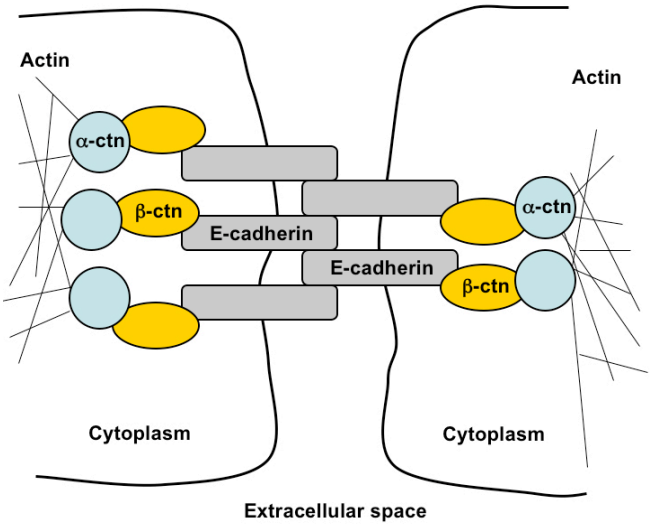


Figure I-1. **Generalized structure of adherens junctions**
The molecular constituents of adherens junctions are the cadherin and catenin proteins. Cadherins span the plasma membrane and bind to cadherins on neighboring cells. The cadherin intracellular domain binds β-catenin (β-ctn), which links to the actin cytoskeleton through α-catenin (α-ctn).

Adherens junctions are composed of cadherin and catenin proteins (Figure I-1). Cadherins are single-pass transmembrane glycoproteins that bind homotypically to cadherins on neighboring cells in a calcium-dependent manner (Angst et al., 2001). The

intracellular tail of cadherins binds β-catenin, which then recruits α-catenin and links to the actin cytoskeleton. As such, cadherin-mediated contacts link the cytoskeletons of neighboring cells and impart a structural rigidity to cell-cell contacts. E-(epithelial)cadherin is the predominant cadherin family member expressed in epithelial cells.

**3. The canonical Wnt pathway: soluble ligands promote signaling through the cell contact protein β-catenin.**

In addition to its adhesive role at the plasma membrane, β-catenin can function as a transcriptional activator when localized to the nucleus (Figure I-2). A key constraint on β-catenin-mediated transcription is the stability of β-catenin in the cytoplasm. In the absence of soluble Wnt factors, cytosolic 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 degraded by the proteasome. Notably, this active degradation mechanism keeps cytosolic concentrations of β-catenin very low.

Signaling events that inhibit this degradation machinery, such as those initiated by a subset of Wnt family ligands, stabilize β-catenin. This allows β-catenin to accumulate and translocate to the nucleus, where it binds to the Tcf/Lef family of transcription factors. Together, this bipartite transcription factor induces expression of genes including *cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999) and *c-myc* (He et al.,

1998). The activation of gene transcription by β-catenin:Tcf/Lef complexes is generally referred to as β-catenin signaling; when Wnt ligands are the agonist of β-catenin signaling, this process is known as Wnt signaling.

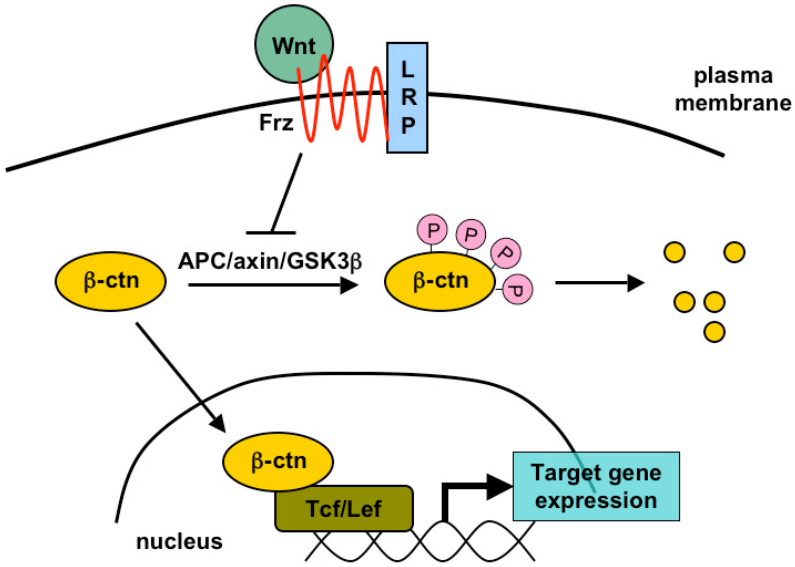


Figure I-2. **The canonical Wnt signaling pathway**
In the absence of Wnt ligands, cytosolic β-catenin (β-ctn) is phosphorylated by a multiprotein complex consisting of APC, axin, and GSK3β. Phosphorylated β-catenin is then degraded by the proteasome, keeping cytosolic concentrations of β-catenin low. When Wnt ligands bind to the co-receptor complex of Frizzled (Frz) and LRP 5/6, the cytosolic degradation machinery is inhibited, allowing β-catenin to accumulate in the cytoplasm and translocate to the nucleus. In the nucleus, β-catenin binds to the Tcf/Lef family of transcription factors and mediates expression of target genes including *cyclin D1* and *c-myc*.

## 4. E-cadherin and β-catenin in normal and pathological contexts

E-cadherin and β-catenin play prominent roles in both embryonic development and carcinogenesis (Clevers, 2006; Halbleib and Nelson, 2006; Wijnhoven et al., 2000). Morphogenetic and oncogenic signals are transmitted by both the adhesive function of cadherins and the nuclear signaling activity of β-catenin. Because of the functional effects that E-cadherin and β-catenin exert on cellular processes, extracellular cues - or

mutations that mimic these cues - regulate cell behaviors by regulating cadherin-mediated cell-cell interactions and β-catenin signaling.

*4.1. De-regulation of β-catenin signaling drives proliferation.*

Mutations that abnormally stabilize β-catenin and hard-wire Tcf/Lef transcription into a constitutively activate state occur in a diverse range of cancer types, implying a functional link between β-catenin signaling and tumor development. One such mechanism commonly found in breast cancers is autocrine secretion of β-catenin signaling agonists, including Wnt ligands (Bafico et al., 2004). Confirming that overexpression of β-catenin agonists can induce transformation, mammary-tissue-specific overexpression of Wnt-1 induces adenocarcinomas in mouse models (Tsukamoto et al., 1988). Consistent with these findings, studies using stabilized mutants of β-catenin or Tcf/Lef-VP16 fusion constructs have affirmed the capacity of β-catenin signaling to transform established cell lines and primary cells (Aoki et al., 1999; Kolligs et al., 1999; Orford et al., 1999).

In fact, antagonizing β-catenin signaling appears to be an effective method to curb the growth of cancer cell lines afflicted by elevated levels of nuclear β-catenin. Inhibitors of soluble Wnt factors decrease cell growth of human breast cancers that exhibit autocrine Wnt signaling (Bafico et al., 2004). Furthermore, overexpression of proteins – such as full-length E-cadherin or a truncated mutant that retains β-catenin binding – sequester stabilized β-catenin at the plasma membrane, precluding its association with Tcf/Lef transcription factors and effectively inhibiting proliferation of

colorectal cancer cell lines (Gottardi et al., 2001; Orsulic et al., 1999; Sadot et al., 1998).

Although the role of β-catenin in hyperproliferation of cancer cells is well established, the role of β-catenin and Tcf/Lef transcription factors in cell cycle progression of *normal* mammalian cells is only recently becoming apparent. Immunohistochemical data have shown that epithelial precursor cells in the intervillus regions of the small intestine may require β-catenin signaling for self-renewal (van de Wetering et al., 2002). In addition, Tcf4 knock-out mice lack proliferating stem cells and possess only differentiated villus cells, suggesting a causal role for Tcf/Lef in governing stem cell lineage commitment (Korinek et al., 1998). In addition to intestinal epithelia, Tcf/Lef signaling is involved in lineage commitment of human epidermal stem cells (Chenn, 2002; Chenn and Walsh, 2002; Hari et al., 2002; Zhu and Watt, 1996; Zhu and Watt, 1999), hematopoietic stem cells (Reya et al., 2003) and embryonic stem cells (Kielman et al., 2002). In all these cases, the upstream ligands that regulate β-catenin signaling are either Wnt or unidentified.

*4.2. Cadherins suppress tumorigenesis.*

In general, the attenuation of cell-cell adhesion plays a critical role in both early and late stages of oncogenesis (Wijnhoven et al., 2000). At early steps, reduced intercellular adhesion may attenuate contact-inhibition of proliferation, permitting unregulated cell division and tumor formation; at later stages, reduced cell-cell adhesion is often associated with invasion, metastasis, and poor patient prognosis (Christofori and Semb, 1999). In particular, expression of E-cadherin is frequently lost in cancers by

transcriptional inactivation (Giroldi et al., 1997; Hennig et al., 1996; Ji et al., 1997), but it is not clear whether loss of E-cadherin is a prerequisite for cancer progression or merely a consequence of the dedifferentiation that occurs during cancer progression (Wijnhoven et al., 2000). Since re-expression of E-cadherin inhibits invasion (Vleminckx et al., 1991) and tumorigenicity (Navarro et al., 1991) of some cancers, loss of E-cadherin may have a dual effect, permitting motility and invasion, as well as relaxing the constraints on proliferation (Sasaki et al., 2000).

The growth-suppressive effects of cadherins has been attributed to both sequestration of β-catenin outside of the nucleus (Sasaki et al., 2000; Stockinger et al., 2001) and the attenuation of receptor tyrosine kinase (RTK) (Grazia Lampugnani et al., 2003; Lampugnani et al., 2006; Qian et al., 2004; Takahashi and Suzuki, 1996). By inhibiting proliferation, cadherins may also play a role in contact inhibition of proliferation, whereby cells growth arrest even in the presence of mitogenic ligands (Motti et al., 2005; St Croix et al., 1998; Stockinger et al., 2001). Thus, cadherin-mediated cell-cell contacts may antagonize intracellular signaling pathways and subsequent cell responses that are initiated by soluble factors.

**5. Current unresolved questions involving crosstalk between soluble factors and cell-cell interactions**

The classical agonists of β-catenin transcriptional activity are the Wnt ligands (Figure I-2). However, it is becoming apparent that some ligands which activate RTKs,

including the epidermal growth factor (EGF), also provoke β-catenin signaling (Lu et al., 2003; Muller et al., 2002). If indeed non-Wnt ligands such as EGF can induce β-catenin transcriptional activity, it is unclear whether they utilize the canonical Wnt mechanism that stabilizes cytoplasmic β-catenin. In the case of EGF, this question is particularly interesting because EGF is known to inactivate GSK3β (Eldar-Finkelman et al., 1995), the kinase which primes cytosolic β-catenin for degradation (Aberle et al., 1997). Investigating this mechanism may shed insight on whether β-catenin is a primed or non-primed substrate of GSK3β (Ding et al., 2000; Liu et al., 2002). If EGF-mediated transactivation of β-catenin does not involve Wnt-like mechanisms such as the stabilization of β-catenin, what mechanisms are important? One possibility is that EGF transactivates β-catenin by modulating the adhesive and transcriptional properties of β-catenin through tyrosine phosphorylation (Harris and Peifer, 2005).

Whatever the mechanism of EGF-mediated β-catenin transactivation, it would also be interesting to test whether Wnt ligands and RTK ligands can co-regulate β-catenin:Tcf/Lef transcription. Some reports have suggested that specific signals downstream of RTKs, including constitutively-active Ras, can cooperate with constitutive inhibition of GSK3β to induce synergistic β-catenin signaling (Chen et al., 2000; Desbois-Mouthon et al., 2001). However, constitutive activation or inhibition of signaling pathways is clearly different from intracellular signals mediated by soluble ligands, precluding assessment of whether RTK and Wnt ligands co-regulate Tcf/Lef transcriptional activity.

In cancer, hyperactive β-catenin signaling drives unchecked proliferation (Clevers, 2006). Because the targets of β-catenin transcription include proteins that are ubiquitously required for cell cycle progression (e.g., cyclin D1, c-myc), the untested hypothesis remains that β-catenin signaling is important for proliferation of *normal* cells. Correlations between serum-mediated proliferation and Tcf/Lef transcriptional activity have been demonstrated in an engineered mammary cell system (Stockinger et al., 2001); however, the expression of a c-Fos:estradiol receptor fusion protein in these cells precludes an assessment of whether β-catenin nuclear activity is involved in proliferation, since c-Fos itself is itself critically involved in cell cycle control (Cook et al., 1999). Thus, it remains to be tested whether β-catenin signaling is involved in proliferation of normal cells, and if so, whether non-Wnt ligands utilize β-catenin:Tcf/Lef transcription to regulate passage through the cell cycle.

Because β-catenin signaling can be attenuated by binding to E-cadherin at the plasma membrane, the interplay between proliferative signals mediated by β-catenin and contact-induced, anti-proliferative signals may regulate growth. One cellular process that may be regulated by this mechanism is contact inhibition of proliferation, a property of normal cells that is often lost during tumorigenesis. As such, in the context of confluent, growth-arrested, epithelial cell monolayers, do cadherins antagonize β-catenin signaling and thereby inhibit proliferation? To test the hypothesis that E-cadherin regulates the growth of normal cells, it may be necessary to develop quantitative assays to measure the association of E-cadherin and β-catenin. Such quantitative measurements may help

distinguish between contact-mediated growth suppression and alternative mechanisms that concomitantly block proliferation of normal cells at high density.

Finally, although cell-cell interactions may regulate signaling initiated by soluble factors, the converse is also true. In particular, EGF signaling in carcinoma cells promotes the dissociation of cell-cell junctions, as seen in epithelial-mesenchymal transition (EMT) (Boyer et al., 1997; Edme et al., 2002; Lu et al., 2003), a process whereby tumor cells lose their epithelial characteristics and acquire invasive mesenchymal phenotypes. Although hyperactive EGF signaling induces cell scatter in epithelial cells (Khoury et al., 2001), it is not clear whether the soluble factor EGF initiates similar phenomena in normal epithelia, or whether EGF cooperates with other signaling pathways to induce synergistic responses.

**6. Current results**

In this report, we have investigated the molecular networks that control fundamental cellular processes including proliferation, adhesion, and multicellular organization. Chapter II elucidates how the soluble factor EGF promotes proliferation through the cell-cell contact protein β-catenin. In fact, transactivation of β-catenin:Tcf/Lef target genes is an essential signal for EGF-mediated proliferation of normal cells. Because Wnt ligands are the classical activators of β-catenin:Tcf/Lef transcription, Chapter III compares and contrasts the mechanisms by which Wnt 3a and EGF activate β-catenin signaling in cancer cells overexpressing EGFR. These chapters

illustrate the sophisticated molecular circuitry that regulates activation of β-catenin:Tcf/Lef transcription and highlight the importance of this process for proliferation of normal cells.

One of the key mechanisms regulating β-catenin signaling may be tuning the ability of E-cadherin to bind β-catenin. Thus, in Chapter IV, a quantitative method for measuring the association of endogenous E-cadherin and β-catenin is developed. In two case studies closely related to cancer cell biology, we use this quantitative method to observe the regulation of adherens junctions *in vivo*. Because E-cadherin can attenuate β-catenin signaling, this suggests that E-cadherin:β-catenin interactions may mediate growth suppression of normal cells at high density, a property of normal cells that is often lost during tumorigenesis. In Chapter V, evidence for both contact-dependent and density-dependent mechanisms of growth inhibition in normal cells is presented.

Finally, in Chapter VI, we probe the role of soluble ligands in promoting aggregation of individual epithelial cells into multicellular structures with extensive intercellular adhesions. We demonstrate that EGF and other soluble factors synergistically govern the cell-cell interactions that guide multicellular organization. Notably, this behavior resembles the program initiated during metastatic cancer, thus illustrating the flexibility of the epithelial phenotype even in non-cancerous cells.

Together, these studies illustrate how the topology of molecular signaling networks can couple environmental cues to regulate fundamental cellular functions.

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