Keep your dreams alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination, and dedication. Remember all things are possible for those who believe.

- Gail Devers
1.1 Wnt Signaling

The normal development of tissues, body shape and size are strictly coordinated. Secreted growth factors signal between cells to regulate the key physiological processes of cellular proliferation and differentiation. However, misregulation of signaling pathways could lead to developmental defects during embryogenesis and different kinds of diseases in adults. Detailed investigation of growth factor signaling will have implications in understanding the molecular basis of disease onset and progression. Wnt represents one of the growth factors important in embryonic development and various diseases (Rao and Kuhl, 2010).

1.2 Nature of Wnt and secretion

Wnt family of Morphogenetic proteins are secreted, palmitoylated, cysteine rich, glycoproteins. Wnt proteins carry an N-terminal signal peptide and are secreted in the extracellular medium, and they are relatively less soluble. Its low soluble nature is attributed to a specific post translational modification i.e., cysteine palmitoylation, which is essential for Wnt activity (Willert et al., 2003). Primary amino acid sequence of the protein contains 23 to 25 cysteine residues that are highly conserved between species (Tanaka et al., 2000).

In the year 2000, it was reported that a Drosophila gene required for Wnt-secretion, termed porcupine, displays homology with acyl-transferases, enzymes that acylates many substrates in the endoplasmic reticulum (Hofmann, 2000). It is believed that Drosophila porcupine and its worm homolog mom-1 encode the enzyme that is responsible for Wnt palmitoylation (Zhai et al., 2004). Recently, another conserved gene in Drosophila was discovered that is essential for Wnt secretion, named Wntless (wls) (Banziger, 2006; Bartscherer et al., 2006). The gene encodes a seven-pass transmembrane protein that is conserved from worm (mom-3) to human.
(liWLS) and primarily present in the Golgi apparatus and secretory vesicles (Bartscherer and Boutros, 2008; Eaton, 2008; Hardin and King, 2008). In the absence of Wls/ev Wnts are retained inside the Wnt producing cells.

1.3 Importance of Wnt signaling

Wnt signaling controls many processes including cell fate specification, cell proliferation, cell polarity and cell migration, which are critical for embryogenesis and development (Logan and Nusse, 2004). Misregulation of Wnt pathway is a cause for various diseases including colorectal cancers (CRCs) (Bienz and Clevers, 2000).

1.4 Milestones in history of Wnt signaling

There are many key events in the field of Wnt signaling that have helped elucidating the currently known pathway. Here are a few major milestone discoveries.

In 1982, Roel Nusse and Harold Varmus discovered a gene in mice tumor cells that was getting activated by a tumor-causing virus i.e. mouse mammary tumor virus (MMTV), they named it the integration 1 (Int1) gene. Soon thereafter, the Int1 gene was found to be homologous to the wingless (wg) gene in Drosophila (Munemitsu et al., 1995; Nusse and Varmus, 1982; Nusslein-Volhard and Wieschaus, 1980). This family of genes was subsequently renamed as Wnt, which originates from the merger of the two genes wingless and Int1. Wnt signaling is a pathway highly conserved from worms to humans (Nusse, 1991).

In the year 1987, it was demonstrated that Drosophila wg, which controls segment polarity during larval development, is a homolog of mouse Wnt1 gene (Rijsewijk et al., 1987). In the same year, McMahon and Moon observed
the phenomenon of a duplication of the body axis in Xenopus following injection of mouse Wnt1 mRNA into ventral blastomeres of embryos at the 4-cell stage. This provided a rapid and convenient assay to study components of the Wnt pathway and its effect in vertebrates (McMahon and Moon, 1989).

In late 80s and early 90s, truncation mutations in the gene Adenomatous polyposis coli (APC) were reported in patients of familial adenomatous polyposis coli (FAP) and sporadic colorectal cancers (Ashton-Rickardt, 1989; Groden, 1991).

In the year 1990, it was shown that spatial expression of the Drosophila segment polarity gene armadillo (arm) could post transcriptionally be regulated by wingless. In this study they found that even though arm RNA is uniformly distributed in embryos, ARM protein was found at higher levels in regions that contain wingless, and local increases in ARM protein required the wingless activity (Riggleman et al., 1990). They also found that two other segment polarity genes, porcupine and Dishevelled (Dvl), are essential for wingless to function.

Molecular cloning of β-catenin, the vertebrate homolog of Drosophila arm, was achieved in the year 1991 (McCrea et al., 1991). Genetic epistasis experiments further indicated that wg signaling operates through inactivating the zw3 repression. In addition, it was also shown that zw3 is the Drosophila homolog of mammalian glycogen synthase kinase-3 (Siegfried et al., 1992).

Later on, in the year 1993, through immunoprecipitation experiments β-catenin was shown to interact directly with APC (Rosin-Arbesfeld et al., 2000). Two years later, the effect of exogenous expression of APC on the amount and distribution of endogenous β-catenin in a colorectal cancer cell line containing mutant APC, was examined. Expression of wild-type APC
caused a pronounced reduction in the endogenous $\beta$-catenin levels in SW480 colorectal cancer cell line. This reduction was due to an enhanced rate of $\beta$-catenin degradation. Truncated mutant APC protein, characteristic of those associated with many CRCs lacked this activity. Mutational analysis also revealed that the central region of the APC protein, which is often deleted in tumors, was responsible for the down-regulation of $\beta$-catenin. These results suggested that, in the CRCs, the tumor-suppressor activity of APC may be compromised due to a defect in its ability to regulate $\beta$-catenin (Munemitsu et al., 1995).

In year 1995, The Nobel Prize in Physiology or Medicine was awarded to Nüsslein-Volhard, Wieschaus and Lewis for their discoveries of the genetic control of early embryonic development in Drosophila by Wnt.

In 1996, while performing quantitative immunoblot studies of FAP patients, it was found that in absence of wild type functional APC protein, $\beta$-catenin accumulates into the nucleus (Inomata et al., 1996). In the same year, it was also demonstrated that Xenopus GSK3 (Xgsk-3) regulates the stability of $\beta$-catenin by directly interacting and phosphorylating it at the conserved N-terminal region. They further showed that overexpression of dominant negative form of Xgsk-3 in Xenopus embryo lead to the nuclear accumulation of $\beta$-catenin (Yost et al., 1996).

In the same year, using a yeast two-hybrid screen it was shown that Lymphocyte enhancer factor (LEF-1) interacts with $\beta$-catenin. When co-expressed, both LEF-1 and $\beta$-catenin were found to exist in a complex. In the same study, they also found that microinjection of LEF-1 mRNA into Xenopus embryos induced axis duplication, a phenotype similar to $\beta$-catenin overexpression (Behrens, 1996).
In year 1997, mutations in β-catenin were identified in human melanoma and colon cancers (Korinek, 1997; Lin and Perrimon, 1999; Morin, 1997; Rubinfeld, 1997). In same year, stable and active β-catenin-T cell factor (TCF) complex was found in the nuclei of APC−/− colon carcinoma cells. They found increased transcriptional activity using Tcf reporter gene assay. Reintroduction of APC abolished the transcriptional transactivation by reducing the level of β-catenin (Korinek, 1997).

In 1997, the three-dimensional structure of the ARM repeat, which is a conserved stretch of a 42 amino acids, of β-catenin was determined, and the structure suggested that ARM repeat forms a super helix of helices with a positively charged groove, which provide the interface for interactions with other proteins (Huber et al., 1997). It was also demonstrated that phosphorylation of β-catenin targets it for subsequent ubiquitination by the E3 ligase β-transducin repeat containing protein (β-TrCP), and for further proteasome dependent degradation (Aberle et al., 1997).

Later a group of soluble proteins, called soluble Frizzleds (SFRP2 and FRZB), was identified as the extracellular antagonist of Wnt ligands (Finch, 1997).

Microinjection of Axin mRNAs into Xenopous embryo lead to inhibition of dorsal axis formation in embryos, which suggested that Axin acts as a negative player of Wnt signaling (Zeng, 1997). Later, He et. al. identified the proto oncogene myc as a target of the Wnt signaling pathway. They demonstrated that reintroduction of functional APC in CRCs led to the suppression of myc expression, and in contrast, overexpression of β-catenin lead to myc activation (He, 1998).

In 1998, a report suggested that mice lacking TCF-4 displayed a reduction of epithelial stem-cell compartments in the small intestine, suggesting the role of TCF-4 -β-catenin complex in intestinal epithelial homeostasis by stem cells
In the same year, Axin1 and Axin2 were found to be interacting with β-catenin, GSK3-β and APC, and promoting GSK3-β-dependent phosphorylation and further degradation of β-catenin. Using microinjection experiments in Xenopus embryos, it was shown that cunductin (Axin2) interferes with Wnt-induced axis formation (Behrens, 1998). In support of Behren’s study, Keiji Itoh et. al. found that the axis determination in Xenopus involves biochemical interactions of Axin, glycogen synthase kinase 3 and β-catenin (Itoh et al., 1998).

In the same year, Groucho was discovered as a corepressor of LEF–TCF transcription factors. It was found that dTcf can function as either an activator or a repressor of wg-responsive genes depending on the state of the wg signaling pathway and thus the availability of ARM. They found dTcf, in the absence of Wnt, forms a complex with groucho and functions as a repressor (Cavallo, 1998).

In the year 1999, it was demonstrated that ectopic expression of CK1 induces a complete second axis, similar to that of Wnt, and the study also suggested Dvl as a binding partner and substrate for CK1 and through various epistasis experiments they positioned CK1 between Dvl and GSK3-β (Peters et al., 1999).

In 1999, two groups found that GPI-anchored heparin sulphate proteoglycans, Dally in Drosophila and Knypek in zebrafish, as a negative regulators of Wnt signaling (Lin and Perrimon, 1999; Tsuda, 1999). In the same year, Wnt-inhibitory factor-1 WIF, was demonstrated to be a secretory protein, which interacts and inhibits Wnt ligands activity (Hsieh, 1999).

In 2000, Marcel Wehrli et. al. showed that arrow function is essential in cells which receives wg signals, and it acts upstream of Dvl. arrow encodes a single-pass transmembrane protein, that becomes a part of the receptor
complex with Frizzled (Fz) class of proteins. This was the first report identifying a co-receptor for the Wnt ligand (Wehrli, 2000).

In the year 2001, low-density lipoprotein receptor-related protein (LRP) (the vertebrate homolog of Drosophila arrow) was found to be inducing Wnt signaling by recruiting Axin to the plasma membrane. They also found that LRP and Axin can form the in vivo complex, which is increased under Wnt3a induced condition, and that this complex was found to be dependent on GSK3-β activity (Mao, 2001). In 2005, Sclerostin (SOST) was identified as a secreted, Wnt inhibitor that binds to LRP5 and LRP6. This study also showed that the loss of sclerostin (SOST) leads to the activation of Wnt signaling (Li, 2005).

In 2007, there were many discoveries in the field of Wnt signaling. Dvl polymerization and recruitment of Axin was demonstrated to be an important event for transducing the signal (Schwarz-Romond, 2007). Also, LGR5 was identified as a Wnt target gene and stem cell marker in the small intestine and colon (Barker, 2007).

In the same year, it was demonstrated that two microRNAs, miR-15 and miR-16, are regulated by the Wnt-β-catenin pathway and control Nodal signaling. In their study, they found that in Xenopus laevis microRNAs miR-15 and miR-16 limit the size of the organizer by targeting the Nodal type II receptor Acvr2a. Endogenous miR-15 and miR-16 are ventrally enriched as they are negatively regulated by the dorsal Wnt/β-catenin pathway (Martello, 2007).

Currently, three different pathways are believed to be operating upon Wnt receptor activation: a) the canonical Wnt/β-catenin cascade b) the non-canonical β-catenin independent pathway, that is further divided into two
distinct branches, the Planar Cell Polarity pathway and the Wnt/Ca\textsuperscript{2+} pathway (Fig 1.1).

![Canonical Wnt pathway](image)

**Fig 1.1 Canonical Wnt signaling.** (Left panel) In the absence of Wnt ligand, the serine/threonine kinases, CK1 and GSK3-\(\beta\), phosphorylate \(\beta\)-catenin, which leads to its degradation by the proteasome. When Wnt is bound to the cognate receptors, the degradation complex is inhibited, due to Fz-dependent recruitment of Dvl and LRP-dependent recruitment of Axin. As a consequence, \(\beta\)-catenin is stabilized and accumulates in the nucleus, where in association with TCF-4, it transactivates the transcription of Wnt responsive genes (right panel) (adapted from Clevers 2006).

1.5 The canonical Wnt signaling / \(\beta\)-catenin cascade

Canonical Wnt signaling is mediated by \(\beta\)-catenin, a protein consisting of 781 a.a. and has Mol wt 89 KDa. In humans, it is encoded by the CTNNB1 gene (Kraus et al., 1994). In Drosophila, the homologous protein of \(\beta\)-catenin is called ARM (Barkai and Leibler, 1997).
β-catenin was originally identified as a protein localized at cell adherens junctions, where it helps to bridge the cytoplasmic tail of cadherins to α-catenin and the actin cytoskeleton (Hulskens et al., 1994; McCrea et al., 1991). Besides being a vital component in cell adhesion, β-catenin is also a key signaling molecule in the canonical Wnt pathway (Moon et al., 2004; Polakis, 2000). Deregulation of β-catenin activity is associated with cancer and other human diseases in adults (Moon et al., 2002; Peifer and Polakis, 2000). Constitutive activation of β-catenin-dependent transcriptional activity is found in most colon cancers, which is due to inactivating mutations in the tumor suppressors APC and Axin, or activating mutation in β-catenin itself (Bienz and Clevers, 2000; Kinzler and Vogelstein, 1996). APC mutations that lead to β-catenin accumulation were found in more than 80% of colon cancers, while in some cases of hepatoblastomas and pilomatricoma, β-catenin has been found to be mutated (Bienz and Clevers, 2000; Moon et al., 2004; Polakis, 2000). In recent times, the Wnt/β-catenin pathway has emerged as a critical regulator of stem cell proliferation and differentiation (Reya and Clevers, 2005), and has been shown to play role in bone-density syndromes as well as in Alzheimer's disease (Moon et al., 2004). Therefore, β-catenin is a rationale target for various drug development programs, and there has been great interest in understanding its structure, function and regulation.

1.6 Structure of β-catenin

β-catenin protein contains a central structural core of 12 ARM repeats (residues 138–664) (Huber et al., 1997). Each ARM domain contains 42 amino acids, usually rich in the positively charged amino acids. These repeats generate a positively charged groove that spans the entire super helical ARM repeat region. ARM regions constitute the binding surface for the majority of (more than 20) β-catenin interacting partners, many of them are critical for
cell adhesion and Wnt signaling (Daniels and Weis, 2002; Eklof et al., 2001; Graham et al., 2000; Graham et al., 2002; Huber et al., 1997; Huber and Weis, 2001; Xing et al., 2003). Amino acid sequences of β-catenin terminal domains are less conserved than the ARM repeat domain. Both the N and C-terminal domains are sensitive to mild protease digestion, and it is believed that they can not form any stably folded structure by themselves (Huber et al., 1997). However, β-catenin terminal domains mediate a subset of protein-protein interactions, which in association with the ARM repeat domain, enables β-catenin to function as a scaffold for multiprotein assemblies. The N-terminal domain of β-catenin connects the β-catenin/E-cadherin complex to α-catenin, which is a key regulator of the actin cytoskeleton (Drees et al., 2005; Nagafuchi, 2001; Yamada et al., 2005). β-catenin is phosphorylated at the N terminus by kinases and recognized by the β-TrCP ubiquitin ligase and further targeted for ubiquitin mediated degradation (Jiang and Struhl, 1998; Wu et al., 2003). In brief, the phosphorylation status at the N terminus determines β-catenin’s stability and association with adherence junction component (Fig 1.2).

![Diagram of β-catenin structure]

**Fig 1.2 Primary structure of β-catenin.** The central domain of β-catenin consists of 12 ARM repeats (represented in empty rectangular boxes), which interact in an overlapping and mutually exclusive manner with APC, TCF, E-cadherin. The N terminus contains multiple phosphorylation sites, mutation or deletion of these potential phosphorylation sites at the amino terminus produces constitutively active β-catenin protein. The carboxyl terminus contains a transcriptional activation domain (adapted from Willert and Roel Nusse, 1998).
In the nucleus, TCF family members interact with β-catenin through ARM repeats 3–10, and anchor β-catenin to specific promoters. In addition to the TCF binding region, the first ARM repeat (R1) and ARM repeat 11 to the C terminus (R11-C) has been identified as important regions for the transactivation of Wnt target genes (Stadeli et al., 2006; Willert and Jones, 2006). β-catenin R1 interacts with BCL9, which in turn recruits Pygopus, an important transcriptional coactivator in Wnt signaling (Kramps et al., 2002). The β-catenin R11-C region has been shown to interact with many transcriptional co-activators, such as parafibromin, Brg1, CBP/p300, MED12, as well as transcriptional inhibitors such as ICAT and Chibby (Barker et al., 2001; Mosimann et al., 2006; Sierra et al., 2006; Tago et al., 2000; Takemaru et al., 2003; Takemaru and Moon, 2000).

Structure of β-catenin protein has been solved by various groups using X-ray crystallography. However, the various studies have used either β-catenin ARM repeat domains or β-catenin in complex with other interacting proteins (Daniels and Weis, 2002; Eklof et al., 2001; Graham et al., 2000; Graham et al., 2002; Huber et al., 1997; Huber and Weis, 2001; Xing et al., 2003). In 2008, Yi Xing et. al. solved the crystal structure of the full-length zebrafish and human β-catenin at 3.4 and 2.2 Å resolution, respectively (Xing et al., 2008).

1.7 Events during Wnt signaling

Wnt signaling can be discussed by dividing the cascade into three different steps. 1) Wnt reception at the membrane 2) Post-translational regulation of β-catenin by cytoplasmic destruction complex. and 3) β-catenin translocation to the nucleus and transactivation of Wnt responsive genes
1.8 Wnt reception at the membrane

In canonical Wnt signaling, secreted Wnt binds to its cognate receptors, the Fz and LRP5/6 co-receptor, and formation of this ternary complex at the plasma membrane activates the pathway (Fig 1.1). Cytoplasmic tail of Fz has been shown to associate with Dishevelled (Dsh/Dvl), a cytoplasmic transducer that functions upstream of β-catenin and the kinase GSK3-β. Wnt signaling leads to phosphorylation of Dvl (Wallingford, 2005). However, it is not very clear how precisely the binding of Wnt to Fz receptor regulates interaction between Fz-Dsh, and it is also not known how Dvl phosphorylation is controlled during Wnt response or how phosphorylated Dvl functions in Wnt signal transduction pathway. Few recent studies have demonstrated that the co-receptor LRP5/6 interacts with Axin through five phosphorylated PPP(S/T)P repeats in the cytoplasmic tail of LRP (Davidson, 2005; Zeng, 2005). Wnts are believed to induce the phosphorylation of the cytoplasmic tail of LRP, thus regulating the binding of Axin. GSK3-β phosphorylates the PPP(S/T)P motif, whereas casein kinase I-γ (CK1γ) phosphorylates multiple motifs close to the GSK3-β sites (Fig 1.1).

It also remains unclear whether Wnt controls GSK3-β-catenin mediated phosphorylation of LRP5/6 (Zeng, 2005) or CK1γ is the kinase regulated by Wnt (Davidson, 2005). When bound to their respective membrane receptors, one line of thought also suggests that heterodimerization of Dvl and Axin through their respective DIX domains may cooperatively mediate downstream events (Schwarz-Romond, 2007).

1.9 Post translational regulation of β-catenin by cytoplasmic degradation complex

Stability of β-catenin in the cytoplasm is controlled by the degradation complex. In the degradation complex Axin, which acts as a scaffold protein,
interacts directly with β-catenin and other components of degradation complex, APC, and the two kinase families (CK1α, -δ, -ε and GSK3α and -β) (Price et al, 2006). When Wnt receptors are not engaged with Wnt ligand, CK1 and GSK3α/β sequentially phosphorylate β-catenin by a dual kinase mechanism at a series of highly conserved Ser/Thr residues near its N terminus (Fig 1.2). In this phosphorylation event, CK-1 first phosphorylates β-catenin at Ser45, this creates priming site for GSK3-β phosphorylation at the Thr41, Ser37 and Ser33. Phosphorylated β-catenin is recognized by the F box/WD repeat protein β-TrCP, a component of E3 ubiquitin ligase complex. As a consequence, β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome (Aberle et al., 1997).

Upon receptor activation by Wnt ligands, the intrinsic kinase activity of the degradation complex for β-catenin is inhibited. It is unclear how this occurs, but it likely involves the Wnt-induced recruitment of Axin to the phosphorylated PPP(S/T)P site in the tail of LRP and/or binding of Dvl to the Fz.

1.10 β-catenin translocation to the nucleus and transactivation of Wnt responsive genes

β-catenin is a protein that shuttles between nucleus and cytoplasm. This may help in relaying the Wnt signal in an effective manner. Many attempts have been made to discern the mechanism of β-catenin transport into and out of the nucleus. The import of β-catenin is shown to be independent of Ran and other nuclear import factors (Fagotto et al., 1998; Yokoya et al., 1999). This appears partly due to the fact that β-catenin resembles importin-β at the three dimensional level (in containing structurally related ARM or HEAT repeats), and importin-β also can enter the nucleus in a Ran independent manner (Lee et al., 2000). However, better functional characterization is required to understand the mechanisms involved. The export of β-catenin, on the other
hand, occurs in a Ran-dependent but CRM-1-independent manner (Henderson and Fagotto, 2002; Hendriksen et al., 2005). A role for other β-catenin interacting partners, such as APC, Axin and TCF, in both import and export has also been suggested (Henderson and Fagotto, 2002). Particularly, APC is shown to play a role in the export of β-catenin from the nucleus and concomitant degradation in the cytoplasm (Rosin-Arbesfeld et al., 2003; Rosin-Arbesfeld et al., 2000). Colorectal cancer patients are believed to have a compromised APC-mediated export function. Deletion analysis also indicated that the C-terminal region has an overlapping import and export activity (Koike et al., 2004). In 2005, Jolita Hendriksen et al. showed that β-catenin is exported out of nucleus under Wnt-1 induced condition by RanBP3, independent of APC and CRM1 (Hendriksen et al., 2005).

A clear picture is still lacking as to how the transport of β-catenin is precisely regulated. Although the transport of β-catenin across the nuclear membrane is critical for the control of Wnt signaling, little is known about the mechanism involved in either nuclear import or export.

Both fly and worm genomes encode a single TCF protein, the vertebrate genome harbors four Tcf/LEF genes. TCF factors bind their cognate motif in an unusual fashion, i.e., in the minor groove of the DNA helix, while inducing a bend of over 90°. TCF target sites are highly conserved between the four vertebrate TCF/LEF proteins and Drosophila TCF. These sites are similar to (AGATCAAAGG) (van de Wetering et al., 1997). TCF reporter plasmids such as pTOPflash (Korinek et al., 1997) are widely used to measure activation of Wnt pathway, consist of repeats of TCF binding motifs cloned upstream of a minimal promoter. In the absence of Wnt signals, TCF acts as a transcriptional repressor by forming a complex with Groucho/Grg/TLE proteins (Cavallo, 1998; Roose, 1998). During Wnt activation, the interaction of β-catenin with the N terminus of TCF (Behrens, 1996; Molenaar, 1996; van de Wetering et al., 1997), transiently converts it into an activator (Fig 1.3). β-
catenin physically displaces Groucho from TCF/LEF complex (Daniels and Weis, 2005; Molenaar, 1996; van de Wetering et al., 1997). The recruitment of β-catenin to TCF target genes affects local chromatin architecture; C terminus of β-catenin has been shown to bind with transcription co-activators, histone acetylase CBP and Brg-1 (Stadeli et al., 2006), and this region is considered to possess the trans-activator function as revealed by transient reporter gene assays (Molenaar, 1996; van de Wetering et al., 1997).

Two dedicated nuclear partners of the TCF/β-catenin complex, Legless/Bcl9 and Pygopus, were recently found by genetic screens in Drosophila (Hendriksen et al., 2005; Kramps, 2002; Parker et al., 2002; Thompson et al., 2002). Mutations in these genes result in phenotypes similar to wingless (Hoffmans et al., 2005) and overexpression of both genes promotes TCF/β-catenin activity in mammalian cells (Thompson et al., 2002). Bcl9 helps in bridging Pygopus to the N terminus of β-catenin, and the formation of this trimeric complex has been implicated in nuclear import/retention of β-

Fig 1.3 β-catenin inside the nucleus. Inside the nucleus de-phospho form of β-catenin binds to the TCF at the N terminus this binding in turn releases Groucho suppression over TCF and allows activation of Wnt target genes (adapted from Bio base biological database).
catenin (Townsley et al., 2004), but this could also directly contribute to the ability of β-catenin to transactivate transcription. Although most, if not all, Wnt signaling events in Drosophila appear to be dependent on Bcl9 and Pygopus, it is currently unclear if this holds true in vertebrate development.

### 1.12 Non-canonical planar cell polarity (PCP) pathway

The non-canonical signaling pathway is also known PCP pathway, which is independent of β-catenin-mediated transcriptional activation (McDonald and Silver, 2009). In this pathway, the Wnt signal is received by Fz receptor. Fz, and several other PCP effectors, including Strabismus, Diego and Prickle, work in coordination to regulate the asymmetric accumulation of a complex of proteins at the plasma membrane that includes Dvl (Wallingford et al., 2002; Klein and Mlodzik, 2005). The non-canonical Wnt signaling pathway, regulates cell polarity, cell movements during gastrulation, and is mediated primarily by the PDZ and DEP domains of Dvl (Wallingford and Habas, 2005).

At the level of Dvl, two independent parallel pathways lead to the downstream activation of the intracellular small GTPases Rho and Rac (Eaton et al., 1996; Peranen et al., 1996; Fanto et al., 2000; Habas et al., 2003; Tahinci and Symes, 2003). The first pathway signals to Rho, and occurs through the molecule DAAM1 (Dishevelled associated activator of morphogenesis 1) (Habas et al., 2001). This Rho pathway leads to the activation of the Rho-associated kinase ROCK, which mediates cytoskeletal re-organization (Marlow et al., 2002; Veeman et al., 2003). A second pathway activates Rac, which in turn stimulates JNK activity (Fig 1.4) (Li et al., 1999; Boutros et al., 1998; Yamanaka et al., 2002; Habas et al., 2003). Both these pathways control the reorganization of actin cytoskeleton (Habas et al., 2003; Tahinci and Symes 2003). However, the nature of Dvl function in the PCP cascade, as in the canonical pathway, remains largely unknown.
Fig 1.4 Schematic representation of the non-canonical Wnt signaling pathway. Non-canonical Wnt signaling is transduced through Fz receptor without engagement of LPR5/6. The PCP (left) pathway leads to cytoskeletal changes through activation of the small GTPases Rho and Rac. The Wnt-Ca2+ pathway (right panel) operates through Fz mediated activation of heterotrimeric G-proteins, which leads to the release of intra-cellular Ca2+, and this process involves Dvl, calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC) (adapted from Habas and Dawid 2005).

1.13 Ca2+ signaling pathway

Another β-catenin independent, non-canonical pathway is the Wnt/Ca2+ pathway, which may actually influence the function of both the canonical and PCP pathways (Hocking et al. 1999; Park et al. 1999; Slusarski et al. 2003). Wnt/Fz, via the trimeric G-protein, activates phospholipase C, leading to the generation of diacylglycerol and IP3, which in turn generates calcium fluxes. Release of intracellular calcium results in the activation of various intracellular calcium-sensitive enzymes such as protein kinase C (PKC), calcium-calmodulin-dependent kinase II (CamKII) and the calcium-sensitive phosphatase calcineurin (Fig 1.4 right panel) (Habas et al. 2007). In Xenopus embryos, expression of a Dsh mutant lacking the DIX (DshΔDIX) domain causes an increase in intracellular calcium flux, CamKII activation, and PKC
translocation to the plasma membrane. Conversely, loss of function of Dvl results in decreased membrane translocation and decreased kinase activity of ectopically expressed PKC (Slusarski et al. 2003).

1.14 Nuclear pore complex

The nucleus is an important organelle in the eukaryotic cell. It contains the genetic material and directs cellular activity by regulating gene expression. The nuclear pore complex (NPC) is found on the nuclear envelope. This structure represents the gate between nucleoplasm and cytoplasm, mediating the traffic of macromolecules between the cytoplasm and nucleoplasm (Meier et al., 2008; Xu et al., 2007). The NPC is a large multi-protein complex, which comprises multiple copies of about 30 different proteins called nucleoporins (Rout and Aitchison, 2000; Cronshaw et al., 2002). The molecular masses of mammalian and yeast NPCs are estimated to be 125 and 50 MD, respectively. Ultrastructural analysis of the NPC reveals that its basic architecture is conserved among vertebrates (Kiseleva et al., 1996), yeast (Allen and Douglas, 1989), and plants (Roberts and Northcote, 1970; Fiserova et al., 2009). Vertebrate NPC subunits form a doughnut-shaped channel with an eightfold radial symmetry that can be divided into three parts: a nuclear basket, a central pore, and cytoplasmic filaments (Fig 1.5).

FxFG repeat binds directly to the transport receptors and thereby mediating active transport through the NPC (Ryan and Wente, 2000). FG repeats also play a role in NPC permeability, preventing non-karyophilic proteins larger than 40 kD from entering or exiting from the nucleus by simple diffusion (Patel et al., 2007). Nucleoporins, which are members of the Trp-Asp (WD) repeat family, generate a β propeller structure that is thought to be important for the assembly of large multiprotein complex (Smith and de, 1999). In the NPC, WD repeats appear to mediate the assembly of NPC scaffold
subdomains (Rabut et al., 2004), and facilitate interactions between transport complexes and the NPC.

![Current NPC model with its major structural components](image)

**Fig 1.5 Current NPC model with its major structural components.** NPC has a basket-like structure with eight-fold symmetry, which can be divided into 3 parts 1) cytoplasmic ring and fibers which control the import and export of various molecules at cytoplasmic side, 2) central ring, which governs the selective passage of nucleo-cytoplasmic transport and 3) Nuclear ring and nuclear basket, which control the import and export of various molecules at the nuclear side. Nup358 is localized towards the cytoplasmic face of NPC on cytoplasmic filaments (adapted from Pante et al., 2000; Pante et al., 1994).

The NPC is well characterized in vertebrates and yeast, and their nucleoporins share structural motifs and specific sequences. Transmembrane domains are found in a small number of nucleoporins. In vertebrate cells, GLYCOPROTEIN OF 210 kD (gp210), PORE MEMBRANE PROTEIN OF 121 kD (Pom121), and NUCLEAR DIVISION CYCLE1 (NDC1) have been identified as integral pore membrane proteins (Cronshaw et al., 2002). They appear to function as a scaffold for the NPC, and following mitosis they are involved in reconstructing the nuclear pore in the newly formed nuclear envelope (Guttinger et al., 2009).

NPCs are usually stable throughout interphase but disassemble into subcomplexes during mitosis. At the end of mitosis each sub complex is sequentially recruited to the nuclear periphery, in association with nuclear
envelope reformation (Daigle et al., 2001; Bodoor et al., 1999). In vertebrate NPCs, three sub complexes have been described: the Nup62 sub complex (Nup62, Nup58, Nup54, and Nup45); the Nup107-160 sub complex (Nup160, Nup133, Nup107, Nup96, Nup75, Nup43, Nup37, Seh1, Sec13, and ALADIN); and the Nup93 sub complex (Nup205, Nup188, Nup155, Nup93, and Nup35). Each sub complex performs specific function (Tran and Wente, 2006) (Fig 1.6).

**Fig 1.6 Schematic representation of the relative position of the major nucleoporin subcomplexes in metazoans.** Localization of protein (delimited by dashed line) in not known precisely within or around the NPC, Protein names in black indicate proteins having homologs in fungi, whereas those in red indicate proteins having no homologs but structural analogues in fungi. Lines between sub-complexes indicate putative interactions, whereas double lines indicate confirmed interactions (adapted from Bapteste et al., 2005). Note that Nup358 is localized on the cytoplasmic face of the NPC.
1.15 Nup358 (or Ran binding protein 2)

Nup358, also called Ran binding protein 2 (RanBP2), is a giant nuclear pore protein with a molecular weight 358 KDa, and is localized to the cytoplasmic side of the NPC (Yokoyama et al., 1995) (Fig 1.5). It is a protein with well-defined structural modules such as four RanBP1-like Ran binding domains (RanBD), leucine-rich region (LRR), a zinc finger domain (ZnF), a cyclophilin-homology domain (CHD) (Wu et al., 1995). Nup358, being a Ran binding protein and nuclear pore protein, is believed to regulate nucleo-cytoplasmic transport (Fig 1.7).

![Diagram of human Nup358](image)

*Fig 1.7 Schematic representation of human Nup358 (3224 a.a).* Nup358 with its characterized functional domains and different truncation of full length Nup358 used in our lab (adapted from Murawala, et al. 2009).

The amino terminus of Nup358 possesses a leucine rich region (LRR), through which Nup358 interacts with microtubules (Joseph and Dasso, 2008). RNAi mediated knockdown of Nup358 affected polarized stabilization of microtubules during directed cell migration, confirming the *in vivo* role of Nup358 in regulating interphase microtubules (Joseph and Dasso, 2008). The amino terminus of Nup358 also has a tetratricopeptide repeat (TPR) domain spanning amino acids from 60–93, which is believed to play a role in protein-protein interactions. Nup358 has eight zinc finger motifs that bind to Ran-GDP (Yaseen and Blobel, 1999). Carboxy terminus of Nup358 contains internal repeats (IR), which is shown to interact with SUMO modified RanGAP1 and Ubc9 (Saitoh et al., 1997). Nup358 functions as SUMO E3
ligase (mediated through the IR region) and SUMOylates various proteins such as DNA-unwinding enzyme topoisomerase II (Reverter and Lima, 2005; Dawlaty et al., 2008). During mitosis, Nup358, in association with RanGAP1, is targeted to kinetochores in a microtubule-dependent manner (Joseph et al., 2002). Nup358 has been shown to be essential for kinetochore maturation as well as in proper kinetochore-microtubule interactions during mitosis (Joseph et al., 2004). At the extreme C terminus, it has cyclophilin homology domain (CHD), which contains the *cis-trans* prolyl isomerase activity, and functions as a selective chaperone for folding of red/green opsin in retina (Ferreira et al., 1996; Ferreira et al., 1997).

In 2009, our group showed that Nup358 interacts with APC, and ectopic expression of the middle region of APC is sufficient to recruit endogenous Nup358 to the plus ends of microtubules. Furthermore, Nup358 was shown to cooperate with kinesin-2 to regulate the localization of APC to the cell cortex through a nuclear-transport-independent mechanism and thereby playing a novel role in cell polarization. Very recently, Nup358 was also implicated in different pathogenic conditions, various group showed that siRNA mediated Nup358 depletion results in inhibition of HIV-1 infection (Zhang et al., 2010; Ocwieja et al., 2011). Nup358 depletion was found to be causing cell death with defects in classical nuclear localization signals (NLS) mediated protein import, nuclear export signals (NES) mediated protein export, and mRNA export. The N-terminal Nup358 fragment, containing the NPC binding domain, three phenylalanine-glycine repeats, and one Ran binding domain (RBD), restored transport and viability. The data suggested that a critical function of Nup358 is to capture recycling RanGTP-importin-β complexes at cytoplasmic fibrils to allow for adequate NLS-mediated import of cargos (Hamada et al., 2011).
1.16 Ran and nucleo-cytoplasmic transport

Ran is an abundant GTPase that is highly conserved in eukaryotic cells and has been implicated in many important cellular processes, especially in determining the directionality of nucleocytoplasmic transport during interphase. However, it is also critical for other cellular processes, including mitotic spindle assembly and post-mitotic nuclear envelope assembly (Sazer and Dasso 2000; Joseph, 2006).

Ran, like other GTPases, switches between a GTP bound and a GDP bound form. These transitions are regulated by differentially distributed regulators of Ran i.e. a guanine nucleotide exchange factor (GEF) termed RCC1 (regulator of chromosome condensation 1) (Bischoff and Ponstingl, 1991) nuclear and the GTPase-activating protein RanGAP1 cytoplasmic (Bischoff et al., 1995). The hydrolysis by RanGAP1 requires an additional factor, RanBP1, which possesses a RanGTP binding domain and assists the process of export of cargos into the cytoplasm (Bischoff et al., 1995; Bischoff and Gorlich, 1997). Nup358 has four RanBP1-like domains and is therefore believed to regulate nucleo-cytoplasmic transport like RanBP1 (Wu et al., 1995).

Differential localization of the regulators of Ran is predicted to create a steep RanGTP gradient across the nuclear envelope: RanGTP levels being higher in the nucleus and lower in the cytoplasm (Fig 1.8). This asymmetry dictates the nucleo-cytoplasmic transport by regulating the assembly and disassembly of import and export complexes (Gorlich and Kutay, 1999; Macara, 2001). The transport of macromolecules across the NE is generally mediated by a family of RanGTP binding receptors, termed `karyopherins'. These include importin-β and CRM-1 (chromosome region maintenance 1), which mediate the import and export of a characteristic set of cargos containing nuclear localization signals (NLSs) and nuclear export signals (NESs), respectively.
Fig 1.8 Receptor-Mediated nucleo-cytoplasmic Transport. Import receptor (left) recognizes and binds NLS-containing cargos. The complex proceeds to the nucleoplasm through the FG channel of the NPC. In the nucleus, the import complex encounters high RanGTP, maintained by the Ran guanine nucleotide exchange factor (RanGEF). Binding of RanGTP to the importin releases the cargo into the nucleus. Export receptor (right) binds to its cargo (NES) in the nucleus in the presence of RanGTP. The trimeric complex crosses the FG hydrophobic channel and reaches to the cytoplasm. Here in the presence of the RanGAP1 and the cofactor RanBP1/RanBP2, the export complex dissassociates upon RanGTP hydrolysis (adapted from Xylourgidis and Fornerod, 2009).

In the cytoplasm, where the RanGTP level is low, the import complex is formed when NLS-containing cargo is recognized by importin-α and assembled with importin-β. The import complex travels through the NPC into the nucleus, where it disassembles following binding of importin-β to the abundant nuclear RanGTP. Conversely, NES bearing export cargo binds CRM1 and RanGTP in the nucleus and translocates through the NPC. In the cytoplasm, RanGTP hydrolysis by RanGAP1 and RanBP1/RanBP2 disassembles the complex, releasing the export cargo. The RanGDP resulting from GTP hydrolysis in the cytoplasm is recycled back to the nucleus by the
RanGDP binding protein NTF2 (nuclear transport factor 2). RanGTP thus acts as a positional cue defining the nuclear compartment, and directs the disassembly and assembly of import and export complexes, respectively (Fig 1.8). Importin-β cycles back to the cytoplasm in a RanGTP-dependent manner, whereas importin-α requires an additional factor, CAS (cellular apoptosis susceptibility protein), for its recycling. Both CRM1 and NTF2 can relocate to the nucleus and cytoplasm, respectively, independently of RanGTP.

1.17 APC

In humans, APC gene is predicted to encode a protein of 2843 amino acids (Mol. wt. 312 KDa) with multiple functional domains. Mutational studies in mice and cultured cells have established APC as a suppressor of canonical Wnt signaling (Fodde et al., 1994; Oshima et al., 1995; Su et al., 1992). Through different domains APC interacts with the various important proteins, such as β-catenin, Axin, the cytoskeletal regulators EB1 and IQGAP1, and the Rac guanine-nucleotide-exchange factor (GEF) Asef1, it also associates with microtubules. Various studies have suggested the role of APC in cell adhesion, migration, organization of the actin and microtubule networks, spindle formation and chromosome segregation.

Mammalian APC has an oligomerization domain, an ARM repeat-domain, a 15 or 20 residue repeat domain important for binding to β-catenin, SAMP repeats for Axin binding, a basic domain for microtubule binding and C-terminal domains that bind to EB1 and DLG proteins.

APC gene is a tumor suppressor gene, which was initially identified by its link to familial and sporadic forms of colorectal cancer. Germline mutations in the APC gene have been demonstrated in most cases of familial adenomatous polyposis, an inherited form of colorectal cancer (Groden et al.,
1991; Joslyn et al., 1991; Nishisho et al., 1991). In more than ~80% cancer-linked to APC, mutations occur in a central region of APC, that is referred to as the mutation cluster region, often leading to the loss of C-terminal regions (Fig 1.9) (Beroud and Soussi, 1996).

Mutational loss of APC results in a truncated protein, incapable of binding Axin and other regulatory proteins, and thus leads to stabilization and further accumulation of $\beta$-catenin inside the nucleus, which in turn leads to Wnt-independent activation of TCF/LEF and Wnt responsive genes, e.g. c-Myc and cyclinD1 (Barker and Clevers, 2006; Morin, 1997; Korinek et al., 1997; Reya and Clevers, 2005).

Many studies suggested that APC may play a regulatory role in exporting $\beta$-catenin into the cytoplasm and its enhanced degradation (Henderson and Fagotto, 2002; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000).

1.18 Nuclear export of APC and cancer

Nuclear export of APC has been shown to be dependent on CRM1/Exportin receptor pathway (Henderson and Fagotto, 2002; Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). APC contains at least five different NESs. Two classic Rev-type NESs (that closely resemble the HIV-1 Rev protein) are located at the N terminus (Henderson, 2000; Neufeld et al., 2000), and three others are near the central region within the $\beta$-catenin binding domain (Rosin-Arbesfeld et al., 2000). Comparative analysis of NES revealed that N-terminal NES1 sequence to be the strongest and well conserved signal (Galea et al., 2001). A transiently expressed form of APC (1–1309 a.a), the most common mutant APC in cancer (Polakis, 2000), is also primarily cytoplasmic but shifts to the nucleus under leptomycin B treated condition (a CRM1 inhibitor) and by site-directed mutations in NES1 (Galea et al., 2001; Henderson, 2000). While it is possible that the actual rate of nuclear
import/export is reduced in cancer cells, the conservation of nuclear shuttling activity in cancer may be important for cell survival or growth. For a long time people thought that \( \beta \)-catenin being a shuttling protein (without strong NES) can rely on APC for its export.

Fig 1.9 Schematic diagram of full length and one of the C-terminally truncated APC proteins. Full length wild type protein possesses oligomerization domain at its extreme N-terminus, next to it an ARM repeats are present. In the center it possesses 15 and 20 amino acid repeats which binds to \( \beta \)-catenin, central region also has SAMP repeats which binds to the Axin. Central region of APC spanning from (1284-1580 a.a) is mutation cluster region. More than 95% of colorectal cancer patients have mutation in this region of APC which results in the C-terminally truncated proteins (adapted from Koji Aoki and Makoto M. Taketo 2007).

The first clue that \( \beta \)-catenin can exit the nucleus independently of APC came from the analysis of a Xenopus \( \beta \)-catenin mutant (\( \Delta \)19), that disrupts its binding to APC, LEF-1 and E-cadherin. This \( \beta \)-catenin mutant was primarily
nuclear but capable of nuclear export (Prieve and Waterman, 1999). Eleftheriou et al. (2001) used in vitro assays with digitonin-permeabilized SW480 colon cancer cells, which express high levels of nuclear β-catenin, and showed that the endogenous β-catenin exited the nucleus independently of APC (Eleftheriou et al., 2001). These findings suggested that, in human cancer cells, β-catenin nuclear export occurs primarily independent of APC.

Transport of β-catenin across the nuclear membrane is critical for the control of Wnt signaling, many attempts have been made to discern the mechanism of β-catenin transport across the nuclear membrane. But still a clear picture is lacking as to how the transport of β-catenin is precisely regulated. In present study we have tried to understand the regulation of β-catenin by a nucleoporin Nup358.

During the characterization of Nup358 and β-catenin interaction, we came across an interesting observation. We found that β-catenin possesses the ability to interact with metal ions such as Nickel, in our study we have tried to understand the Nickel binding properties of β-catenin and possible role of this interaction in Wnt signaling.

1.19 Importance of studying metal toxicity

Our global environment consists of ample amount of various metals. Metals have played important role in industrial development and technological advances. Most metals are not destroyed because of their very high half-life, and they start accumulating in the environment at an enhanced pace, due to the growing demands of modern society. The global distribution of metals in the environment is of great concern because of the carcinogenic properties of many of the metals. Only few of the current research focus in the area of metal toxicity in the global environment. Research in this field is equally
important because of increasing concern of metal toxicity, which will allow us to understand the molecular mechanism of pathogenic effects of the metals and metal induced carcinogenesis.

1.20 Metal carcinogenesis

Prolonged exposure to the transition elements such as Nickel (II), Chromium (VI), or Cobalt (II) has long been known to increase the incidence of cancers among affected individuals. Toxic metals are strong activators of stress-signaling pathways. Apart from activating stress signaling pathways by inducing DNA damages, heavy metals can also bring about epigenetic changes in DNA, such as change in histone modifications and DNA methylation, which further results in altered gene expression patterns.

It appears that, in general, metal carcinogenesis requires the formation of specific metal complexes, which can affect the cells in numerous ways such as chromosomal damage, inhibiting the function of proteins and inappropriate activation of signal transduction pathways (Salnikow and Zhitkovich, 2008).

1.21 Uptake, distribution, and retention of nickel

There are different forms of nickel element present in nature, which differ in their carcinogenic properties significantly; this reflects the differences in their cellular uptake, transport, distribution and retention (Fig 1.10). Capacity to deliver Ni (II) ions to specific cells and target molecules largely depends on the physical and chemical properties of metal such as solubility, particle structure, size, and redox activity of various nickel derivatives.

Phagocytosis is considered to be the most effective mechanism of nickel uptake, which has also been shown to be dependent on both the size and the
surface electric charge of the particles (Heck and Costa, 1982; Costa et al., 1981; Kuehn et al., 1982).

![Fig 1.10 Schematic representation of the uptake and cellular interactions of Ni (II) derived from nickel compounds](image)

1) Once Ni (II) get into the cell from different ways such as phagocytosis, Ca2+ channels or by diffusion. 2) It forms variety of complexes with different ligands, such as amino acids, peptides, proteins, and glutathione, some of which are redox active and catalyze ROS production; the major effect is hypoxic stress due to Ni (II) interference with iron transport and iron-dependent hydroxylases. 3) The nuclear Ni (II) and Ni (II)-generated ROS interact with DNA and histones, causing promutagenic DNA damage (enhanced through inhibition by Ni (II) of DNA repair enzymes, and epigenetic alterations resulting from Ni (II)-induced DNA hypermethylation, histone, hypoxacylation and structural damage, and transcription factors activation (Kasprzak et al., 2003).

### 1.22 Human exposure to nickel and symptoms

Exposure of nickel to human occurs primarily via inhalation and ingestion and is particularly high among nickel painting and metallurgy workers. Wearing or handling of jewelry, coins, or utensils, that are made-up from nickel alloys may result in cutaneous nickel absorption (Hostynek, 2002).
Another source of human nickel exposure is dietary where some foods, especially of plant origin, might contain around 1 mg Ni/kg. Industrial exposure to nickel occurs predominantly in mining, refining, alloy production, electroplating, and welding companies. In 1990, the International Committee on nickel Carcinogenesis suggested nickel as a carcinogen (International Agency for Research on Cancer 1990).

Exposure to nickel compounds can produce a various adverse effects on human health, ranging from contact dermatitis to lung fibrosis, cardiovascular and kidney diseases.

1.23 Molecular mechanisms of action

The most serious concerns related to carcinogenic activity of nickel compounds and its human epidemiology have been studied in detail in various experimental animal and cell culture models, based on the experimental evidences various groups, have postulated different molecular mechanisms of nickel carcinogenesis.

Like other transition element, nickel has wide range of effects on the target cells such as genotoxic effect, epigenetic effect, inhibition or activation of transcription factors and inhibition of function of important cellular proteins.

1.24 Genotoxic effect

Similar to the other transition element, nickel can exert its carcinogenic effect on the cells by several ways. For example, nickel compounds generate specific morphologic chromosomal damage. CHO cells treated with nickel shows chromosomal abnormalities in cultures (Conway and Costa, 1989). In these cells, nickel compounds were also found to be weak inducers of sister chromatid exchanges (SCE), especially in heterochromatin region (Sen and
Costa, 1986). In cultured human lymphocytes, a nearly two-fold increase in SCEs was observed after treatment of Ni (II) sulfate (Sahu et al., 1989). Exposure to Ni$_2$S$_2$ also resulted in a significant increase of micronuclei formation in human lymphocytes (Arrouijal et al., 1990). In addition to chromosomal damage, DNA–protein cross-links and oxidative DNA base damage were observed in cells exposed to Ni (II) (Patierno et al., 1985; Kasprzak, 1991). In rat kidney epithelial (NRK) cells infected with MuSVts110 retrovirus, Ni (II) has been shown to induce insertion mutation of a 70 base pair-long sequence of DNA (Chiocca et al., 1991).

1.25 Epigenetic effects

Further better understanding of molecular mechanisms of nickel carcinogenicity was gained by the study showing that nickel compounds increase the extent of DNA methylation that leads to the inactivation of gene expression (Lee et al., 1995). In this elegant study, the position of the gpt transgene on the chromosome was found to be important, since exposure of cells to nickel compounds resulted in hypermethylation of the transgene when it was located near heterochromatin, and when the gene was far from the heterochromatin the effect was not seen.

In addition to gene silencing by hypermethylation, an inhibitory effect of nickel on histone H4 acetylation in vitro has been shown in both yeast as well as mammalian cells (Broday et al., 2000). The acetylation at Lys-12 and Lys-16 in yeast was found to be inhibited by nickel. These data again suggested that nickel causes epigenetic alteration, further leading to aberrant gene expression.
1.26 Binding of nickel with cellular proteins

Mass spectrophotometry studies of Ni-NTA pull down sample from cell lysates have identified several nickel binding proteins which belong to stress signaling pathways (Heiss et al., 2005). Various studies have also highlighted the importance of nickel binding with proteins and its effect on the cellular machinery.

1.27 Binding with HIF-1 transcription factor

HIF-1α is the hypoxia-inducible subunit of the HIF-1 transcription factor. It binds with the HIF-1β subunit and transactivates hypoxia responsive genes (Semenza, 2000; Semenza, 2001), such as Glut1 and Glut3 (glucose transporters 1 and 3) and most of the glycolytic enzymes (Carmeliet et al., 1998). During tumor progression, HIF-1 facilitates angiogenesis, which is essential for survival of the tumor at low oxygen level (Folkman, 1971; Semenza et al., 1994). In case of hypoxic cancer and stromal cells, HIF-1 transactivates various growth factors including VEGF, FGF, PAI-1, adrenomedullin, nitric oxide synthase (NOS), and transferrin. These factors stimulate endothelial cell proliferation, migration, invasion, and angiogenesis (Rolfs et al., 1997; Garayoa et al., 2000; Melillo et al., 1995).

The Gene Chip microarray analysis also revealed that genes coding for glycolytic enzymes, glucose transporters, and other hypoxia-inducible genes regulated by HIF-1, were induced by nickel in normal but not in HIF-1α-null mouse embryonic cells (Salnikow et al., 2003; Salnikow et al., 2002). Like hypoxia, nickel also causes transactivation of the hypoxia-inducible genes through the induction of HIF-1 transcription factor in a reactive oxygen species (ROS)-independent manner (Salnikow et al., 2000).
In normoxic conditions, the HIF-1α protein is rapidly degraded by the proteasomal machinery, and therefore is maintained at low levels (Sutter et al., 2000). Under hypoxic conditions, the degradation stops and HIF-1α is accumulated, whereas reoxygenation results in a resumption of its degradation (Jewell et al., 2001). Therefore, HIF-1 plays the role of an oxygen sensor. In the presence of O2, proline hydroxylase, a Fe(II)-dependent enzyme, hydroxylates HIF-1α at Pro-564 (Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001). The hydroxylated proline residue is recognized by the von Hippel–Lindau protein (VHL), a component of the E3 ubiquitin ligase complex (Ohh et al., 2000). The interaction with VHL leads to ubiquitination and subsequent degradation of HIF-1α. In hypoxic conditions, Pro-564 is not hydroxylated; HIF-1α protein does not bind to VHL that leads to its accumulation in the cytoplasm. HIF-1α hydroxylation requires O2 and the Fe (II)-dependent enzyme, proline hydroxylase. In addition, Asn-803 in the C-terminal transactivation domain of HIF-1α is hydroxylated by a factor inhibiting HIF-1 (FIH-1) under normoxic conditions, causing abrogation of the HIF-1α/p300 interaction (McNeill et al., 2002; Lando et al., 2002). This reaction also requires oxygen as a substrate and iron as a co-factor. Under hypoxic stress, i.e., in the absence of Asn-803 hydroxylation, p300 (an acetyltransferase) binds to HIF-1 and transcriptionally activates HIF-1-dependent genes. Thus, in order to activate the HIF-1-dependent pathways, Ni (II) should stabilize HIF-1α protein and facilitate HIF-1 interaction with p300. This could happen only when hydroxylation of the proline and asparagine residues in HIF-1α is prevented. The key factor for the hydroxylase activity is Fe (II). Therefore, Ni (II) is likely to induce hypoxia through the depletion of cellular iron or competitive inhibition of proline hydroxylase activity (Fig 1.10).
1.28 Effect of nickel binding with carriers

At the physiological pH, the strength of Ni (II) interactions with proteins depends on the nature of amino acid residues, their relative position, and their accessibility in the protein, in certain conditions, deprotonated peptide nitrogen may also coordinate Ni (II) ions. The greatest affinity for Ni (II) has been shown by the histidyl (via imidazole nitrogen) and cysteinyl residues (via the sulfhydryl group) in proteins, and mainly by the Xaa-Yaa-His (or XYH in one-letter code; X and Y stand for any amino acid) motif at the N-terminus. This motif coordinates Ni (II) in a square planar ring that is created by (a) The terminal amino group; (b) deprotonated nitrogen atoms of the two subsequent peptide bonds; and (c) the imidazole nitrogen of the histidine residue at the third position (Templeton and Sarkar, 1985; Predki et al., 1992; Donaldson et al., 2001). The very strong affinity of Ni (II) for His is widely used for purification of recombinant proteins having N-terminal hexa-histidyl tags on NTA-agarose containing immobilized Ni (II) ions (Crowe et al., 1994).

Serum Albumin is the major carrier of nickel in blood plasma (Van and Sunderman, Jr., 1972; Asato et al., 1975). The primary Ni (II)-binding site of serum albumin has been identified and characterized as the N-terminal XYH motif: e.g., DAHKSEVA in human, DTHKSEVA in bovine, or EAHKSEIA in rat albumin (Callan and Sunderman, Jr., 1973; Laussac and Sarkar, 1984). A secondary Ni (II)-binding site, likely to involve His-105, His-146, and/or His-247 in the folded molecule, has also been identified in human, bovine, and porcine albumins (Bal et al., 1998). A very small pool of Ni (II) - ligands in blood plasma binds to the amino acids (e.g., Histidine).

A major fraction of non-exchangeable form of nickel is present in nickeloplasmin, which is a Ni (II)-containing α2-macroglobulin (Nomoto et al., 1971; Nomoto and Sunderman, Jr., 1988). The nickel content of
nickeloplasmin because of strong binding is not readily exchangeable with free Ni (II), and hence nickeloplasmin seems to be not involved in the extracellular transport of nickel (Nomoto and Sunderman, Jr., 1988).

The results from nickel equilibrium studies have suggested that a Ni (II)–L-histidine complex is the most important form, through which nickel transport across the cell membrane occurs, whereas Ni (II)–albumin complex is the form which is responsible for systemic transport of nickel (Glennon and Sarkar, 1982).

1.29 Effect of Nickel binding with Regulatory proteins

In year 1995, DAN gene was discovered in the Neuroblastoma. DAN gene encodes a protein which possesses Ni (II)-binding motifs (PHSHAHPHP) in the C-terminal region (Kondo et al., 1995). This motif allows native DAN protein to be isolated on a Ni (II) affinity resin. The expression of DAN is significantly reduced in transformed cells and it has been demonstrated that DAN has tumor-suppressive activity (Ozaki et al., 1995). Cell cycle analysis revealed that overexpression of the DAN gene product causes retardation of cellular growth by blocking entry into the S phase. Thus, possible interaction of DAN protein with Ni (II) may impair cell cycle regulation in nickel-treated cells.

Another Ni (II) binding protein is pNiXa, a serine protease inhibitor (serpin) abundant in Xenopus oocytes and embryos. It has similarity to tissue plasminogen inhibitor (Sunderman, Jr. et al., 1996). The serpin pNiXa has the histidine-rich domain -HRHRHEQQGHDSAKHGH- and forms six-coordinate octahedral Ni (II) complexes. Binding of Ni (II) to pNiXa may lead to the loss of protein function and cause embryotoxicity and tissue injury. The importance of proteases like tissue plasminogen in carcinogenesis is well known (Kotyza et al., 1998). Xenopus laevis oocytes and embryos also
contain a 40 kDa protein that can be isolated on Ni (II)-agarose (Antonijczuk et al., 1995). This nickel-binding protein shows similarity to eukaryotic aldolases and is 96% identical with human aldolase A. These data suggest that aldolase A could also be a target for nickel toxicity. Similarly, lipovitellin 2β appears to be a Ni (II)-binding protein (Grbac-Ivankovic et al., 1994).

Nuclear transport of proteins and different cargos across the nuclear membrane is essential for signal transduction. One of the key soluble receptor of nuclear transport, importin α has been shown to be a Ni (II)-binding protein (Gorlich et al., 1994). Binding of Ni (II) with the importin α have made people to think the possibility of nickel being the regulator of the nuclear transport of proteins and various transcription factors further, detailed studies are required to understand the mechanism.

Similar to the several other transition metal cations Ni (II), can also bind to the iron regulatory protein-1 (IRP-1), a key regulator of iron homeostasis (Oshiro et al., 2002). Binding of nickel to the IRP-1, modulates its activity by replacing iron at the fourth labile position in the 4Fe-4S cluster. IRPs are mainly involved in controlling RNA stability or the translation rate of proteins involved in Iron metabolism, e.g. transferrin receptor and ferritin. When cellular iron is inadequate, IRP-1 loses one Fe with the formation of a 3Fe-4S cluster and this state has little enzymatic (aconitase) activity. The latter effect was also found to be produced by Ni (II), which may signal for iron scarcity and thus contribute to the hypoxic response (Oshiro et al., 2002).

In general, besides certain specific effects, the ability of Ni (II) to react with a number of proteins raises the possibility that nickel may significantly change the conformation of protein in complex, and thus change their functions and cellular homeostasis, which can further lead to various pathogenic effects.
1.30 Effect of nickel binding with structural proteins

Ni (II) has very little affinity with DNA for complex formation, (Kasprzak et al., 1986). Therefore, inside the nucleus structural components of the chromatin i.e. histones and protamines are the major target for nickel binding. (Bal et al., 2000) At physiological pH, strong Ni (II)-binding motifs have been found in protamine P2 and in core histones H3 and H2A, and a weak one in histone H4. Protamine P2 contains the classic XXH N-terminal motif, –RTHGQSHYRR– (Bal et al., 1997). The histone H3 motif –CAIH– is located in a hollow, metal-accessible structure of the core histone octamer, while the –TESHHKAKGK– motif of histone H2A is located near the end of its C-terminal tail (Bal et al., 1995; Luger et al., 1997). The weak Ni (II) coordination by histone H4 is offered by the –AKRHRK– motif, located around His-18 in the N-terminal tail of this histone (Bal et al., 1995; Zoroddu et al., 2002). Binding of Ni (II) by histone H3 in the histone tetramer was confirmed in vitro experimentally (Bal et al., 1999). The sequestration of Ni (II) by histone tetramer (H3/H4)2 and histone H2A has been evaluated using numerical models and found to be substantial even in the presence of high physiological concentrations of the competing cellular ligands histidine and glutathione (Bal et al., 2000; Krezel et al., 2003).

1.31 Nickel-induced oxidative damage

In comparison to the other metal ions such as copper, iron, cobalt and other redox-active metals, nickel generates relatively low, but quantifiable levels of ROS in cells (Salnikow et al., 2000; Salnikow et al., 1994; Huang et al., 1994) (Fig. 1.10). Both soluble Ni (II) chloride and insoluble Ni3S2 enhanced the formation of intracellular ROS after 6 h of exposure. After 18 h, more ROS were observed at the nucleus when cells were exposed to Ni3S2 (Huang et al., 1994). In addition to the direct measurement of free radicals, depletion of the antioxidant glutathione represents another marker of oxidative stress. In
vivo, hepatic glutathione levels diminished greatly after Ni (II) injection (Rodriguez et al., 1991; Herrero et al., 1993). Likewise, glutathione was found to be depleted by nickel in cultured cells (Salnikow et al., 1994; Li et al., 1993). The nickel-resistant cells had their basal levels of glutathione nearly twice as high as the wild-type cells. These results indicate that the resistant cells gain some defense mechanisms against oxidative stress during prolonged Ni (II) exposure.

The oxidative effects of nickel depend on its ability to form the Ni (III)/Ni (II) redox couple around pH 7.4. This is possible only when Ni (II) is complexed by some natural ligands, such as peptides and proteins, especially those which form square planar nickel complexes, e.g., GGH, or GGGG (Bal et al., 2000). An important result of reactions of such Ni (II) complexes with oxygen species, e.g., endogenous O2 or H2O2, is generation of not only the hydroxy radical OH (or an oxo-cation NiO2+), but also other oxygen-, carbon-, and sulfur-centered radicals originating from the ligands.

1.32 Nickel-induced Protein damage

Along with the oxidative damage, protein oxidation is also believed to be mechanistically involved in a wide variety of adverse effects, including cancer (Stadtman and Berlett, 1988; Requena et al., 2001; Stadtman, 1993). Nickel (II), like many of the other transition metals, may promote oxidative modification of both free amino acids (Stadtman, 1993) and the amino acid residues in proteins. In the latter, major targets are the side chains of Cys, His, Arg, Lys, and Pro, residues. The sulphydryls are commonly oxidized to disulfides, but they may be turned into sulfino-, sulfeno-, and sulfono-derivatives as well; the. The histamine imidazole may be oxidized to aspartic acid, asparagine, or 2'-OH-His. Arg is converted to γ-glutamic-semialdehyde; Lys, to 2-amino-adipic-semialdehyde; and Pro is turned into glutamic acid, pyroglutamic acid, γ-aminobutyric acid, and γ-glutamic-semialdehyde.
According to reports from Stadtman’s laboratory, glutamic and amino adipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins (Requena et al., 2001).

The formation and rearrangements of radical intermediates arising in the oxidation process of protein molecules also result in protein fragmentation and intra- and inter-protein cross-linking. Because of that, proteins also may become cross-linked with DNA. It seems obvious that such kind of oxidative effects on proteins must be widely pathogenic. The site-specificity of Ni (II) and other metals-mediated protein damage depends on the coordination of transition metal ions by proteins and peptides predominantly through the imidazole, sulfhydryl, and the deprotonated peptide bond and side chain nitrogens, followed by generation of ROS (e.g., from metabolic H2O2) at these particular sites. Therefore, Trp, Tyr, Phe, and Met, which are also sensitive to oxygen radicals, but do not bind Ni (II) under physiological conditions, are less likely to be targeted by metal-catalyzed oxidation (Stadtman, 1993). They may, however, be damaged if they are located close to the metal binding site.

A good illustration of both oxidative and conformational effects of Ni (II) on polypeptides is the one observed in experiments with a 15-mer peptide, RTHGQSHYRRHCSR-amide (HP21−15), modeling the N-terminal sequence of human protamine P2 (Bal et al., 1997; Bal et al., 2000). When bound to the RTH-end of this peptide, Ni (II) catalyzes oxidation by H2O2 of not only Arg-1 and His-3, but also of Tyr-8. The reason for this is a strong structuring effect of Ni (II) on the peptide ligand (Bal et al., 2000). This effect brings Tyr-8 close to the metal center. It also shifts all the positive Arg side chains to one side of the molecule. Thus, by imposing conformational changes on its ligand, the bound Ni (II) can cause oxidative damage on a
particular target, and also control the function of the ligand, e.g., increase the DNA–peptide binding (Bal et al., 1997; Bal et al., 2000).

1.33 DNA damage

In nuclear chromatin, the DNA molecule, having an abundance of phosphate anions and nitrogen and oxygen donor groups, is an ideal binding partner for metal cations, including Ni (II). The chromatin proteins can bind Ni (II) strongly (Bal et al., 1998; Bal et al., 1997; Zoroddu et al., 2002). This helps to explain why, following in vivo exposure, heavy metals, including nickel, are found in cell nuclei (Berg, 1986; Kasprzak and Poirier, 1985; Peskin and Shlyahova, 1986). The generation of O$_2^-$ and H$_2$O$_2$, was also detected in cell nuclei (Peskin and Shlyahova, 1986). Hence, the bound metal can catalyze ROS generation in the cell nucleus and thus facilitate oxidative damage to DNA and other nuclear components, as it has been shown experimentally (Coogan et al., 1989). Important targets for metals are also mitochondria and mitochondrial DNA (Kasprzak and Poirier, 1985). The major oxidative effects in DNA associated with exposure of experimental animals and cultured cells to nickel and other transition metals include strand scission, depurination, cross-linking, and base modifications (Coogan et al., 1989).

1.34 Cross-linking

The most common effect of toxic metals in chromatin observed in vitro and in vivo is DNA–protein cross-linking (Klein et al., 1991; Standeven and Wetterhahn, 1991). Generally, metal ions can generate DNA–protein cross-links mainly in two ways either by bringing both, in mixed ligand complexes or by inducing the formation of strong covalent bonds directly between DNA and the proteins. The formation of cross-links in chromatin may result in morphologic aberrations of chromosomes. Such aberrations were observed, in lymphocytes of workers exposed to nickel and chromium compounds. In
CHO cells, chromosomal alterations caused by Ni (II) were predominantly localized in the protein-rich, heterochromatic region of the X chromosome (Huang et al., 1995).

1.35 Alteration of cell signaling pathways

Various studies have suggested the mechanism of metal toxicity and effect on the target cells by inducing the important signaling cascade few of them are discussed below.

1.35.1 NF-κB signaling

Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1, E-selectin) are endothelial surface molecules that have been shown to play a role in leukocyte recruitment to the sites of inflammation during contact hypersensitivity. ICAM-1, VCAM-1, and ELAM-1 were found to be up-regulated by Ni (II) in cultured human umbilical vein endothelium cells (HUVEC) (Goebeler et al., 1993). The induction of adhesion molecules by Ni (II) requires de novo mRNA and protein synthesis. Up-regulation could be blocked by kinase inhibitor H-7 but not staurosporine, suggesting involvement of phosphorylation independent of protein kinase C. Moreover, pre-treatment for 24h with Ni (II) caused hypo-responsiveness to IL-1 and TNF-α upon restimulation, suggesting that Ni (II) and these cytokines might to some extent share a common pathway of activation. When the transcriptional mechanisms underlying gene-inductive effects of nickel were studied, NF-κB transcription factor was found to be involved in the induction of adhesion molecules. A strong increase of NF-κB binding with DNA was found after stimulation of HUVEC with Ni (II) or Co (II) (Goebeler et al., 1995). NF-κB plays an important role as a transcription factor in apoptosis and inflammation. It is known that activation of NF-κB by nickel causes significant modulation of cellular and tissue responses. In addition,
activation of NF-κB explains nickel-induced allergic effects and contact skin hypersensitivity in humans (Goebeler et al., 1993).

1.3.5.2 p53 pathway

Mutations in p53 are the most common genetic alterations found in human cancer (Hernandez-Boussard et al., 1999). The p53 gene was reported to be mutated in human kidney epithelial cells transformed by nickel (Maehle et al., 1992). However, no mutations in the p53 gene were found in 10 analyzed nickel-induced rat renal tumors (Weghorst et al., 1994). This has led to another question as to whether p53 mutations are essential for nickel-induced neoplastic transformation. The acute treatment of human cells with Ni (II) induced the expression of wild-type p53 protein, but not of mutant p53 (Salnikow et al., 1999). Another report suggested the induction of p53 protein by Ni (II) acetate in CHO cells (Shiao et al., 1998). It is not clear so far whether the induction of p53 protein results from nickel-assisted DNA damage, or from stabilization of p53 by other factors as reported for HIF-1 (An et al., 1998). Despite the initial induction of p53 in cells kept in Ni (II)-containing media, the functional activity of p53 decreased when the cell were transformed. Apart from this, in human and rodent cells transformed by nickel, a shift in the balance of HIF-1-dependent transcription versus p53-dependent transcription has also been demonstrated (Salnikow et al., 1999).

1.3.5.3 Wnt/β-catenin Signaling

Fe (II) transition metal ion has been shown to activate the Wnt Signaling in CRCs line with non-functional APC or β-catenin mutations (Brookes et al., 2008). However, in the study performed in the normal cell lines with Cd (II) has also demonstrated that Cd2+ induces E-cadherin disruption and nuclear translocation of β-catenin in sub-confluent cultured kidney proximal tubule cells (PTC). Induced nuclear β-catenin under Cd2+ treated condition resulted
in an increased Wnt response measured by TOP reporter assays (Chakraborty et al., 2010).

1.3.5.4 Oxidative stress signaling

Different oxidizing reagents have also been shown to activate the β-catenin and FOXO mediated stress signaling pathways, which leads to transcriptional activation of SOD (superoxide dismutase), measured by SOD reporter assays (Essers et al., 2005).

Genesis of Work

As our previous study uncovered the functional interaction between Nup358 and APC, we sought to check what if, Nup358 also interacts with β-catenin (an effector molecule of canonical Wnt3a/β-catenin signaling) and regulate its function in Wnt signaling.

The objectives of the study were;

1. **Characterization of the interaction between Nup358 and β-catenin.**
2. **Investigating the functional significance of Nup358-β-catenin interaction in Wnt signaling.**

While studying the Nup358-β-catenin interaction, serendipitously we found that β-catenin possessed metal binding property, and the third objective was based on this observation.

3. **Characterization of a novel metal binding property of β-catenin.**