Specific Armadillo Repeat Sequences Facilitate β-Catenin Nuclear Transport in Live Cells via Direct Binding to Nucleoporins Nup62, Nup153, and RanBP2/Nup358 * 1

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Background: The nuclear localization of β-catenin is directly linked to its cancer causing activity.

Results: Armadillo repeats (10–12) mediate nuclear transport of β-catenin through direct interaction with specific nuclear pore complex proteins.

Conclusion: β-Catenin can function like a nuclear transport receptor in its ability to translocate independently through the nuclear pore complex.

Significance: β-Catenin may transport specific binding partners between the nucleus and cytoplasm in response to Wnt signaling.

β-Catenin transduces the Wnt signal from the membrane to the nucleus, and certain gene mutations trigger its nuclear accumulation, leading to cell transformation. β-Catenin was previously hypothesized to shuttle between the nucleus and cytoplasm through classical Ran/export pathways, which was previously hypothesized to play a role in the import/export activity. Using both in vivo FRAP and protein knock-down of endogenous Nup358 and Nup62 impeded the nuclear import but displayed no change in the nuclear export activity. We show for the first time that specific Arm repeats of β-catenin interact directly with the nucleoporins Nup62, Nup98, and Nup358. This interaction facilitates the nuclear export of β-catenin. Moreover, a proteomics screen identified RanBP2/Nup358 as a binding partner of Arm R10–12, and β-catenin was confirmed to interact with endogenous and ectopic forms of Nup358. We further demonstrate that knock-down of endogenous Nup358 and Nup62 impeded the rate of nuclear export of β-catenin to a greater extent than that of importin-β. The Arm R10–12 sequence facilitated transport even when β-catenin was bound to the Arm-binding partner LEF-1, and its activity was stimulated by phosphorylation at Tyr-654. These findings provide functional evidence that the Arm domain contributes to regulated β-catenin transport through direct interaction with the NPC.

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This article has been withdrawn by the authors. Further analysis revealed that the published data in Fig. 3C describing the binding of the R3-8 Arm fragment of β-catenin to Nup62 were in error. In addition, several lanes of the immunoblots in Fig. 3 were spliced together for presentation without clear indication of this fact.

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GTPase for transport (10–12). We and others observed that a fraction of β-catenin can exit the nucleus indirectly in complex with other proteins (e.g. APC, Kank, LZTS2, Axin) that do access the CRM1/exportin-1 route, at least when these proteins are overexpressed in cells (13–17). However, when its expression is induced transiently by Wnt signaling or chronically by cancer-linked mutations, the majority of β-catenin exits the nucleus independent of CRM1, exogenous soluble factors, and Ran-GTPase (12, 18). Additionally, the nuclear import of β-catenin occurs independently of Ran-GTPase and the importins (10, 11), although LEF-1 has been implicated in its import via the importin pathway (19). Notably, the receptor independent pathway for nuclear transport of β-catenin has not yet been resolved.

Structurally, β-catenin comprises a helical folded 12 Armadillo (Arm) repeat sequence flanked by unstructured N and C termini (20, 21) (see Fig. 1A). It was previously proposed (and frequently assumed in the literature) that the β-catenin Arm domain mediates nuclear transport, as Arm repeats 9–12 adopt a very similar conformation to importin-β HEAT repeats 4–8, which mediate nuclear transit through direct contact with the NPC (22, 23). However, there is currently little evidence to support a role for the Arm repeat domain in β-catenin nuclear transport or for interaction with NPC components. First, the complete Arm repeat sequence (R1–12), or a combination of the Arm and C terminus (R8–12 + C), displayed only very weak transport activity in a Xenopus oocyte microinjection assay (18) (see Fig. 1B). It was previously proposed (and supported by a study by Koike et al. (22) could not measure activity of the Arm sequence alone. In combination with C-terminus, R10–12 contribute to transport of β-catenin-His-Y654F in cell permeabilization assays to NIH 3T3 cells. In terms of evidence for binding to Nups, Fagotto et al. (10) showed in vitro that β-catenin could bind directly to the FG repeats of a single yeast nucleoporin, Nup1p, however, they did not assess the FG repeats of mammalian Nups normally contacted by transport receptors. Moreover, the same laboratory later rescinded their claims and reported that β-catenin does not bind to Nup FG repeats (25). More recently, Hendriksen et al. (26) cited unpublished data that the Arm domain of β-catenin could immunoprecipitate certain nucleoporins from Xenopus laevis oocytes, but no testing for a direct interaction between β-catenin and NPC components was performed.

In this report, we tested different β-catenin Arm sequences and show for the first time that the Arm domain is highly active for transport, and that this activity maps primarily to Arm repeats 10–12. We further demonstrate differential transport activity of other Arm repeat sequences and show evidence for a direct interaction between β-catenin Arm sequences and the FG repeats of specific nucleoporins (Nups) implicated in protein transport. The live cell photobleaching assay data are further compounded by evidence that silencing specific Nups slows transport of β-catenin. These findings outline a model for the nuclear transport of β-catenin.

Materials and Methods

Plasmids

All β-catenin-GFP plasmids were constructed by PCR amplification of the β-catenin cDNA and cloning into pEGFP-N1 between KpnI and BamHI restriction sites. All constructs were confirmed by sequencing (see supplemental Table S1 for primer details). β-Catenin-WT-MBP, LEF1-ΔHMG, and GFP-importin-β constructs were kind gifts of Prof. Kozo Kaibuchi (27), Dr. Marian Waterman (28), and Dr. Ian Ellenberg (29). β-Catenin-His-Y654E and Y654F cDNAs were kindly supplied by Dr. Antonio Garcia de Herreros (30), and used as templates to construct GFP-tagged forms as outlined above. To construct additional maltose-binding protein (MBP) expression plasmids, the β-catenin cDNA insert was removed from the pMAL-C2 vector with the BamHI digest and replaced with PCR-amplified β-catenin fragments (see supplemental Table S1 for primer details). pYFP-CRM1 (31) and pRev-WT-GFP were described previously (32). GST-Nup62-N and GST-Nup153-C were gifts from Dr. Shige H. Yoshimura (33). Nup98-GST was cloned by PCR amplifying the insert and cloning into the pGEX4T-1 vector at EcoRI and SalI sites (see supplemental Table S1).

Antibodies

Antibodies were purchased from Santa Cruz, New England Biolabs, GE Healthcare, Transduction Laboratories, mAb414, rabbit IgG (Sigma), anti-MBP (New England Biolabs), anti-GST (GE Healthcare), anti-HA (Covance), anti-LEF1 pAb (Santa Cruz), anti-MBP (Gibco), and Nup358 mAb (Santa Cruz) were used.

Cell Culture, RNA Interference, and Transfections

NIH 3T3 cells were routinely maintained in 75-cm² tissue culture flasks by passaging with trypsin/EDTA. At 24 h prior to transfection, cells were seeded on coverslips in a 6-well tray or 2-well chamber slides (Nunc) and transfected at ~50% confluence using Lipofectamine according to the manufacturer's instructions. Cells were treated with 5.2 ng/ml of leptomycin B (LMB) for 3 h prior to fluorescence recovery after photobleaching (FRAP). Mouse Nup358 (sc-36381) and Nup62 siRNA (sc-36108) sequences were ordered from Santa Cruz. NIH 3T3 cells were seeded at 50% confluence for 24 h prior to transfection. 3 μg of siRNA was transfected using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells were given fresh media after 5 h of transfection. After 48 h these cells were transfected with β-catenin-WT-GFP or β-catenin fragments using FuGENE (Promega) according to the manufacturer's protocol. 72 h post-transfection, cells were imaged (for FRAP assay), immunostained, or lysed for Western blot assay.

Live Cell FRAP Assay

FRAP analysis was performed on an Olympus FV1000 confocal laser scanning microscope using ×60 water objective; zoom = 2, scanning speed = fast, image format = 512 × 512, laser power during pre- and post-bleach scanning = ~5–7% to minimize loss of fluorescence intensity, and laser power during bleaching = 100%. The microscope chamber was set and equilibrated to 37 °C and 5% CO₂.
Nuclear Export/Import FRAP Data Acquisition—3 pre-bleach images of the whole cell were acquired. 90% of the cytoplasm/nucleus for export/import FRAP was bleached for 9–12 s (depending on the construct and stability of GFP tag in these cells). Post-bleach imaging was done in 3 stages: first stage, 30 frames at the fastest interval; second stage, 30 frames at 1.2-s intervals; third stage, 30 frames at 10-s intervals. Fluorescence intensities for the cytoplasm, nucleus, and background were acquired using Olympus Fluoview software and exported to a Microsoft Excel file.

Data Analysis—Background values were subtracted from cytoplasmic and nuclear fluorescence intensities. To compare the rates of nuclear export/import between different samples, fluorescence data were expressed as a cytoplasmic/nuclear ratio (for nuclear export) or nuclear/cytoplasmic ratio (for nuclear import). For each cell data set, the pre-bleach ratio was set to 100%, the time for the first post-bleach image was set to 0 s, and the recovery curve was adjusted to start at 25% recovery at time 0 (this was the closest average value). The average of the data for at least 10 cells from 2 to 4 experimental repeats was plotted. Initial export/import rates for the first 30 s was analyzed in GraphPad Prism 5.0 using linear regression analysis. Other kinetic analysis was done using the single association curve in GraphPad Prism 5.0 with the curve weighted by 1/Y2.

Immunoprecipitation

Cells were cross-linked by incubating the flask with 1% paraformaldehyde. Cell pellet was washed 3 times with PBS and resuspended in lysis buffer (containing fresh protease inhibitor mixture from Roche Diagnostics). Lysates were spun at 13,000 × g for 10 min at 4 °C. The supernatant was quantified using a Bradford assay. 50 μl of protein G-agarose (Amersham Biosciences) (for mouse monoclonal anti-catenin) or protein A-Sepharose (for rabbit anti-GFP antibody) was added to 500 ng of purified MBP or β-catenin-MBP for 1 h at 4 °C, washed once with TEDM buffer (TEDM = 20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2), and blocked with 5% BSA/TEDM for 1 h at 4 °C. 500 ng of purified GST or Nup-GST fragments were added to blocked amylase resin and incubated for 2 h at 4 °C. The resin was washed once with RIPA buffer and twice with TEDM + 0.25 mM NaCl. The resin was eluted with 10 μl of maltose elution buffer and Laemmli buffer. The eluate was loaded and separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The membrane was first probed with anti-GST antibody and then with anti-MBP antibody. Similar exposures were quantified with Adobe Photoshop. β-Catenin-WT-MBP and MBP were always included as controls and for quantification purpose. For each assay, background signal from MBP protein was subtracted and the ratio of β-catenin-WT-MBP to MBP was set to 100%. Each experiment was repeated 2–4 times.

Western Blotting

NIH 3T3 cells were seeded in a T25 flask and transfected with β-catenin-GFP fragments using Lipofectamine (as described in supplemental Methods). 48 h post-transfection cells were harvested using trypsin and lysed in RIPA buffer for 20 min on ice (containing fresh protease inhibitor mixture from Roche Diagnostics). Lysates were spun at 13,000 × g for 10 min at 4 °C. The supernatant was quantified using a Bradford assay. 50 μg of total cell lysate was separated on a 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk/PBS and immunoblotted with anti-GFP antibody (1:1000 from Roche Diagnostics) and antimouse HRP antibody (1:5,000 from Sigma).

In Vitro Binding Assay

MBP fusions of β-catenin were expressed and purified from DH5α bacteria, and glutathione S-transferase (GST)-tagged FG repeats of Nup62, -98, and -153 were expressed and purified from BL21 cells (for details of protein purification refer to supplemental Methods). 10 μl of amylase resin (New England Biolabs) was coated with ~500 ng of purified MBP or β-catenin-MBP for 1 h at 4 °C, washed once with TEDM buffer and blocked with 5% BSA/TEDM for 1 h at 4 °C. 500 ng of purified GST or Nup-GST fragments were added to blocked amylase resin and incubated for 2 h at 4 °C. The resin was washed once with RIPA buffer and twice with TEDM + 0.25 mM NaCl. The resin was eluted with 10 μl of maltose elution buffer and Laemmli buffer. The eluate was loaded and separated by SDS-PAGE and then transferred onto nitrocellulose membrane. The membrane was first probed with anti-GST antibody and then with anti-MBP antibody. Similar exposures were quantified with Adobe Photoshop. β-Catenin-WT-MBP and MBP were always included as controls and for quantification purpose. For each assay, background signal from MBP protein was subtracted and the ratio of β-catenin-WT-MBP to MBP was set to 100%. Each experiment was repeated 2–4 times.

Statistical Analysis

Statistical analysis was performed on the data using the STATVIEW program (version 5; SAS Institute). Drug treatment and transfection comparisons were analyzed by one-way analysis of variance. Statistics for FRAP assays were calculated using the repeated measure of analysis (one-way analysis of variance) over multiple time points and post hoc test was used to compare significant differences between constructs. Results were considered significant when p < 0.05. The Student’s unpaired t test was also used.

RESULTS

The Arm Repeats 10–12 of β-Catenin Display Strong Nuclear Export Activity in Living Cells—It was previously speculated that specific Arm repeats (9–12) of β-catenin (Fig. 1A) contribute to its nuclear transport due to a structural resemblance to the transport-active HEAT repeats of importin-β (22, 23). However, published analyses of the β-catenin Arm region have provided no clear answer due to contradictory data (18, 22, 24). To compare the activity of different Arm regions of β-catenin, we constructed GFP-tagged Arm repeat fragments for use in FRAP assays in NIH 3T3 cells. This noninvasive technique...
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enabled us to compare both nuclear export and import rates of β-catenin sequences in living cells. The GFP-tagged peptides were all beyond the size limit (~45 kDa) for passive diffusion through the NPC and correct expression of each construct was verified by Western blotting (supplemental Fig. S1).

β-Catenin can exit the nucleus via the CRM1 pathway when bound to specific partners that contain an nuclear export sequence (13–17). However, the major export route of β-catenin is independent of CRM1 (35). To ensure that only CRM1-independent export was analyzed, cells were treated with the CRM1 inhibitor LMB for 3 h prior to photobleaching. To measure nuclear export, the GFP-based fluorescence of transfected cells was bleached in the cytoplasm and the rate of fluorescence recovery was then quantified for up to 360 s (Fig. 1B and supplemental Fig. S2). For ease of comparison of transport rates, the different fluorescence recovery curves were plotted and shown as the cytoplasmic:nuclear (C/N) ratio (see “Materials and Methods”) for the first 150 s (Fig. 1C). We confirmed our previous result showing the rapid CRM1-independent nuclear export of wild-type (WT) β-catenin relative to a 58-kDa tomato-dimer fluorescent protein control (36). The export rates of the β-catenin subfragments were then assessed from recovery slopes by linear regression analysis of the first 30 s, which time transport was least influenced by nuclear retention or re-equilibration. A comparison clearly showed that the Arm repeat sequences R1–8 and R3–8 were least efficiently exported, and recovered in the cytoplasm up to 80% of the pre-bleach cytoplasmic intensity, and displayed a similar recovery curve to that seen for the full Arm R1–12 sequence. A comparison of the initial export rates (summarized in Fig. 1D) distinguished Arm repeats R10–12 as the primary export sequence. To underscore the specificity of this activity, the export kinetics of the 7-repeat Arm domain from APC protein was tested and found to be relatively slow for nuclear export and comparable in activity to β-catenin Arm repeats R1–8 and R3–8 (Fig. 1, B and C). This is the first systematic analysis of the β-catenin and APC Arm repeats in live cells, and reveals both differential export activity of different Arm sequences and direct evidence that Arm sequence R10–12 comprises the strongest nuclear export function. The nuclear export activity of the R10–12 was further confirmed using an

FIGURE 1. Arm repeats (R10–12) of β-catenin mediate nuclear export. A, a schematic diagram of the primary β-catenin protein domains: N terminus (1–141 amino acids), Arm repeats 1–12 (141–664 amino acids), and C terminus (665–781 amino acids). B, NIH 3T3 cells were transfected with β-catenin-GFP fragments (WT, R1–12, R1–8, R3–8, R10–12, APC arm), YFP-CRM1 or td-tomato (58-kDa negative control) as indicated. After 48 h, cells were pre-treated with 5.2 ng/ml of LMB for 3 h before nuclear export FRAP analysis. Confocal images are shown of cells before bleach and after >90% of the cytoplasm was bleached (shown up to 160s when nearing plateau). Side panel shows relative export activity. C, mean recovery curves for all constructs were calculated as the cytoplasmic to nuclear ratio, set to 100% based on pre-bleach values and compared with td-tomato (negative control). Each curve represents an average of at least 10 cells from 2 to 4 experiments. D, nuclear export rate (mean ± S.D.) was calculated for the first 30 s using one-phase association equation and linear regression analysis on GraphPad Prism software. See supplemental Fig. S2 and Table S2 for a more detailed kinetic analysis.
β-Catenin Arm R10–12 Mediates Rapid Bidirectional Transport, Whereas β-Catenin Arm R3–8 Is More Biased toward Nuclear Import Than Export—A previous study by Krieghoff and colleagues (24) used FRAP analysis to study β-catenin transport, but did not distinguish between nuclear import and export rates and did not compare different Arm repeat sequences. We next employed FRAP analysis to test the nuclear import kinetics of the GFP-tagged Arm repeats by bleaching the nuclear fluorescence with a high-power laser and tracking the rate of nuclear recovery over 150 s. Note that all transfected cells analyzed displayed an equivalent and moderate level of nuclear and cytoplasmic GFP expression. Arm sequences R1–12 and R10–12 were found to be capable of rapid nuclear import at a rate slightly faster than wild-type β-catenin (Fig. 2, A–C). Interestingly, the import rates of β-catenin wild-type and Arm (R1–12 or R10–12) sequences were ~40–50% slower than that of importin-β, a benchmark import receptor. This suggests that importin-β is slightly more active at the NPC, and indeed a previous study indicated that importin-β could effectively compete with β-catenin for transport across the nuclear pores (22). Further analysis revealed that β-catenin Arm sequence R3–8 was also imported into the nucleus relatively efficiently (Fig. 2, A and B), and at a faster rate than it was exported (compare graphs in Figs. 1D and 2B, see supplemental Fig. S4). These findings pinpoint the Arm region R10–12 as the optimal bidirectional interaction element, and identify R3–8 as a strong high-efficiency nuclear import sequence (sus import) of different Arm sequences (wild-type, R3–8, R1–12, and R10–12). Wild-type β-catenin and Nups at the NPC.

The Arm Repeats of β-Catenin Mediate Import Active FG Repeats of Nucleoporins—The receptor-independent transport of β-catenin is reminiscent of transport receptors such as importin-β and NTF2 that traverse the NPC by binding to multiple FG repeats of nucleoporins such as Nup62, Nup98, and Nup153 (37). An interaction of wild-type β-catenin with yeast Nup1p was originally reported by Fagotto et al. (10) but later contradicted by the same group who could not confirm this interaction (25). It is possible that yeast Nup1p is not the best binding partner for β-catenin in vivo. To address this issue, we first performed a cross-linking experiment, using an antibody highly specific for nucleoporin FG repeats to pull down binding partners in cross-linked SW480 colon tumor cell extracts. As shown in Fig. 3A, the immunoprecipitation assay detected a weak in vivo interaction with β-catenin, suggesting a physiological interaction between β-catenin and Nups at the NPC.

Next, we tested whether the transport active Arm repeats of β-catenin identified here by the FRAP assay could bind directly in vitro to specific NPC regions thought to mediate active passage of transport receptors, i.e. the FG repeats of Nup62, Nup98, and Nup153. β-Catenin sequences (wild-type, R3–8, and R10–12) were purified from bacteria as MBP fusions and coated onto amyllose resin beads. GST-tagged forms of the Nup FG repeats were then prepared and compared for binding to the MBP-β-catenin sequences (protocol outlined in Fig. 3B). There was very little background binding of GST-Nups to beads coated with MBP alone (Fig. 3C, first and fourth lanes). Wild-type β-catenin-MBP was found to bind consistently above background to the FG repeats of Nup62, -98, and -153 (e.g. second lane). In Fig. 3D, the percentage binding of MBP-β-catenin sequences to FG repeats was calculated by normalizing against MBP, and setting wild-type β-catenin-MBP values to 100% (see “Materials and Methods”). The Arm repeats of β-catenin were bound to Nup FG repeats, although to varying degrees.
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It is interesting to note that Arm repeats R3–8 showed differential binding to these Nups (bound Nup62 much more strongly than Nup153), which might possibly correlate with its slow rate of nuclear export relative to import (Fig. 2). These data provide the first clear demonstration, using purified components, that full-length β-catenin and its Arm repeat domain can bind directly to specific nucleoporin FG repeats. This is highly consistent with a direct transport route for β-catenin through the NPC.

The Arm Repeat Sequence R10–12 Binds to FG Repeats of RanBP2/Nup358—The Arm repeat R10–12 region displayed the strongest nuclear export and import activities (Figs. 1 and 2), and thus we used this sequence to screen for other potential binding partners that might influence its nuclear transit activ-

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**A**

IP: IgG mAb Input

| β-catenin | Nup62 |
|-----------|-------|
| 414       |       |

**B**

![Diagram](image)

| β-catenin coated beads | Nup-GST | β-catenin bound to Nup-GST |
|------------------------|---------|---------------------------|

**C**

| GST-Nup 62-FxFG | GST-Nup 98-FxFG | GST-Nup 153-FxFG | anti-MBP |
|-----------------|-----------------|-----------------|----------|

**D**

![Graph](image)

**E**

**Ectopic IP**

| IP: IgG | β-catenin Input |
|---------|-----------------|
| anti-Nup358-FG-HA (37 kDa) | β-catenin (long exposure) |
| β-catenin-GFP | β-catenin (short exposure) |

| Nup358-FG-HA | + + |
| β-catenin-WT-GFP | + + |

**F**

**Endogenous IP**

| IP: IgG | Nup358 Input |
|---------|--------------|
| β-catenin | Nup358 |

**G**

**Ectopic IP**

| IP: IgG | GFP antibody Input |
|---------|--------------------|
| Nup358-FG-HA | YFP-CRM1 |
| GFPImportin-β | β-catenin-GFP |

| Nup358-FG-HA | + + + + |
| YFP-CRM1 | + - - - |
| GFPImportin-β | - + - - |
| β-catenin-GFP | - - + - |
ity. MBP-β-catenin R10–12-coated beads were incubated with total cell lysate from NIH 3T3 cells and candidate binding partners were identified by mass spectrometry (see supplemental Fig. S5). This approach identified several new binding partners, of which RanBP2/Nup358 was the most promising new transport partner. RanBP2/Nup358 is a mobile nucleoporin that locates at the cytoplasmic filaments of the NPC. We confirmed that β-catenin interacts with Nup358 by immunoprecipitation, where endogenous full-length β-catenin was pulled down by Nup358 antibody (see Fig. 3F). In addition, the FG repeat fragment of Nup358 was transiently expressed in cells and shown to specifically immunoprecipitate β-catenin (Fig. 3E). To compare β-catenin-Nup358 binding with that of transport receptors, we co-transfected the FG repeats of Nup358 with β-catenin-GFP, GFP-importin-β, or YFP-CRM1 and immunoprecipitated using anti-GFP antibody. As anticipated, a modest and comparable interaction was observed between Nup358-FG repeats and the three transporter proteins (Fig. 3G). Taken together, these results suggest that β-catenin interacts with the FG repeats of Nup358 (cytoplasmic filaments), Nup62 (central channel), Nup98 and Nup153 (nuclear basket) and traverses the NPC without the aid of transport receptors (see model in Fig. 5H).

Silencing of Nup358 and Nup62 Slows Nuclear Export and Import of β-Catenin—No previous study has yet tested the functional influence of specific Nups on β-catenin transport. To evaluate whether Nup358 regulates β-catenin transport and silenced its expression by siRNA in NIH 3T3 cells, achieving a selective loss of up to 90% (Fig. 4G and supplemental Fig. S6). We then studied the live cell transport kinetics of GFP-tagged β-catenin Arm R10–12 in response to silencing Nup358, as shown in Fig. 4A, silencing Nup358 decreased nuclear export (Figs. 4B and C). Thus, Nup358 binds to the Arm R10–12 sequence and contributes to its transport dynamics.

Next, we tested whether loss of either Nup358 or Nup62, which are located at the cytoplasmic face and central channel of the NPC, respectively, impacted the transport of full-length β-catenin. FRAP analysis of β-catenin-GFP expressed in NIH 3T3 cells revealed that relative to control siRNA, the knockdown of Nup358 caused a significant ~45% reduction in nuclear export rate (Fig. 4, D and E), and a ~50% reduction in import rate of β-catenin (Figs. 4, F and G, and 5E). A similar knockdown of mouse Nup62 was performed in NIH 3T3 cells and the effect on kinetics of β-catenin-GFP were evaluated. Nup62 was effectively silenced as verified by Western blot (Fig. 5G), and its loss had no effect on β-catenin steady-state distribution (see pre-bleach image in Fig. 5A). However, the Nup62 knockdown caused a ~30% reduction in export rate (Fig. 5, A, B, and E) and a ~20% decrease in nuclear import rate (Fig. 5, C–E) of β-catenin. To compare with another transport receptor, we silenced Nup62 and Nup358 and studied the effect on nuclear import of GFP-importin-β. We found that loss of neither nucleoporin had any significant effect on the transport rate of importin-β (Fig. 5F and supplemental Fig. S7).

These findings suggest that both Nup62 and Nup358 contribute, with a degree of selectivity, to the trafficking of β-catenin through the NPC (Fig. 5H). We note in particular that loss of Nup358 had a more pronounced effect on β-catenin than did loss of Nup62, implicating an important role of the NPC cytoplasmic filaments in the transport process. The changes observed in nuclear transport rate are quite significant and show that the nuclear transport rate of β-catenin was slowed by ~50% by silencing Nup358 (Figs. 4, C and D), and 45% by silencing Nup62 (Fig. 4, D and E). This is the first evidence to demonstrate that specific nucleoporins affects nuclear shuttling of proteins.

![Withdrawn](January 28, 2016)
This form of LEF-1 binds β-catenin at Arm repeats R3–8, which leaves R10–12 potentially available for export (Fig. 6). We co-transfected LEF-1ΔHMG with different β-catenin-GFP sequences (Fig. 6B) and scored the localization of LEF-1ΔHMG in co-transfected cells by microscopy. We found that wild-type β-catenin-GFP shifted staining of LEF-1ΔHMG from the nucleus to cytoplasm in 85% of cells, compared with just 20% of cells co-transfected with control or Nup358 siRNA. In contrast, transient expression of the Arm R10–12 sequence, which does not bind to LEF-1, displayed a similar background activity to GFP control (Fig. 6B). This data suggests that Arm repeats R10–12 can contribute to transport of cargo out of the nucleus even when proteins are bound to the adjacent Arm repeat sequences of β-catenin.

Tyrosine Mutations in Arm R10–12 Sequence (Tyr-654) Show Differential Transport Activity—We recently showed that the S45A mutation, which blocks glycogen synthase kinase-3β phosphorylation of the N terminus of β-catenin, has no significant effect on nuclear transport (38). Src-mediated phosphorylation of tyrosine 654, which lies within the Arm R10–12 sequence, could alter the stability or binding properties of β-catenin-GFP. We therefore examined the effect of a Tyr-654→Phe (Tyr-654F) mutation on the nuclear transport of β-catenin-GFP. As shown in Fig. 6C, the Tyr-654F mutation decreased nuclear export activity by 86% compared with wild-type β-catenin-GFP. Importantly, the Tyr-654F mutation did not affect the nuclear transport of wild-type β-catenin-GFP, suggesting that the tyrosine phosphorylation of Arm R10–12 plays a critical role in regulating nuclear export of β-catenin.
sequence (see Fig. 7A), has been shown to dissociate β-catenin from E-cadherin at the plasma membrane (30), and to stimulate Wnt signaling and initiate APC-dependent tumorigenesis in mice (39). We tested whether mutagenesis of this residue affects β-catenin nuclear transport, by performing live cell FRAP analysis of mutants Y654E (phosphomimic) and Y654F (nonphosphorylated) in full-length β-catenin-GFP. Unexpectedly, both mutations caused a ~50% increase in the import rate of β-catenin, and displayed comparable T₁/₂ values (Fig. 7, B, C, and F). When analyzed for export, however, Y654E showed a similar nuclear export rate to that of wild-type β-catenin-GFP, whereas Y654F was significantly slower (Fig. 7, D, E, and G). We conclude that perturbation of a single residue in the active R10–12 sequence is sufficient to modulate β-catenin transport, and that shifts in phosphorylation of Tyr-654 can impact on nuclear export dynamics.

**DISCUSSION**

β-Catenin can shuttle between three distinct subcellular locations: the nucleus, cytoplasm, and plasma membrane. In live cells it was previously shown by photobleaching assays that the Arm domain, a known protein interaction site, contributes to retention of β-catenin in the nucleus and at cell:cell junctions through binding to LEF-1/TCF transcription factors and cad-
herins, respectively (24, 36, 38). Here, we have re-assessed the potential role of the Arm repeat domain to mediate active transport of \( \beta \)-catenin. We show for the first time in live cells that the 12-repeat Arm region retains the same degree of nuclear import/export activity as full-length \( \beta \)-catenin, and we mapped the optimal bidirectional transport function to Arm repeats R10–12. The R10–12 sequence was found to bind to the FG repeats of Nup98 and Nup153 (located on the nuclear face of the NPC), Nup62 (central channel), and Nup358 (cytoplasmic face of the NPC). Thus, \( \beta \)-catenin uses the same transport route as nuclear transport receptors, and displayed similar transport kinetics as importin-\( \beta \) and CRM1. In functional assays, it was further revealed that silencing of Nup62 or -358, which are located at different positions within the NPC, diminished the import/export rate of \( \beta \)-catenin. This provides the first evidence supporting the conjecture that the \( \beta \)-catenin Arm R9–12 region might contact the NPC, based on its folding similarities to the importin-\( \beta \) HEAT repeats (23). These new lines of evidence, when considered together, strongly support a role for the Arm domain in contributing to \( \beta \)-catenin nuclear transit through direct and transient contacts with the NPC, and resolves some of the discrepancies in the literature that likely arose from different methodologies applied to measure transport and Nup binding (10, 25).

Our results, furthermore, elucidate differences between different Arm sequences, exemplified by \( \beta \)-catenin Arm repeats R3–8 and the APC Arm domain, which displayed a significantly slower export rate than \( \beta \)-catenin Arm R10–12. The differential transport activity (import > export) of the Arm R3–8 sequence correlated with a difference in its binding to specific Nups, showing a pronounced preference for Nup62 relative to Nup153. This may suggest that passage and direc-
tionality of the Arm R3–8 sequence across the NPC is affected by an affinity gradient, as previously reported for importin-β (40). The various Arm repeats vary substantially in sequence identity but adopt a typical helical fold (21); the differences between R10–12 and R3–8 could thus arise from the exposure of specific amino acids at the helical surface. Indeed, NPC-contacting domains of transport receptors are generally presumed to be surface-located hydrophobic patches and are dependent on protein conformation (reviewed in Ref. 41). In this context we note that changes to a single residue (Tyr-654) of the R10–12 sequence were sufficient to accelerate importin-β-catenin import to a rate comparable with that of importin-β (Fig. 7).

Conversely, a nonphosphorylatable Y654F mutation significantly decreased the export rate of β-catenin. This highlights the potential for certain post-translational modifications to impact on the β-catenin Wnt signaling function through modulation of transport efficacy.

It is interesting to note that silencing Nup62 and Nup358/RanBP2 did not affect the steady state localization of β-catenin-GFP (pre-bleach panels in Figs. 4 and 5), but did impact on the kinetics of nuclear transport. In particular, despite effective silencing of both Nups, the loss of Nup358 caused a far more consistent disruption of β-catenin import/export than loss of Nup62. At the NPC, Nup62 exists in a complex with Nup45, Nup54, and Nup58 in the central channel (42, 43) and also interacts with Nup153, Nup214, and Nup358 (44). Therefore, the loss of Nup62 may be more readily compensated by other Nups. The silencing of Nup62 was recently reported to slow the nuclear export of HIV Rev viral RNA particles, although actual transport dynamics were not measured (45).

Nup358 is the major component of cytoplasmic filaments and its roles in docking/undocking of transport substrates is less readily compensated for by other Nups. This may explain why knockdown of Nup358 elicited a stronger effect on trans-
Arm-mediated β-Catenin Nuclear Transport

port of β-catenin in this study. Studies employing both in vitro (46, 47) and in vivo (48) assays have shown that siRNA-mediated loss of Nup358 can cause a reduction in importin-α/β and transportin-mediated import of proteins such as HIV Rev. In addition, recent studies found that Nup358 promotes nuclear import of proteins in a selective transport receptor-specific manner (49), which partly involves the capture and recycling of RanGTP-importin-β complexes at the cytoplasmic fibrils of the NPC (50). We observed no change in import rate for total GFP-importin-β after knockdown of Nup62 or Nup358 (supplemental Fig. S7), suggesting that only its Ran-dependent transfer of nuclear localization signal-containing cargo is dependent on Nup358 docking. We propose that β-catenin binds transiently to sequential Nups during its passage through the NPC. Because the knockdown of Nup358 caused a selective 50% decrease in nuclear export/import rate of β-catenin, we speculate that Nup358 is important for β-catenin docking/un-docking at NPC cytoplasmic fibrils during the nuclear transport process. In a previous study the Arm repeats of β-catenin were implicated in a RanGTP-dependent export pathway. It was proposed that the CRM1 export co-factor, RanBP3, binds to the Arm repeats of β-catenin in a RanGTP-stimulated fashion and exports the transcriptionally active, de-phosphorylated pool of β-catenin out of the nucleus independent of the CRM1 export pathway (26). This resulted in reduced transcriptional activity of β-catenin and reduced effects of Wnt signaling in HeLa and Drosophila melanogaster. Although this paper was intriguing, we could not replicate the findings and changes in translocation of de-phosphorylated similar conditions (i.e., in LiCl-treated cells) after overexpression of Arm (supplemental Fig. S8). Instead, we observe a phospho-β-catenin, which we (supplemental Fig. S8) and others have found to be nonspecific (51).

The Arm domain is the main protein interaction region of β-catenin, and it is targeted by a range of key binding and/or retention partners including LEF-1/TCFs, APC, and cadherins (52). The majority of these factors bind within the first 8 repeats, and thus are likely to mask the Arm R3–8 sequence, but it was not obvious if the Arm R10–12 would remain functional. To address this question we co-transfected cells with various β-catenin sequences and a nuclear nonretained mutant of LEF-1 (Fig. 6), and found that the full-length Arm could indeed function to export nuclear-localized LEF-1 to the cytoplasm. Thus, R10–12 remains accessible and active even when β-catenin is bound to its Arm-binding cargo. This has implications for the role of β-catenin as a potential transporter of various cargo (19, 53). A key distinction between β-catenin and other transport receptors is that β-catenin is not regulated by Ran-GTPase and thus, rather than dissociate from its cargo after crossing the NPC, it is likely to move with its protein partners along extended transport routes between the nuclear chromatin and the plasma membrane. In summary, we propose that Arm R10–12 contributes to shuttling of β-catenin between the nucleus and cytoplasm by directly contacting FG repeats of critical nucleoporins, and that when stabilized by Wnt signaling or cancer mutations, β-catenin is likely to utilize this transport pathway and function as a protein-specific nuclear transport receptor.

Acknowledgments—We thank Prof. Eric Fearon for β-catenin-FLAG constructs and Prof. Kozo Kaibuchi for the β-catenin-WT-MBP plasmid, and members of our laboratory for helpful discussions. We thank Dr. Antonio García de Herreros for β-catenin Tyr-654 mutant cDNAs.

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Supplementary Methods:

Expression and purification of recombinant proteins: β-catenin-MBP fusion constructs were transformed into DH5α cells and purified as follows. 10 ml of overnight starter culture was used to inoculate 200 ml of 2YT+ampicillin broth at 37°C until OD595 reached 1.0 (~2-3 h). IPTG was added to a final concentration of 0.3 mM and incubated at 37°C for 5 h. Bacterial cells were spun down and the pellet washed with 40 ml of cold PBS. Cells were resuspended in 10 ml H-buffer containing fresh protease cocktail inhibitor (Roche) (H-buffer=TEDM buffer+0.5M NaCl; TEDM = 20 mM Tris pH8.0, 1 mM EDTA, 1 mM DTT, 1 mM MgCl2) and sonicated. Cells were spun at 20,000g/30min/4°C. The cell lysate was loaded onto 1ml Amylose resin column (NEB) pre-equilibrated with H-buffer. The column was washed with 2cv of H-buffer, followed by 10cv of TEDM buffer. The bound protein was eluted with E-buffer (TEDM+36mg/ml maltose+protease inhibitor) and dialysed extensively against TEDM buffer.

Nup153-GST and Nup62-GST were gifts from Dr. Yoshimura (2) and were transformed into BL21 cells. 10 ml of overnight culture was used to inoculate 200 ml of 2YT+ampicillin broth. Cells were cultured for 3 h at 37°C and induced with 0.1 mM IPTG at 37°C for 3 h. Cells were washed with cold PBS and lysed in 10 ml lysis buffer (50 mM Tris pH8.3, 0.5 M NaCl, 1 mM EDTA, 2 mM DTT and fresh protease inhibitor cocktail-Roche), sonicated for 180 s using microtip sonicator (50% output power) at 4°C. The supernatant was loaded onto 1ml Glutathione-Sepharose 4B column (GE Healthcare) pre-equilibrated with lysis buffer. The column was washed with lysis buffer, 10cv wash buffer (100mM Tris pH 8.3, 0.1M NaCl, 1mM EDTA, 2mM DTT) and eluted with elution buffer (wash buffer+50mM glutathione+protease inhibitors).

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**Supplementary Table S1: Primers used for cloning**

| Construct | Amino Acids | Forward primer | Reverse primer | Cloning site |
|-----------|-------------|----------------|----------------|--------------|
| β-catenin-WT-GFP | 1-781 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-Y654E-GFP | 1-781 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-Y654F-GFP | 1-781 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-R1-12-GFP | 132-695 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-R1-8-GFP | 132-467 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-R3-8-GFP | 218-467 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| β-catenin-R10-12-GFP | 520-664 | GTCGACGGTACCCATGGCTACTCAAGCTGAGTTTGATG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | KpnI and BamHI |
| APC Arm-GFP | 334-900 | GTAGACGGTCCCGAATGCTTGGGATCC | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | Sall And BamHI |
| β-catenin-WT-MBP | 1-781 | ACCTGGGATCCGTTGCTACTGATCAAACCAAG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | BamHI |
| β-catenin-R3-8-MBP | 218-467 | ACCTGGGATCCGTTGCTACTGATCAAACCAAG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | BamHI |
| β-catenin-R10-12-MBP | 520-664 | ACCTGGGATCCGTTGCTACTGATCAAACCAAG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | BamHI |
| β-catenin-R10-12-Rev | 520-664 | ACTGGGATCCGTTGCTACTGATCAAACCAAG | ACCCGTGGATCCCGAGGTCACTGATCAAACCAAG | BamHI |
| Nup-98-GST | 15-512aa | GCCGGATCGATCCCGCTTGGTCAAGCTGATCAAACCAAG | GCCGGATCGATCCCGCTTGGTCAAGCTGATCAAACCAAG | EcoRI and Sall |

**Supplementary Table S2: FRAP kinetic analysis** (using one phase association equation)

| Fragment | Amino acid | Treatment | Direction | $R^2$ | Rate constant(K) | Half-time | Plateau |
|----------|------------|-----------|-----------|-------|-----------------|-----------|---------|
| WT-GFP   | 1-781      | LMB       | Export    | 0.994 | 0.014           | 49.5      | 86.55   |
| R1-12    | 132-695    | LMB       | Export    | 0.996 | 0.017           | 38.52     | 89.8    |
| R3-8     | 218-467    | LMB       | Export    | 0.991 | 0.013           | 51.84     | 54.03   |
| R10-12   | 520-664    | LMB       | Export    | 0.996 | 0.014           | 49.7      | 89.59   |
| APC-arm  | 334-900    | LMB       | Export    | 0.9923| 0.023           | 30.42     | 50.56   |
| WT-GFP   | 1-781      | Untreated | Import    | 0.992 | 0.013           | 51.2      | 95.74   |
| R1-12    | 132-695    | Untreated | Import    | 0.983 | 0.024           | 28.37     | 88.22   |
Supplementary Figure legends:

Supplementary Figure S1: Western Blot showing integrity of β-catenin-GFP fragments. β-catenin-GFP fragments were transfected in NIH 3T3 cells and cell lysates were run on the gel and immunoblotted with anti-GFP antibody.

Supplementary Figure S2: Comparing nuclear export of β-catenin sequences +/- LMB. NIH 3T3 cells were transfected with β-catenin-GFP fragments: (A) WT (1-781aa), (B) Arm repeats R1-12 (132-695aa), (C) Arm repeat R10-12 (520-664aa), (D) Arm repeats R1-8 (132-467aa), (E) Arm repeats R3-8 (218-467aa). Mean recovery curves from untreated cells (dotted line) or LMB treated cells (solid line) were calculated as cytoplasmic to nuclear ratio, which was pre-set to 100% based on pre-bleach values. Each curve represents an average (+ std. dev) of at least 10 cells from 2-4 experiments.

Supplementary Figure S3: Rev Assay confirming CRM1 independent export activity of Arm repeat R10-12. (A) β-catenin fragment Arm repeat R10-12 was cloned into Rev1.4-GFP vector and transfected in T47D cells along with Rev(1.4)-GFP and Rev(1.4)NES3 as controls. 48h post transfection cells were treated with respective drugs for 3h, fixed and stained with Hoechst. Representative cell images are shown. (B) Transfected cells (>150 cells) were scored for nuclear (N), nuclear/cytoplasmic (N/C) or cytoplasmic localization and plotted on a graph. This assay is elsewhere described in detail (Ref. 1), and in brief only constructs with an active export sequence display movement from nucleus to cytoplasm after actinomycin D treatment. LMB had little effect here, except for the Rev NES control.

Supplementary Figure S4: Comparison of nuclear export and import rates of β-catenin sequences. NIH3T3 cells were transfected with β-catenin-GFP fragments: WT (1-781aa), Arm repeats R1-12 (132-695aa), Arm repeats R10-12 (520-664aa) and Arm repeats 3-8 (218-467aa) were subjected to nuclear export or import FRAP and analysed as above. Mean recovery curves (+ sd) from 10 cells from 2-4 experiments after nuclear export (dotted lines) or import (solid line) are shown.
**Supplementary Figure S5: Identification of potential β-catenin Arm R10-12 binding partners.** (A) Purified beads coated with MBP (lane 1) or Arm R10-12-MBP (lane 2) were incubated with total cell lysate from NIH3T3 cells. After extensive washing the beads were run on a 4-20% gradient gel. Marked boxes indicate the bands excised for Mass Spectrometry analysis. (B) List of additional candidate binding partners identified by Mass Spec with relative scores.

**Supplementary Figure S6: Loss of Nup62 and Nup358 does not affect nuclear envelope integrity or GFP diffusion rate at NPC.** (A) NIH 3T3 cells were transfected with control or Nup62 siRNA and immunostained with Nup153 pAb (Abcam). A representative image is shown. Loss of Nup62 did not diminish nuclear rim staining of Nup153. (B) NIH 3T3 cell lysates from cells transfected with control or Nup62 siRNAs (left panel) and Nup358 siRNAs (right panel) were separated by SDS-PAGE and immunoblotted with antibody (Convance). Western blot shows that specific loss of Nup62 does not affect expression of other FG-repeat Nups recognized by the antibody. (C) NIH 3T3 cells transfected with control or Nup358 siRNA and immunostained for mAb414 antibody, revealing that the nuclear envelope remained intact. (D) NIH 3T3 cells transfected with control or Nup358 siRNA and immunostained for Nup62 showing that nuclear envelope is intact. Representative cell images are shown. (E) NIH 3T3 cells transfected with control or Nup62 siRNAs and pEGFP-N1 vector. At 72 h post-transfection cells were subjected to nuclear import FRAP for GFP and the average diffusion curves from 8 cells were plotted on the graph. The loss of Nup62 did not alter GFP diffusion across the NPC. Similar data were seen for Nup358 (not shown).

**Supplementary Figure S7. Knockdown of Nup62 or Nup 358 does not alter the import rate of GFP-importin-beta.** The approach for import FRAP of GFP-importin-beta was similar to that described in legends to Figures 4 and 5. (A) After bleaching GFP-importin-beta nuclear fluorescence its recovery was measured over 360 s in cells transfected with control or Nup62 siRNA. (B) Recovery import curves were plotted and no significant difference observed. (C) Similar FRAP assay was performed in presence of control or Nup358/RanBP2 siRNA and
recovery curve was plotted (D). Again no effect on import. Note that the knockdowns were routinely validated in parallel by Western blot (examples shown in Figures 4 and 5).

**Supplementary Figure S8. Evidence against a role of RanBP3 in nuclear export of dephospho β-catenin.** (A) β-catenin 8E7 mAb recognizes non-specific nuclear protein. NIH 3T3 were transfected with control or β-catenin siRNA and stained with β-catenin 8E7mAb (top panel) or total β-catenin mAb (Transduction Labs) (bottom panel). Phalloidin-FITC was used a control. (B) SW480 cells were transfected with control or RanBP3 siRNA and stained with total β-catenin mAb (Transduction Labs). Representative images are shown suggesting that silencing RanBP3 does not affect localization of β-catenin (top panel). Western Blot to confirm knockdown of RanBP3 (bottom panel). (C) NIH 3T3 cells were co-transfected with β-catenin-S45A-GFP and RFP or RanBP3-RFP plasmids. Cell images showing that β overexpression does not induce export of β-catenin S45A mutant. (D) NIH 3T3 cells were transfected with YFP or RanBP3-YFP. 48 h post transfection cells were treated with 40mM LiCl for 6h and then immunostained with total β-catenin mAb (Transduction Labs). >300 cells (from at least 2 experiments) were scored for a loss of nuclear β-catenin and presented in a graph (bottom panel). Ectopic RanBP3 did not stimulate nuclear export of dephospho-β-catenin.
Supplementary Figure S1

\[ \beta\text{-catenin-GFP fragments} \]

| kDa | WT | R1-12 | R1-8 | R3-8 | R10-12 |
|-----|----|-------|------|------|--------|
| 200 |    |       |      |      |        |
| 115 |    |       |      |      |        |
| 96  |    |       |      |      |        |
| 51  |    |       |      |      |        |
| 37  |    |       |      |      |        |

WB: anti-GFP

WITHDRAWN
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Comparing nuclear export of β-catenin sequences +/- LMB by FRAP

Supplementary Figure S2
Rev(1.4)-GFP export assay confirms activity of β-catenin Arm 10-12 sequence

Supplementary Figure S3
Comparison of nuclear import and export rates of β-catenin sequences

Supplementary Figure S4

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January 28, 2016
Supplementary Figure S5

A

|     | MBP | MBP (R10-12) | Marker (kDa) |
|-----|-----|--------------|--------------|
| 1   |     |              |              |
| 2   |     |              |              |
| 3   | 260 | 160          | 80           |
| 4   |     |              |              |
| 5   |     |              |              |
| 6   |     |              |              |
| 7   | 410 | 80           |              |

R10-12 bands were used to identify binding partners by mass spec.

B

| Binding partners for R10-12                                      | Score |
|---------------------------------------------------------------|-------|
| Myb binding protein 1a (MBB1A)                                | 97    |
| Leucyl-tRNA synthetase                                         | 81    |
| Mini-chromosome maintenance complex 2 (MCM2)                  | 49    |
| cullin-associated and neddylation-dissociated 1 (CAND1)       | 49    |
Loss of Nup62 or Nup358/RanBP2 does not affect nuclear envelope staining or GFP diffusion rate at the NPC

A

control siRNA

Nup62 siRNA

B

siRNA: Nup62 control

RanBP2

Nup358

Nup214

Nup153

Nup62

actin

C

Ctrl siRNA

RanBP2 siRNA

D

E

Effect of Nup62 siRNA on Nuclear Import/ diffusion of GFP

% Recovery Fluorescence

0 20 40 60 80 100 120

50 100 150 200 250 300 350 400

Recovery Time (s)

Supplementary Figure S6
Knockdown of Nup358 or Nup62 has no effect on import rate of GFP-importin-β

A) Nuclear import of importin-β-GFP

|            | Pre-bleach | Bleach  | Post-bleach |
|------------|------------|---------|-------------|
|            | 0s         | 40s     | 80s         | 160s        | 360s       |
| control    |            |         |             |             |            |
| siRNA      |            |         |             |             |            |
| Nup62      |            |         |             |             |            |
| siRNA      |            |         |             |             |            |

B) % fluorescent recovery (N/C ratio)

Time (s)

C) Nuclear import of importin-β-GFP

|            | Pre-bleach | Bleach  | Post-bleach |
|------------|------------|---------|-------------|
|            | 0s         | 40s     | 80s         | 160s        | 360s       |
| control    |            |         |             |             |            |
| siRNA      |            |         |             |             |            |
| Nup358     |            |         |             |             |            |
| siRNA      |            |         |             |             |            |

D) % fluorescent recovery (N/C ratio)

Time (s)

Supplementary Figure S7
Evidence against a role for RanBP3 in nuclear export of dephospho-β-catenin

A 8E7 active β-catenin Mab NIH 3T3 cells

control siRNA

β-catenin siRNA

control siRNA

β-catenin total antibody

Evidence that the 8E7 Mab is β-catenin cross-reacted

C NIH 3T3 RFP

β-catenin (S45A)

GFP

RFP

RFP-RanBP3

D YFP endogenous β-catenin Hoechst

YFP RanBP3-YFP

% cells showing loss of nuclear β-catenin

Supplementary Figure S8

WITHDRAWN January 28, 2016
Specific Armadillo Repeat Sequences Facilitate β-Catenin Nuclear Transport in Live Cells via Direct Binding to Nucleoporins Nup62, Nup153, and RanBP2/Nup358

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