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Review Article

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Role of Wnt/β-catenin-Snail axis in epithelial-mesenchymal transition towards formation of colorectal cancer

KM Taufiqul Arif, Barun Kanti Biswas*

Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh

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Abstract

β-catenin is one of the key components in the Wnt signaling pathway which is responsible for the development of colonesphere and E-cadherin cell adhesion complex. Activation of canonical Wnt signaling pathway followed by the activation of β-catenin/TCF/LEF transcription complexes have been shown to target genes involved in epithelial mesenchymal transition (EMT) programs. Moreover, downregulation of E-cadherin expression is a hallmark of EMT and is mediated by β-catenin thorough the inhibition of the E-cadherin promoter. This review will depict the above mentioned processes that are co-ordinated by β-catenin, towards EMT and the formation of colon cancer via transcriptional repression of SNAIL family proteins under hypoxic condition.

Keywords: β-catenin, colonesphere, E-cadherin, epithelial mesenchymal transition, Snail

1. Introduction

Colorectal tumors are amongst the most common human neoplasms. Wnt signaling pathway plays a central role in the etiology of this cancer (Bienz and Clevers **2000**). Signaling by the Wnt family of secreted glycolipoproteins is one of the fundamental mechanisms that direct cell proliferation, polarization and fate during embryonic development and tissue homeostasis (Logan and Nusse **2004**), (MacDonald *et al.* **2009**). Wnt/β-catenin signaling is intricately involved in the growth and maintenance of colonospheres (Bienz and Clevers **2000**), (Kanwar *et al.* **2010**) and dysregulation of this signaling has been implicated in epithelial cancers particularly colorectal cancer (CRC) (Cadigan and Peifer **2009**). A key member of the Wnt signaling cascade is β-catenin. Cytosolic β-catenin pool is stabilized by Wnt factors which then associates with T cell factor/lymphoid enhancer factor-1 (TCF/LEF-1) proteins in the nucleus to initiate the transcription of Wnt target genes (Schneikert and Behrens **2007**).

In CRC, mutation of β-catenin (in 10% of cases) causes de-regulation of intracellular β-catenin levels which leads to the nuclear accumulation of β-catenin (Morin *et al.* **1997**). This causes aberrant and constitutive expression of Wnt target genes, and thus the development of CRC (Kim *et al.* **2005**). Interestingly, then interaction between β-catenin and cytoplasmic domain of E-cadherin, provides a connection to the actin cytoskeleton through its binding to α-catenin. However, in aggressive CRC with down-regulated E-cadherin, it is unclear whether the loss of this interaction with β-catenin promotes TCF-dependent transcription. Numerous mechanisms of E-cadherin repression like gene mutations and promoter hypermethylation have also been reported in CRC. However, transcriptional repression of E-cadherin and associated up-regulation of Snail is also considered to play a part in the progression of CRC (Klaus and Birchmeier **2008**). Analysis of Snail in human CRC has shown that 78% of the tumor samples examined overexpressed this protein (Wheeler

Corresponding author: KM Taufiqul Arif**,** Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh
E-mail: taufiq0571@gmail.com Cell: +88-01816-688651.

et al. 2001). In primary CRC Snail 2/Slug expression has been shown to be positive in 37% of cases, which correlated significantly with metastasis of CRC. Evidence for ZEB-1, SIP1 and Twist mediated repression of E-cadherin has not yet been established in CRC (Natawala *et al.* 2008).

Wnt signaling was found to restrain Snail phosphorylation, ubiquitination following degradation, allowing it to amass and induce EMT. These special effects were concealed following Snail knockdown (Yook *et al.* 2005). Additionally, under hypoxic condition, a decrease in E-cadherin expression, increased Snail expression and nuclear translocation of β-catenin consistent with Wnt pathway activation were reported (Imai *et al.* 2003). Snail, induced by hypoxia in CRC cells had undergone EMT due to overexpression showed higher self-renewal ability (Hwang *et al.* 2011). Consequently, activation of the Wnt pathway stabilizes the Snail protein through the same mechanism that stabilizes β-catenin (Yook *et al.* 2005). It is possible that in CRC cells, Wnt/β-catenin signaling represses E-cadherin in parallel through LEF1/β-catenin and stabilized Snail eventually results in EMT.

2.Wnt/β-catenin signaling: Off state

In this evolutionarily conserved pathway (**Fig 1**), Wnts act through stabilization and nuclear translocation of β-catenin (Logan and Nusse 2004), (Cadigan and Waterman 2012). Wnt ligand–receptor interactions can trigger a set of signals that direct cell behavior in several ways: cell proliferation, differentiation, polarity and mobility, survival and self-renewal (Hsu *et al*. 1999), (Cadigan and Waterman 2012). Regulation of cytosolic β-catenin by phosphorylation/degradation by Wnt is the fundamental event of Wnt signaling (MacDonald *et al.* 2009). The scaffolding protein Axin uses separate domains to interact with GSK3β, CK1α, and β-catenin, and directs sequential phosphorylation of β-catenin at serine 45 by CK1α (Logan and Nusse 2004), (Parker *et al.* 2007). Following this, GSK3β comes to the site and phosphorylates at threonine 41, serine 37, and serine 33 (Daugherty and Gottardi 2007), (Huang and He 2008). Two serine/threonine phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) neutralize the action of GSK3β, CK1α in the Axin/APC complex. PP1 dephosphorylates Axin and disassemble the Axin complex whereas PP2A dephosphorylates β-catenin (Luu *et al.* 2004), (Klaus and Birchmeier 2008). Axin contains a regulator of G protein signaling (RGS) domain that interacts with the large multifunctional scaffolding protein APC that binds β-catenin. Indeed, both Axin and APC are tumor suppressor genes. These core Axin complex components share a common goal of ensuring β-catenin phosphorylation and degradation (Dahmen *et al.* 2001), (Cadigan and Peifer 2009). In colorectal cancer APC mutations are found omnipresent (Clevers 2006) however, APC gene was originally discovered as the genetic cause for familial adenomatous polyposis (FAP). FAP patients develop numerous colorectal polyps in early maturity. Devoid of any interference, several polyps can grow into carcinomas and metastatic colorectal cancers (Behrens and Lustig 2004). However, this whole versatile system (**Fig 1**) is branded as the "destruction complex" (Clevers 2006), (MacDonald *et al.* 2009), (MacDonald and He 2012), (Stamos and Weis 2013). Other proteins such as Wilms tumor gene on the X chromosome (WTX) may have roles in degradation of β-catenin. WTX binds to Axin, APC, β-catenin, and E3 ubiquitin ligase β-TrCP to manipulate β-catenin ubiquitination even though its biochemical role remains unidentified. Diversin, another Axin-binding protein, supports β-catenin degradation by recruiting CK1ε to phosphorylate β-catenin (Logan and Nusse 2004), (MacDonald *et al.* 2009). β-catenin phosphorylation at serine 33 and 37 creates a binding site for β-TrCP that leads ubiquitination of β-catenin and its degradation by proteasome eventually (**Fig 1**) (Aberle *et al.* 1997), (Daugherty and Gottardi 2007), (Klaus and Birchmeier 2008). Ubiquitination of β-catenin is reduced in Wnt expressing cells and completely abolished when the GSK3β-phosphorylation consensus motif in β-catenin is mutated (Aberle *et al.* 1997). Mutations of β-catenin and adjoining serine and threonine residues help to escape it from phosphorylation and degradation which is repeatedly found in cancers (MacDonald *et al.* 2009). In the nucleus, transcriptional repressors such as Groucho/BCL9 (Bauer and Willert 2012), HDAC (Gordon and Nusse 2006) are bound to TCF, thereby blocking expression of target genes. At WREs, CtBP is required for silencing Wnt targets in fly (Cadigan and Peifer 2009).

3.Wnt/β-catenin signaling: On state

Phosphorylation of LRP5/6 by Wnts and stabilization of β-catenin are the fundamental events for receptor activation (**Fig 2**) (Mao *et al.* 2001), (Luu *et al.* 2004), (Tamai *et al.* 2004). Role of Fz is required for Wnt-induced LRP6 phosphorylation, and compulsory Fz-LRP6 alliance is adequate to set off LRP6 phosphorylation (Zeng *et al.* 2005). Fz function is usually linked to Dsh/Dvl (Habas and Dawid 2005), a cytoplasmic scaffolding protein that may directly interact with Fz. Indeed, Fz-Dvl interaction and Dvl function are critical for Wnt-induced LRP6 phosphorylation (Zeng *et al.* 2005), (MacDonald *et al.* 2009). Because Dvl interacts with Axin (Habas and Dawid 2005), and is required for Axin recruitment to the plasma membrane during Wg signaling or in Fz overexpression one model stipulates that Fz-Dvl

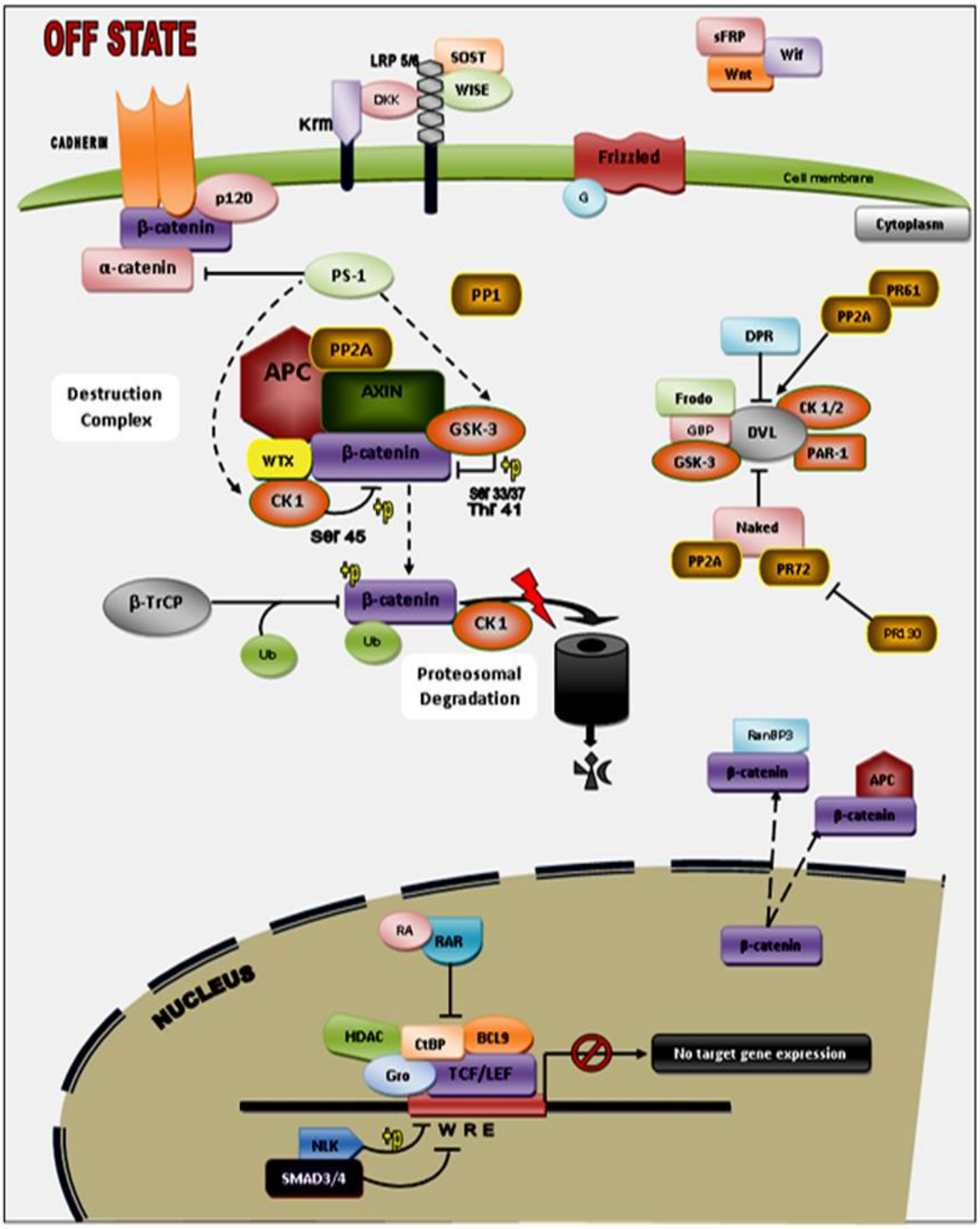

Fig 1. The canonical Wnt/β-catenin signaling: Off state. In the absence of Wnt ligands, β-catenin is recruited into the destruction complex with APC and Axin. Following phosphorylation of β-catenin by the kinases CK1 and GSK3β, and subsequent ubiquitylation by β-TrCP (β-transducin repeat-containing protein, an E3 ubiquitin ligase), β-catenin is proteasomally degraded. Low cytoplasmic levels of β-catenin ensure transcriptional repression of Wnt target genes by recruitment of the corepressor Groucho, CtBP, NLK, HDAC to TCF/LEF transcription factors.

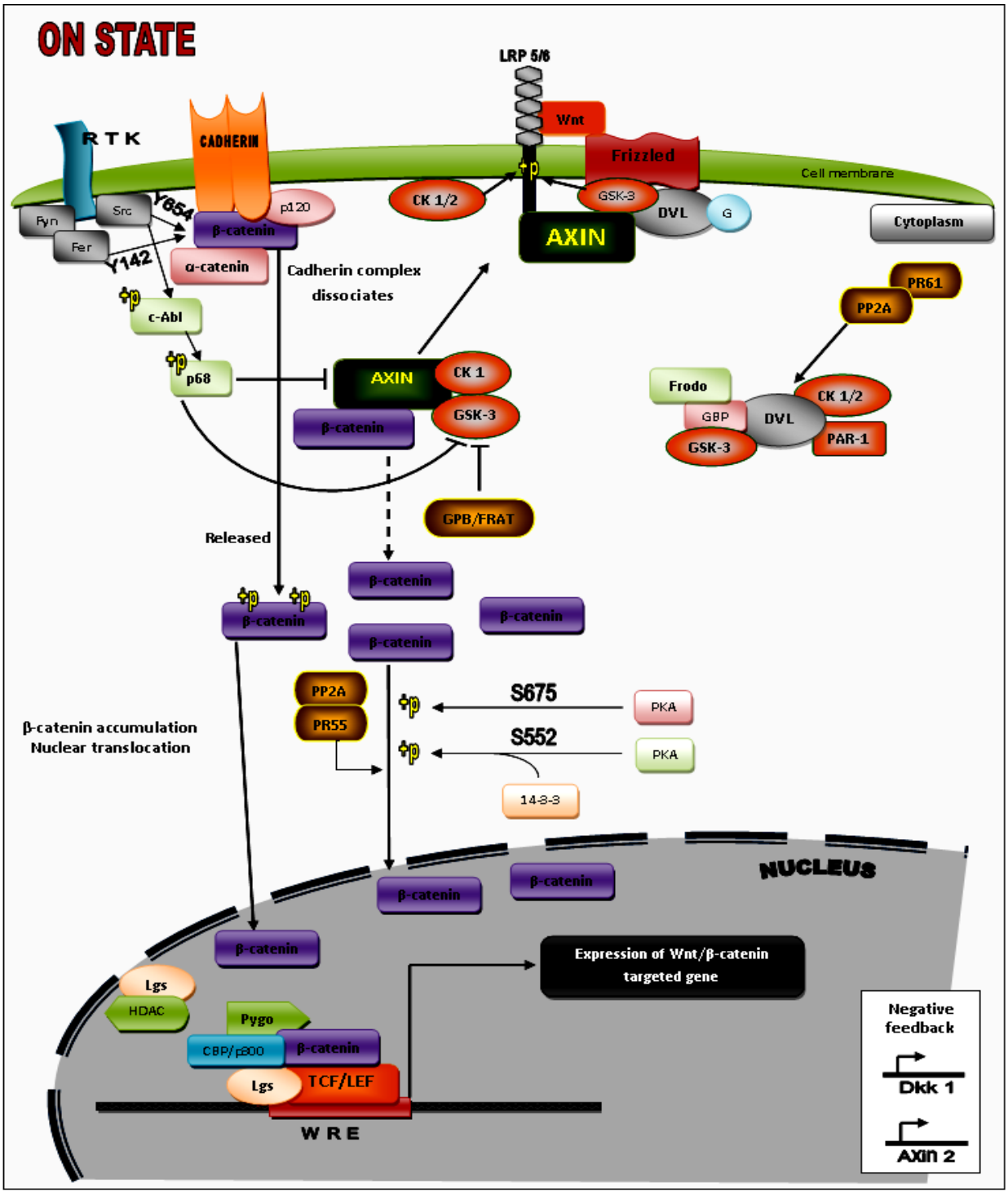

Fig 2. Wnt/β-catenin signaling ON state. The Wnt ligand binds to Fz and LRP5/6 receptors to form a Fz-LRP5/6 complex. Dally and Kny can also bind Wnt and enrich Wnt concentration locally or help Wnt gradient distribution. Rspo proteins and Norrin are secreted agonists that bind to LRP5/6 and/or Fz to activate Wnt-catenin signaling. Formation of the Fz-LRP6 complex via Dvl promotes LRP6 phosphorylation by GSK3 and CK1 And other CK1. Fz binds Dvl and phosphorylated LRP6 recruits Axin to the plasma membrane, resulting in inhibition of β-catenin phosphorylation/degradation by an as yet unknown mechanism. Fz-LRP6-Dvl aggregation may be involved. LRP6 association with caveolin may promote its endocytosis and signaling. Translocation of Axin is facilitated by MACF1. Trimeric G proteins may act between Fz and Dvl, may associate with Dvl and Axin. Stabilized β-catenin is translocated to the nucleus where it binds TCF/LEF and recruits coactivators such as the Lgs/Pygo complex, CBP/p300 to initiate RNA transcription and elongation. TCF-catenin controls the expression of many genes that affect cell proliferation (i.e., c-myc, cyclin D1) and differentiation and also the expression of Wnt signaling inhibitory proteins (i.e., Dkk1, Axin2) that act as a negative feedback loop (He *et al.* 2004; Logan and Nusse 2004; MacDonald *et al.* 2009; Stamos and Weis 2013).

recruitment of the Axin-GSK3β complex initiates LRP6 phosphorylation by GSK3β (Zeng *et al.* **2005**). Wnt activation inhibits the phosphorylation of β-catenin by GSK3β, thus prevents its ubiquitylation and subsequent proteasomal degradation (Polakis **2000**). β-catenin is thereby stabilized thus repression of Wnt targets is relieved when sufficient β-catenin enters nuclei (Cadigan and Peifer **2009**) and forms nuclear complexes with the TCF/LEF-1 family of transcription factors (Behrens and Lustig **2004**), inducing a genetic program that can lead to cell transformation (Polakis **2000**), (Henderson and Fagotto **2002**). Nuclear accumulation of β-catenin is also observed in cancers resulting from mutations in the β-catenin, APC or Axin genes (Polakis **2000**). The APC tumor suppressor binds to β-catenin and the scaffold protein Axin to form a complex promoting GSK3β phosphorylation of β-catenin. Inherited mutations in the APC gene cause FAP, and >80% of colorectal cancers carry mutations with inactivated APC protein (Miyoshi and Hennighausen **2003**). Majority of these mutations target the 'mutation cluster region' in the center of APC gene manufacturing a truncated protein incompetent of binding Axin and other regulatory proteins. Mutated APC can bind to β-catenin but cannot stimulate degradation due to its failure to bind Axin (Henderson and Fagotto **2002**), (Cadigan and Peifer **2009**).

4.Wnt/β-catenin mediated dysregulation of cell-cell adhesion and EMT

Pathways that control cell-adhesion and morphogenesis have been classically viewed as distinct pathways (Amin and Vincan **2012**). However, latest efforts have exposed multiple levels of cross-talk between cell adhesion and pathways that regulate morphogenesis. Disruption of epithelial polarity is anticipated to displace signaling molecules from their exact cellular localization which ultimately hinder the signaling pattern. Conversely, any altered form of morphogenetic signaling will escort defects in cell-adhesion and the synchronization of cell movements (Parker *et al.* **2007**), (Amin and Vincan **2012**). EMT is initiated by extracellular signals such as those from components of the ECM or soluble growth factors. This signaling leads to the activation of small GTPases and Src families of proteins that promote the disassembly of junctional complexes and changes in cytoskeletal organization (Gottardi *et al.* **2001**). The downstream consequence of such signals leads to the activation of transcriptional regulators likes Snail and Slug and the repression of E-cadherin. Down regulation of E-cadherin abolishes the E-cadherin-β-catenin interaction at the adherens junctions (AJs) and allows the entry of β-catenin in the nucleus where it can participate in canonical Wnt signaling. These processes translate into the loss of apical-basal polarity and cell-cell contacts allowing detachment from surrounding cells. Cells undergoing EMT also digest the basal membrane via the action of MMPs to further facilitate migration (Amin and Vincan **2012**).

Canonical Wnt pathway activation and the resultant activation of β-catenin/TCF/LEF transcription complexes have been shown to target genes involved in EMT programs and cell migration (Logan and Nusse **2004**), (Parker *et al.* **2007**). E-cadherin is a component of the AJ and the loss of E-cadherin expression is a hallmark of EMT. The downregulation of E-cadherin is required for morphogenetic processes where cellular rearrangement occurs by changing polarity and cell-cell contacts. Downregulation of E-cadherin is mediated by β-catenin and LEF-1 binding to and inhibiting the E-cadherin promoter (Conacci-Sorrel *et al.* **2002**). Wnt signaling has been shown to downregulate E-cadherin during brain development in mice and a correlation between reduced E-cadherin and elevated nuclear β-catenin have also been observed in human cancers (Conacci-Sorrell *et al.* **2003**). Furthermore, canonical Wnt signaling directly affects EMT as it leads to the accumulation of the EMT transcriptional regulators Snail and Slug. Downregulation of E-cadherin marks the onset of the EMT during gastrulation and neural crest development and plays a key role in the progression of primary tumors to metastasize (Yang and Weinberg **2008**). The Snail protein plays a key role in EMT during development and transformation, in large part through direct repression of E-cadherin transcription. Three E-box sequences near the E-cadherin proximal promoter bind Snail and presumably mediate repression (Kato and Shimmura **2008**). In keratinocytes, Wnt/β-catenin signaling was found to repress E-cadherin additively with Snail (Parker *et al.* **2007**). Interestingly, despite a direct role for LEF-1/β-catenin in cultured cells, LEF-1 null mice still develop large guard hair follicles and repress E-cadherin suggesting a redundant mechanism. This other mechanism may involve Wnt regulation of Snail stability. Snail proteins contain a conserved phosphorylation motif that causes Snail turnover to be regulated by the same GSK-3β-dependent phosphorylation and ubiquitin-dependent proteasomal destruction that regulates β-catenin stability (Amin and Vincan **2012**). Consequently, activation of the Wnt pathway stabilizes the Snail protein through the same mechanism that stabilizes β-catenin (Yang and Weinberg **2008**).

LEF-1 and β-catenin also upregulate Slug transcription (Conacci-Sorrell *et al.* **2003**) which leads to the repression of E-cadherin. In addition to the down-regulation of E-cadherin, Wnt/β-catenin signaling is capable of regulating many other aspects of EMT.

During EMT, MMPs act to dismantle the local basement membrane (Gottardi *et al.* **2001**), (Yang and Weinberg **2008**). Activation of β-catenin/TCF-4 leads to upregulation of MT1-MMP/MMP-14, MMP-7 and MMP-26 in a variety of cancer cell types (Hajra and Fearon **2002**). Similarly, the degradation of the ECM is mediated by Urokinase which has been shown to be under the control of β-catenin and is expressed at high levels at the invasive front of carcinomas. Wnt target gene activation following APC loss (the key early event in the development of sporadic colorectal cancers) leads to the activation of Integrin and Rho pathway clusters in mice (Logan and Nusse **2004**), (Amin and Vincan **2012**). In colon cancer cells, the over-expression of Fzd7 induces TCF-reporter transcriptional activity (Daugherty and Gottardi **2007**) whilst Fzd7 knockdown leads to the inhibition of canonical Wnt signaling-mediated epithelial patterning (Amin and Vincan **2012**). Interestingly, Fzd7 is itself a β-catenin/TCF target gene. Notably, these studies suggest a possible role for Fzd7 in canonical upregulation of invasion factors via the canonical Wnt pathway. Indeed, Fzd7 upregulates EMT transcription factor such as Slug via the canonical Wnt pathway (Yang and Weinberg **2008**). Furthermore, β-catenin/TCF-4 activation in colon cancers has been shown to regulate molecules involved in migration and in Xenopus fibroblasts β-catenin activity upregulates fibronectin. Presumably the Wnt branch of the Wnt pathway activated by Fzd7 is dependent on the tissue and cellular context. Indeed, recent evidence suggests regulation by the ECM in colorectal cancer (Amin and Vincan **2012**).

5. State of affairs: SNAIL

The Snail family of zinc-finger transcrip¬tion factors consist of Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc), which shares an evolutionary conserved role in mesoderm formation in vertebrates. These molecules are composed of a highly conserved carboxy-terminal region containing four to six C2H2-type zinc fingers which is responsible for mediating sequence-specific interactions with DNA promoters con¬taining an E-box sequence (CAGGTG). The amino termini of all vertebrate Snail family members contain the evolutionarily conserved SNAG (Snail/Gfi) domain which is indispensable for transcriptional repression. However, SNAG domain is absent in Drosophila Snail which has a consensus PxDLSx motif and exerts its repressive function through the interaction with the co-repressor CtBP. Hence, Drosophila and vertebrate Snail use either a SNAG domain or a CtBP binding motif to repress gene expression (Nieto **2002**). Snail also directly recruits repressor complex to repress gene expression. Snail interacted with a co-repressor complex Sin3A, and histone deacetylases HDAC1 and HDAC2 to repress E-cadherin expression by modification of local chromatin structure (Peinado *et al.* **2004**). Moreover, other co-repressors like CtBP can also regulate the activity of Snail proteins. Snail exerts global effects on epithelial cell gene expression, and therefore regulates EMT, cell polarity, stem cell-like properties and cell survival or apoptosis (Wu and Zhou **2010**). The function that Snail genes are best known for is the induction of a phenotypic change called epithelial to mesenchymal transition (EMT). Snail-induced EMT converts epithelial cells into mesenchymal cells with migratory properties that contribute to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumors. Snail-induced EMT is partly occurred due to the direct repression of E-cadherin transcription both during development and tumor progression. Since, the lack of E-cadherin expression in tumors is regarded to be a marker of a poor clinical result, the repressors of E-cadherin are considered as markers of malignancy and as targets for anti-invasive drugs (Nieto **2002**), (Barrallo-Gimeno and Nieto **2005**).

6. Regulation of Snail

Snail is a highly unstable protein and is dually regulated by cellular location and protein stability. Expression of Snail is synchronized by an integrated and complex signaling network at the transcriptional and post-transcriptional level; this network includes integrin-linked kinase (ILK), PI3K, mitogen-activated protein kinases (MAPKs), GSK3β and NFκB pathways (De Craene *et al.* **2005**). Receptor tyrosine kinase signaling such as fibroblast growth factor (FGF) or epidermal growth factor (EGF) induces Snail expression by suppressing the actions of GSK3β. Surprisingly, there are numerous signaling pathways involved in embryonic development that can regulate Snail expression. For instance, the TGFβ/Smad pathway induces EMT in hepatocytes, epithelial and mesothelial cells by directly binding to the Snail promoter. Additionally, Notch signaling organizes two distinct mechanisms that act in synergy to control the expression of Snail (Wu and Zhou **2010**). First, Notch directly upregulates Snail expression by recruiting the Notch intracellular domain to the Snail promoter and second, Notch potentiates hypoxia-inducible factor 1α (HIF-1α) recruitment to the lysyl oxidase (LOX) promoter and promotes the hypoxia-induced upregulation of LOX which protects Snail from protein degradation. Furthermore, Wnt can suppress the activity of GSK3β (Bachelder *et al.* **2005**) and thus stabilize Snail and β-catenin at the protein level (Yook *et al.* **2005**). Snail expression and protein level can also be regulated by the NFκB pathway via tran¬scriptional and post-translational mecha¬nisms. First, Snail expression is directly activated by the NFκB

homologue Dorsal in Drosophila. NFκB also binds the human Snail promoter between -194 and -78 bp and increases the transcription of Snail. In addition, GSK3β inhibition stimulates the transcription of Snail by activating the NFκB pathway. In our recent study, we found that the inflamma¬tory cytokine TNFα is the major signal that induces Snail stabilization. Stabilization of Snail by TNFα/ NFκB is mediated by the transcriptional initiation of CSN2 which hinders the phosphorylation and ubiquitylation of Snail by disrupting the binding of Snail with GSK3β and β-TrCP, and results in the eventual stabilization of Snail in a non-phosphorylated and non-ubiquity¬lated functional state (Barrallo-Gimeno and Nieto **2005**), (Wu and Zhou **2010**).

Cytoplasmic Snail has a very short half-life as it is targeted for ubiquitin-mediated proteasome degradation by GSK3β-induced phos¬phorylation and Snail has to translocate to the nucleus to exert its efficacy. The subcellular localization of Snail can be modulated by phosphory-lation involving the p21-activated kinase 1 (PAK1). PAK1 phosphorylates Snail at S246 and favors the nuclear localization of Snail thus enhances its transcription activity. The nuclear entry of Snail in Zebrafish embryos is controlled by the expression of the zinc transporter LIV1, activator of STAT3 (Yang *et al.* **2005**). Export of Snail is controlled by phosphorylation of a ser-rich sequence adjacent to the nuclear export sequence (NES). GSK3β phosphorylates the NES of Snail and induces its export to the cytoplasm. Exportins, like CRM1 are involved in exporting phosphorylated Snail from the nucleus to the cytoplasm (Dominguez *et al.* **2003**).

7. E-cadherin down-regulation and EMT: role of transcriptional repressors

The gene encoding the adherens junction protein E-cadherin is considered to be the paradigmatic epithelial gene (Barrallo-Gimeno and Nieto **2005**). Experiments with transgenic animal models have determined that loss of this gene is associated to higher invasion and metastasis. Forced expression of E-cadherin in cultured tumor cells deficient for this protein induces a more epithelial phenotype decreasing migration; moreover, ectopic expression of this protein also prevents the transcription of mesenchymal genes. Therefore, E-cadherin loss is normally considered the main hallmark of EMT. In general, E-cadherin is not expressed in mesenchymal cells as a consequence of the action of transcriptional repressors (Berx and van Roy **2009**). Functional E-cadherin found in adherens junctions is highly stable but is much more labile if it is associated with the cytoskeleton. Therefore, all the epithelial cells are not equally sensitive to the EMT triggering stimuli since cells with E-cadherin in very stable adherens junctions will not be affected by these stimuli. A repression in the E-cadherin gene expression can cause a slow or no down-regulation of E-cadherin protein in epithelial cells and therefore only these cells with revised adhesiveness are fit to E-cadherin down-regulation and go through EMT (Bezdekova *et al.* **2012**). However, in mesenchymal cells, expression of E-cadherin gene (CDH1) is typically modulated by numerous transcriptional repressors by binding to particular sequences in the CDH1 promoter which contain a core 5′-CACCTG-3′ and are denominated E-boxes. In E-cadherin deficient tumor cells, mutation of these elements stimulates CDH1 gene expression by interfering the binding of specific transcriptional repressors (Ohkubo and Ozawa **2004**). Snail factor is capable of binding these elements and repressing E-cadherin, consequently inducing an EMT (De Herreros *et al.* **2010**). Therefore, a plethora of other transcriptional factors have been characterized as CDH1 repressors and EMT inducers although only six are capable of directly interacting with the CDH1 promoter. Besides KLF8 which binds to GT boxes (Wang and Shang **2013**), the other five repressors associate to the E-boxes; these are the two members of the Snail family (Snail/Snail1 and Slug/Snail2) (Cano *et al.* **2000**), (Hajra and Fearon **2002**), the two Zeb proteins (Zeb1 and 2) and the bHLH protein E47. The bHLH family displays a common protein structural domain consisting of two parallel amphipatic α-helices linked by a loop that is required for dimerization. Transcription factors like bHLH bind to DNA as homo- or heterodimers through a consensus E-box site (5′-CANNTG-3′). Among these factors, only E47 (also known as TCF3) has been found to be capable of binding and repressing CDH1 (Perez-Moreno *et al.* **2001**). However, the relevance of this factor in EMT has been very little studied in addition to its initiation by stimuli triggering this renovation.

8. Snail proteins as essential regulators of EMT

Snail prevents the expression of epithelium-specific genes for example Muc1, PTEN, Occludin, Claudin and some nuclear factor receptors (Vitamin D receptor, HNF-1α) (Peinado *et al.* **2004**). All the Snail targets are not related to EMT. EMT and Snail are related to a range of cancer hallmarks for instance uncontrolled growth, resistance to apoptotic signals and additionally evasion from immunosurveillance. For example, while exposed to ionizing radiation or pro-apoptotic cytokines, cell lines with overexpressed Snail show lower apoptosis (Kajita *et al.* **2004**). Moreover, Snail also enables breast cells to become tumor-initiating cells (Mani *et al.* **2008**) and promotes immunosupression in

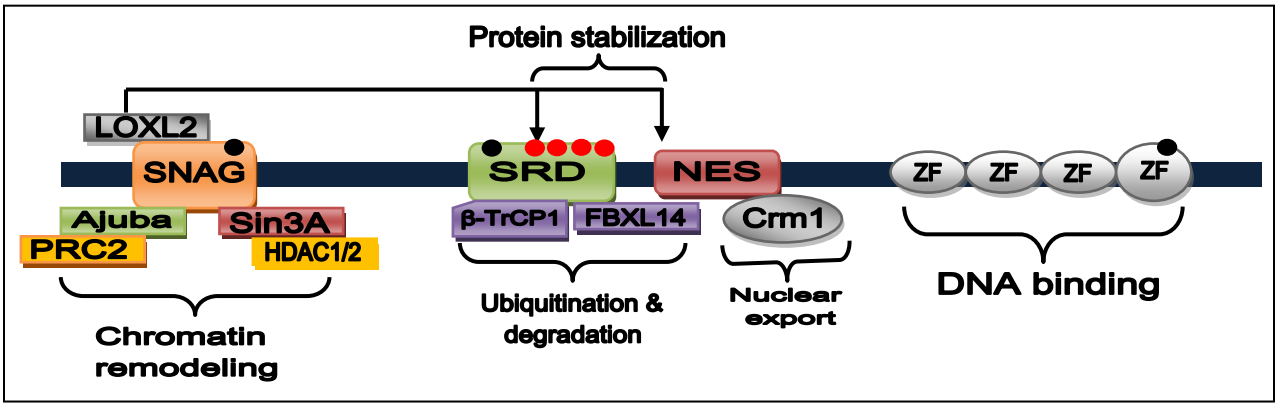

Fig 3. Structure of Snail protein: the different domains of Snail protein and the interactors binding to each of them. Phosphorylation sites are indicated as dots: black, if they stimulate Snail action; red, if they inhibit it. The arrow initiating at LOX protein labels the Snail1 amino acids putatively modified by this enzyme.

melanoma cells (Poser et al. 2001). The precise targets of Snail involved in these effects are currently unknown. The N-terminal half of Snail is responsible for the fine control of this transcriptional factor activity. This part of the protein (in human or murine Snail comprising amino acids 1–150) is much more divergent and holds several related sequences. First, the N-terminus SNAG subdomain of all vertebrate Snail proteins, is essential for co-repressors binding: the chromatin remodeling factors histone deacetylases (HDAC) 1 and 2 and the Polycomb group of proteins 2 (PRC2). The interaction of these proteins with Snail is not direct but mediated by Sin3A and Ajuba respectively (**Fig 3**). The SNAG domain in Drosophila is more than a binding site for CtBP rather is required for repression, which is absent in the mammalian genes. These evidence suggest that even though the repressor activity of the SNAIL family members has been evolutionary conserved, they use a variety of mechanisms depending on either SNAG or CtBP binding elements (Peinado *et al.* **2004**).

Snail co-immunoprecipitates with a Smad 2/3 complex and that this complex is present on the CDH1 promoter when transcription of this gene is repressed. The CDH1 promoter contains a Smad-binding element (SBE) close to one of the E-boxes; a similar proximity of E-boxes and SBE has also observed for other Snail target genes (Vincent *et al.* **2009**). Snail not only represses epithelial genes but also stimulates mesenchymal gene transcription. The speculative idea was, stimulatory effects of Snail are dependent on the repression of E-cadherin and the release of transcriptional factors retained by this protein; accordingly E-cadherin over-expression prevents Snail induction of mesenchymal genes. Still, transcription stimulation cannot be exclusively explained by E-cadherin inhibition since genetic interference of CDH1 transcription does not promote the activation of mesenchymal genes to the same extent as Snail expression. In addition, effects of Snail on mesenchymal genes are detected even in cells defective for expression of E-cadherin (Ohkubo and Ozawa **2004**). It has also been reported that Snail interacts with β-catenin in the nucleus (Stemmer *et al.* **2008**) promoting transcriptional activation of Wnt target genes, signifying that Snail, at least in some conditions, might work as a direct activator. The central part of the Snail proteins is involved in the regulation of protein stability and localization. Different phosphorylation motifs, for instance, Snail phosphorylation on Ser 104 and 107 by GSK3β have been allocated in this domain which might facilitate Snail nuclear way out, revealing a nuclear export sequence in between amino acids 132 and 143. After entering the cytosol, additional phosphorylation on Ser 96 and 100 by the same protein kinase (Zhou *et al.* **2004**) induces Snail binding to β-TrCP1 ubiquitin ligase leading to its ubiquitination and degradation. The above mentioned phosphorylation is neutralized by the action of the small C-terminal domain phosphatase (SCP) which stabilizes Snail in the nucleus (Dominguez *et al.* **2003**). Snail can also experience phosphorylation in other residues that positively control its action, such as, Snail repression of E-cadherin and interaction with Sin3A co-repressor are stimulated if Ser 11 and 92 are phosphorylated by protein kinase A and CK2 respectively. Moreover, Snail can be phosphorylated at the C-terminus by PAK1 (Dominguez *et al.* **2003**) resulting in increased protein retention in the nucleus. It has also been shown that the interaction of Snail with Lysil oxidase-like 2 (LOXL2) also affects its repressive function through the oxidation of Lys 98 and 137 by this enzyme (Peinado *et al.* **2005**).

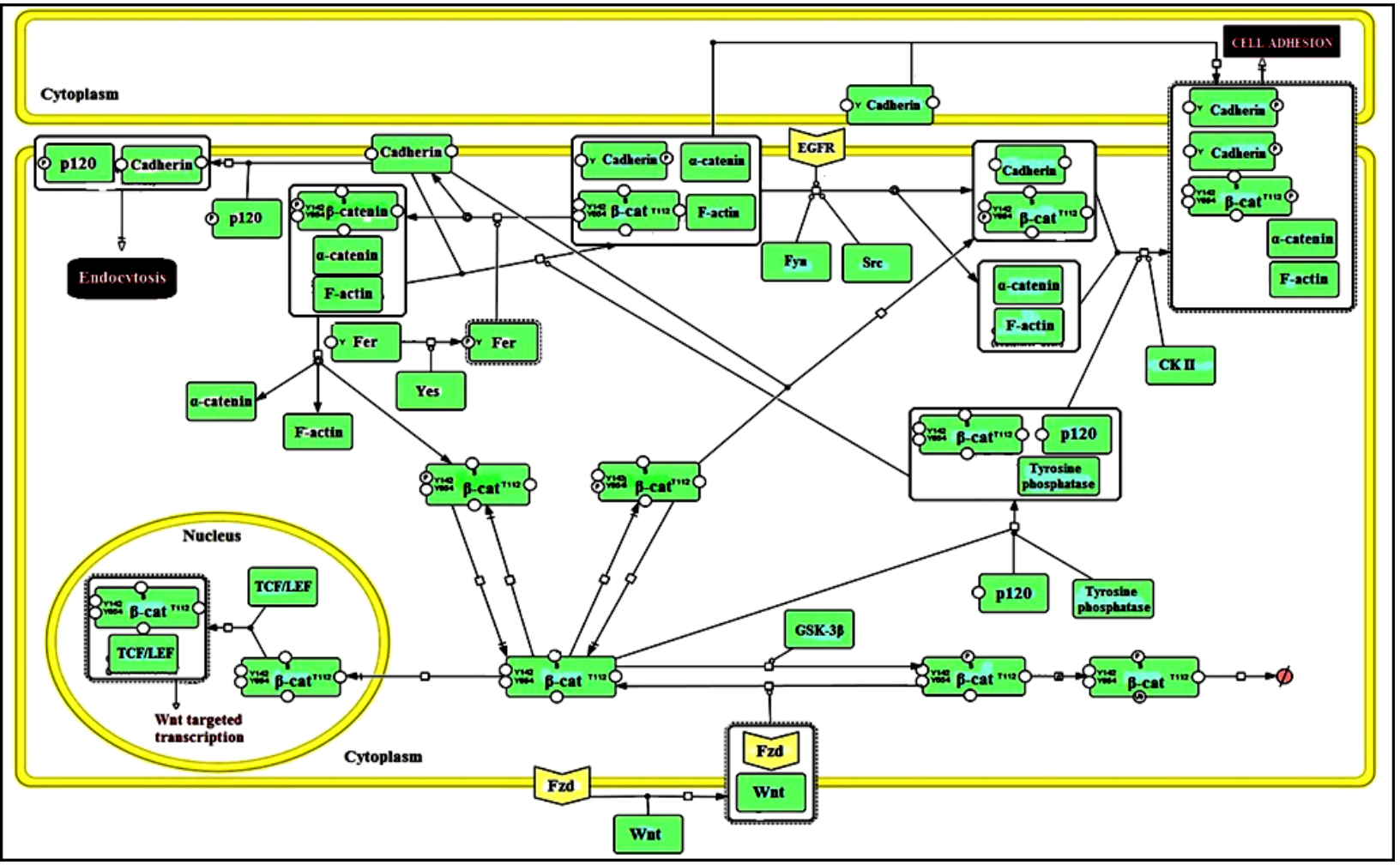

Fig 4. Relationship between Wnt/β-catenin,cadherin/catenin-mediated cell adhesion complex.

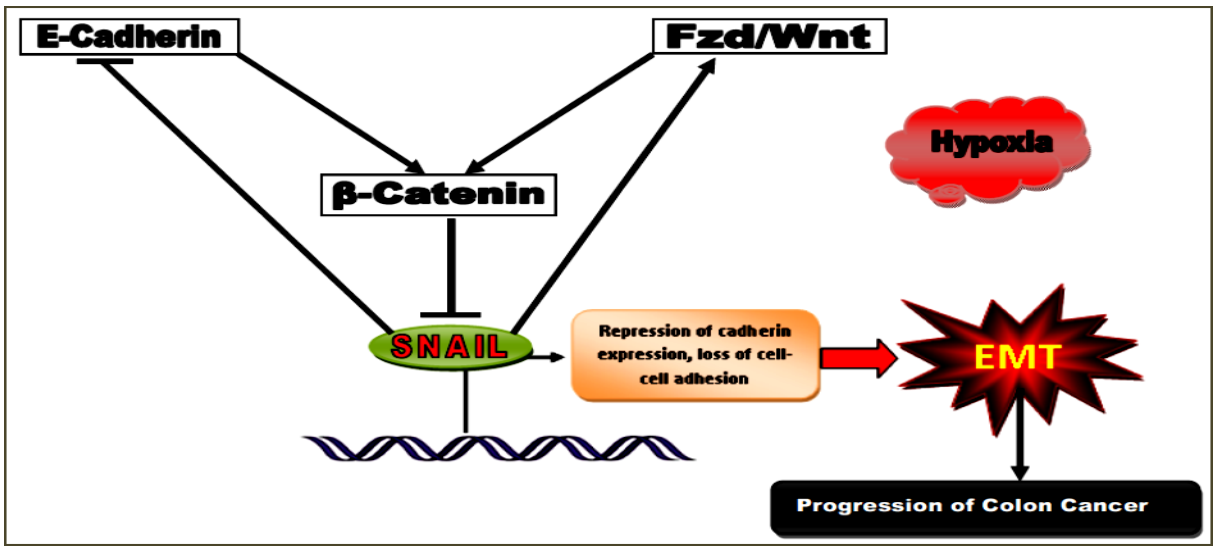

Fig 5. Orchestration of β-catenin mediated EMT and progression of colon cancer via transcription suppressor SNAIL.

Compared to Snail, the biochemical characteristics of the rest of the members of the SNAIL family Slug and Smuc, have been little studied. In comparison to Snail, Slug shows less E-cadherin repressing potency and binds to E-boxes in this promoter with lower affinity (Yu *et al.* **2010**). Repression also requires the interaction of histone deacetylases with the N-terminal regulatory domain. A small sequence in this domain activates transcription in the fusion proteins of GAL4 (Peinado *et al.* **2004**). In the central part of Slug there is a lack of most of the phosphorylation sites (Lee *et al.* **2011**). Although it is not phosphorylated and therefore it does not bind to the ubiquitin ligase β-TrCP1, the stability of Slug is firmly controlled. Half-life of Xenopus Slug is controlled by the Fbxl14 ortholog Partner of Paired (Ppa) that interacts with a hydrophobic sequence in the central part of the molecule. Moreover, Slug protein has also been shown to be a target of the Mdm2 ubiquitin ligase. Binding of this enzyme to Slug takes place through amino acids 27–66 and is dependent on p53 that also associates to Slug in a neighbor sequence (amino acid 21–27) (Conacci-Sorrell *et al.* **2003**). Smuc also shares an analogous C-terminal DNA binding domain structure and an N-terminal regulatory domain. The three proteins of this family present an almost identical SNAG subdomain placed in the N-terminal. However, it is not quite established that proteins for instance Ajuba or Sin3A interacting to the SNAG sequence in Snail, also associate to Slug or Smuc. The DNA-binding domain shows great similarity and most of the Snail target promoters can also be bound by Slug in vitro with a lower affinity. In opposition, a small number of cases have been reported for a Slug target that cannot be bound by Snail (Kataoka *et al.* **2000**).

9. E-Cadherin/β-Catenin, Snail and Epithelial-Mesenchymal Transition (EMT)

Components of cadherin-catenin complex serve a role in activating several key signal transduction networks (**Fig 4**). E-cadherin/β-catenin protein complexes are actively involved in epithelial to mesenchymal (EMT) and mesenchymal to epithelial (MET) transitions which have great impact on development, tissue organization and progression of cancer. Basically, EMT is illustrated by differentiated epithelial cells that undergo a phenotypic conversion that gives rise to the matrix-producing fibroblasts and myofibroblasts (Kalluri and Weinberg **2009**). Cell contacts are critical determinants of EMT. A loss of markers like zonula occludens-1 (ZO-1), cytokeratin and especially E-cadherin from epithelial cells result in gaining the mesenchymal phenotype with expression of mesenchymal proteins including vimentin, α-smooth muscle actin (α-SMA), fibroblast-specific protein-1 (FSP1), and production of interstitial matrix components type I collagen and fibronectin (Zeisberg and Neilson **2009**).

Loss of E-cadherin likely promotes β-catenin release and facilitates EMT, although E-cadherin expression can reverse the transformed phenotype (Thiery *et al.* **2009**). β-catenin plays an important role in the TGF-β1 and cell contact-dependent, synergistic induction of EMT. Where TGF-β1 is absent, E-cadherin along with β-catenin is quickly degraded following contact disassembly. However, β-catenin dissociation from epithelial contacts and its stabilization in the cytoplasm are TGF-β1 induced which make it available for nuclear import (Thiery **2002**), (Thiery and Sleeman **2006**), (Thiery *et al.* **2009**). So the loss of cell-cell adhesion triggers EMT and is associated with diseases involving EMT. However, Snail, Slug, and Twist are the hallmark initiating change of EMT. They can repress E-cadherin transcription which is influenced by β-catenin. β-catenin can also be released in response to loss of cadherin mediated adhesion. The typical binding of β-catenin to E-cadherin and α-catenin illustrates a link between E-cadherin cell-adhesion complex and the intracellular cytoskeleton. These connections got proteolytically cleaved following the loss of E-cadherin and result in release of free β-catenin into cytoplasm in a manner free from canonical Wnt signaling. In fact, the Wnt signaling network and the cadherins appear to compete for the same pool of intracellular β-catenin (Heuberger and Birchmeier **2010**), (Talbot *et al.* **2012**). Again, while Wnt signal is absent, cytoplasmic β-catenin pool is stabilized by GSK3β (Bachelder *et al.* **2005**), and the activity of β-catenin is controlled by APC. Mutations in APC that eventually prevents degradation of β-catenin are very common in sporadic colorectal cancers. Additionally, approximately 10% of colorectal cancers carry mutations in the GSK3β phosphorylation site located in the N-terminus of β-catenin. In colon cancer, APC mutations and β-catenin mutations are mutually exclusive, because of role behind the stabilization of β-catenin and in constitutive β-catenin /TCF transcriptional activity. β-catenin is an E-cadherin binding protein and thus plays an important role in cell-cell adhesion. E-cadherin repression is thought to play a major role in the abnormal manifestation of EMT in epithelial-derived cancer types (Batlle *et al.* **2000**), (Hajra and Fearon **2002**). From development to cancer progression, Snail, the zinc finger transcription factor, has been concerned in E-cadherin repression via its binding to elements in the E-cadherin promoter (Nieto **2002**). Indeed, Snail plays an obligatory role during development in driving the EMT programs that mark gastrulation as well as neural crest development (Cano *et al.* **2000**), (Nieto **2002**), (Nelson and Nusse **2004**). In a similar, but misdirected manner, neoplastic cells nominate Snail chore to adopt a

mesenchymal cell-like invasive phenotype that characterizes their aberrant behavior (Fujita *et al.* **2003**). Although Snail plays a critical role in both physiologic and pathologic EMT (Fujita *et al.* **2003**), E-cadherin repression frequently occurs in tandem with activation of the Wnt signaling cascade (He *et al.* **2004**), (Nelson and Nusse **2004**). The Snail transcript encodes a series of β-catenin-like canonical motifs that maintain its phosphorylation, ubiquitination by GSK3β and β-TrCP respectively and especially proteasomal degradation via a Wnt-1-driven route. Since mounting evidence indicates that Wnt signals can impact on multiple cell functions in neoplastic tissues (He *et al.* **2004**), these findings support a model wherein activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype by controlling β-catenin/TCF- and Snail-dependent transcriptional programs in tandem fashion. Mutations in Axin, APC or Snail gene itself might possibly stabilize Snail in a same manner of β-catenin facilitate the development of carcinogenic states (Cano *et al.* **2000**), (Hajra and Fearon **2002**). However, because increasing evidence supports important roles for the activation of canonical Wnt signaling in normal as well as neoplastic states (He *et al.* **2004**), inappropriate activation of Snail-dependent transcriptional programs are not necessarily constrained to pathological processes that require somatic mutations in the Snail gene itself or accessory molecules that regulate its phosphorylation and degradation (Yook *et al.* **2005**). β-catenin, upon its association with LEF-1, can perform mutually with Snail to suppress E-cadherin transcription via LEF-1/β-catenin interactions with sequences upstream of the E-cadherin promoter. Snail acting as a transcriptional repressor of E-cadherin which potentially facilitates the intracellular transfer of E-cadherin-bound β-catenin to a Wnt-stabilized "signaling" pool. Inappropriate activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype not only via effects on β-catenin/TCF-dependent transcription but also through the ability of the Wnt-GSK3β signaling cascade to activate Snail-driven transcriptions. Indeed, activities of Snail extend beyond the regulation of EMT-related processes to include cell death and growth (Vega *et al.* **2004**).

10. Conclusion

β-catenin is a key player in the Wnt signaling pathway and cellular adhesion system that regulates the development and maintenance of colonspheres throughout the animal kingdom. It can potentially activate EMT in colon epithelial cells through repressing Snail protein activities in large part through direct activation of Wnt pathway and the repression of E-cadherin transcription (**Fig 5**). However, how Snail induces de-differentiation is unclear, and whether Snail can introduce similar stemness traits in CRC is also undecided. Because of Snail or other EMT transcription factors are non-targetable by current therapeutic approaches, the identification of Snail-regulated genes that can be targeted by drugs may lead to the development of novel therapeutic strategies against EMT-related malignancy and importantly, the epigenetical events which could be responsible for the regulation of Snail should be uncovered.

11. Conflict of interest

The authors declare that they have no conflict of interest.

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