# Defining the molecular basis for the β-catenin and CDC73 interaction

by

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ABSTRACT

RNA polymerase II (Pol II) is required for expression of protein coding genes. To initiate gene transcription, Pol II is recruited to gene promoters by transcription factors. Gene specific transcription factors, including β-catenin, a component of the Wnt signaling pathway, are used to regulate gene expression in response to developmental or environmental cues. Previous studies reported that β-catenin binds to the central region of CDC73, a subunit of the Polymerase Associated Factor 1 complex (PAF). PAF associates with Pol II during transcription elongation to stimulate processive elongation and co-transcriptional histone modification. Genetic studies have shown that CDC73 is required for expression of Wnt signaling pathway genes. In addition, such studies have hypothesized that the interaction between β-catenin and CDC73 was used to recruit Pol II to genes through PAF. In this work I explored how the CDC73•β-catenin interaction is used to regulate gene expression. My data indicates that CDC73 uses its middle region to associate with either PAF or β-catenin but cannot associate with both proteins simultaneously. Both CDC73 and β-catenin weakly interact with nucleic acids meaning that this complex might need another protein factor to mediate their interaction with DNA. To solve the structure of the β-catenin•CDC73 complex, purification strategies require some optimization to isolate a stable complex. Solving the structure of β-catenin•CDC73 complex will define the exact nature of their interaction. Moreover, it will allow for cellular studies of β-catenin•CDC73 and PAF to understand if the complexes are differentially used to regulate gene expression. Together, the work presented in this thesis provide new insights on the β-catenin•CDC73 interaction in comparison with the interaction of CDC73 with PAF.

Thesis Supervisor: Seychelle Vos
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# Defining the molecular basis for the β-catenin•CDC73 interaction

## 1. Introduction

RNA polymerase II (Pol II) is required for expression of protein coding genes. To initiate gene transcription, Pol II is recruited to gene promoters by transcription factors. Gene specific transcription factors, including β-catenin, a component of the Wnt signaling pathway, are used to regulate gene expression in response to developmental or environmental cues (Clever 2006).

β-catenin comprises of a very structured middle region arranged in 12 arm repeats (known as the armadillo repeats) linked to a less well-structured N- and C-terminal domain (Xing et al. 2008). The C-terminal end of the armadillo repeat twelve is flanked by an additional helix (arm C, Xing et al. 2008). The structured region of β-catenin has been reported to interact with various proteins involved in different molecular activities such as Wnt signaling pathway, cell adhesion, and chromatin remodeling (reviewed in Valenta et al. 2012).

β-catenin is a core component of the canonical Wnt signaling pathway. The translocation of β-catenin into the nucleus is regulated by the extracellular binding of Wnt to Frizzled (Fz)/LRP transmembrane receptors. In the absence of Wnt, the β-catenin destruction complex composed of GSK3, CK1, APC, and Axin phosphorylates β-catenin using the kinases GSK3 and CK1. Phosphorylated β-catenin is recognized and ubiquitinated by β-TrCP, a component of the E3 ubiquitin ligase complex. After ubiquitination, β-catenin is targeted for a rapid degradation by the proteosome preventing its translocation to the nucleus and activation of gene expression of Wnt

target genes (Clever 2006, Figure 1A top). When Wnt binds the Fz/LRP coreceptor complex, it allows for the interaction of Fz with Dishevelled (Dsh), a cytoplasmic protein that acts early in the Wnt signaling transduction. The interaction between Fz and Dsh then stimulates the phosphorylation of Dsh (Clever 2006, Figure 1B). The binding of Wnt to Fz/LRP coreceptor complex also induces the phosphorylation of the intracellular domain of LRP by GSK3 and CK1γ kinases which triggers the docking of Axin to the nuclear membrane by LRP (Figure 1A bottom). The binding of Axin to the LRP cytoplasmic tail and Dsh subsequently stimulates other events downstream of Wnt signaling transduction (Clever 2006). Axin functions as a scaffold to the destruction complex. Therefore, with Axin removed from the destruction complex, β-catenin becomes more stable and translocates into the nucleus (Clever 2006, Figure 1A bottom). In the nucleus, β-catenin interacts with TCF/LEF (T cell factor/lymphoid enhancer factor family), transcription factors that help to anchor β-catenin to the nucleus and stimulate Wnt target genes expression during embryonic and stem-cell development (Logan & Nusse 2004, Nusse 2008, Clever 2006, Figure 1A bottom).

β-catenin is also found at the cell membrane in complex with alpha-catenin and E-cadherin to provide a cell adhesion function (Bullions and Levine 1998). It is intriguing that a cell adhesion protein also activates gene expression functioning as a transcription factor in the nucleus. In efforts to understand the function of β-catenin as a cytosolic and nuclear protein, many studies have identified binding partners of β-catenin (reviewed in Valenta et al. 2012). One previously reported binding partner of β-catenin is Cell Division Cycle 73 (CDC73, 218-263), a subunit of the Polymerase Associated Factor 1 complex (PAF) (Mosimann et al. 2006/2009, Takahashi et al. 2011). Human PAF complex is a six-subunit complex (CTR9, CDC73, LEO1, PAF1 RTF1, and WDR61/SKI8) which associates with Pol II during transcription elongation to stimulate processive

elongation and co-transcriptional histone modification (Van Oss et al. 2017, Vos et al. 2018/2020). After Pol II escapes the promoter region, it pauses aoorund 50-150 nucleotides downstream of transcription start site due to the association and stabilization of the complex by DRB sensitivity-inducing factor (DSIF, a complex formed by SPT4 and SPT5) and negative elongation factor (NELF) (Wada et al. 1998, Yamaguchi et al. 1999, Narita et al. 2003, Core and Adelman 2019). Pol II is released from the promoter-proximal region into processive elongation by phosphorylation of the positive transcription elongation factor b (P-TEFb, which includes the kinases CDK9 and CYCLIN T1) to Pol II, DSIF and NELF (Wada et al. 1998, Vos et al. 2018). Phosphorylation of these complexes results in NELF release and association of elongation factors, including SPT6 and PAF (Liu et al. 2009, Sdano et al. 2017, Vos et al. 2018). With additional elongation factors, PAF can enhance the rate of transcription 5-10x over the rate of basal Pol II rate (Vos et al. 2020).

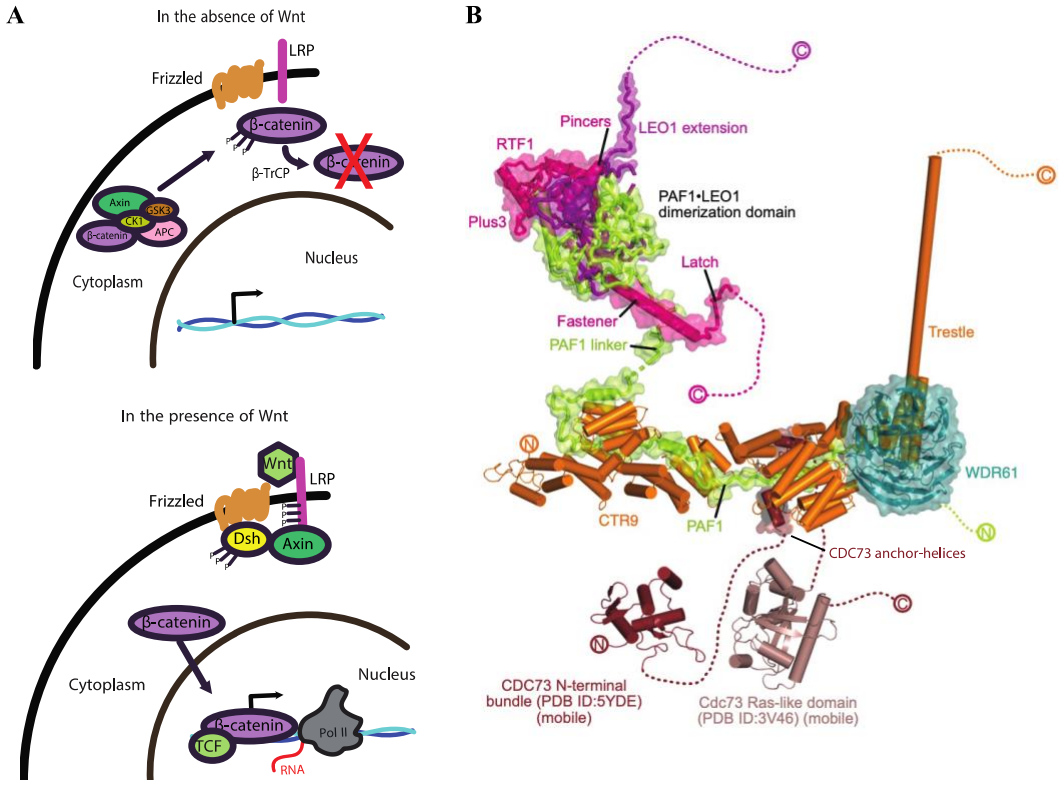


**Figure 1.** β-catenin and CDC73 cellular roles and structure. A. Schematic showing the flow of the Wnt signaling pathway in the absence (top) and presence (top) of Wnt secreted molecules. B. PAF complex structure displaying the binding interface of CDC73 with CTR9 (Vos et al. 2020).

Recent structural work has determined how CDC73 interacts with the rest of the PAF complex (Deng et al. 2018, Vos et al. 2018/2020, Chen et al. 2022, Figure 1B). The CDC73 middle region (217-263) interacts with CTR9 through its anchor helices, the same region previously reported to interact with β-catenin (Vos et al. 2020, Mosimann et al. 2006). The CDC73 anchor helices are linked to the N- and C-terminal domains which densities are not observed when PAF is bound to Pol II, likely due to the flexibility of the domains (Vos et al. 2020). The C-terminal domain has a RAS-like fold. Knockdown of the CDC73 C-terminus results in the reduction of PAF association with chromatin (Amrich et al. 2012). CDC73 is heavily mutated in jaw and thyroid cancers. Most mutations associated with these cancers lie in the N-terminal region of the protein (Newey et al. 2010, Rozenblatt-Rosen et al 2005, Yart et al. 2005, Sun et al. 2017). CDC73 cancer associated phenotypes appear to be specific to CDC73 because no mutations in other PAF subunits are known to give rise to these types of cancer.

Genetic studies have shown that CDC73 is required for expression of Wnt responsive genes when in association with β-catenin (Mosimann et al. 2006). Previous studies have reported a mutant form of the Lgs ($Lgs^{17E}$), the Drosophila homolog of BCL9 (B-Cell CLL/Lymphoma 9) (Kramps et al. 2002). This mutant was found to weaken the binding of Arm to the Arm, Lgs, Pan, and Pygo complex (Drosophila homolog of β-catenin, BCL9, TCF/LEF, Pygo) which consequently causes notched wings. Co-overexpression of hyrax (hyx, Drosophila homolog of CDC73) with the $Lgs^{17E}$ mutant via P-elements or by UAS-hyx transgene reverted the dominant-negative effect of $Lgs^{17E}$ (Mosimann et al. 2006). Hyx mutants reduce expression of Wingless (Wg, Drosophila homolog for Wnt) pathway target genes, such as Distal-less (Dll), and Wg

deficiency phenotypes. Overexpression of the human homolog (CDC73, also known for parafibromin) in $Lgs^{17E}$ defected cells also reverted $Lgs^{17E}$ phenotype which indicates a similar function of hyx/CDC73 in Wnt Signaling pathway. Pulldowns and Western blots revealed that β-catenin•hyx (Drosophila) and β-catenin•CDC73 (human) physically associate. Together, it was hypothesized that the CDC73•β-catenin interaction mediates the recruitment of PAF, and by association, Pol II, to Wnt responsive genes (Mosimann et al. 2006). However, structure and biochemical work on the PAF complex and CDC73•β-catenin interaction have shown that both β-catenin and PAF interact with the CDC73 anchor helices. It is unclear whether β-catenin and PAF interact simultaneously with CDC73 to regulate gene expression. In the work presented in this thesis, I explored how CDC73 interacts with β-catenin and PAF. This work is an important step to understanding how β-catenin regulates Wnt specific gene expression and how cancer related CDC73 mutations are related to its interaction with PAF and β-catenin.

# 2. Methods

### 2.1 Molecular cloning

Full length CDC73 was obtained from Vos et al. (2020) and was used as a template for PCR amplification of the CDC73 N- and C-terminal domains (residues 1-111 and 336-531, respectively) and the CDC73 anchor-helices (residues 213-263). CDC73 N- and C- terminal domains were inserted into vector 1C (N-terminal 6×His-Maltose binding protein ($His_6$-MBP) tag followed by tobacco etch virus (TEV) protease cleavage site) by ligation independent cloning (LIC) (Gradia et al. 2017). The CDC73 anchor-helices were inserted into *E. coli* and baculovirus expression vectors 14C and 438C, respectively, (N-terminal MBP and $His_6$-MBP tag followed by TEV protease cleavage site) by LIC.

The gene for full length β-catenin was amplified from human K562 cDNA. Full-length β-catenin was inserted into baculovirus expression vector 438B (N-terminal $His_6$ tag followed by a TEV protease cleavage site) by LIC. A N-terminal truncation of β-catenin, corresponding to residues 138-781, was amplified by PCR and inserted into *E. coli* expression vectors 1B and 14B (N-terminal $His_6$ tag followed by a TEV protease cleavage site) by LIC.

Vectors 14B $His_6$-β-catenin (138-781) and 14C MBP-CDC73 (213-263) were combined by restriction enzyme cloning to create a co-expression vector. Mutations were introduced into the co-expression construct and the individual constructs via PCR and sequence- and ligation-independent cloning (SLIC, Jeong et al. 2012).

$His_6$-β-catenin (138-691) and CDC73 (213-263) were fused together via glycine-serine linkers with lengths of 5, 10, or 20 amino acids (L5, L10, and L20 constructs, respectively; Table 1). The genes were combined by SLIC in the 1B vector. $His_6$-β-catenin (residues 138-781) and CDC73 (213-263) were combined by SLIC in the 1B vector (the NO-linker construct) as well.

All clones were verified by Sanger sequencing.

**Table 1.** Linker sequences designed to create β-catenin & CDC73 fusion constructs

| Construct Name | Linker Amino Acid Sequence |
|---|---|
| **L5** | SGSGS |
| **L10** | GSSGSGGGSG |
| **L20** | GASGNGSGSGSNGSSGGGSG |

### 2.2 Protein expression

Expression vectors encoding CDC73 1-111 and 336-531 were transformed and expressed in Bl21 (DE3) LOBSTR RIL and Bl21 (DE3) RIL cells, respectively. For each construct, five liters of expression were prepared in 5, 2 L baffled flasks with 1 L 2xYT media, 100 µg/mL kanamycin, 34 µg/mL chloramphenicol per flask, and each inoculated with 10 mL of an overnight culture. Cells were grown at 37ºC, 160 rpm until optical density 600 nm ($OD_{600}$) reached 0.4-0.6. Protein expression was induced by adding 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown for an additional 16-18 hrs at 18ºC. Cells were collected by centrifugation, resuspended in lysis buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix (2µM pepstatin A, 0.7µM leupeptin, 1mM phenylmethylsulfonyl fluoride, 2.8mM benzamidine, 100% ethanol)), snap frozen in liquid nitrogen, and stored at -80ºC until purification.

CDC73 213-263 construct was transformed and expressed in Bl21 (DE3) Rosetta 2 pLysS cells. Five liters of expression were prepared in 5, 2 L baffled flasks with 1 L 2xYT media, 100 µg/mL ampicillin, 34 µg/mL chloramphenicol per flask, and each inoculated with 10 mL of an overnight culture. Cells were grown at 37ºC, 160 rpm until $OD_{600}$ reached 0.4-0.6. Protein

expression was induced by adding 0.5 mM of IPTG and cells were grown for an additional 4 hrs at 37ºC. Cells were collected by centrifugation, resuspended in lysis buffer (500 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix), snap frozen in liquid nitrogen, and stored at -80ºC until purification.

Both β-catenin 138-781 and the co-expression construct of CDC73 213-263 and β-catenin 138-781 were transformed and expressed in Bl21 (DE3) LOBSTR RIL. For each construct, five liters of expression were prepared in 5, 2 L baffled flasks with 1 L 2xYT media, 100 µg/mL kanamycin and ampicillin respectively, 34 µg/mL chloramphenicol per flask, and each inoculated with 10 mL of an overnight culture. Cells were grown at 37ºC, 160 rpm until $OD_{600}$ reached 0.4-0.6. Protein expression was induced by adding 0.5 mM IPTG and cells were grown for an additional 16-18 hrs at 18ºC. Cells were collected by centrifugation, resuspended in lysis buffer (500 and 300 mM NaCl respectively, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix), snap frozen in liquid nitrogen, and stored at -80ºC until purification.

β-catenin (residues 138-691 and 138-781) and CDC73 213-263 fusion constructs (L10 and NO-linker, respectively) were transformed and expressed in Bl21 (DE3) Rosetta 2 pLysS. For each construct, 10 liters of expression were prepared in 10, 2 L baffled flasks with 1 L 2xYT media, 100 µg/mL kanamycin, 34 µg/mL chloramphenicol per flask, and each inoculated with 10 mL of an overnight culture. Cells were grown at 37ºC, 160 rpm until $OD_{600}$ reached 0.4-0.6. Protein expression was induced by adding 0.5 mM IPTG and cells were grown for an additional 16-18 hrs at 18ºC. Cells were collected by centrifugation, resuspended in lysis buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 20 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix), snap frozen in liquid nitrogen, and stored at -80ºC until purification.

**2.3 Purification of CDC73 1-111 and 336-531**

All steps were performed at 4ºC unless otherwise specified. Cell pellets were thawed and sonicated with a Branson 450 sonicator on ice for 5 minutes, at 70% power with bursts of 0.6s on, 0.4s off. Cell lysates were clarified by centrifugation (30 minutes, 86,756xg, A27 rotor). The clarified lysate was applied to a 5 mL HisTrap column (Cytvia) equilibrated in lysis buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix). The column was washed with ~25 column volumes of lysis buffer, followed by 5 column volumes of high salt wash buffer (800 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT), and another 5 column volumes of lysis buffer. The nickel column was developed over 30 minutes via a gradient from 0%-100% nickel elution buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 500 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 10% SDS-PAGE and Coomassie staining. Fractions containing CDC73 were pooled, mixed with 1.5 mg of $His_6$-TEV protease, transferred to the SnakeSkin dialysis tubing (7 kDa MWCO), and dialyzed for ~12-16 hrs against 1L of lysis buffer. The protein was removed from the SnakeSkin dialysis tubing and a sample was saved for gel analysis. The rest of the protein was applied to a 5 mL HisTrap column equilibrated in lysis buffer to remove TEV protease, the $His_6$-MBP tag, and uncleaved protein. The flowthrough was collected. TEV and any uncleaved protein were eluted with nickel elution buffer. The elution was also collected, monitored with Bradford, and saved for gel analysis. The input, flowthrough, and elution samples were evaluated by 15% SDS-PAGE and Coomassie staining. The flowthrough was concentrated to 2-4 mL using a 3 kDa or 10 kDa MWCO Amicon Millipore 15 mL concentrator for CDC73 1-111 or 336-531 constructs, respectively. The concentrated

protein was applied to a S75 16/600 column (Cytvia) equilibrated in SE buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 15% SDS-PAGE and Coomassie staining. Appropriate fractions were pooled, concentrated in 3 kDa or 10 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Units, aliquoted, snap frozen, and stored at -80ºC. The A280 absorbance was measured with spectrophotometer (Nanodrop One, Thermo Scientific), and the amount of protein was quantified using the Beer-Lambert law

$$A = \varepsilon lc \qquad \text{(Equation 1)}$$

where the Extinction coefficient ($\varepsilon$) was estimated in SnapGene 6.0.1/6.0.2.

**2.4 Purification of β-catenin 138-781**

All steps were performed at 4ºC unless otherwise specified. Cell pellets were thawed and sonicated with a Branson 450 sonicator on ice for 5 minutes, at 60% power with bursts of 0.6s on, 0.4s off. Cell lysates were clarified by centrifugation (30 minutes, 86,756xg, A27 rotor). The clarified lysate was applied to a 5 mL HisTrap column (Cytvia) equilibrated in lysis buffer (500 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix). The column was washed with lysis buffer for ~25 column volumes, followed by 5 column volumes of high salt wash buffer (1 M NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT), and another 5 column volumes wash of lysis buffer. The nickel column was developed over 30 minutes via a gradient from 0%-100% nickel elution buffer (500 mM NaCl, 20 mM Na-HEPES pH 7.4, 500 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. Fractions containing β-catenin were pooled, mixed with 1.5 mg of $His_6$-

TEV protease, transferred to the SnakeSkin dialysis tubing (7 kDa MWCO), and dialyzed for about 12-16 hrs against 1L of lysis buffer. The protein was removed from the SnakeSkin dialysis tubing and a sample was saved for gel analysis. The rest the protein was applied to a 5 mL HisTrap column equilibrated in lysis buffer to remove TEV protease, the $His_6$-tag, and uncleaved protein. The flowthrough was collected. TEV and any uncleaved protein was eluted with nickel elution buffer. The elution was also collected, monitored with Bradford, and saved for gel analysis. The input, flowthrough, and elution samples were evaluated by 10% SDS-PAGE gel and Coomassie staining. The flowthrough was concentrated to 2-4 mL using a 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit. The concentrated protein was applied to a S200 16/600 column (Cytvia) equilibrated in SE buffer (500 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. Appropriate fractions were pooled, concentrated in 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit, aliquoted, snap frozen, and stored at -80ºC. The protein was concentrated down to ~100-200 μM. The A280 absorbance was measured with spectrophotometer (Nanodrop One, Thermo Scientific), and the amount of protein was quantified using equation 1.

**2.5 Purification of β-catenin 138-781 and CDC73 213-263 complex**

All steps were performed at 4ºC unless otherwise specified. Cell pellets were thawed and sonicated with a Branson 450 sonicator on ice for 5 minutes, at 60% power with bursts of 0.6s on, 0.4s off. Cell lysates were clarified by centrifugation (30 minutes, 86,756xg, A27 rotor). The clarified lysate was applied to a 5 mL HisTrap column (Cytvia) equilibrated in lysis buffer (300 or 150 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix). The column was washed with lysis buffer for about 25 column volumes,

followed by 5 column volumes of high salt wash buffer (800 or 600 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT), and another 5 column volumes wash of lysis buffer. The nickel column was developed over 30 minutes via a gradient from 0%-100% nickel elution buffer (300 or 150 mM NaCl, 20 mM Na-HEPES pH 7.4, 500 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT) into an amylose column (Cytvia). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. The amylose column was washed with 5 column volumes of lysis buffer followed by the elution of the column with 2 column volumes of amylose elution buffer (300 mM or 150 mM NaCl, 20 mM Na-HEPES pH 7.4, 116 mM Maltose, 10% (v/v) glycerol, 1 mM DTT). Fractions peak from both elution were pooled, mixed with 1.5 mg of $His_6$-TEV protease, transferred to the SnakeSkin dialysis tubing (7 kDa MWCO), and dialyzed for about 12-16 hrs against 1L of lysis buffer. The protein was removed from the SnakeSkin dialysis tubing and a sample was saved for gel analysis. The rest the protein was applied to a 5 mL HisTrap column equilibrated in lysis buffer to remove TEV protease, the $His_6$/MBP tag, and uncleaved protein. The flowthrough was collected. TEV and any uncleaved protein was eluted with nickel elution buffer. The elution was also collected, monitored with Bradford, and saved for gel analysis. The input, flowthrough, and elution samples were evaluated by 10% SDS-PAGE gel and Coomassie staining. The flowthrough was concentrated to 2-4 mL using a 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit. The concentrated protein was applied to a S200 16/600 column (Cytvia) equilibrated in SE buffer (300 or 150 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. Appropriate fractions were pooled, concentrated in 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit, aliquoted, snap frozen, and stored at -80ºC.

The purification of β-catenin 138-781 and CDC73 213-263 complex was performed as described above, except that the proteins were first individually eluted from nickel and amylose resins, respectively. The elution of each protein was mixed with 1.5 mg of $His_6$-TEV protease, transferred to the SnakeSkin dialysis tubing (7 kDa MWCO), and dialyzed for about 12-16 hrs against lysis buffer with 150 mM NaCl. The A280 absorbance was measured with a spectrophotometer (Nanodrop One, Thermo Scientific), and the amount of protein was quantified using the equation 1.

**2.6 Purification of β-catenin 138-781 and CDC73 213-263 fusions**

All steps were performed at 4ºC unless otherwise specified. L10 and NO-linker fusion constructs cell pellets were thawed and sonicated with a Branson 450 sonicator on ice for 5 minutes, at 60% power with bursts of 0.6s on, 0.4s off. Cell lysates were clarified by centrifugation (30 minutes, 158,114xg, A27 rotor). The clarified lysate was applied to a 5 mL HisTrap column (Cytvia) equilibrated in lysis buffer (300mM NaCl, 20mM Na HEPES pH 7.4, 20mM imidazole pH 8.0, 10% (v/v) glycerol, 1x protease inhibitor cocktail mix, 1mM DTT). The column was washed with lysis buffer for about 25 column volumes, followed by 5 column volumes of high salt wash buffer (1 M NaCl, 20 mM Na-HEPES pH 7.4, 20 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT), another 5 column volumes wash of lysis buffer and low salt buffer (100 mM NaCl, 20 mM Na HEPES pH 7.4, 20 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT). The nickel column was developed over 30 minutes via a gradient from 0%-100% nickel elution buffer (100 mM NaCl, 20 mM Na-HEPES pH 7.4, 400 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT) into an amylose column (Cytvia). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. Fractions containing the complex were pooled, mixed with 1.5 mg of

$His_6$-TEV protease, transferred to the SnakeSkin dialysis tubing (7 kDa MWCO), and dialyzed for about 12-16 hrs against 1L of low salt buffer. The protein was removed from the SnakeSkin dialysis tubing and a sample was saved for gel analysis. The rest the protein was concentrated to 2-4mL and applied to a 5 mL HisTrap column tandem to a S column equilibrated in low salt buffer. The two columns were washed with 10 column volumes of low salt buffer. The flowthrough was collected. The nickel column was removed, and the S column was washed with another 2 column volumes of low salt buffer. The S column was eluted via a gradient from 0-100 mL of high salt buffer. Peak fractions of S column elution were collected and saved for gel analysis. TEV and any uncleaved protein was eluted from the HisTrap column with nickel elution buffer, collected, monitored with Bradford, and saved for gel analysis. The input, nickel tandem to S column flowthrough, and nickel/S column individual elution samples were evaluated by 10% SDS-PAGE gel and Coomassie staining. The nickel column tandem to S column flowthrough sample was concentrated to 2-4 mL using a 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit. The concentrated protein was applied to a S200 16/600 column (Cytvia) equilibrated in SE buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT). Peak fractions were collected and evaluated by 10% SDS-PAGE gel and Coomassie staining. Appropriate fractions were pooled, concentrated in 30 kDa MWCO Amicon Millipore Ultra-15 Centrifugal Filter Unit, aliquoted, snap frozen, stored at -80ºC or used immediately for crystallography. The protein was concentrated to ~5-7 mg/mL. The A280 absorbance was measured with a spectrophotometer (Nanodrop One, Thermo Scientific), and the amount of protein was quantified using the equation 1.

**2.7 β-catenin and CDC73 mutants reciprocal pull-down**

β-catenin (138-781) and CDC73 (213-263) mutant constructs were transformed and expressed in Bl21 (DE3) LOBSTR RIL. For each construct, 25 mL of expression were prepared with 25 mL 2xYT media, 100 µg/mL ampicillin, 34 µg/mL chloramphenicol, and each inoculated with 250 µL of an overnight expression culture. Cells were grown at 37ºC and 160 rpm until $OD_{600}$ reached 0.4-0.6. Protein expression was induced by adding 0.5 mM IPTG and cells were grown for an additional 16-18 hrs at 18ºC. Cells were collected by centrifugation, resuspended in lysis buffer (300 mM NaCl, 20 mM Na-HEPES pH 7.4, 30 mM imidazole pH 8.0, 10% (v/v) glycerol, 1 mM DTT, 1x protease inhibitor cocktail mix), and used immediately for pull-downs. For the pull-downs, all steps were performed at 4ºC unless otherwise specified.

Resuspended cell pellets were sonicated 3 times with a Branson 450 sonicator on ice for 10s, with constant power, total output set to 2, and resting cells for 2 minutes between sonication. Cell lysates were clarified by centrifugation (30 minutes, 21,300xg). 10 µL of the clarified lysate was removed, mixed with 30 µL of lysis buffer and 10 µL of loading buffer, and saved for gel analysis. The rest of the supernatant was added to 150 µL of either nickel or amylose beads equilibrated in lysis buffer and incubated for 15 minutes using a rotating device. The beads were spun down at 2348xg for 30 seconds. The supernatant was removed, and the beads were washed 3 times with 1mL of lysis buffer. After the last wash, the protein was eluted by adding 30 µL of either nickel (300 mM NaCl, 20 mM Na-HEPES, 500 mM imidazole, 10% (v/v) glycerol, 1 mM DTT) or amylose (300 mM NaCl, 20 mM Na-HEPES, 116 mM Maltose, 10% (v/v) glycerol, 1 mM DTT) elution buffer and incubated at room temperature for 2 minutes. The beads were spun down and 15 µL of the supernatant were removed and mixed with 5 µL of loading buffer and saved for gel analysis.

The rest of the supernatant from the elution of each resin were removed and added to the opposite fresh resin (e.g.: nickel eluates were applied to amylose resin and amylose eluates were applied to nickel resin). The beads from the first pulldown round were washed with additional 500 µL of lysis buffer to remove any residual protein that might be attached to the beads. The beads were spun down, and the supernatant were transferred to the tubes with the rest of the elution. The beads were incubated with protein for 15 minutes using a rotating device. The beads were spun down supernatant was removed, and the beads were washed 3 times with 1 mL lysis buffer. After the last wash, the protein was eluted in the same way as stated above. 30 µL of the elution supernatant was removed and mixed with 10µL of loading buffer for gel analysis. All samples (supernatant, elution from both rounds of pulldowns) were analyzed on a 10% SDS PAGE gel and stained with Coomassie blue.

**2.8 β-catenin and CDC73 reciprocal pull-down**

β-catenin (138-781) and CDC73 (213-263) constructs were transformed into *E. coli* DH10αEMBacY to produce bacmids (Berger et al. 2015). Bacmid DNA was isolated from verified white colonies via alkaline lysis, isopropanol, and ethanol precipitation. Bacmid DNA was incubated with Xtreme Gene transfection mix for 30 minutes and then applied to $1x10^6$ *Spodoptera frugiperda* (Sf9). Within 2-4 days, YFP expressing colonies were visible and the virus was harvested by gently removing the media (V0 virus). V1 virus was produced by adding approximately 0.15-3 mL of V0 virus to 25 mL of Sf21 cells at a concentration of $1x10^6$ cells/mL. Cells were monitored each day for viability, cell counts, and diameter. 48-72 hrs after the day after proliferation arrest (DPA), cells were collected by centrifugation (238xg, 15 minutes) and the supernatant was recovered (V1 virus). 12.5-25 µL of V1 virus was applied to 50-100 mL of

*Trichoplusia ni (*Hi5) cells for protein expression. Cell pellets were used to test expression levels and protein interaction between β-catenin and CDC73.

Harvested cells were resuspended with lysis buffer (500 mM NaCl, 20 mM Na-HEPES, 30 mM imidazole, 10% (v/v) glycerol, 1 mM DTT, protease inhibitor cocktail mix). The pull-down was done in the same way as described above for the pull-downs of mutants. However, resuspended cell pellets were sonicated 3 times on ice for 10s, with constant power, total output set to 3, and resting cells for 2 minutes between each sonication step. In addition, the concentration of the NaCl in amylose elution buffer was 800 mM instead of 300 mM and 200 µL of bead slurry was used.

**2.9 Crystallization**

The purified L10 and NO-linker fusion constructs were freshly prepared for crystallization screens. The protein was dialyzed against crystallography buffer (100 mM NaCl, 10 mM Na HEPES pH 7.4, 1 mM TCEP pH 7.0) in a 10 kDa MWCO Slide-A-Lyzer MINI Dialysis Unit for 12-16 hrs at 4ºC. The first crystal hits were identified by using sparse matrix crystal trays from Qiagen (PACT), Hampton Research (PEG RX HT and Index HT), and Molecular Dimensions (JCSG+ and Morpheus). The crystallization of these proteins was done by the sitting drop vapor diffusion technique performed by Art Robbins Phoenix robot (drop: 0.15 µL protein and 0.15 µL mother liquor; well solution: 70 µL). The concentration of the proteins for the crystallization screen were ~5mg/mL. In some well conditions, crystals appeared after 24 h and continued to grow over the course of 2-6 days, and others appeared after a week and grew for another 2-6 days. The first crystal hits and their respective condition details can be found in the Table 2-3 (Appendix).

To optimize crystal hits from sparse matrix screens (Table 2-3), hanging drop crystal trays were set. Concentration of the proteins were around the concentration of the protein from the first crystal hits. 2 μL drops were prepared (1 μL protein; 1 μL mother liquor) and hanged above a 500 μL mother liquor solution. Crystals appeared after 24 hrs - 7 days and continued to grow over the course of 2-6 days. Identified hits and respective growing conditions can be found in Table 4-5 (Appendix).

**2.10 Fluorescence anisotropy binding assays with β-catenin and nucleic acids**

β-catenin was first dialyzed for approximately 4 hrs against its FA buffer (100 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT) in a 10 kDa MWCO Slide-A-Lyzer MINI Dialysis Unit. The protein was retrieved from the dialysis unit and used directly for fluorescence anisotropy assays. 25-mer 5' 6-FAM labelled double-stranded DNA (TCCATCAGAATTCGGATGAACTCGG) obtained from IDT, 20-mer 5' 6-FAM labelled single-stranded DNA (GAACCCACUCGGAGCCAGCA) and 12-mer 5' 6-FAM labelled RNA (AGGGAACCCACU) obtained from Sigma, were dissolved in water, and stored in -80ºC until use. β-catenin was serially diluted in half-log steps in FA buffer. β-catenin (9 μL, 10 μM - 0.1 nM final concentration) and nucleic acids (3 μL, 100 μM stock concentration, and 5 nM final concentration) were mixed on ice and incubated for 10min. The assay was brought up to a final volume of 30 μL and incubated at room temperature in the dark for 20 minutes (final condition: 30 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 3 mM $MgCl_2$, 0.05mg/mL BSA, 0.27mM DTT). 18μl of the reaction was transferred to a Greiner 384-well black flat bottom small volume plate. Fluorescence anisotropy was measured at room temperature with a Tecan Spark plate reader with an excitation wavelength of 470 ± 20 nm, an emission wavelength of 518 ±

20 nm, and a gain of 100. The experiment was performed in triplicates and binding curves were fitted using a single site quadratic binding equation as defined below in MATLAB.

$$y = \left(\frac{B_{max}\times([x]+[L]+K_{d,app}-\sqrt{([x]+[L]+K_{d,app})^2-4([x]\times[L])})}{2\times[L]}\right) \qquad \text{(Equation 2)}$$

where Bmax is the maximum specific binding, L is the nucleic acids concentration, x is the concentration of the protein (in this case, β-catenin) , and Kd, app is the apparent dissociation constant for the protein and DNA (Vos et al. 2016). Error bars are the standard deviation from the mean of three experimental replicates. Experiments were performed independently from each other using different prepared buffers and protein aliquots.

**2.11 Fluorescence anisotropy binding assays with CDC73 termini and nucleic acids**

CDC73 1-111 and 336-531 was used directly in the fluorescence anisotropy assay. The same nucleic acids used in the β-catenin fluorescence anisotropy assay were used for CDC73 termini. Both proteins were serially diluted in half-log steps in FA buffer (150 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 1 mM DTT). CDC73 1-111 (6 µL, 196 µM - 1.96 nM final concentration) and CDC73 336-531 (6 µL, 21.2 µM - 0.52 nM final concentration) were each mixed with nucleic acids (3 µL, 100 µM stock concentration, and 5 nM final concentration) on ice and incubated for 10 min. The assay was brought up to a final volume of 30 µL and incubated at room temperature in the dark for 20 minutes (final conditions: 30 mM NaCl, 20 mM Na-HEPES pH 7.4, 10% (v/v) glycerol, 3 mM MgCl2, 0.05mg/mL BSA, 0.27mM DTT). 18µl of the reaction was transferred to a Greiner 384-well black flat bottom small volume plate. Fluorescence anisotropy was measured and analyzed as described above.

### 2.12 Sequence conservation analysis

Protein sequences of β-catenin and CDC73 were selected using the BLAST algorithm (NCBI, Altschul, et al. 1990). The non-redundant protein sequences database was used to find homologs of *Homo sapiens* β-catenin and CDC73 protein sequences using the blastp algorithm and standard parameters (Table 4). Protein sequence alignments were performed on the MAFFT server and visualized in Jalview 2.11.1.7 (Katoh et al. 2002, Waterhouse et al. 2009). For specific CDC73 domains (213-263 and 336-531), sequence alignment and identity were performed using the Alignment tool in SnapGene (version 6.0.2). Philogenetic trees were made using phyML program from NGPhylogeny website (version 3.3_1, Guindon et al. 2010). The Baysean algorithm was used to build phylogenetic trees using the sequence alignment data retrieved from MAFFT. The trees were rooted and boostrapping statistical tests were performed to build branches.

### 2.13 AlphaFold2 structure prediction

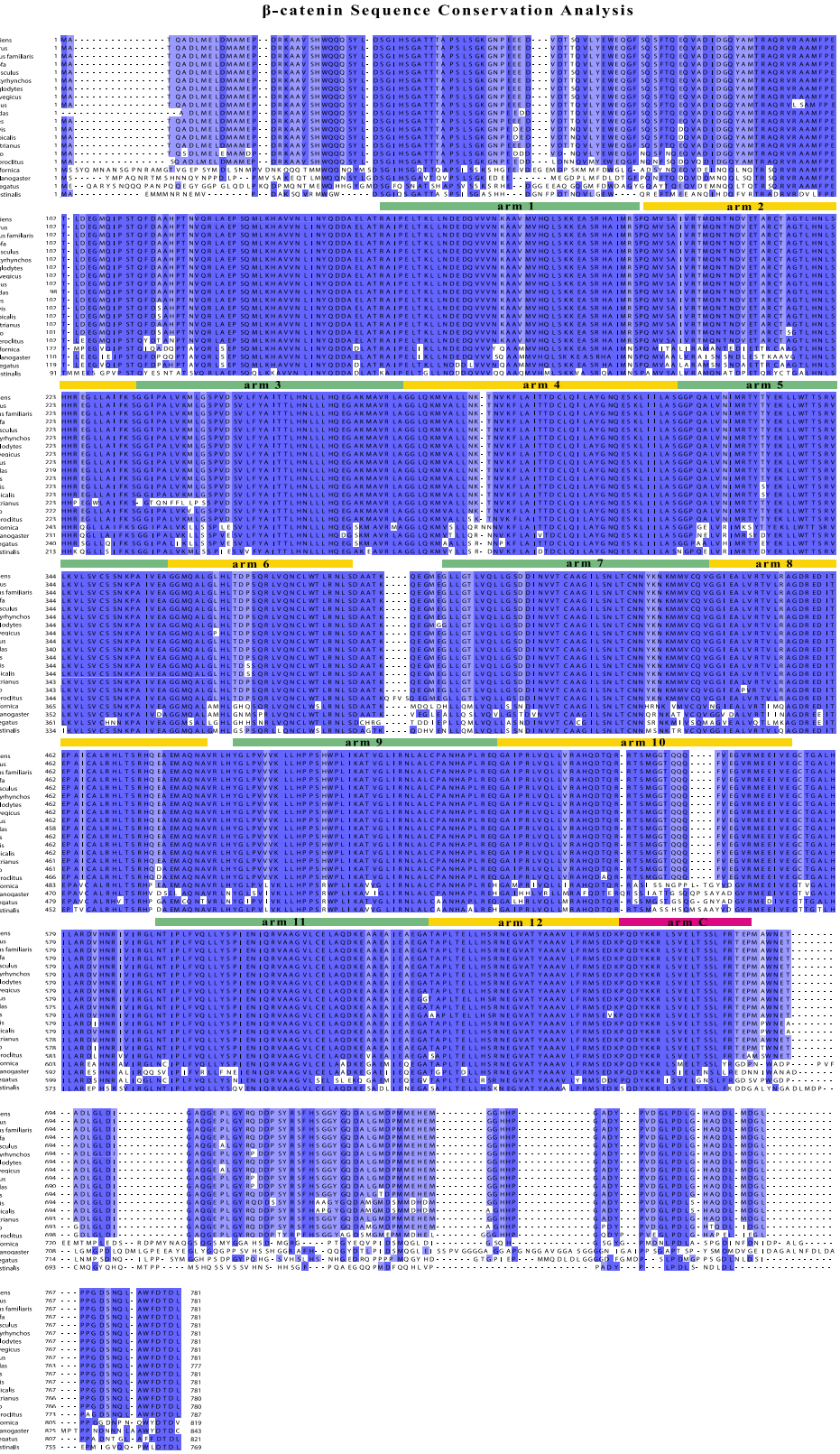
Protein structure prediction was performed using AlphaFold2 (Jumper, J et al. 2021 and Varadi, M et al. 2022). The sequence of β-catenin (residues 150-781) and CDC73 (residues 188-469) were input as two chains. Calculations were run using the google CoLab AlphaFold2 installation (February 2022). The predicted structure was visualized and analyzed in Pymol 2.5.2 (Schrödinger LLC).

# 3. Results

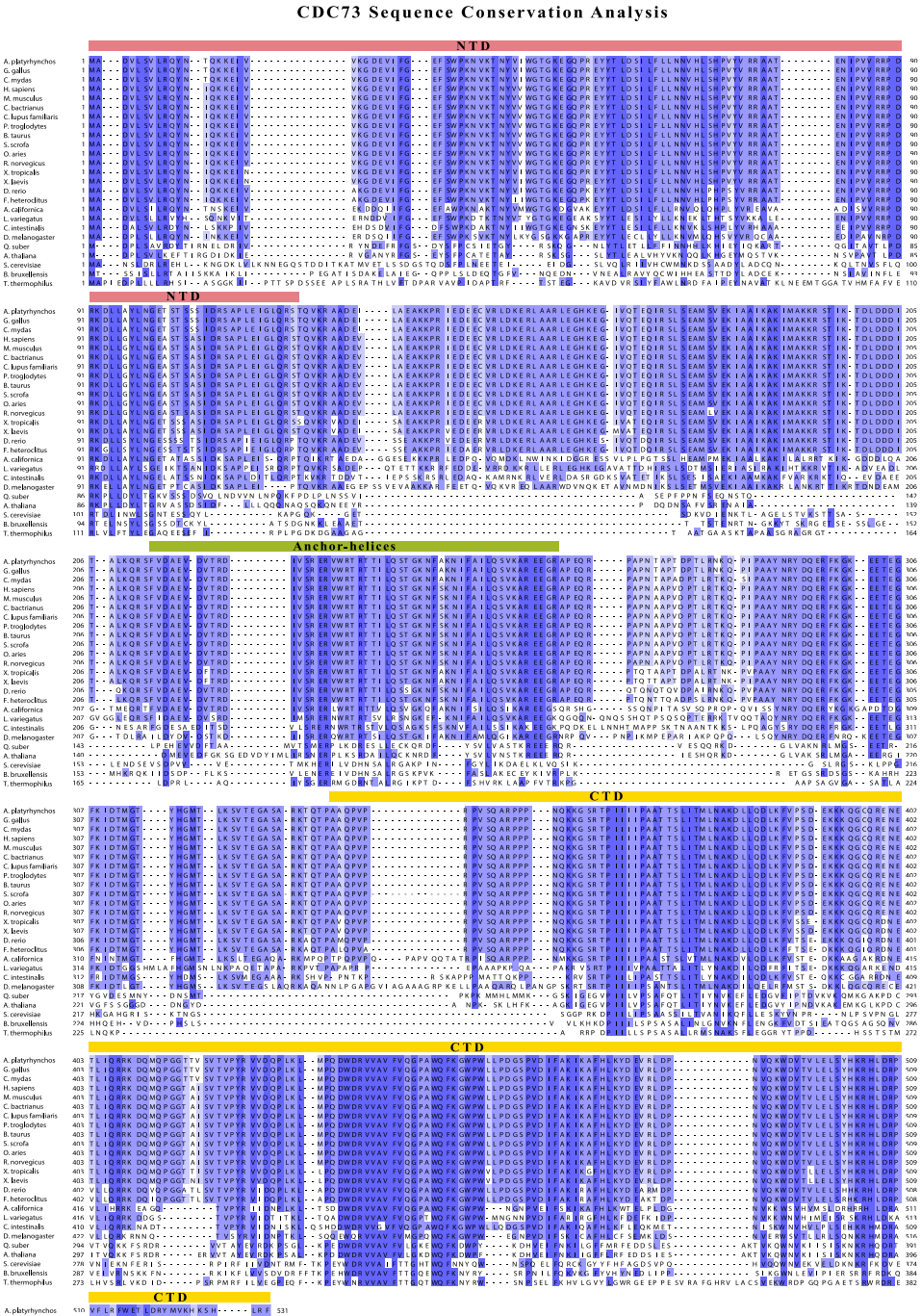
### 3.1 Sequence conservation analysis of β-catenin and CDC73

To analyze the conservation of β-catenin and CDC73, I selected representative protein sequences using the BLAST algorithm (Methods). Sequences were aligned to generate multiple sequence alignments for each protein (MAFFT, Katoh et al. 2002). Twenty sequences were selected for β-catenin and twenty-five sequences for CDC73. β-catenin is broadly conserved among vertebrates (~97% identity between *Danio rerio* and *Homo sapiens,* Figure 2A). CDC73 is found in all eukaryotic lineages analyzed however, its sequence diverges significantly between vertebrate and nonvertebrate lineages (~27% identity between *S. cerevisiae* and *Homo sapiens* versus 91% identity between *Danio rerio* and *Homo sapiens*, Figure 2B). Specifically, the CDC73 N-terminal domain is only observed in metazoans as previously described (Mosimann et al. 2006), whereas the CDC73 C-terminal domain is conserved among all eukaryotes (~29% identity between *S. cerevisiae* and *Homo sapiens* versus 91% identity between *Danio rerio* and *Homo sapiens*). The CDC73 anchor helices (213-263) are present in all organisms, however, sequence identity for this region is weak for *Saccharomyces cerevisiae* (residues 156-207 – PDB 7DKH) and *Thermothelomyces thermophilus* (residues 155-227 – PDB 6AF0) versus human (approximately 24% and 15%, respectively).

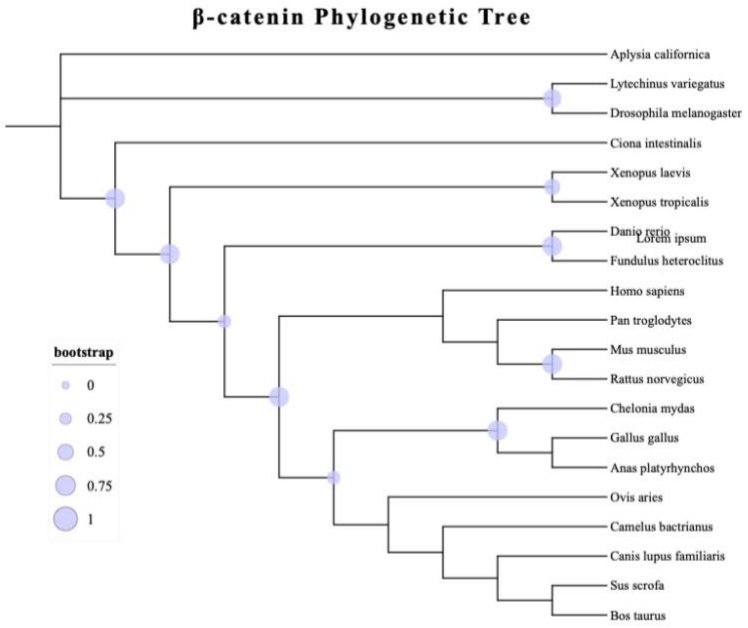
A.


β-catenin Sequence Conservation Analysis
arm 1
arm 2
arm 3
arm 4
arm 5
arm 6
arm 7
arm 8
arm 9
arm 10
arm 11
arm 12
arm C

B.


CDC73 Sequence Conservation Analysis
NTD
NTD
Anchor-helices
CTD
CTD
CTD

C.




D.

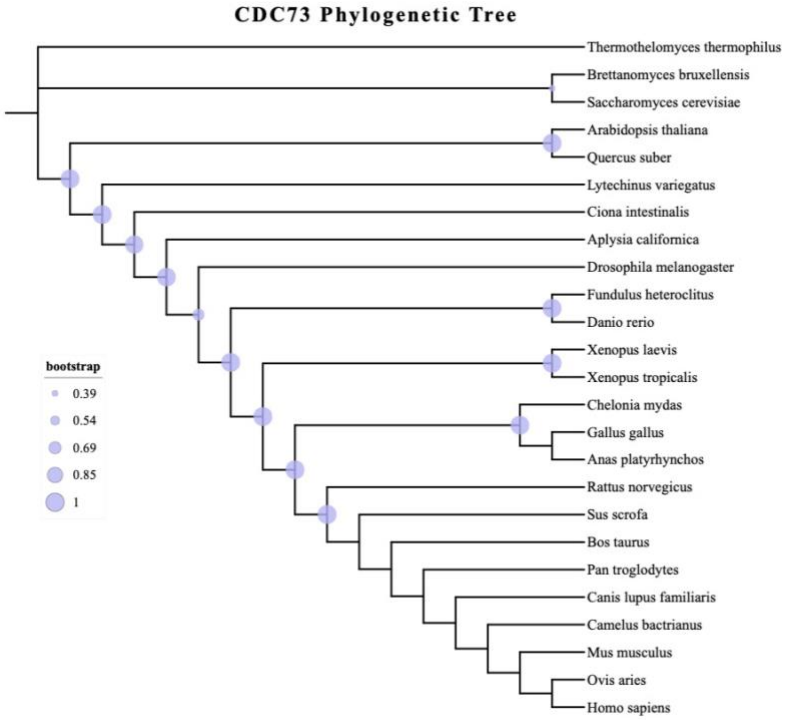



**Figure 2.** β-catenin and CDC73 sequence conservation analysis A-B) Multiple sequence alignment by MAFFT of β-catenin and CDC73 (colored by percentage identity; darker shades of blue indicate higher conservation A) β-catenin sequence conservation analysis (alternate green and yellow rectangles represents β-catenin armadillo repeat 1-12). B) CDC73 sequence conservation analysis (CDC73-NTD: red rectangle; CDC73-CTD: yellow; CDC73 anchor-helices: green) C-D) Phylogenetic tree of β-catenin and CDC73.

### 3.2 Predicted structure of β-catenin and CDC73

To gain an understanding of how β-catenin and CDC73 may interact at the atomic level, I used AlphaFold2 multimer as implemented in google CoLab (Jumper, J et al. and Varadi, M et al. 2021) to predict the structure of the β-catenin (150-781) and CDC73 (188-469) complex (Figure 3A). AlphaFold2 predicted that β-catenin and CDC73 form a complex using hydrophobic residues between part of the twelfth armadillo repeat of β-catenin (arm 12) and a helix on C-terminus of the armadillo repeat (arm C), specifically residues 649-683, and the CDC73 anchor helix (residues 245-259; Figure 3B). AlphaFold2 also predicted the well-structured and unstructured regions of both proteins (Figure 3C). Most of β-catenin has good pLDDT confidence scores (Figure 3D). However, the pLDDT scores for the C-terminus of β-catenin (686-781) are low (close to 40) which is a region known for being unstructured (Figure 3C). On the other hand, CDC73 was predicted with low confidence score and most of the protein had pLDDT scores of ~40 (Figure 3 C-D). However, CDC73 anchor-helices and C-terminus domain, more specifically the residues 246-263 and 357-467 respectively, had better pLDDT scores (60-80 for CDC73 anchor-helices and close to 90 for the C-terminus domain; Figure 3C). This implies that interactions between the twelve repeat of β-catenin and CDC73 anchor-helices are confidently predicted by AlphaFold2. However, solving the structure of the complex is essential to determine the exact nature of the interaction between β-catenin and CDC73.

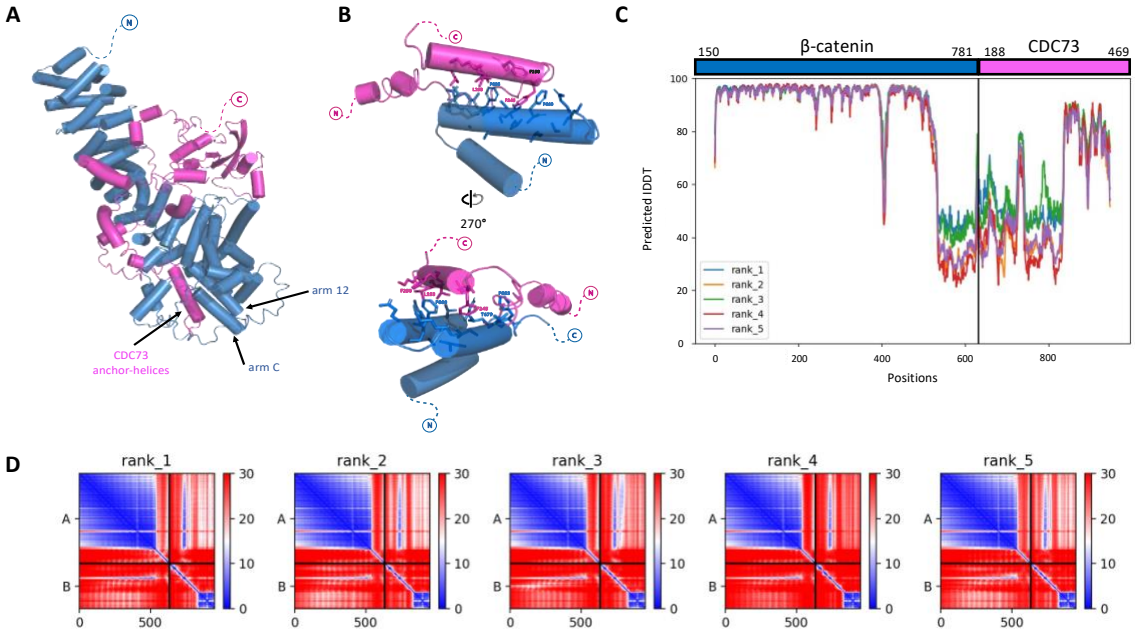



**Figure 3.** AlphaFold2 structure prediction and analysis. A) AlphaFold2 predicted structure of β-catenin and CDC73 complex (blue: β-catenin armadillo repeat-12 + helix C 637-686; green: CDC73 anchor helices 213-263; structure from rank 1 prediction). B) Two different angles of the zoom in version of β-catenin and CDC73 predicted structure showing the interacting residues. C) AlphaFold2 per-residue confidence score of β-catenin and CDC73 complex predicted structure (pLDDT). D) Predicted Aligned Error (PAE) for five different predicted structures of β-catenin and CDC73 complex.

Using the AlphaFold2 structure of β-catenin and the CDC73 anchor helices, I designed and tested whether point mutations (single, double, and triple combinations) at the interface of the interaction of these proteins would disrupt their interactions (β-catenin: F660R/A, T673R/A, F689R/A; CDC73: F245R/A, F250R/A, L253R/A; Figure 3B). β-catenin 138-781 and CDC73 213-263 mutants were co-expressed, and pull-downs were performed to determine if they can be pulled-down together. The results of the reciprocal pull-downs were inconclusive. Based on gel images, $His_6$-β-catenin was not present anymore after the second elution while it was present in the first round of elutions. This was independented of whether the complex was pulled-down using either amylose or nickel beads (data not shown). It would be necessary to perform these pull-downs again or other binding experiments in order to make any conclusions. In addtion, structure determination would be essential to identify the specific interactions between β-catenin and CDC73.

### 3.3 Mutually exclusive binding of CDC73 anchor helices with PAF1 complex and β-catenin

To determine whether β-catenin and CDC73 form a complex, we co-expressed these proteins in *Trichoplusia ni* cells and examined whether the complex can be pulled down together. Using nickel and amylose beads, $His_6$-β-catenin and MBP-CDC73 were isolated as a complex in a reciprocal pull-down experiment (Figure 4A – left). Using the same beads, MBP-CDC73 and PAF complex with a tag MBP-$His_6$ on CTR9 (CTR9-MBP-$His_6$) were also reciprocally pulled-down together (Figure 4A – right). All the elements of the PAF complex were pulled down with CDC73 but β-catenin was not after mixing β-catenin with PAF (Figure 4B). This data suggests that the CDC73 interaction with β-catenin and PAF may be mutually exclusive.

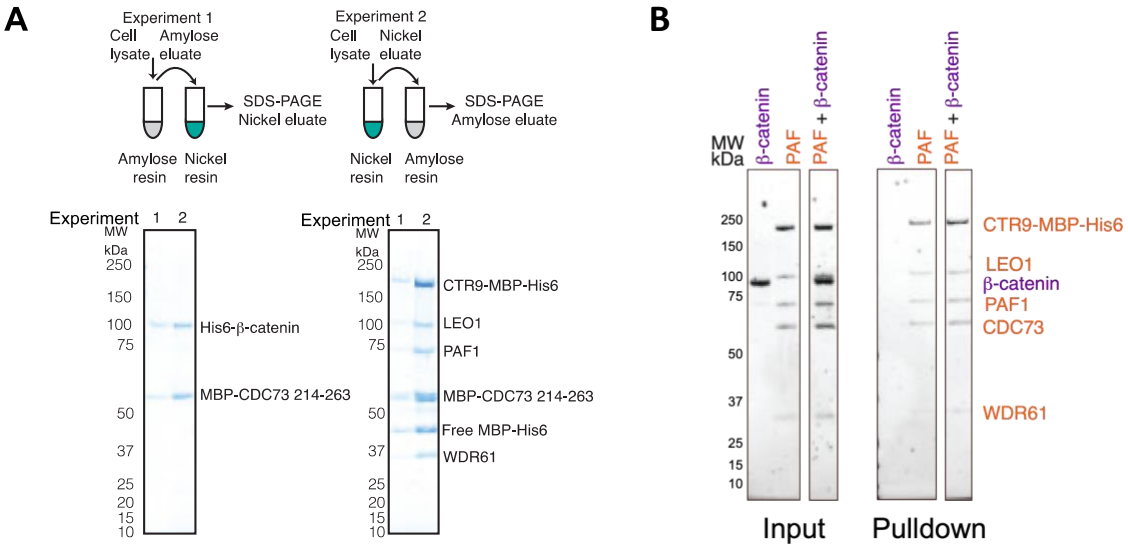



**Figure 4.** CDC73 cannot be pull-down with β-catenin and PAF simultaneously. A) Reciprocal pull-down of CDC73 with either β-catenin (left) and PAF (right). Pull-down of the PAF complex (CTR9-MBP-$His_6$) shows that CDC73 interacts with PAF and β-catenin mutually exclusively.

### 3.4 Efforts towards determining a structure of the β-catenin and CDC73 complex

Human β-catenin (138-781) and CDC73 (213-263) were previously reported to constitute the minimal β-catenin-CDC73 complex (Mosimann et al. 2006). I thus co-expressed β-catenin (138-

781) and CDC73 (213-263) in *E. coli* cells and attempted to purify the complex using affinity, ion exchange, and size exclusion chromatography (Methods). Unfortunately, the complex disassociated over the course of the purification (Figure 5A). Co-expression and purification of the full-length proteins also resulted in complex dissociation (data not shown). In a final attempt to form the complex, I purified the individual protein domains, however, CDC73 213-263 was highly insoluble in isolation (data not shown).

To stabilize the transient β-catenin (138-781) and CDC73 (213-263) complex, fusions containing both proteins were designed (Figure 5B). It was previously reported that the region spanning the C-terminal armadillo repeat (arm 12) and part of the C-terminal domain (helix C) of β-catenin bound to the middle region of CDC73 (Mosimann et al. 2006). I thus designed fusion constructs that appended CDC73 to the C-terminus of β-catenin. The first construct consisted of β-catenin (138-781) directly fused to CDC73 213-263 (NO-linker construct). A second construct was designed using only the structured region of β-catenin (138-691) that was C-terminally connected to CDC73 213-263 via a glycine-serine linker (GSSGSGGGSG; L10 construct). Both fusion constructs were expressed in *E. coli* cells and purified by affinity, ion exchange, and size exclusion chromatography (Figure 5C). The highly purified protein was used for sparse matrix crystallization experiments. Several crystallization conditions were identified in the sparse matrix screens and were further optimized. (Figure 5D; Table 2-5).

Mass spectrometry analysis revealed a co-purifying contaminant of an almost equal mass to our construct. The *E. coli* contaminant was the bifunctional polymyxin resistance protein ArnA. ArnA is a histidine rich protein known to associate with nickel resin. Producing the protein in Bl21(DE3) LOBSTR RIL cells would be a solution as these cells are designed to minimize the expression of ArnA and other commonly found contaminants in *E. coli* (Kasper et al. 2013).

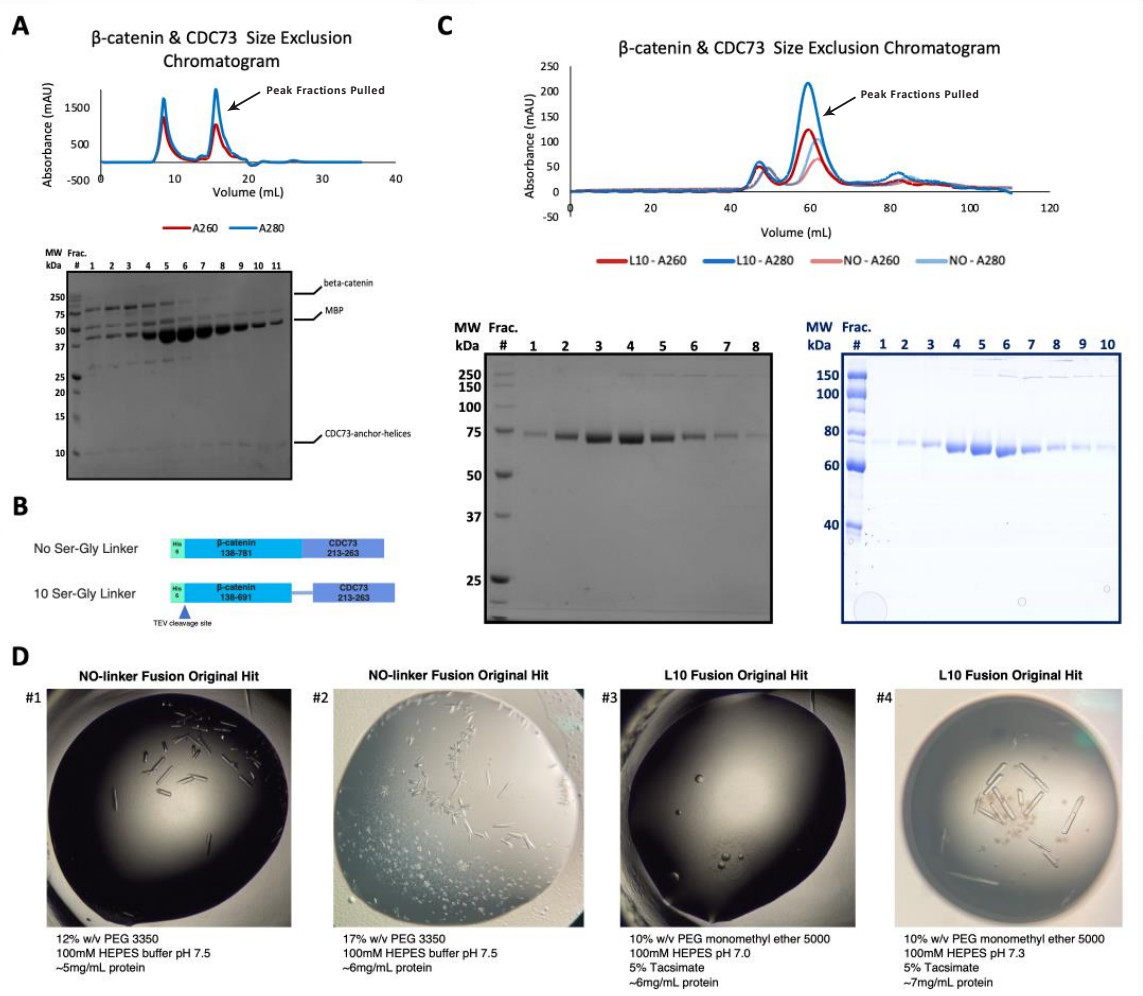



**Figure 5.** β-catenin and CDC73 purification and crystallization. A) Size exclusion chromatography purification of the co-expressed β-catenin and CDC73 (300mM NaCl). B) β-catenin and CDC73 fusion constructs designed. C) Size exclusion chromatography purification of β-catenin and CDC73 NO-linker (bottom left) and L10 fusion constructs (bottom right); top: chromatogram of the purification in the gels at the bottom (C). D) Crystal hits of β-catenin and CDC73 NO-linker and L10 fusion; crystal hit from PEG RX HT sparse matrix screen (#1); crystal hit from optimizing the #1 hit (#2); crystal hit from Index HT sparse matrix screen (#3); crystal hit from optimizing the #3 hit (#4).

### 3.5 β-catenin and CDC73 N- and C- terminal domains weakly interact with nucleic acids

I performed fluorescence anisotropy (FA) binding assays to determine if β-catenin and CDC73 interact with nucleic acids. β-catenin (138-781), CDC73 N-terminal (1-111), and CDC73 C-terminal domains (336-531) were purified to homogeneity (Figure 6A). I next tested whether they interact with either double-stranded DNA, single-stranded DNA, and RNA. We found that all these

domains bind nucleic acids weakly with $K_d$ values greater than 1 µM (Figure 6B-D; Appendix - Table 6).

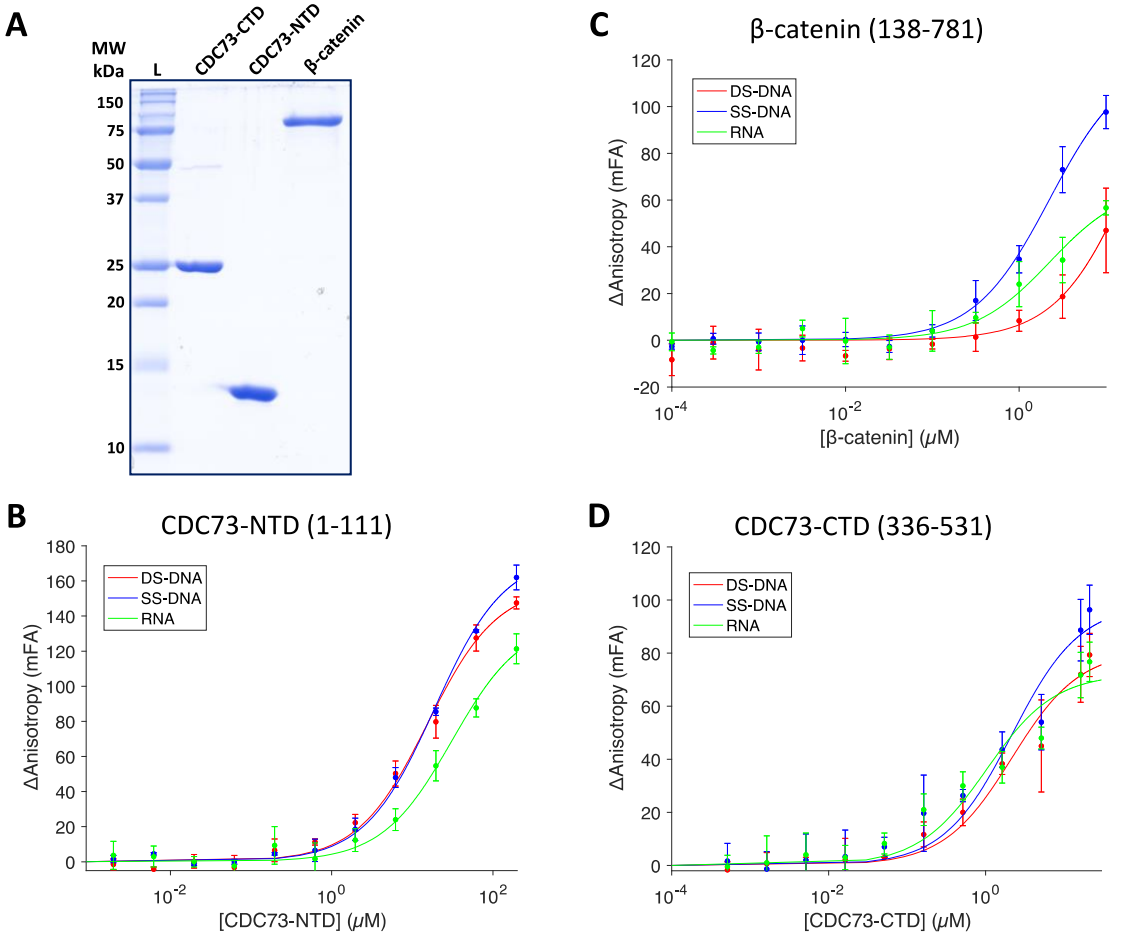



**Figure 6.** β-catenin and CDC73 interaction with nucleic acids. A) SDS-PAGE gel showing the purified proteins used in the FA assays. B-D) Fluorescence anisotropy titration shows that β-catenin, CDC73 N- and C-terminal domains bind nucleic acids weakly ($K_{d,app}$ > 1 µM). Points represent the mean of three independent experiments and error bars are the standard deviation between the replicates.

# 4. Discussion

Here I aimed to study the molecular basis for the β-catenin•CDC73 interaction. Both proteins are highly conserved across vertebrates. CDC73 additionally interacts with PAF, an important transcription elongation factor. Preliminary data described here suggests that CDC73 forms mutually exclusive complexes with either β-catenin or PAF. These findings contrast previous studies where it was suggested that the interaction between β-catenin and CDC73 mediates the recruitment of PAF and Pol II to Wnt target genes (Mosimann et al. 2006).

Previous experiments and AlphaFold2 predictions performed here suggest β-catenin armadillo repeat twelve and CDC73 anchor-helices interact via a hydrophobic patch (Mosimann et al. 2006). To validate these results and define the residues required for the interaction, I aimed to solve the structure of the complex by X-ray crystallography. Unfortunately, β-catenin and CDC73 disassociate throughout standard protein purification procedures. To obtain a stable complex, I fused β-catenin and CDC73. These constructs have not yet yielded an optimal sample due to sample degradation. Transcriptional co-activator with PDZ-binding motif (TAZ) is a factor that has been previously reported to mediate the interaction between β-catenin and CDC73 (Tang et al. 2018). TAZ could be used to potentially stabilize the β-catenin and CDC73 complex. A structure of β-catenin and CDC73 will define the exact nature of the interaction between β-catenin and CDC73 complex, providing a platform to investigate how CDC73 specifically interacts with either PAF or β-catenin. In the future, ChIP-seq experiments should be performed in the same mouse or human stem cells with β-catenin, CDC73, and PAF to ascertain whether the complexes reside simultaneously at the same genes or whether the complexes function at different stages of transcription.

Together, CDC73 appears to form mutually exclusive complexes with PAF and β-catenin. Understanding how these distinct complexes are used to regulate specific gene expression programs is an area for further investigation and will help explain how cancer associated mutations in CDC73 impact gene expression.

# 5. References


Altschul, Stephen F., et al. “Basic Local Alignment Search Tool.” *Journal of Molecular Biology*, vol. 215, no. 3, 1990, pp. 403–410., https://doi.org/10.1016/s0022-2836(05)80360-2.

Amrich, Christopher G., et al. “Cdc73 subunit of Paf1 complex contains C-Terminal Ras-like domain that promotes association of Paf1 complex with chromatin.” *Journal of Biological Chemistry*, vol. 287, no. 14, 2012, pp. 10863–10875., https://doi.org/10.1074/jbc.m111.325647.

Andersen, Kasper R., et al. “Optimized *E. Coli* expression strain LOBSTR eliminates common contaminants from His-tag purification.” *Proteins: Structure, Function, and Bioinformatics*, vol. 81, no. 11, 2013, pp. 1857–1861., https://doi.org/10.1002/prot.24364.

Berger, Imre, and Arnaud Poterszman. “Baculovirus expression: old dog, new tricks.” *Bioengineered*, vol. 6, no. 6, 2015, pp. 316–322., https://doi.org/10.1080/21655979.2015.1104433.

Bullions, Linda C., and Arnold J. Levine. “The role of beta-catenin in cell adhesion, signal transduction, and cancer.” *Current Opinion in Oncology*, vol. 10, no. 1, 1998, pp. 81–87., https://doi.org/10.1097/00001622-199801000-00013.

Chen, Feilong, et al. “Crystal structure of the core module of the yeast Paf1 complex.” *Journal of Molecular Biology*, vol. 434, no. 2, 2022, p. 167369., https://doi.org/10.1016/j.jmb.2021.167369.

Clevers, Hans. “Wnt/β-Catenin signaling in development and disease.” *Cell*, vol. 127, no. 3, 2006, pp. 469–480., https://doi.org/10.1016/j.cell.2006.10.018.

Core, Leighton, and Karen Adelman. “Promoter-proximal pausing of RNA Polymerase II: A nexus of Gene Regulation.” *Genes & Development*, vol. 33, no. 15-16, 2019, pp. 960–982., https://doi.org/10.1101/gad.325142.119.

Deng, Pujuan, et al. “Transcriptional elongation factor Paf1 core complex adopts a spirally wrapped solenoidal topology.” *Proceedings of the National Academy of Sciences*, vol. 115, no. 40, 2018, pp. 9998–10003., https://doi.org/10.1073/pnas.1812256115.

Gradia, Scott D., et al. “MacroBac: new technologies for robust and efficient large-scale production of recombinant multiprotein complexes.” *Methods in Enzymology*, 2017, pp. 1–26., https://doi.org/10.1016/bs.mie.2017.03.008.

Guindon, Stéphane, et al. “New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyMlL 3.0.” *Systematic Biology*, vol. 59, no. 3, 2010, pp. 307–321., https://doi.org/10.1093/sysbio/syq010.

Jeong, Jae-Yeon, et al. “One-step sequence- and ligation-independent cloning as a rapid and versatile cloning method for functional genomics studies.” *Applied and Environmental Microbiology*, vol. 78, no. 15, 2012, pp. 5440–5443., https://doi.org/10.1128/aem.00844-12.

Jumper, John, et al. “Highly accurate protein structure prediction with AlphaFold.” *Nature*, vol. 596, no. 7873, 2021, pp. 583–589., https://doi.org/10.1038/s41586-021-03819-2.

Katoh, Kazutaka, et al. “MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform.” *Nucleic Acids Research*, vol. 30, no. 14, 2002, pp. 3059–3066., https://doi.org/10.1093/nar/gkf436.

Kramps, Thomas, et al. “Wnt/Wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear β-Catenin-TCF Complex.” *Cell*, vol. 109, no. 1, 2002, pp. 47–60., https://doi.org/10.1016/s0092-8674(02)00679-7.

Liu, Ying, et al. “Phosphorylation of the transcription elongation factor Spt5 by yeast Bur1 kinase stimulates recruitment of the PAF Complex.” *Molecular and Cellular Biology*, vol. 29, no. 17, 2009, pp. 4852–4863., https://doi.org/10.1128/mcb.00609-09.

Logan, Catriona Y., and Roel Nusse. “The Wnt signaling pathway in development and disease.” *Annual Review of Cell and Developmental Biology*, vol. 20, no. 1, 2004, pp. 781–810., https://doi.org/10.1146/annurev.cellbio.20.010403.113126.

Mosimann, Christian, et al. “Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with β-Catenin/Armadillo.” *Cell*, vol. 125, no. 2, 2006, pp. 327–341., https://doi.org/10.1016/j.cell.2006.01.053.

Mosimann, Christian, et al. “The role of Parafibromin/Hyrax as a nuclear Gli/Ci-interacting protein in Hedgehog target gene control.” *Mechanisms of Development*, vol. 126, no. 5-6, 2009, pp. 394–405., https://doi.org/10.1016/j.mod.2009.02.002.

Narita, Takashi, et al. “Human transcription elongation factor NELF: identification of novel subunits and reconstitution of the functionally active complex.” *Molecular and Cellular Biology*, vol. 23, no. 6, 2003, pp. 1863–1873., https://doi.org/10.1128/mcb.23.6.1863-1873.2003.

Newey, Paul J., et al. “Cell division cycle protein 73 homolog (*cdc73*) mutations in the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and parathyroid tumors.” *Human Mutation*, vol. 31, no. 3, 2010, pp. 295–307., https://doi.org/10.1002/humu.21188.

Nusse, Roel. “Wnt signaling and stem cell control.” *Cell Research*, vol. 18, no. 5, 2008, pp. 523–527., https://doi.org/10.1038/cr.2008.47.

Rozenblatt-Rosen, Orit, et al. “The parafibromin tumor suppressor protein is part of a human Paf1 Complex.” *Molecular and Cellular Biology*, vol. 25, no. 2, 2005, pp. 612–620., https://doi.org/10.1128/mcb.25.2.612-620.2005.

Sdano, Matthew A, et al. “A novel SH2 recognition mechanism recruits Spt6 to the doubly phosphorylated RNA Polymerase II linker at sites of transcription.” *eLife*, vol. 6, 2017, https://doi.org/10.7554/elife.28723.

Sun, Wei, et al. “Crystal structure of the N-Terminal domain of human CDC73 and Its Implications for the hyperparathyroidism-jaw tumor (HPT-JT) syndrome.” *Scientific Reports*, vol. 7, no. 1, 2017, https://doi.org/10.1038/s41598-017-15715-9.

Takahashi, Atsushi, et al. “SHP2 tyrosine phosphatase converts parafibromin/Cdc73 from a tumor suppressor to an oncogenic driver.” *Molecular Cell*, vol. 43, no. 1, 2011, pp. 45–56., https://doi.org/10.1016/j.molcel.2011.05.014.

Tang, Chao, et al. “Transcriptional co-activator functions of YAP and TAZ are inversely regulated by tyrosine phosphorylation status of parafibromin.” *IScience*, vol. 1, 2018, pp. 1–15., https://doi.org/10.1016/j.isci.2018.01.003.

Valenta, Tomas, et al. “The many faces and functions of β-Catenin.” *The EMBO Journal*, vol. 31, no. 12, 2012, pp. 2714–2736., https://doi.org/10.1038/emboj.2012.150.

Van Oss, S. Branden, et al. “Emerging insights into the roles of the Paf1 complex in gene regulation.” *Trends in Biochemical Sciences*, vol. 42, no. 10, 2017, pp. 788–798., https://doi.org/10.1016/j.tibs.2017.08.003.

Varadi, Mihaly, et al. “Alphafold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models.” *Nucleic Acids Research*, vol. 50, no. D1, 2021, https://doi.org/10.1093/nar/gkab1061.

Vos, Seychelle M, et al. “Architecture and RNA binding of the human negative elongation factor.” *eLife*, vol. 5, 2016, https://doi.org/10.7554/elife.14981.

Vos, Seychelle M., et al. “Structure of activated transcription complex Pol II–DSIF–PAF–SPT6.” *Nature*, vol. 560, no. 7720, 2018, pp. 607–612., https://doi.org/10.1038/s41586-018-0440-4.

Vos, Seychelle M., et al. “Structure of complete Pol II–DSIF–PAF–SPT6 transcription complex reveals RTF1 allosteric activation.” *Nature Structural & Molecular Biology*, vol. 27, no. 7, 2020, pp. 668–677., https://doi.org/10.1038/s41594-020-0437-1.

Vos, Seychelle M., et al. “Structure of paused transcription complex Pol II–DSIF–NELF.” *Nature*, vol. 560, no. 7720, 2018, pp. 601–606., https://doi.org/10.1038/s41586-018-0442-2.

Wada, T., et al. “DSIF, a novel transcription elongation factor that regulates RNA Polymerase II processivity, is composed of human SPT4 and SPT5 homologs.” *Genes & Development*, vol. 12, no. 3, 1998, pp. 343–356., https://doi.org/10.1101/gad.12.3.343.

Waterhouse, A. M., et al. “Jalview Version 2--a multiple sequence alignment editor and analysis workbench.” *Bioinformatics*, vol. 25, no. 9, 2009, pp. 1189–1191., https://doi.org/10.1093/bioinformatics/btp033.

Xing, Yi, et al. “Crystal structure of a full-length β-catenin.” *Structure*, vol. 16, no. 3, 2008, pp. 478–487., https://doi.org/10.1016/j.str.2007.12.021.

Yamaguchi, Yuki, et al. “NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA Polymerase II elongation.” *Cell*, vol. 97, no. 1, 1999, pp. 41–51., https://doi.org/10.1016/s0092-8674(00)80713-8.

Yart, Armelle, et al. “The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA Polymerase II.” *Molecular and Cellular Biology*, vol. 25, no. 12, 2005, pp. 5052–5060., https://doi.org/10.1128/mcb.25.12.5052-5060.2005.

# 6.1 Appendix

**Table 2.** Sparse matrix first crystal hits for L10 fusion construct

| Hit screen/well position | Component type | Component name | Concentration | Units | pH |
|---|---|---|---|---|---|
| **Index HT - B6** | Precipitant | Sodium di-hydrogen phosphate | 0.49 | M | |
| | Precipitant | di-Potassium hydrogen phosphate | 0.91 | M | |
| **Index HT - F3** | Salt | Tacsimate | 5 | %v/v | |
| | Buffer | HEPES | 0.1 | M | 7 |
| | Precipitant | Polyethylene glycol monomethyl ether 5,000 | 10 | %w/v | |
| **Index HT - H4** | Salt | tri-Ammonium citrate | 0.2 | M | |
| | Precipitant | PEG 3350 | 20 | %w/v | |
| **Morpheus - A3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Divalent | 0.06 | M | |
| **Morpheus - B3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Halogens | 0.09 | M | |
| **Morpheus - C3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |
| **Morpheus - C5** | Precipitant | P550MME_P20K | 30 | %w/v | |
| | Buffer | Morpheus buffer 2 | 0.1 | M | 7.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |
| **Morpheus - C7** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 2 | 0.1 | M | 7.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |
| **Morpheus - C9** | Precipitant | P550MME_P20K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |
| **Morpheus - C10** | Precipitant | EDO_P8K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |

| | | | | | |
|---|---|---|---|---|---|
| **Morpheus - D7** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 2 | 0.1 | M | 7.5 |
| | Complex ingredient | Morpheus Alcohols | 0.12 | M | |
| **Morpheus - D10** | Precipitant | EDO_P8K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus Alcohols | 0.12 | M | |
| **Morpheus - E9** | Precipitant | P550MME_P20K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus Ethylene glycols | 0.12 | M | |
| **Morpheus - E10** | Precipitant | EDO_P8K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus Ethylene glycols | 0.12 | M | |
| **Morpheus - F10** | Precipitant | EDO_P8K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |
| | Complex ingredient | Morpheus Monosaccharides | 0.12 | M | |
| **Morpheus - G3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Carboxylic acids | 0.1 | M | |
| **Morpheus - G7** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 2 | 0.1 | M | 7.5 |
| | Complex ingredient | Morpheus Carboxylic acids | 0.1 | M | |
| **Morpheus - H3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Amino Acids | 0.01 | M | |
| **PACT - A4** | Buffer | SPG buffer | 0.1 | M | 7 |
| | Precipitant | Polyethylene glycol 1,500 | 25 | %w/v | |
| | | | | | |
| **PACT - A5** | Buffer | SPG buffer | 0.1 | M | 8 |
| | Precipitant | Polyethylene glycol 1,500 | 25 | %w/v | |
| **PACT - B6** | Precipitant | Polyethylene glycol 1,500 | 25 | %w/v | |
| | Buffer | MIB buffer | 0.1 | M | 9 |
| **PACT - F4** | Precipitant | Polyethylene glycol 3,350 | 20 | %w/v | |

| | | | | | |
|---|---|---|---|---|---|
| | Salt | Potassium thiocyanate | 0.2 | M | |
| | Buffer | BIS-TRIS prop | 0.1 | M | 6.5 |
| **PEG RX HT - F3** | Salt | di-Sodium malonate | 0.1 | M | |
| | Buffer | TRIS | 0.1 | M | 8 |
| | Polymer | Polyethylene glycol 1,000 | 30 | %w/v | |
| **PEG RX HT - F7** | Salt | Sodium chloride | 0.1 | M | |
| | Buffer | BIS-TRIS prop | 0.1 | M | 9 |
| | Polymer | Polyethylene glycol 1,500 | 25 | %w/v | |
| **PEG RX HT - F10** | Salt | tri-Ammonium citrate | 0.2 | M | |
| | Buffer | Imidazole | 0.1 | M | 7 |
| | Polymer | Polyethylene glycol monomethyl ether 2,000 | 20 | %w/v | |

**Table 3.** Sparse matrix first crystal hits for NO-linker fusion construct

| Hit screen/well position | Component type | Component name | Concentration | Units | pH |
|---|---|---|---|---|---|
| **JCSG+ECO - C4** | Buffer | HEPES | 0.1 | M | 7 |
| | Precipitant | PEG 6000 | 10 | %w/v | |
| **JCSG+ECO - D2** | Salt | Magnesium chloride | 0.2 | M | |
| | Buffer | HEPES | 0.1 | M | 7.5 |
| | Precipitant | PEG 400 | 30 | %V/V | |
| **JCSG+ECO - G4** | Additive | trimethylamine N-oxide | 0.2 | M | |
| | Buffer | TRIS | 0.1 | M | 8.5 |
| | Precipitant | PEG monomethyl ether 2000 | 20 | %w/v | |
| **Morpheus - C3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus nitrate-phosphate-sulfate | 0.09 | M | |
| **Morpheus - F3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Monosaccharides | 0.12 | M | |
| **Morpheus - G3** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 1 | 0.1 | M | 6.5 |
| | Complex ingredient | Morpheus Carboxylic acids | 0.1 | M | |
| **Morpheus - G11** | Precipitant | GOL_P4K | 30 | %w/v | |
| | Buffer | Morpheus buffer 3 | 0.1 | M | 8.5 |

| | | | | | |
|---|---|---|---|---|---|
| | Complex ingredient | Morpheus Carboxylic acids | 0.1 | M | |
| **PACT - A4** | Precipitant | PEG 1500 | 25 | %w/v | |
| | Buffer | SPG buffer | 0.1 | M | 7 |
| **PACT - A5** | Precipitant | PEG 1500 | 25 | %w/v | |
| | Buffer | SPG buffer | 0.1 | M | 8 |
| **PACT - A6** | Precipitant | PEG 1500 | 25 | %w/v | |
| | Buffer | SPG buffer | 0.1 | M | 9 |
| **PACT - B6** | Precipitant | PEG 1500 | 25 | %w/v | |
| | Buffer | MIB buffer | 0.1 | M | 9 |
| **PACT - D6** | Precipitant | PEG 1500 | 25 | %w/v | |
| | Buffer | MMT buffer | 0.1 | M | 9 |
| **PACT - H9** | Precipitant | PEG 3500 | 20 | %w/v | |
| | Salt | Potassium Sodium tartrate | 0.2 | M | |
| | Buffer | BIS-TRIS prop | 0.1 | M | 8.5 |
| **PEG RX HT - C6** | Buffer | HEPES | 0.1 | M | 7.5 |
| | Polymer | PEG 3350 | 12 | %w/v | |
| **PEG RX HT - F3** | Salt | di-Sodium malonate | 0.1 | M | |
| | Buffer | TRIS | 0.1 | M | 8 |
| | Polymer | PEG 1000 | 30 | %w/v | |
| **PEG RX HT - G2** | Buffer | Tacsimate | 2 | %v/v | 7 |
| | Organic (volatile) | 2-Propanol | 5 | %v/v | |
| | Buffer | Imidazole | 0.1 | M | 7 |
| | Polymer | Polyethylene glycol 3,350 | 8 | %w/v | |
| **PEG RX HT - H12** | Additive | Dextran sulfate sodium salt 5,000 | 3 | %w/v | |
| | Buffer | BICINE | 0.1 | M | 8.5 |
| | Polymer | Polyethylene glycol 20,000 | 15 | %w/v | |

**Table 4.** Sparse matrix optimized crystal hits for L10 fusion construct

| **Index HT - Well F3** | | | |
|---|---|---|---|
| **Well** | **PEG monomethyl ether 5000 (w/v)** | **HEPES buffer pH*** | **Tacsimate (%)** |
| **B5** | 12.5 | 7.3 | 5 |
| **C5** | 10 | 7.3 | 5 |
| **C4** | 10 | 7.1 | 5 |
| **C1** | 10 | 6.1 | 5 |

| PACT - Well A5 | | | |
|---|---|---|---|
| **Well** | **PEG 1500 (w/v)** | **SPG buffer*** | |
| **C3 -C6** | 25 | 7.8-8.4 | |
| **D1-D6** | 22.5 | 7.4-8.4 | |
| **PEG RX HT - Well F10** | | | |
| **Well** | **PEG monomethyl ether 2000 (w/v)** | **Imidazole pH*** | **tri-Ammonium citrate (M)** |
| **C4** | 20 | 7 | 0.2 |
| **C6** | 20 | 7.4 | 0.2 |
| **D5** | 17.5 | 7.2 | 0.2 |
| **D6** | 17.5 | 7.4 | 2 |
| **PEG RX HT - Well F7** | | | |
| **Well** | **PEG 1500 (w/v)** | **Sodium Chloride (mM)** | **BIS-TRIS propane pH9 (mM)** |
| **D2** | 22.5 | 75 | 100 |
| | | | |
| ***Final concentration:** 100mM/well | | | |
| ****SPG buffer:** sodium dihydrogen phosphate monohydrate, glycine, succinic acid | | | |

**Table 5.** Sparse matrix optimized crystal hits for NO-linker fusion construct

| JCSG +ECO D2 | | | |
|---|---|---|---|
| **Well** | **PEG 400** | **HEPPES buffer pH**** | **Magnesium Chloride (mM)** |
| **A5** | 35 | 7.7 | 200 |
| **Pact B6** | | | |
| **Well** | **PEG 1500** | **MIB buffer pH*** | |
| **C2** | 25 | 8.6 | |
| **PEG RX HT C6** | | | |
| **Well** | **PEG 3350** | **HEPPES buffer pH**** | |
| **A4** | 17 | 7.5 | |
| **A6** | 17 | 7.9 | |
| **B5** | 14.5 | 7.7 | |
| **B6** | 14.5 | 7.9 | |
| | | | |
| ***MIB buffer:** sodium malonate dibasic monohydrate, imidazole, boric acid | | | |
| ****Final concentration:** 100mM/well | | | |

**Table 6.** β-catenin, CDC73-NTD and -CTD FA $K_{d,app}$ values and standard deviations

| Protein | ds-DNA (μM) | ss-DNA (μM) | RNA (μM) |
|---|---|---|---|
| **β-catenin** | 7.9 ± 5.04 | 2.4 ± 0.72 | 2.4 ± 1.86 |
| **CDC73-NTD** | 15.8 ± 2.8 | 19 ± 1.44 | 31.1 ± 6.04 |
| **CDC73-CTD** | 2.96 ± 1.92 | 2.24 ± 0.49 | 1.23 ± 0.57 |

**Table 7.** List of organisms used in the sequence conservation analysis

| **β -catenin** | | |
|---|---|---|
| | **Species** | **Sequence ID** |
| **1** | Anas platyrhynchos | NP_001297337.1 |
| **2** | Aplysia californica | NP_001191600.1 |
| **3** | Bos taurus | NP_001069609.1 |
| **4** | Camelus bactrianus | XP_010944179.1 |
| **5** | Canis lupus familiaris | NP_001131124.1 |
| **6** | Chelonia mydas | EMP31599.1 |
| **7** | Ciona intestinalis | NP_001027779.1 |
| **8** | Danio rerio | NP_571134.2 |
| **9** | Drosophila melanogaster | AAF45687.2 |
| **10** | Fundulus heteroclitus | XP_021170246.2 |
| **11** | Gallus gallus | AAB80856.1 |
| **12** | Homo sapiens | CAA79497.1 |
| **13** | Lytechinus variegatus | AAC06340.1 |
| **14** | Mus musculus | NP_001159374.1 |
| **15** | Ovis aries | NP_001295519.1 |
| **16** | Pan troglodytes | JAA29668.1 |
| **17** | Rattus norvegicus | NP_445809.2 |
| **18** | Sus scrofa | NP_999532.1 |
| **19** | Xenopus laevis | NP_001084045.1 |
| **20** | Xenopus tropicalis | NP_001016958.1 |
| **CDC73** | | |
| | **Species** | **Sequence ID** |
| **1** | Anas platyrhynchos | XP_027319063.1 |
| **2** | Aplysia californica | XP_005088839.1 |
| **3** | Arabidopsis thaliana | OAP05073.1 |

| | | |
|---|---|---|
| **4** | Bos taurus | NP_001178201.1 |
| **5** | Brettanomyces bruxellensis | VUG18658.1 |
| **6** | Camelus bactrianus | XP_010972340.1 |
| **7** | Canis lupus familiaris | NP_001074976.1 |
| **8** | Chelonia mydas | XP_037763777.1 |
| **9** | Ciona intestinalis | XP_002120818.2 |
| **10** | Danio rerio | AAI65395.1 |
| **11** | Drosophila melanogaster | ABD58935.1 |
| **12** | Fundulus heteroclitus | XP_035993817.1 |
| **13** | Gallus gallus | NP_001026436.1 |
| **14** | Homo sapiens | NP_078805.3 |
| **15** | Lytechinus variegatus | XP_041480918.1 |
| **16** | Mus musculus | NP_666103.1 |
| **17** | Ovis aries | XP_027832064.1 |
| **18** | Pan troglodytes | XP_001167188.1 |
| **19** | Quercus suber | POE82962.1 |
| **20** | Rattus norvegicus | NP_001019940.1 |
| **21** | Saccharomyces cerevisiae | QHB10554.1 |
| **22** | Sus scrofa | XP_020919948.1 |
| **23** | Thermothelomyces thermophilus | XP_003660519.1 |
| **24** | Xenopus laevis | NP_001079512.1 |
| **25** | Xenopus tropicalis | NP_001016031.2 |

**Table 8.** Primers

| **Oligoname** | **Sequence (5´- 3´)** |
|---|---|
| **beta-catenin-NO-Linker-FP** | AGAAGAAGGGCGTTAATAACATTGGAAGTGGATAACGGATCCGAATTC |
| **beta-catenin-NO-Linker-RP** | ACCTCAGCATCCACCATTGCCAGGTCAGTATCAAACCAGGCCAG |
| **CDC73-NO-Linker-FP** | CCTGGTTTGATACTGACCTGGCAATGGTGGATGCTGAGG |
| **CDC73-NO-Linker-RP** | TCCGTTATCCACTTCCAATGTTATTAACGCCCTTCTTCTCTGGCTTTTAC |
| **Linker20-FP** | CGGATCCGGCAGCAACGGATCCAGCGGAGGCGGATCCGGTGCAATGGTGGATGCTGAGG |
| **Linker20-RP** | ATCCGTTGCTGCCGGATCCGCTGCCGTTTCCTGATGCGCCATTCCAAGCCATTGGCTCTGTTC |
| **Linker10-FP** | CAGCGGATCCGGAGGCGGATCCGGTGCAATGGTGGATGCTGAGG |
| **Linker10-RP** | ATCCGCCTCCGGATCCGCTGCTGCCATTCCAAGCCATTGGCTCTGTTC |

| | |
|---|---|
| **Linker5-FP** | GGAATAGCGGATCCGGAAGCGCAATGGTGGATGCTGAGG |
| **Linker5-RP** | ATTGCGCTTCCGGATCCGCTATTCCAAGCCATTGGCTCTGTTC |
| **CDC73-F245R-FP** | ACGAACAACTATCTTACAAAGCACAGGAAAGAATCGGTCCAAGAACATTC |
| **CDC73-F245R-RP** | TTTCCTGTGCTTTGTAAGATAGTTGTTCG |
| **CDC73-F250A-L253A-FP** | CAAAGCACAGGAAAGAATTTTTCCAAGAACATTGCCGCAATTGCCCAATCTGTAAAAGCCAGAGAAG |
| **CDC73-F250R-L253R-FP** | CACAGGAAAGAATTTTTCCAAGAACATTCGGGCAATTCGGCAATCTGTAAAAGCCAGAGAAG |
| **CDC73-F250-L253-RP** | GAAAAATTCTTTCCTGTGCTTTGTAAGATAGTTGTT |
| **CDC73-F245A-F250A-L253A-FP** | CTTACAAAGCACAGGAAAGAATGCCTCCAAGAACATTGCCGCAATTGCCCAATCTGTAAAAGCCAGAGAAGAAGGGC |
| **beta-catenin-F660-RP** | CAAAACAGCAGCTGCATATGTCGC |
| **b-catenin-F660A-FP** | CATATGCAGCTGCTGTTTTGGCTCGAATGTCTGAGGACAAG |
| **b-catenin-F660R-FP** | CATATGCAGCTGCTGTTTTGCGACGAATGTCTGAGGACAAG |
| **beta-catenin-T679-F683-RP** | AGCTCAACTGAAAGCCGTTTCT |
| **beta-catenin-T679A-F683A-FP** | CTTTCAGTTGAGCTGGCTAGCTCTCTCGCTAGAACAGAGCCAATGG |
| **beta-catenin-T679R-F683R-FP** | GCTTTCAGTTGAGCTGCGAAGCTCTCTCCGAAGAACAGAGCCAATGGCTT |
| **CDC73_1-111_FP** | TACTTCCAATCCAATGCAATGGCGGACGTGCTTAGC |
| **CDC73_1-111_RP** | TTATCCACTTCCAATGTTATTACTTCTGTCTATACTTGCCGATGTTGACG |
| **CDC73_336-531_FP** | TACTTCCAATCCAATGCAGCCCAGCCAGTACCAAGAC |
| **CDC73_336-531_RP** | TTATCCACTTCCAATGTTATTAGAATCTCAAGTGCGATTTATGCTTTACC |
| **CDC73_214-263_FP** | TACTTCCAATCCAATGCA ATGGTGGATGCTGAGGTAGATGTGACC |
| **CDC73_214-263_RP** | TTATCCACTTCCAATGTTATTA ACGCCCTTCTTCTCTGGCTTTTAC |
| **beta-catenin_138-781_FP*** | TACTTCCAATCCAATGCAAACTTGATTAACTATCAAGATGATGCAGAACTTG |
| ***CDC73_214-263_RP was used as reverse primer to amplify beta-catenin 138-781 | |

## Molecular Cloning

### Constructs

- $His_6$-β -catenin (138-781)
- $His_6$-MBP-CDC73 1-111 and 336-531
- $His_6$-β -catenin (138-781) and CDC73 (213-263) fusion (L10 and NO-linker) constructs
- $His_6$-β -catenin (138-781) and MBP-CDC73 (213-263) co-expression construct (WT)
- $His_6$-β -catenin (138-781) and MBP-CDC73 (213-263) co-expression construct (mutant)
- MBP-CDC73 (213-263) co-expression construct (mutant)
- $His_6$-β -catenin (138-781) (mutant)

### Materials

- Dimethyl sulfoxide (DMSO)
- dNTPs (10mM)
- dCTP (25mM)
- 5x Phusion buffer (New England BioLabs)
- Phusion DNA polymerase (New England BioLabs)
- DpnI (New England BioLabs)
- T4 DNA ligase (New England BioLabs)
- T4 DNA ligase buffer (New England BioLabs)
- T4 DNA polymerase (Novagen or lab made)
- 10x T4 DNA polymerase buffer (Table 13)
- Sterile water
- Plasmid DNA of interest
- Primers
- LB plates and media
- 34 mg/mL Kanamycin or Ampicillin
- Gentamycin
- X-Gal
- IPTG
- EDTA
- NaCl
- $MgCl_2$
- DTT
- Glucose
- DNAse
- NaOH
- KOAc pH 5.5
- Tris pH 8.0
- DH5α cells
- DH10αEMBacY cells
- 10x Cutsmart buffer (New England BioLabs)
- 10x 3.1 buffer (New England BioLabs)
- NotI (New England BioLabs)

- AsiSI (New England BioLabs)
- PacI (New England BioLabs)
- SwaI (New England BioLabs)
- PmeI (New England BioLabs)
- 100% isopropanol
- 70% ethanol
- 0.2 mL tube
- 1.5mL Eppendorf tube

**Procedure**

- Design appropriate primers with a Tm between ~60°C.

**PCR Mix (50µL)**

- 1 µL Forward primer (100µM)
- 1 µL Reverse primer (100µM)
- 1 µL Template DNA (50 ng)
- 2.3 µL DMSO
- 10 µL 5x Phusion buffer
- 1 µL dNTPs
- 0.5 µL Phusion DNA polymerase
- 33.2 µL water (35.5 µL without DMSO)

**PCR Program**

**2-step**
1. 95°C 5min
2. 95°C 10s
3. 72°C 6min
5. Repeat to 2 34x
6. 72°C 10 min
7. 12°C ∞

**3-step**
1. 95°C 5min
2. 95°C 1min
4. 60°C 30sec
3. 72°C 6min
5. Repeat to 2 34x
6. 72°C 10 min
7. 12°C ∞

1. Perform either 2- or 3- step PCR and with or without DMSO to identify the best condition to amplify your construct.
2. After PCR, add 1.3µL DpnI and incubate at 37°C for one hour to digest the mother plasmid.

3. Run entire PCR reaction on a 1% (w/v) agarose EtBr TAE with a ladder.
4. Excise the properly sized band and gel extract product. Elute with 25µL water.

**One-Step SLIC Reaction**

Mix the following components in a PCR tube:

- ~100ng PCRed vector
- 1 µL 10x T4 DNA polymerase buffer
- 0.5 µL T4 DNA polymerase
- Water to 10µL

**SLIC reaction procedures (fusion constructs, mutant constructs)**

Day 1

1. Incubate components at room temperature for 10 minutes.
2. Transfer tube to ice for 10 minutes.
3. Add entire SLIC reaction to 100 µL chemically competent DH5α cells.
4. Incubate on ice for 25min.
5. Heat shock at 42°C for 45 secs.
6. Cool down on ice for 2min.
7. Add 1mL of LB media.
8. Shake at 37°C, 500 rpm for 1-2 hrs.
9. Spin the cells down at 3434xg for 5min.
10. Discard supernatant and resuspend cells gently in the remaining LB medium (~100 µL).
11. Plate the cells on plate with appropriate antibiotic (ampicillin).
12. Spread with glass beads.
13. Incubate at 37°C overnight.

Day 2

1. Pick at least 3 single colonies and grow 5 mL cultures with appropriate antibiotic selection at 37°C shaking 160 rpm overnight. Make sure to streak out colonies.

Day 3

1. Harvest cells by centrifugation
2. Prepare DNA using a mini-prep DNA purification kit. The DNA should be eluted from the columns in water. Typically, 35 µL of water is sufficient.
3. Measure DNA concentration with Nanodrop.
4. Prepare sequencing reactions with appropriate sequencing primers to confirm that the gene is present. Mix:
    a. 2-5 µL DNA (500-1000 ng of DNA)
    b. 1 µL 100 µM primer
    c. Water to 15 µL

**Combining 14C-CDC73 (insert) and 14B-β-catenin (vector) mutants construct into one vector**

**Vector digestion**

Enzymes

- 14C CDC73 - NotI + AsiSI (insert)
- 14B β-catenin - NotI + PacI (vector)

**Digestion reaction mix (20µL)**

- vector (~500ng)
- insert (5 molar excess of insert to vector)
- 2µL of CutSmart (1X)
- 2µL Enzyme 1
- 2µL Enzyme 2
- water to 20µL

**Digestion reaction procedure**

1. Incubate the digestion mix for at least 6hrs at 37°C.
2. Phosphatase treatment of the vector.
    a. 1µL of Antarctic phosphatase.
    b. 2µL of Antarctic phosphatase buffer.
3. Incubated the vector and the phosphatase for 30min at 37°C.
4. Run entire digestion reaction (both vector and insert) on a 1% (w/v) agarose EtBr TAE with a ladder.
5. Excise the properly sized band and gel extract product. Elute with 25µL water.
6. Store DNA at -20°C.

**Ligation reaction mix (20 µL)**

- 2µL of T4 ligase buffer
- 1µL of T4 ligase
- insert (3-5 molar excess of insert to vector)
- 50ng vector
- Water to 20µL

**Ligation reaction procedure**

<u>Day 1</u>

1. Incubate the mix for 1hr at room temperature.
2. Transform 5µL of the reaction into a 100 µL chemically competent DH5α cells.
3. Step (4-13) of SLIC reaction procedure described above

<u>Day 2</u>

1. Step (1) of SLIC reaction procedures described above

Day 3

1. Step (1-3) of SLIC reaction procedures described above
2. Prepare restriction digest to determine if clone is positive. NotI/AsiSI/HindIII usually produce good fragments. Check in SnapGene before conducting the digest.
    a. Restriction Digest Mix (20 µL)
        i. 2-5 µL DNA (~500 ng)

ii. 2 µL restriction enzyme 1
iii. 2 µL restriction enzyme 2
iv. 2 µL restriction enzyme 3
v. 2 µL 10x CutSmart buffer
vi. water to 20 µL

b. Incubate at 37ºC for 1 hr. Add 2 µL of 6x DNA loading dye to each sample.
c. Run samples on 1xTAE gel. Load 20 µL of sample. Run gel at 120 V.

3. For positive clones, prepare sequencing reactions with appropriate sequencing primers.
   a. Mix:
      i. 2-5 µL DNA (500-1000 ng of DNA)
      ii. 1 µL 100 µM primer
      iii. Water to 15 µL
   b. Send samples for Sanger Sequencing.
4. For positive clones, start a large 25 mL culture. Mix 25 mL of LB media with ampicillin in a 250-500 mL Erlenmeyer flask.
5. Grow overnight, shaking at 37ºC, 160-180 rpm.
6. Harvest cells by centrifugation in a 50 mL falcon (3434xg, 10 min).
7. Resuspend in 1 mL of miniprep resuspension buffer.
8. Transfer to 4, 1.5 mL tubes, ~300 µL of resuspended cells per tube.
9. Treat each tube as a separate miniprep and follow miniprep protocol.
10. Elute DNA with 35 µL water from each column. Combine the eluate from the 4 columns into a single 1.5 mL tube. Measure DNA concentration.
11. Submit for complete Sanger Sequencing as described in step 3. Store DNA in -20ºC.

**Combining β-catenin (vector) and CDC73 (insert) WT into 438B/C vector for expression in insect cells**

**Vector digestion**

Enzymes

- 14C CDC73 - PmeI (insert)
- 14B β-catenin – SwaI (vector)

**Digestion reaction mix - vector (50µL)**

- vector (~2µg)
- 5µL 10x 3.1 buffer
- 2µL SwaI
- water to 50µL

**Digestion reaction mix - vector (50µL)**

- vector (~2µg)
- 5µL 10x CutSmart buffer
- 2µL PmeI
- water to 50µL

**Digestion reaction procedure**

7. Incubate the digestion mix for 2-6hrs at 37°C (SwaI 25°C).

8. Run entire digestion reaction (both vector and insert) on a 1% (w/v) agarose EtBr TAE with a ladder.
9. Excise the properly sized band and gel extract product. Elute with 25µL sterile water.
10. Store DNA at -20°C.

**DNA Polymerase treatment of vector and insert DNA**
Use only LIC-qualified T4 DNA polymerase.

The LIC vectors and insert DNA must be treated in two separate reactions. Combine the following in 0.2 mL PCR tubes:

Insert
- 5-14.6 µL insert DNA (~500 ng)
- 2 µL 10x T4 DNA polymerase buffer
- 2 µL dCTP (25 mM)
- 1 µL 100 mM DTT
- 0.4 µL T4 DNA polymerase
- Water to 20 µL

Vector
- 5-14.6 µL vector DNA (~150-500 ng)
- 2 µL 10x T4 DNA polymerase buffer
- 2 µL dGTP (25 mM)
- 1 µL 100 mM DTT
- 0.4 µL T4 DNA polymerase
- Water to 20 µL

**Procedure**
1. Incubate reaction at 25°C for 40 min in thermocycler.
2. Heat inactivate T4 polymerase at 75 °C for 20 min in thermocycler.
3. Store reactions at -20°C or proceed immediately to LIC annealing steps.

**LIC Annealing**
1. Combine 2 µL insert DNA from T4 reaction with 2 µL LIC vector DNA from T4 reaction in 0.2 mL tube.
2. Incubate reaction at room temp. for 20-60 min.
3. Store reactions at -20°C or proceed to transformation steps.

**Transformation**
Day 1
1. Add entire LIC reaction to 25-50µL competent *E. coli* DH5α cells
2. Step (4-13) of SLIC reaction procedure described above

Day 2
1. Step (1) of SLIC reaction procedures described above

Day 3

1. Step (1-3) of SLIC reaction procedures described above
2. Prepare restriction digest to determine if clone is positive. NotI/XhoI/XbaI usually produce good fragments. Check in SnapGene before conducting the digest.
    a. Restriction Digest Mastermix (10, 20 µL reactions, 200 µL final volume)
        i. 2-5 µL DNA (~500 ng) x 10 (do not add here, this is to ensure water volume is adequate)
        ii. 5 µL restriction enzyme 1
        iii. 5 µL restriction enzyme 2
        iv. 20 µL 10x CutSmart buffer
        v. water to 200 µL
    b. Mix 15-18 µL of mastermix with 2-5 µL of plasmid in a 0.2 mL tube.
    c. Incubate at 37ºC for 1 hr. Add 2 µL of 6x DNA loading dye to each sample.
    d. Run samples on 1xTAE gel. Load 20 µL of sample. Run gel at 120 V.
3. For positive clones, prepare sequencing reactions. Initially, sequence with forward or reverse primers (i.e., PolH or SV40) to confirm that the gene is present.
    a. Mix:
        i. 2-5 µL DNA (500-1000 ng of DNA)
        ii. 1 µL 100 µM PolH, SV40 or MBP read through primer
        iii. Water to 15 µL
    b. Send samples for Sanger Sequencing.
4. Step (4-11) of CDC73 and β-catenin mutants ligation reaction.
5. When the DNA has no mutations, etc. proceed with making bacmids.

**Recombination of plasmid DNA into *E. coli* DH10αEMBacY to produce bacmids**

1. Add ~0.25–1 µg of the construct plasmid to 100 µL electrocompetent DH10αEMBacY cells. Miniprepped DNA should always be in water to avoid complications with the electroporation.
2. Incubate on ice for 10 min.
3. Transfer to chilled electroporation cuvette (0.1 cm width). Pulse, 25 µF, 1.8 kV. If the instrument has an "arc" warning, the salt concentration is too high in your DNA. Try the electroporation again using less DNA. "Arced" cells will not grow.
4. Add 1 mL LB media and transfer to 15 mL culture tube. Grow 5 hrs - overnight, shaking at 37˚C.
5. Plate ~25-150 µL cells (amount depends on cell competence and size of your construct) on LB agar plate containing appropriate antibiotic (gentamycin for 438 series), 150 µg/mL X-Gal and 1 mM IPTG. Leave remaining culture at 37°C, shaking overnight.
6. Incubate plates at 37˚C for 1.5 day.
7. If too few colonies are present, plate 10-100 µL of the remaining culture + 90-0 µL LB.
8. Pick white colonies with inoculation loop or pipette tip and make a single streak on fresh agar plate with appropriate antibiotic (gentamycin), X-Gal and IPTG.
9. Dip the same loop/tip into 5 mL fresh LB with appropriate antibiotic. Finish streaking the portion on the plate.
10. Select at least 4 white colonies for each construct (positive integration events). Select one blue colony as a negative control.
11. Grow plates 1.5 days at 37˚C and liquid cultures overnight at 37˚C, 160-180 rpm.

**Isolation of bacmid DNA from verified white colonies via alkaline lysis/isopropanol/ethanol precipitation**

1. Harvest entire 5 mL culture by centrifugation for streaked colonies that appear white. Discard supernatant.
2. Resuspend pellet in 250 µL resuspension buffer (Table 13).
3. Transfer to 1.5 mL tube.
4. Add 250 µL lysis buffer (Table 13) and invert 3-5 times.
5. Add 350 µL neutralization buffer (Table 13) and invert tube 3-5 times.
6. Spin lysed cells at 21300xg for 10 min in tabletop centrifuge at room temperature.
7. Transfer supernatant to fresh 1.5 mL tube.
8. Spin at 21300xg for 10 min to remove remaining precipitates at room temperature.
9. Transfer supernatant to fresh 1.5 mL tube.
    a. Label the lid with EtOH resistant pen.
10. Add 700 µL isopropanol (-20°C or RT) and invert tube 3-5 times. Incubate at -20˚C for 1hr-overnight or 1–2 hrs at -80°C to precipitate the DNA.
11. Centrifuge at 21300xg for 30 min at 4°C in tabletop centrifuge.
12. Carefully remove supernatant and discard. Add 500 µL 70% ethanol (-20°C or room temperature). Spin at 21300xg for 10 min at 4˚C.
13. Carefully remove ethanol and discard.
14. Add 30 µL 70% ethanol to cover the DNA pellet completely and store precipitated DNA at -20˚C until ready to transfect insect cells (or room temperature if it will be used a few hours).

**Table 13.** Buffers for bacmid isolation DNA

| **10x T4 DNA polymerase buffer** | **Cell Resuspension buffer** | **Lysis Solution** | **SDS Neutralization Solution** |
|---|---|---|---|
| 100mM Tris pH 8.0 | **50mM Tris pH 8.0** | 0.2M NaOH 1% | 4M KOAc pH 5.5 |
| 500mM NaCl | **10mM EDTA** | | |
| 100mM $MgCl_2$ | **50mM Glucose** | | |
| 10mM DTT | **0.1mg/mL DNAse free Rnase A** | | |
| | **Store at 4°C** | | |

# Expression and Purification of H. sapiens $His_6$-MBP-CDC73 1-111 and 336-531, $His_6$-β -catenin (138-781), $His_6$-β -catenin (138-781) and MBP-CDC73 (213-263) co-expression construct, $His_6$-β -catenin (138-781) and CDC73 (213-263) fusion (L10 and NO-linker) constructs

**Materials**

1. 2xYT media
2. 34 mg/mL kanamycin
3. 30 mg/mL chloramphenicol
4. LB plates with 34µg/mL chloramphenicol and 30µg/mL kanamycin
5. 2L baffled flasks
6. 1M IPTG
7. 5M NaCl
8. 1M Na-HEPES pH 7.4
9. 2M imidazole pH 8.0
10. Reducing agent (DTT)
11. Glycerol
12. Protease inhibitor cocktail mix (PI)
13. 5mL $Ni^{2+}$ His-Trap column (Cytvia)
14. Sterile 10 mL serological pipettes
15. Filtered (0.2µ) $ddH_2O$
16. 20% (v/v) ethanol
17. Peristaltic pump
18. S75 column (Cytvia)
19. S200 column
20. S column
21. Amylose column
22. Maltose
23. Sterile 1.5mL Eppendorf tubes
24. 50mL falcon tube

<u>Day 1</u>

Transform H. sapiens:

- $His_6$-MBP-CDC73 1-111, $His_6$-β -catenin 138-781, and $His_6$-β -catenin 138-781 and MBP-CDC73 213-263 co-expression construct into Bl21 (DE3) LOBSTR RIL (100µL).
- $His_6$-MBP-CDC73 336-531 into Bl21 (DE3) RIL cells (100µL).
- $His_6$-β -catenin 138-781 and CDC73 213-263 fusion constructs into Bl21 (DE3) Rosetta 2 pLysS cells (100µL).

1. Add 50-100ng of plasmid to 100µL cells.
2. Incubate cells and DNA on ice for 25 minutes.
3. Heat shock at 42°C for 45s.
4. Chill on ice for 2min.
5. Add 1mL LB media to cells. Outgrow cells for minimum of 1 hour at 37°C.

6. Plate and spread 200 µL of the transformation on LB plate with 34µg/mL chloramphenicol and 30µg/mL kanamycin ($His_6$-MBP-CDC73 1-111, $His_6$-MBP-CDC73 336-531, $His_6$-β-catenin 138-781 and CDC73 213-263 fusions) or ampicillin ($His_6$-β -catenin 138-781, $His_6$-β -catenin 138-781 and MBP-CDC73 213-263 co-expression construct).
7. Incubate plate at 37°C overnight.

Day 2

1. Remove plate(s) from incubator and store at 4°C until the end of the day.
2. Inoculate 100mL 2xYT media in sterile 250-500mL culture flask with several colonies from transformation plate and 34µg/mL chloramphenicol and 30µg/mL kanamycin. Incubate cells, shaking (160rpm) at 37°C overnight.
3. Prewarm 5L of 2xYT media overnight at 37°C.
4. Prepare lysis buffer (Table 8-11) without any reducing agent or protease inhibitor.

Day 3

1. Remove starter culture from 37°C incubator.
2. Poor pre-warm in 2L baffled flasks and add 34µg/mL chloramphenicol and 30µg/mL kanamycin.
3. Inoculate media with 10mL of overnight culture. Grow cells at 37°C, shaking (160 rpm). Measure OD600 every thirty minutes after inoculation. When cells reach OD600 0.4-0.6, induce protein expression by adding 0.5mM IPTG (usually 1.5-2hrs after inoculation).
4. Allow for protein expression until the next morning by growing cells for another 16-18 hrs shaking at 37°C.
5. Transfer cells to 1L centrifuge tubes.
6. Spin cells for 15-20 minutes at 3470xg, 4°C. While cells are spinning, add protease inhibitors and reducing agent to the lysis buffer (Table 8-11) prepared on Day 2.
7. Decant media from cell pellet. Place centrifuge bottles on ice. Add 10-20mL lysis buffer (Table 8-11).
8. Resuspend cells gently by using a sterile 10mL serological pipette. Avoid adding air bubbles to the cells. Continue to pipette until cells and buffer are homogenously mixed.
9. Add resuspended cells to another bottle containing pelleted cells. Continue until all cell pellets are resuspended in lysis buffer (Table 8-11). If cells will be stored, continue with step 10. If used immediately for purification, advance to Protein Purification steps.
10. Pour liquid nitrogen in liquid nitrogen containers. Transfer cells to a 50mL falcon tubes and transfer tubes the liquid nitrogen container.
11. Store cell pellets at -80°C.

**Protein Purification**

Day 0

1. Prepare buffers (without DTT and PIs) as described in Table 8-11 and store at 4°C.
2. Charge a 5mL $Ni^{2+}$ His-Trap column. (CV= column volume(s)).
    a. Run 5CV of filtered ddH2O over the column at a rate of 1.5-3 mL/min.
    b. Apply 5CV Acidic Salt Buffer (Table 12) to the column.
    c. Apply 0.5CV Nickel Sulfate Buffer (Table 12) to the column. Collect flow through.

d. Apply 5CV Acidic Salt Buffer (Table 12) to remove unbound $Ni^{2+}$. Collect flow through and dispose of in appropriate waste bin.

Day 1

1. Add protease inhibitors and reducing agent to lysis, high salt, nickel elution and Size Exclusion Chromatography (SEC) buffer (Table 8-10). Filter (0.2µ) and store at 4°C.
2. Finish charging and equilibrate the 5mL $Ni^{2+}$ His-Trap column for protein preparation. Use a peristaltic pump in the cold room or FPLC to charge and equilibrate the columns. (CV= column volume(s)).
    a. Apply 5CV nickel elution buffer (Table 8-10) to remove weakly bound $Ni^{2+}$.
    b. Equilibrate column with 5CV lysis buffer (Table 8-11). Store column at 4°C.
3. Chill a large centrifuge with an A27 rotor (4°C).
4. Remove cells from -80°C and thaw pellets at room temperature in a beaker with warm water. When cells are almost fully melted, transfer to metal beaker on ice.
5. When cells are fully melted on ice, sonicate cells using a macrotip (70% power, 5 minutes, 0.6s on, 0.4s off).
6. Transfer lysate to A27 tubes and balance.
7. Centrifuge lysate in an A27 rotor for 30 minutes, 15-20k rpm, 4ºC.
8. Gently transfer the supernatant to a 50mL falcon tube with a 10mL serological pipette.
    a. Save 10µL of the clarified lysate and a fraction of the pelleted for a gel. Add SDS-PAGE loading buffer to the samples and store at 4°C.
9. Apply lysate to prepared 5mL $Ni^{2+}$ His-Trap column via a peristaltic pump in the cold room (flow rate 1.5-3 mL/min).
10. Once column is loaded, transfer column to an FPLC (or continue using the peristaltic pump for the rest of Day 1 purification steps).
11. Wash column with about 25 CV lysis buffer (Table 8-11) or until 260/280 values are close to the input values (1.5 mL/min).
12. Wash column with 5 CV high salt buffer (Table 8-11) followed for another 5 CV lysis buffer (Table 8-11).
13. The nickel column will be eluted over 30 minutes, at a flow rate of 1.5 mL/min via a gradient. Collect 1 mL fractions.
14. Run a 10% SDS-PAGE with peak fractions, supernatant, and pellet.
15. Pool peak fractions and mix with 1.5 mg of $His_6$-TEV protease in a 50 mL falcon tube.
16. Transfer protein(s) to the SnakeSkin tubing with a 10 mL serological pipette. Dialyze the protein overnight at 4ºC against 1L of lysis buffer (Table 8-11).
17. Equilibrate S75 16/600 (CDC73 1-111 and 336-531) or S200 16/600 ($His_6$-β -catenin (138-781), $His_6$-β -catenin (138-781) and MBP-CDC73 (213-263) co-expression construct, $His_6$-β -catenin (138-781) and CDC73 (213-263) fusion (L10 and NO-linker)) in SEC buffer overnight.
    a. Apply 150mL ddH2O to column at 0.5 mL/min followed by 150mL SEC buffer (Table 8-11). Equilibration takes 10hrs.

Day 2

1. Equilibrate a 5 mL $Ni^{2+}$ HisTrap column in lysis buffer ((use the same column from Day 1 or charge a new column; Table 8-11).

2. Remove protein from dialysis tubing and transfer it a 50mL falcon tube. Take a 10µL sample for gel analysis.
3. Apply protein to 5 mL $Ni^{2+}$ HisTrap column equilibrated in lysis buffer (Table 8-11) with a peristaltic pump in the cold room.
    a. Collect flowthrough. Monitor elution by Bradford (10µL FT, 90µL Bradford reagent). Save 10µL for gel.
4. Once there is no detectable signal, elute TEV and uncleaved protein with nickel elution buffer. Collect elution and monitor with Bradford. Save 10µL for gel analysis.
5. Run all samples on SDS-PAGE (15%) to determine efficiency of TEV digest.
6. Concentrate CTD (336-531) FT with a 10kDa MWCO Amicon Millipore 15 mL concentrator to 2-4mL. Use a 3kDa MWCO concentrator for the NTD (1-111) construct. Spin at 3434xg, 4ºC for 10 minutes. Mix protein in the concentrator every 10 minutes.
7. Apply protein to S75 16/600 column in 5 mL loop. Run column at 0.8-1 mL/min. Collect 1 mL fractions.
8. Analyze peak fractions by 15% SDS-PAGE gel.
9. Pool appropriate fractions and concentrate protein with a 10kDa MWCO Amicon Millipore 15 mL concentrator to less than 0.5.-1 mL for CTD. Use a 3kDa MWCO concentrator for the NTD construct.
10. Measure protein concentration by absorption at 280nm using spectrophotometer or any other protein measurement equipment. Calculate protein concentration using equation 1.
11. Aliquot protein (5-10 µL), snap freeze in liquid nitrogen, and store at -80ºC.
12. Strip and store 5mL Ni2+ Hi-Trap columns.
    a. Apply 5CV ddH2O to the column(s).
    b. Apply 5CV Stripping Buffer (Table 12).
    c. Wash with 5CV ddH2O.
    d. Apply 5CV 0.5M NaOH.
    e. Wash with 5CV ddH2O.
    f. Exchange column into 20% ethanol (~2-5CV). Store columns at 4°C.

**Protein Purification (His$_6$-β-catenin (138-781) and MBP-CDC73 (213-263) co-expression construct)**

Day 0

1. In addition to equilibrate a 5 mL $Ni^{2+}$ HisTrap column (step 1 on Day 2 of protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531)), equilibrate an amylose column with ~5CV of water and ~5CV of lysis buffer (Table 10). Store at 4°C.

Day 1

1. After completing step (12) of day 1 of the protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531), place amylose column in line with the $Ni^{2+}$ Hi-Trap column.
2. Apply 50mL of nickel elution buffer (Table 10) to the column tandem to each other. Collect flow through in the event there is more protein in the sample than the amylose column can effectively bind.
3. Remove $Ni^{2+}$ His-Trap column out of line. Wash amylose column with lysis buffer (~2CV).

4. Elute protein from amylose column with amylose elution buffer (Table 10). Collect fractions. Pool peak fractions and prepare samples for overnight dialysis as for the protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531).
5. Proceed with protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531), Day 2.

**Protein Purification (His$_6$-β-catenin (138-781) and MBP-CDC73 (213-263) fusion construct)**

Day 1

1. After completing step (12) wash the column with low salt buffer (Table 11), then proceed with step (13) of protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531), Day 1.
2. Before adding 1.5 mg of His$_6$-TEV protease on step (15) of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531) purification (Day 1), concentrate the protein on a 30 kDa MWCO Amicon Millipore 15 mL (2-4mL) Then, transfer protein to 50mL falcon tube, add 1.5 mg of His$_6$-TEV protease, and proceed with steps (16-17).

Day 2

1. Equilibrate both 5 mL $Ni^{2+}$ HisTrap and S column on low salt buffer (Day 1 of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531) protein purification; Table 11).
2. Apply 50mL of nickel elution buffer (Table 11) to the 5 mL $Ni^{2+}$ HisTrap and S column tandem to each other. Collect flow through.
3. Remove $Ni^{2+}$ His-Trap column out of line and attach it to the peristaltic and elute bound molecules with nickel elution buffer (remove TEV protease, the His$_6$-tag, and uncleaved protein). Monitor elution by Bradford (10µL FT, 90µL Bradford reagent). Save 10µL for gel.
4. Wash S column with lysis buffer (~2CV; Table 11). Elute protein from S column with high salt buffer (Table 11) via gradient (0-100mL). Collect fractions. Pool peak fractions for gel analysis.
6. Run all samples on 10% SDS-PAGE to determine efficiency of TEV digest.
7. Concentrate the flowthrough of the nickel and S column in a 30kDa MWCO Amicon Millipore 15 mL concentrator to 2-5mL.
8. Spin the sample in the centrifuge for 10min, at 4ºC.
9. Proceed with step (7) of protein purification of CDC73 (1-111), CDC73 (336-531), and β -catenin (138-531), Day 2.
10. After quantifying the amount of protein purified, either store the protein at -80ºC or prepare samples for crystallography.

**Table 8.** CDC73-NTD & -CTD purification buffers.

| **Buffer** | **Lysis** | **High salt** | **$Ni^{+2}$ elution** | **SEC** |
|---|---|---|---|---|
| **NaCl** | 300mM | 800mM | 300mM | 150mM |
| **Na-HEPES** | 20mM | 20mM | 20mM | 20mM |
| **imidazole** | 30mM | 30mM | 500mM | |
| **glycerol** | 10% | 10% | 10% | 10% |

| | | | | |
|---|---|---|---|---|
| **DTT** | 1mM | 1mM | 1mM | 1mM |
| **pepstatin A** | 2mM | | | |
| **leupeptin** | 0.7mM | | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | | |
| **benzamidine** | 2.8mM | | | |
| **EtOH** | 100% | | | |

**Table 9.** β-catenin purification buffers.

| **Buffer** | **Lysis** | **High salt** | **Ni+2 elution** | **SEC** |
|---|---|---|---|---|
| **NaCl** | 500mM | 1000mM | 500mM | 500mM |
| **Na-HEPES** | 20mM | 20mM | 20mM | 20mM |
| **imidazole** | 30mM | 30mM | 500mM | |
| **glycerol** | 10% | 10% | 10% | 10% |
| **DTT** | 1mM | 1mM | 1mM | 1mM |
| **pepstatin A** | 2mM | | | |
| **leupeptin** | 0.7mM | | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | | |
| **benzamidine** | 2.8mM | | | |
| **EtOH** | 100% | | | |

**Table 10.** β-catenin (138-781) and CDC73 (213-263) purification buffers.

| **Buffer** | **Lysis** | **High salt** | **Ni+2 elution** | **Amylose elution** | **SEC** |
|---|---|---|---|---|---|
| **NaCl** | 300mM | 800mM | 300mM | 300mM | 150mM |
| **Na-HEPES** | 20mM | 20mM | 20mM | 20mM | 20mM |
| **imidazole** | 30mM | 30mM | 500mM | | |
| **glycerol** | 10% | 10% | 10% | 10% | 10% |
| **DTT** | 1mM | 1mM | 1mM | 1mM | 1mM |
| **Maltose** | | | | 116mM | |
| **pepstatin A** | 2mM | | | | |
| **leupeptin** | 0.7mM | | | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | | | |
| **benzamidine** | 2.8mM | | | | |
| **EtOH** | 100% | | | | |

**Table 11.** β-catenin (138-781) and CDC73 (213-263) fusions purification buffers.

| Buffer | Lysis | High salt | Low salt | Ni+2 elution | SEC |
|---|---|---|---|---|---|
| **NaCl** | 300mM | 1000mM | 100mM | 100mM | 300mM |
| **Na-HEPES** | 20mM | 20mM | 20mM | 20mM | 20mM |
| **imidazole** | 20mM | 20mM | 20mM | 400mM | |
| **glycerol** | 10% | 10% | 10% | 10% | 10% |
| **DTT** | 1mM | 1mM | 1mM | 1mM | 1mM |
| **pepstatin A** | 2mM | | | | |
| **leupeptin** | 0.7mM | | | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | | | |
| **benzamidine** | 2.8mM | | | | |
| **EtOH** | 100% | | | | |

**Table 12.** Buffers for charging nickel columns.

| Stripping Buffer | Nickel Sulfate Buffer | Acidic Salt Buffer |
|---|---|---|
| **0.5M NaCl** | 0.2M NiSO4 | 0.5M NaCl pH 3.0 |
| **50mM EDTA** | | |

# Reciprocal Pull-down of β-catenin 138-781 and CDC73 213-263

**Introduction**

This protocol aims to determine if CDC73 middle region indeed interacts with β-catenin. The goal of this protocol is to co-express wild-type β-catenin 138-781 and CDC73 213-263 and determine if they can be pulled down together. In addition, co-express β-catenin 138-781 and CDC73 213-263 mutants and determine if their interaction can be disrupted.

**Materials**

- 2xYT media
- LB media
- 34 mg/mL ampicillin
- 30 mg/mL chloramphenicol
- LB plates with 34μg/mL chloramphenicol and 30μg/mL ampicillin
- ESF Transfection Medium
- ESF-921 media
- Xtreme Gene 9 transfection reagent
- 1M IPTG
- 5M NaCl
- 1M Na-HEPES pH 7.4
- 2M imidazole pH 8.0
- Reducing agent (DTT)
- Glycerol
- Protease inhibitor cocktail mix (PI)
- Nickel resin (Takara)
- Amylose resin (New England BioLabs)
- Bl21 (DE3) LOBSTR RIL
- Sf21/Sf9 cells
- Hi5 cells
- Filtered (0.2μ) dd$H_2O$
- Maltose
- Sterile 1.5mL Eppendorf tubes
- 50mL falcon tube
- 15 mL falcon tube
- 6-well plate

**Expression of $His_6$-β-catenin 138-781 and MBP-CDC73 213-263 mutants**

Day 1

Transform H. sapiens β-catenin 138-781 and CDC73 213-263 mutant constructs into Bl21 (DE3) LOBSTR RIL cells (100μL).

1. Add 50-100ng of plasmid to 100μL cells.
2. Incubate cells and DNA on ice for 25 minutes.
3. Heat shock at 42°C for 45s.
4. Chill on ice for 2min.

5. Add 1mL LB media to cells. Outgrow cells for minimum of 1 hour at 37°C.
6. Plate and spread 200 µL of the transformation on LB plate with 34µg/mL chloramphenicol and 30µg/mL ampicillin.
7. Incubate plate at 37°C overnight.

Day 2

1. Remove plate(s) from incubator and store at 4°C until the end of the day.
2. Inoculate 5mL 2xYT media in sterile glass tube with several colonies from transformation plate and 34µg/mL chloramphenicol and 30µg/mL ampicillin. Incubate cells, shaking (160rpm) at 37°C overnight.

Day 3

1. Remove starter culture from 37°C incubator.
2. Inoculate 25mL 2xYT media in sterile 250-500mL culture flask with 250µL of the overnight culture, 34µg/mL chloramphenicol, and 30µg/mL kanamycin.
3. Grow cells at 37°C, shaking (160 rpm). Measure OD600 every thirty minutes after inoculation. When cells reach OD600 0.4-0.6, induce protein expression by adding 0.5mM IPTG (usually 2-2.5hrs after inoculation).
4. Allow for protein expression until the next morning by growing cells for another 16-18hrs shaking at 37°C.
5. Transfer cells to 25mL falcon tubes.
6. Spin cells for 15-20 minutes at 3434xg, 4°C.
7. While cells are spinning, prepare buffers. Prepare 1mL of nickel and amylose elution buffer in a 1.5mL Eppendorf tubes and 50mL of lysis buffer in a falcon tube.
8. Decant media from cell pellet. Place falcon tubes on ice. Add 1mL lysis buffer (Table X).
9. Resuspend cells gently. Avoid adding air bubbles to the cells. Continue to pipette until cells and buffer are homogenously mixed.
10. If cells will be stored, continue with step 11. If used immediately for pull-downs, advance to Reciprocal Pull-down steps.
11. Pour liquid nitrogen in liquid nitrogen container. Transfer resuspended cells to 1.5mL Eppendorf tubes and transfer tubes the liquid nitrogen container.
12. Store cell pellets at -80°C.

**Expression of wild-type $His_6$-β-catenin 138-781 and MBP-CDC73 213-263 in insect cells**

Transfection of Sf9 cells with Bacmid DNA

1. Remove ethanol from bacmids and air dry in hood. Add 20 µL of water to each bacmid once dried. Incubate until bacmids are fully dissolved (5-10 min). Transfer 3 µL of each resuspended bacmid into a new 1.5 mL tube. The remaining bacmid DNA should be stored at -20ºC.
2. Plate 1 mL of Sf9 cells ($10^6$ cells/mL) into a 6-well plate (1 well per construct). Incubate at 27ºC for 30 min.
3. While cells are incubating, prepare bacmid for transfection.
    a. Add 100 µL of ESF Transfection Medium to each 3 µL bacmid aliquot. Incubate in hood for 5 min.

    b. Prepare transfection reagent mastermix. Mix 100 µL of ESF Transfection Medium with 6 µL of Xtreme Gene 9 transfection reagent for each construct.
    c. Add 100 µL of transfection reagent mastermix to each bacmid. Incubate for at RT for 30 min.
4. Add an additional 800 µL of ESF-921 media (normal insect cell media) to each bacmid.
5. Gently remove media over Sf9 cells. Replace with 1 mL of bacmid with transfection reagent.
6. Incubate plate at 27ºC for 4hrs-overnight.
7. Add 2 mL of ESF-921 to each transfection to ensure that they do not dry out.
8. YFP expressing colonies should be visible after 2-4 days.
9. Harvest virus (V0) by gently removing media over cells and transferring to a 15 mL falcon tube.

**V1 virus production**

1. Add 0.15-3 mL of V0 virus to 25 mL of Sf21 cells at a concentration of $10^6$ cells/mL.
2. Check cell growth after 24 hrs. Cells should divide one time after virus is added. If cells fail to divide, the virus is too strong and a lower concentration of V0 virus should be used.
3. When cells stop dividing, this is considered the day after proliferation (DPA). Grow cells for an additional 48-72 hrs after DPA. Measure cell viability, cell number, and YFP fluorescence to determine when to harvest cells.
4. Harvest cells. Centrifuge cells, 238xg for 15 min. Transfer supernatant to a fresh 50 mL falcon. Store V1 virus at 4˚C. The virus should be stable for ~1-1.5 years. Use the pelleted cells to perform pull-down experiments.
    a. Resuspend cell pellets in lysis buffer (Table 16-17).
    b. Transfer resuspended cells to a 1.5 mL falcon, freeze in liquid nitrogen and store at -80 °C or perform pull-downs immediately.
5. The V1 virus can be directly used for large scale protein expression in Hi5 cells.

**V2 virus production**

1. Use 50-100 mL Hi5 cells freshly diluted to a concentration of $10^6$cells/mL in a fresh 1L flask.
2. Add 12.5-25 µL of V1 virus (depending on strength and age of virus). It is essential that the cells divide again, so ensure you do not add too much of the virus.
3. Check growth every 24 hrs. Dilute and proceed as described for V1 production.
4. After 25 hrs cells divide once and must be split to two 1L flasks to maintain a density of $10^6$ cells/ mL. Grow cells for an additional 24-48 hrs. Growing longer will not result in more protein but will likely give more degradation products. Cell viability should be greater than 85%. If cells have a lower viability, harvest.
5. Harvest cells by decanting into 50 mL falcon tube. Centrifuge at 238xg for 30 min at 4°C.
6. Bleach supernatant for 5-10 min and discard in sink.
7. Place tubes with cell pellet on ice and resuspend with 1mL lysis buffer (Table 16-17) at 4°C. Transfer resuspended cells to a 1.5 mL falcon, freeze in liquid nitrogen and store at -80 °C or perform pull-downs immediately.

**Reciprocal Pull-down**

1. Thaw cells or use resuspended cells (if used immediately).
2. Sonicate for 10s, power 2, using the micro tip. Allow samples to rest 2 min in between sonication steps. Sonicate a total of 3 times.
3. Centrifuge the lysed cells for 30 min at 4ºC in cold room at 21300xg.
4. Prepare nickel and amylose resin for pulldown experiments.
    A. Gently swirl the resins to create homogenous slurry.
    B. Pipette 1 mL of the resins into 1.5 mL Eppendorf tubes.
    C. Centrifuge at 2348xg for 30s to collect the resin.
    D. Gently remove supernatant.
    E. Add 1 mL of water to resin. Do not use the pipette to resuspend beads. Invert the tubes gently.
    F. Centrifuge at 2348xg for 30s to collect the resin.
    G. Repeat steps E. and F. two more times.
    H. Repeat steps E. And F. Three times with lysis buffer (Table 16-17).
    I. Add lysis buffer (Table 16-17) to return final volume of beads to 1 mL. Transfer 150µL of each set of beads to 1.5 mL Eppendorf tubes.
5. Remove samples from centrifuge. Save 10 µL of the supernatant fraction. Mix the 10 µL supernatant sample with 10 µL of 2x SDS-PAGE loading buffer and 30 µL of lysis buffer
6. Transfer the rest of each sample supernatant into the resin tubes.
7. Incubate samples on rotating device in cold room at 4ºC for 15 min.
8. Wash the beads.
    A. Centrifuge at 2348xg for 30s to collect the resin.
    B. Gently remove supernatant.
    C. Add 1 mL of lysis buffer to resin. Do not use the pipette to resuspend beads. Invert the tubes gently.
    D. Centrifuge at 2348xg for 30s to collect the resin.
    E. Repeat steps C. and D. two more times.
9. After the last wash, eluted the protein by adding 30µL of either amylose or nickel elution buffer (Table 16-17) and incubated in room temperature for 2min.
10. Spin down the beads and remove 15µL of the supernatant and mix them with 5µL of 2x SDS-PAGE loading buffer.
11. Remove the rest of the supernatant from the elution of each resin and added to newly fresh opposite resin for each sample (e.g.: apply nickel eluates to amylose resin and amylose eluates to nickel resin).
12. Wash the beads from the first-round pulldown with 500µL lysis buffer (Table 16-17) to remove any residual protein that might be attached to the beads.
13. Spin the beads, remove the supernatant, and add it to the newly fresh opposite beads.
14. Incubate the beads with protein on the rotating wheel in the cold room at 4ºC for 15min
15. Spin the beads down and remove the supernatant.
16. Wash the beads 3 times with 1mL lysis buffer (Table 16-17) as in the first pull-down round.
17. After the last wash, elute the protein by adding 30µL of the respectively elution buffer (Table 16-17) and incubate it in room temperature for 2min.
18. Spin down the beads, remove 30µL of the supernatant, and mix them with 10µL of 2x SDS-PAGE loading buffer.

19. Run all samples (supernatant, elution from pull-down round 1 and 2) on a 10% SDS PAGE gel. Load:
    A. 2µL of supernatant
    B. 15µL of elution from round 1
    C. 35µL of elution from round 2
20. Stained the gels in instant blue

**Table 16.** $His_6$-β-catenin 138-781 and MBP-CDC73 213-263 mutants' pull-down buffers

| **Buffer** | **Lysis** | **$Ni^{+2}$ elution** | **Amylose elution** |
|---|---|---|---|
| **NaCl** | 300mM | 300mM | 300mM |
| **Na-HEPES** | 20mM | 20mM | 20mM |
| **imidazole** | 30mM | 500mM | |
| **glycerol** | 10% | 10% | 10% |
| **DTT** | 1mM | 1mM | 1mM |
| **Maltose** | | | 116mM |
| **pepstatin A** | 2mM | | |
| **leupeptin** | 0.7mM | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | |
| **benzamidine** | 2.8mM | | |
| **EtOH** | 100% | | |

**Table 17.** Wildtype $His_6$-β-catenin 138-781 and MBP-CDC73 213-263 expressed in insect cells pull-down buffers

| **Buffer** | **Lysis** | **$Ni^{+2}$ elution** | **Amylose elution** |
|---|---|---|---|
| **NaCl** | 500mM | 300mM | 800mM |
| **Na-HEPES** | 20mM | 20mM | 20mM |
| **imidazole** | 30mM | 500mM | |
| **glycerol** | 10% | 10% | 10% |
| **DTT** | 1mM | 1mM | 1mM |
| **Maltose** | | | 116mM |
| **pepstatin A** | 2mM | | |
| **leupeptin** | 0.7mM | | |
| **phenylmethylsulfonyl fluoride** | 1mM | | |
| **benzamidine** | 2.8mM | | |
| **EtOH** | 100% | | |

## Fluorescence Anisotropy Assays of CDC73 1-111, CDC73 336-531, and β-catenin (138-781) with DNA/RNA

**Materials**

- 5M NaCl
- 1M Na-HEPES pH 7.4
- 2M imidazole pH 8.0
- 1M MgCl2
- 10mg/mL BSA
- Reducing agent (DTT)
- Glycerol
- Filtered (0.2µ) dd$H_2O$
- Sterile 1.5mL Eppendorf tubes
- 100µM fluorescently labelled DNA/RNA
- 96-well plate
- 384 well plate
- multichannel pipette

**Procedure**

1. Place a 96-well plate on ice. Prepare buffers (Table 14-15) in 1.5mL Eppendorf tubes
2. Dilute FAM-DNA/RNA to 50 nM. Cover with foil.
3. Serially dilute protein of interest in protein dilution buffer (Table 14-15). Generally, a starting range of 100µM to 10nM (or 10 µM to 1nM) is appropriate. You can best achieve this using half-log dilutions.
4. In the first well of a row on 96-well plate, dilute the protein to 500µM (final 100µM) or 33.3µM (10µM).
5. Make 10 serial dilution by mixing 9.5µL protein with 20.5µL protein dilution buffer (half log dilutions). Leave 12th well with protein dilution buffer
6. Mix 3µL DNA with 6µL protein. Cover with foil and incubate on ice for 10 min.
7. Add 15µL of 2x assay buffer (Table 14-15) and 6µL of $H_2O$ to bring reaction to final volume (30 µL).
8. Transfer plate to room temperature, keep covered. Incubate at room temperature for 20 mins.
9. Transfer 18 µL of each condition to a 384 well plate using a multichannel pipette.
10. Gently tap 384-well plate on all sides or spin in tabletop centrifuge (1000xg for 1 min).
11. Place 384-well plate in plate reader. Perform anisotropy measurement.
12. Read in plate reader with the following settings: 26050 plate Z-height, gain 100/120/80, excitation 470 ± 20 nm, emission 518 ± 20 nm, default number of flashes.
13. Zero data to protein buffer alone, fit the data to the X equation, plot the curves and determine the $K_d$.

**Fluorescence anisotropy assays of β-catenin (138-781) with DNA/RNA buffers**

1. Before starting step (1) above, dialyze the protein in 500mL protein dilution buffer (Table X) in a 10 kDa MWCO Slide-A-Lyzer MINI Dialysis Unit for at least 4 hours at 4ºC to decrease the salt concentration.

2. Remove the protein from the dialysis unit and continue to step (1) in the above procedure steps.

**Table 14.** Fluorescence anisotropy assays of CDC73 1-111 and CDC73 336-531 with DNA/RNA buffers

| Buffer | Protein Dilution | 2x Assay | Final Assay condition |
|---|---|---|---|
| **NaCl** | 150mM | 0mM | 30mM |
| **Na-HEPES** | 20mM | 32mM | 20mM |
| **glycerol** | 10% | 18% | 10% |
| **DTT** | 1mM | 1mM | 0.27mM |
| **MgCl2** | | 6mM | 3mM |
| **BSA** | | 0.1mg/mL | 0.05mg/mL |
| **DNA/RNA** | | | 5nM |

**Table 15.** Fluorescence anisotropy assays of β-catenin (138-781) with DNA/RNA buffers

| Buffer | Protein Dilution | 2x Assay | Final Assay condition |
|---|---|---|---|
| **NaCl** | 100mM | 20mM | 30mM |
| **Na-HEPES** | 20mM | 37.3mM | 20mM |
| **glycerol** | 10% | 18.66% | 10% |
| **DTT** | 1mM | 1mM | 0.27mM |
| **MgCl2** | | 6mM | 3mM |
| **BSA** | | 0.1mg/mL | 0.05mg/mL |
| **DNA/RNA** | | | 5nM |