Chapter 4

Characterization of novel metal binding property of β-catenin
4.1 Molecular cloning of β-catenin in pET30a vector

While performing the study of Nup358 and β-catenin interaction, serendipitously, we discovered that β-catenin possesses a metal binding property. To generate bacterially expressed recombinant protein for in vitro binding studies, we cloned the β-catenin ORF into pET30a vector in order to purify it as N-terminally 6XHis-tagged fusion protein. We could purify the protein using Ni-NTA affinity chromatography in large quantities. Subsequent analyses using western blotting with anti-His antibody and mass spectrometry indicated that β-catenin was expressed as an untagged protein, which was due to flaw in the cloning strategy (Fig 4.1).

![Fig 4.1 Molecular cloning of β-catenin. Mouse β-catenin ORF was released from pEGFP-β-catenin vector by NdeI/BamHI digestion and cloned at the respective sites of pET30a vector. Note that here, the 6XHis present at the N terminus and at the C terminus are lost as a consequence of this cloning strategy. At the N terminus His-tag is lost because of the Nde site was used for cloning and at the C terminus β-catenin comes with its own stop codon TTA.]

4.2 Recombinant β-catenin does not possess 6XHis tag

Due to above mentioned mistake in cloning strategy (Fig 4.1), we wanted to confirm indeed β-catenin is not having 6XHis tag in it. β-catenin was induced in the BL21-pLysS cells along with His-APC (1211-1495 a.a, as a positive control) and GST as a negative control. Soluble fraction of induced lysate was loaded on the gel and western blotting was performed using His-specific antibody. β-catenin did not show any reactivity with His antibody.
confirming the absence of the tag, whereas GST, GST-β-catenin and His-APC showed specific reaction with GST and His antibody (Fig 4.2).

**Fig 4.2 Induced β-catenin does not have the 6XHis tag.** Two hundred milliliter culture of BL21pLysS bacterial cells harbouring plasmid DNA of pGEX6P1, pET30a-β-catenin, pET30-APC (1211-1495 a.a.), were induced by 0.5mM of IPTG. After confirming the induction and solubility, 2ml aliquots were made. Soluble fractions from induced lysate were loaded on 10% gel and western blotting was performed using His-specific antibody. Note that β-catenin did not react with His antibody, suggesting that expressed β-catenin does not have 6XHis tag. Upper band in GST lane in the anti-GST blot possibly represents dimerized GST.

**4.3 Nickel binding activity of β-catenin**

As we could purify untagged protein using Ni-NTA, we concluded that β-catenin would possess some metal binding activity. To validate this conclusion, we expressed GST (as a negative control), GST-β-catenin, His-APC (1211-1495 a.a.) as a positive control, and the untagged β-catenin in BL21pLysS. We could purify GST-tagged as well as untagged β-catenin using Ni-NTA column. As a positive control, His-APC (1211-1495 a.a) was also purified, whereas, GST failed to bind to Ni-column (Fig 4.3A). Furthermore, treatment of Ni-NTA agarose with EDTA, which removes Ni (II) from NTA,
abolished the interaction of purified β-catenin, confirming the specificity of interaction between Ni (II) and β-catenin (Fig 4.3B).

Fig 4.3 β-catenin is a Ni (II) binding protein. (A) Proteins were induced as described for Fig 4.2. The soluble fraction of induced lysate was incubated with Ni-NTA agarose beads for 30 min, and after 3 washes with TBST, the protein was eluted with 250 mM imidazole. Note that β-catenin and GST-β-catenin can be seen in the imidazole elution suggesting that in spite of not having 6XHis, β-catenin binds with Ni (II). (B) Purified proteins were subjected to the NTA (control pull down-NTA P.D.) and Ni-NTA pull down (Ni-NTA P.D.). To check Ni (II) dependent binding of β-catenin. Pull down samples were loaded on gel and coomassie staining was performed to detect the proteins on the gel. Data suggested β-catenin binds to Ni (II) specifically.

4.4 Reactivity of His antibody against the purified proteins

Further to make sure absence of 6XHis tag in pET30a–β-catenin, we purified recombinant GST and GST-β-catenin using glutathione, and β-catenin and His-APC (1211-1495 a.a.) using Ni-NTA. Affinity purified proteins were loaded on the gel and western blotting was performed using His, β-catenin,
or GST specific antibodies. The results further confirm that β-catenin and GST-β-catenin can bind specifically to Ni (II) (Fig 4.4).

**Fig 4.4 β-catenin does not have His tag.** For checking the reactivity of β-catenin against the His antibody, we purified recombinant GST, GST-β-catenin using glutathione, and β-catenin and His-APC (1211-1495 a.a.) using Ni-NTA affinity purification. Equal amount of proteins were loaded on the 10% gel; transferred to the PVDF membrane and western blotting was performed with mouse specific antibodies against the His-tag and β-catenin.

### 4.5 Endogenous β-catenin from SW480 cell lysate can bind to Ni (II)

To validate the Ni (II) binding ability further, we checked if endogenous β-catenin could be purified using Ni-NTA agarose from SW480 and HEK293T cells. We incubated the cell lysate with Ni-NTA, and the bound proteins were eluted with imidazole. Probing the proteins with β-catenin and vinculin antibodies revealed that endogenous β-catenin specifically interacts with Ni-NTA (Fig 4.5). EDTA treated Ni-NTA beads failed to bind with β-catenin suggesting the specific nature of this interaction.
Fig 4.5 Endogenous $\beta$-catenin binds to Ni (II) specifically. SW480 and HEK293T cells were lysed in NP-40 lysis buffer and the soluble fraction was incubated with Ni-free NTA- or Ni-NTA-agarose beads for 2h. The beads were washed 3 times with TBST, Bound proteins were eluted with 250mM imidazole. The eluates were loaded on 10% gel and western blotting was performed with $\beta$-catenin and vinculin specific antibodies. Results indicated that endogenous $\beta$-catenin, but not vinculin, binds to the Ni (II) very specifically. Lower band in the $\beta$-catenin blot possibly represents cleaved $\beta$-catenin.

4.6 Surface plasmon resonance (SPR) studies on $\beta$-catenin interaction with Ni (II)

Thus, the in-vitro and in-vivo experiments suggested that $\beta$-catenin is a Ni (II) binding protein. We used SPR (Surface Plasmon Resonance) for studying the kinetics of the interaction of $\beta$-catenin and Ni (II). The purified untagged $\beta$-catenin; GST-$\beta$-catenin and GST (negative control) were passed on the Ni-NTA chip at various increasing concentrations such as 2.34 nM, 4.69 nM, 9.375 nM, 18.65 nM, or 37.5 nM, 75nm. The study showed that untagged $\beta$-catenin and GST-$\beta$-catenin bind to the Ni (II) in a dose-dependent manner (Fig 4.6) and (Fig 4.7). The analysis of binding affinity data also suggested
that untagged and GST tagged β-catenin bind to Ni (II) very tightly, as indicated by lower $K_d$ dissociation constants (Table 4.1). Further we intended to study the binding with other divalent metal ions such as Fe (II), Zn (II). For that we tried charging NTA chip with these metal ions. But we could not charge the NTA.

**Fig 4.6 Binding of β-catenin to Ni (II).** Surface plasmon resonance analysis was performed to check the binding of untagged β-catenin with Ni (II). The various concentration of untagged β-catenin injected on the nickel charged NTA, in the increasing order indicated at the right of each sensogram overlay. Solid lines represent the global fitting of data to 1:1 Langmuir binding model ($A + B \leftrightarrow AB$; BIA evaluation 4.1).

**Fig 4.7 Binding of GST-β-catenin to the Ni (II).** Surface plasmon resonance analysis was performed to check the binding of GST-β-catenin with Ni (II). The various concentration of GST-β-catenin injected on the nickel charged NTA in the increasing order indicated at the right of each sensogram overlay. Solid lines represent the global fitting of data to 1:1 Langmuir binding model ($A + B \leftrightarrow AB$; BIA evaluation 4.1).
### Table 4.1 Kinetics and affinity data for interaction of β-catenin and GST-β-catenin with Ni (II).

Analysis of kinetics and affinity data suggested that both β-catenin and GST-β-catenin can bind to the Ni (II) very tightly as evident from very low $K_D$. GST-β-catenin binds to Ni (II) stronger than β-catenin. $k_a$, association rate constant; $k_d$, dissociation rate constant; $K_D$, equilibrium rate constant; SE, standard error. Data were calculated by global fitting to a 1:1 Langmuir binding model with drifting baseline (BIAevaluation 4.1).

<table>
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<th>Ligand</th>
<th>Analyte</th>
<th>$k_d(1/s)/k_a(1/Ms)$</th>
<th>SE ($k_d/k_a$)</th>
<th>$K_D$</th>
<th>$\chi^2$</th>
</tr>
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<tbody>
<tr>
<td>Ni (II)</td>
<td>β-catenin</td>
<td>1.08 X 10^{-5}/ 3.8 X 10^{3}</td>
<td>1.27 X 10^{-6}/ 106</td>
<td>2.85 X 10^{-9}</td>
<td>0.128</td>
</tr>
<tr>
<td>Ni (II)</td>
<td>GST-β-catenin</td>
<td>1.66 X 10^{-5}/ 2.31 X 10^{4}</td>
<td>4.40 X 10^{-6}/ 1080</td>
<td>0.719 X 10^{-9}</td>
<td>0.187</td>
</tr>
</tbody>
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#### 4.7 Ni (II) induces β-catenin translocation to the nucleus

To check the functional significance of the interaction between β-catenin and Ni (II), we treated the SW480 cells with various concentrations of NiSO$_4$ such as 50μM, 100μM, 200μM, 300μM, 400μM and 500μM and 24 hrs later, cells were fixed and stained with β-catenin antibody. Immunofluorescence analysis suggested that under Ni (II) treated condition, β-catenin strongly accumulated inside the nucleus, as compared to that in control treated cells. Effect was clearly observed with 500 µM concentration of NiSO$_4$ (Fig 4.8).

#### 4.8 Ni (II) treatment leads to redistribution of β-catenin from the cytosol to nuclei

SW480 cells were untreated or treated with 500μM NiSO$_4$, after 24hrs of treatment cells were lysed in NP40 lysis buffer and equal amount of soluble total protein was loaded on the gel to check the level of β-catenin. Here tubulin was used as loading control. Result suggested that the treatment of Ni (II) does not change the level of the β-catenin (Fig 4.9).
Fig 4.8 Ni (II) induces the nuclear translocation of β-catenin. SW480 cells were treated with 500μM NiSO₄ for 24 hours and cells were immunostained with β-catenin specific mouse antibody. DNA is represented in blue. There was significant difference observed in subcellular localization of β-catenin, in NiSO₄ treated condition increased β-catenin in the nucleus of SW480 cell was observed.

Fig 4.9 Ni (II) treatment does not change the β-catenin level. SW480 cells were treated for 24 hrs with 500μM concentration of NiSO₄ and lysed in NP-40 lysis buffer and soluble fraction was loaded on the gel and Western blotting was performed with β-catenin and tubulin specific antibodies. Results indicated no change in β-catenin level.
4.9 Induced nuclear β-catenin does not alter the Wnt/β-catenin signaling

Further to check the effect of Ni (II) binding on β-catenin function, we measured the transactivation ability of β-catenin using the TCF-4 based luciferase assay. Here, we did not find significant change in β-catenin mediated signaling; this is possible because earlier reports have suggested preferential binding of β-catenin with oxidative stress responsive promoters, which can bring about transcription of oxidative stress responsive genes, such as superoxide dismutase (SOD). We are in process of investigating the read out of stress responsive promoters under nickel induced condition.

![Fig 4.10 Ni (II) induced nuclear β-catenin does not alter Wnt/β-catenin signaling in SW480 cells. SW480 cells were co-transfected with plasmid constructs (TOP and renilla), and 24 hrs later, cells were treated with various concentrations of NiSO₄ as indicated, and after 24 hrs, luciferase reading was taken. The effect of NiSO₄ on β-catenin signaling was examined by measuring the (TOP/renilla) ratio. The results were representative of three independent experiments. Error bars indicate standard deviations; there was no significant difference in β-catenin signaling as compared to control.](image-url)
4.10 Discussion

In our study, we have identified a novel interaction between the Ni (II) and β-catenin (an effector molecule of canonical Wnt signaling). In order to study, the *in vitro* interaction, we cloned the β-catenin ORF into pET30a vector, and because of mistake in the cloning strategy, the recombinant protein did not possess the 6XHis tag, but still we could purify the β-catenin from the induced culture of BL21 pLysS using Ni-NTA agarose. Further identity of the protein was confirmed with mass spectrophotometry study as well western blotting with β-catenin specific antibodies. Absence of 6XHis tag was also confirmed from the DNA sequence analysis of the clone as well as absence of reactivity with His antibody on Western blots.

To validate the metal binding property of β-catenin further, Ni-NTA pull down was performed from lysates of SW480 cells and HEK293T cells. Here, the extent of interaction with Ni varied depending on the amount of the soluble β-catenin present inside the used cell type. SW480 cells having very high amount of the soluble β-catenin (because of APC truncation) showed high extent of binding in the pull down in comparison to HEK293T cells.

Once, we confirmed the binding of β-catenin with Ni (II) in in-vitro and in-vivo condition, to study the binding affinity and kinetics properties of the interactions, we performed surface plasmon resonance (SPR) using Ni-NTA Chip. SPR data suggested that both β-catenin and GST-β-catenin bind tightly with Ni (II) in a dose dependent manner. Other divalent metals such as Cu (II) did not show any binding on the SPR, suggesting us the specific nature of interaction.

Further to check in vivo the functional significance of this interaction, we treated the SW480 cells with Ni (II) sulphate and immunostaining was
performed to check the intracellular localisation of β-catenin. Ni (II) induced nuclear accumulation of β-catenin.

Further to check the transactivation ability of the nuclear β-catenin accumulated under Ni (II) induced condition, we measured the Wnt/β-catenin signaling using TCF-4 based luciferase reporter assay. Here we found no effect on β-catenin signaling. Previously under oxidative stress, β-catenin was shown to associate with FOXO transcription factors and enhance transcription of stress responsive genes. We are in the process of checking the effect of this interaction by using the oxidative stress responsive promoter (Essers et al., 2004).

Taken together, we are the first to report that β-catenin can bind to the Ni (II) metal. Earlier, people have shown the effect of transition elements on various aspects of stress and Wnt signaling pathway but no one has shown any direct metal binding activity by any component of signaling pathway. It is tempting to speculate that some of the reported effect of metals on Wnt signaling could be mediated through a direct binding of the metal to β-catenin. Our study also may hold a great value in possibly understanding the mechanism of Ni (II) induced carcinogenesis. Further investigation is needed to comprehend the underlying effect of the Ni (II)-β-catenin interaction in oxidative stress response and carcinogenesis.