DIRECT UBIQUITINATION OF β-CATENIN BY SIAH-1 AND REGULATION BY THE EXCHANGE FACTOR TBL1

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β-Catenin is a key component of the Wnt signaling pathway that functions as a transcriptional co-activator of Wnt-target genes. Upon UV-induced DNA damage, β-catenin is recruited for poly-ubiquitination and subsequent proteasomal degradation by a unique, p53-induced SCF-like complex (SCF(TBL1)), comprised of Siah-1, Siah-1-interacting protein (SIP), Skp1, Transducin β-like 1 (TBL1) and Adenomatous Polyposis Coli (APC). Given the complexity of the various factors involved and the novelty of ubiquitination of the non-phosphorylated β-catenin substrate, we have investigated Siah-1 mediated ubiquitination of β-catenin in vitro and in cells. Over-expression and purification protocols were developed for each of the SCF(TBL1) proteins, enabling a systematic analysis of β-catenin ubiquitination using an in vitro ubiquitination assay. This study revealed that Siah-1 alone was able to poly-ubiquitinate β-catenin. In addition, TBL1 was shown to play a role in protecting β-catenin from Siah-1 ubiquitination in vitro and from Siah-1-targeted proteasomal degradation in cells. Siah-1 and TBL1 were found to bind to the same armadillo repeat domain of β-catenin, suggesting that poly-ubiquitination of β-catenin is regulated by competition between Siah-1 and TBL1 during Wnt signaling.

β-Catenin is a ubiquitous transcriptional activator in the canonical Wnt signaling pathway involved in cellular processes ranging from embryogenesis, cell proliferation, cell fate and survival to adult stem cell differentiation and oncogenesis (1,2). Upon Wnt stimulation, β-catenin activates T cell factor/Lymphoid enhancer factors (Tcf)/Lef1 initiating the expression of many genes including cyclin D1, c-Myc, Axin2 and vascular endothelial growth factor (VEGF) (1). Mutations in β-catenin and its regulatory factors, such as Adenomatous Polyposis Coli (APC), are associated with increased levels of nuclear β-catenin and in turn, to breast, colorectal, ovarian and other cancers (1,3,4).

Not surprisingly, the level and cellular localization of β-catenin are tightly regulated by a finely tuned balance of post-translational modifications and protein turnover (1,4). The most efficient means to lower the cellular levels of β-catenin is by poly-ubiquitination, leading to degradation in the 26S proteasome. Defects in this protein degradation machinery or mutations in β-catenin that prevent the recognition or processing by this machinery often lead to the stabilization of β-catenin in its oncogenically active state (4).

Poly-ubiquitination involves the serial action of E1, E2 and E3 enzymes, of which the substrate-recruiting E3 ligating enzymes are the most diverse. β-Catenin is recognized and ubiquitinated by a growing number of E3 ligases. Of these, the most well-studied is SCFβ-TrCP, a multi-protein complex that itself is regulated through the canonical Wnt signaling pathway (5,6). In the absence of a Wnt ligand, β-catenin is phosphorylated by Glycogen synthase kinase-3β (GSK3β) and it is the phosphorylated state of the protein that is recognized by SCFβ-TrCP. Under conditions of genotoxic stress, activation of p53 occurs and an additional pathway for β-catenin degradation is initiated. p53 directly induces the expression of Siah-1 and in turn formation of a unique SCF-like complex (SCF(TBL1)) comprised of Siah-1, Siah-1-interacting protein (SIP), Skp1, Transducin β-like 1 (TBL1) and APC (7,8). The physiological significance of Siah-1-targeted degradation of β-catenin is underscored by the discovery that this pathway is directly targeted by the viral oncoprotein latent membrane protein 1 (LMP1) (9). In addition, recent studies identified two drugs, hexachlorophene and isoreserpine, which attenuate the function of β-catenin through activation of Siah-1 and subsequent proteasomal degradation (10,11).
In addition to functioning as part of the SCF(TBL1) complex, Siah-1 alone has been shown to function as an E3 ligase. The Siah-1 RING E2-binding domain is linked to a substrate binding domain that directly recruits, and mediates poly-ubiquitination of many substrates including Deleted in Colorectal Cancer (DCC), Nuclear Co-Repressor (NCoR), c-Myb and synphilin-1 (12-15). The ability of Siah-1 to serve as a simple E3 ligase as well as a component of an SCF-like complex raises the possibility of redundancy in the poly-ubiquitination pathways that lead to degradation of β-catenin.

The involvement of TBL1 in the SCF(TBL1) E3 ligase complex is intriguing. Both TBL1 and its close isoform, TBL1-related protein (TBLR1) have been implicated as exchange factors between nuclear co-activator and corepressor complexes in the regulation of nuclear receptors and transcription factors (16). Moreover, recent evidence shows that TBL1 acts as a co-activator of the Wnt signaling pathway by recruiting β-catenin to the promoter of Wnt target genes and stimulating their expression (17). Thus, TBL1 appears to play a role in both activation and repression of β-catenin activity.

Previous studies by Matsuzawa and coworkers extensively characterized the ubiquitination of non-phosphorylated β-catenin through the action of SCF(TBL1) in cells (8). Here we report a combination of in vitro and cell based assays of β-catenin ubiquitination by SCF(TBL1) and Siah-1 alone. Additionally, we mapped the physical interaction between Siah-1 and β-catenin and analyzed the effect of TBL1 on the Siah-1 mediated ubiquitination of β-catenin. These results highlight the role of TBL1 as a protector of β-catenin activity during Wnt signaling.

Experimental Procedures

Bacterial protein expression and purification. Full length Siah-1 (residues 1-282) was expressed as a His$_6$-maltose binding protein (MBP)-fusion protein. The human cDNA was sub-cloned in pLM302 plasmid (Laura Mizoue, Center for Structural Biology, Vanderbilt University), which contained a 3C-precision protease cleavage site after the MBP tag. The SBD of Siah-1, residues 90-282 was sub-cloned in a pET28a vector (Novagen) with an N-terminal His$_6$-tag containing a thrombin cleavage site. SIP and Skp1 were expressed as His$_6$-constructs from pET28a vectors as previously described (18). UbcH5a was expressed as a His$_6$-fusion construct in a pET15 vector. Full length murine β-catenin (residues 1-781) and four truncation mutant constructs (Nt: residues 1-133), (Nt+arm: residues 1-671), (arm: residues 134-671) and (arm+Ct: residues 1-781) were expressed as glutathione S-transferase (GST)-fusion proteins from pGEX vectors as previously described (19).

The proteins were overexpressed in the Escherichia Coli BL21(DE3) strain. Cells were grown at 37 °C until they reached $A_{600}$ of 0.6-0.8 and were then induced with 0.5 mM isopropyl thiogalactoside (IPTG) for 3-4 hours at 25 °C for Siah-1 constructs, 30 °C for β-catenin constructs and 37 °C for SIP, Skp1 and UbcH5a. Purification of Siah-1, SIP, Skp1 and UbcH5a was performed by Ni-NTA (Qiagen) followed by a Source Q chromatography (18,20). Expression of $^{15}$N-labeled Siah-SBD was carried in minimal medium supplemented with $^{15}$NH$_4$Cl as the sole nitrogen source following the same protocol. β-catenin constructs were affinity purified on Glutathione Sepharose 4B (Amersham) followed by cleavage of the GST-fusion tag, Source Q and size exclusion chromatography, as previously described (19).

Recombinant expression of TBL1 in mammalian 293-6E cells. Full length murine TBL1 (residues 1-526) was sub-cloned into pTT5 vector used for intracellular expression of proteins in mammalian 293 cells (21). N-terminal His$_6$-tag followed by a 3C-precision protease cleavage site was cloned into the XbaI/EcoRI site of pTT5. Then the TBL1 cDNA was inserted into the EcoRI/NotI site of pTT5. Human Embryonic Kidney (HEK) 293-6E cells (Invitrogen) with stably expressing Ebstein-Barr virus nuclear antigen 1 (EBNA1) were grown in suspension in low-calcium-hybridoma serum-free medium (HSFM), under standard conditions at 37 °C and 5% CO$_2$, supplemented with 1% bovine calf serum (BCS), 50 µg ml$^{-1}$ Geneticin, 0.1% Pluronic F-68 (Sigma) and 10 mM HEPES (21). The cells were resuspended in fresh HSFM medium with 1% BCS at a density of 1.0 x 10$^6$ cells ml$^{-1}$ three hours before transfection. TBL1 was transfected using linear Polyethyleneimine (PEI) (Aldrich) into 300 ml of 293-6E cells. 48 hours post transfection, the cell pellet is harvested and stored at -20 °C.

Recombinant His$_6$-TBL1 was affinity purified on a Ni-NTA resin using 25 mM Tris-Cl, pH 7.5, 300 mM NaCl, 5 mM Imidazole, 5 mM β-Mercapto Ethanol (βME) as NiA buffer and 300 mM Imidazole in the NiB buffer. The final yield of purified TBL1 from 293-6E cells is between 4-6 mg/L.

In vitro ubiquitination assay of FL-β-catenin. All ubiquitination experiments were carried out at a final volume of 20 µl including: E1(BostonBiochem) at 52 nM, His$_6$-E2-UbcH5a at 0.6 µM, ubiquitin (BostonBiochem) at 50 µM, His$_6$-MBP-Siah-1 at 0.18 µM, SIP at 1.5 µM,
Skp1 at 1.5 µM, and His6-TBL1 at 1.5 µM. The assay was performed in ubiquitination buffer containing 100 mM NaCl, 1 mM DTT, 5 mM MgCl2 and 25 mM Tris-Cl, pH 7.5 for 0.25 µM β-catenin-FL and (Nt) domain or at pH 8, for β-catenin (arm) and (arm+Ct) constructs. The reactions were activated with 5 mM ATP and incubated for different time periods, as indicated on the specific experiments, at 30 °C. To stop the ubiquitination reaction, the samples were incubated for 15 minutes at 90 °C after the addition of 5 µl SDS-Loading buffer. Reactions were resolved on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen) and detected by a SimplyBlue SafeStain (Invitrogen). Ubiquitination reactions analyzed by Western blotting using C-terminal β-catenin primary antibody (Cell Signaling) and visualized with goat anti-rabbit-horseradish peroxidase (HRP) by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

NMR samples and chemical shift perturbation assay. 15N-1H TROSY HSQC spectra were acquired for 15N-enriched Siah-SBD at 122 μM in 25 mM Tris-Cl, pH 8, 100 mM NaCl and 10 mM βME in 90% H2O/10% D2O. One sample was free Siah-SBD and the second also contained 91.5 µM unlabeled β-catenin-FL. The NMR experiments were performed using a Bruker DRX 800 MHz spectrometer equipped with a CryoProbe. The 15N-1H TROSY HSQC spectra were acquired at 303 K with 128 scans. Data were processed using Topsping 2.0b (Bruker) and analyzed with Sparky (10).

Mapping ubiquitination sites on β-catenin and ubiquitin chain formation by mass spectrometry analysis. Proteins were separated by SDS-PAGE and the gel was stained with SimplyBlue SafeStain (Invitrogen). Individual protein bands were excised, equilibrated in 50 mM NH4HCO3, reduced with DTT (3 mM in 100 mM NH4HCO3, 37 °C for 15 min) and alkylated with iodoacetamide (6 mM in 50 mM NH4HCO3 for 15 min). The gel slice was then dehydrated with acetonitrile and rehydrated with 15 µL 12.5 mM NH4HCO3 containing 0.01 µg/µL modified trypsin (Promega), and trypsin digestion was carried out for >2 hours at 37 °C. Peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid, and dried by vacuum centrifugation and resuspended in 10 µL 0.1% formic acid. LC-MS/MS analysis of the peptides was performed using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 2.0 SR2 instrument control. The peptides were resolved on a fused silica capillary column, 100 µm × 15 cm, packed with C18 resin (Jupiter C18, 5 µm, 300 Å, Phenomenex, Torrance, CA) using a 95 min gradient of increasing acetonitrile with 0.1% formic acid. MS/MS scans were acquired using an isolation width of 2 m/z, and activation time of 30 ms, activation Q of 0.250, and 35% normalized collision energy using 1 microscan and maximum injection time of 100 for each scan. The MS/MS spectra of the peptides were acquired using data-dependent scanning (top-five) with dynamic exclusion (60 sec exclusion, list size = 50, repeat count = 1). Individual MS/MS fragmentation spectra were then searched against the IPI_mouse database (Feb 2008) allowing for complete carbamidomethylation of cysteine, and partial modification by oxidation of methionine. Partial modification of lysine by +114 Da was also selected to detect ubiquitinated peptides (mass shift due to GG ubiquitin sequence that remains after trypsin digestion). All candidate spectra were manually inspected for verification of ubiquitination.

Cell culture, siRNA transfection and Western blot analysis. Human embryonic kidney 293T cells were cultured in DMEM media containing 10% FBS at 37°C in a 5% CO2/95% air atmosphere. For siRNA transfections, 1 × 106 293T cells were seeded into 6-well plates for 12 hours and then transfected with various amount of siRNA using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). Expression vector of Flag-Siah-1 or control empty vector were transfected using Fugene6 (Roche) 24 hours after the transfection with siRNA. Cells were treated, unless otherwise indicated, with 20 mM LiCl. Nuclear and cytosolic extracts were prepared and 5 µg of extract was loaded on SDS-PAGE and followed by Western blot analysis using an enhanced chemiluminescence reagent (Thermo Scientific). siRNAs were synthesized by Dharmacon Research and sequences are used as previously described (17). All antibodies used in the Western blot are commercial available: β-catenin (BD), TBL1 (Abcam), TBLR1 (Bethyl Laboratories), and α-Tubulin (Sata Cruz).

RESULTS

In vitro ubiquitination of β-catenin by the SCF(TBL1) complex. In order to further investigate the molecular basis for β-catenin degradation by the SCF(TBL1) complex, we developed an in vitro ubiquitination assay using purified recombinant proteins. The E2 conjugating enzyme UbcH5a, Skp1, Siah-1, SIP, and the β-catenin substrate were overexpressed and purified from E. coli (18,19,22). A transient expression system was used for the expression of full length
TBL1 in mammalian 293-6E cells (21). The protocols for the in vitro ubiquitination reaction were developed based on previous reports (23), using proteins each purified to >95% homogeneity (Fig. 1A). In order to ensure that the full length β-catenin substrate is properly folded, a circular dichroism (CD) experiment was performed (Fig. S1). The analysis of this spectrum correlates well with the α-helical secondary structure elements observed in the x-ray crystal structure of the protein (24).

The ubiquitination reaction was initiated by addition of ATP after mixing all components and incubated for 60 minutes at 30 °C. Lane 5 in Figure 1B shows the complete reaction with all components of the SCF(TBL1) present. In this reaction, all free β-catenin has been consumed, resulting in formation of a characteristic ubiquitin ladder. Control experiments show no ubiquitination of β-catenin when ubiquitin (lane 2), β-catenin substrate (lane 3) or ATP (lane 4) are excluded from the reactions (Fig. 1B). The absence of Siah-1 also results in no discernable ubiquitination of β-catenin, presumably due to the inability to co-localize the E2-conjugated ubiquitin and the substrate (Fig. 1B, lane 9). In contrast, β-catenin poly-ubiquitination is observed even in the absence of TBL1, Skp1 or SIP (Fig. 1B, lanes 6-8). Together, these results show that the polyubiquitination of β-catenin by SCF(TBL1) can be reconstituted in vitro and only Siah-1 is required for β-catenin poly-ubiquitination.

Siah-1 directly ubiquitinates β-catenin.

The results from the in vitro ubiquitination of β-catenin by the SCF(TBL1) complex were somewhat puzzling given the prevailing view of multi-protein SCF E3 complexes. Ideally, in the absence of any one component, the complex could not assemble and would lose its ubiquitinating activity. Our observation of β-catenin poly-ubiquitination in the absence of TBL1, Skp1 and SIP implied that non-phosphorylated β-catenin can be poly-ubiquitinated by an alternate Siah-1 mediated mechanism. Motivated by reports that Siah-1 serves as a simple E3 ligase and the observation that only Siah-1 is required for the ubiquitination of β-catenin (Fig. 1B, lane 9), a series of in vitro assays were performed to investigate if Siah-1 alone could serve as an E3 ligase for the β-catenin substrate and if so, was the reaction modulated by any of the other SCF(TBL1) proteins.

A time-course of the ubiquitination reaction with Siah-1 alone as an E3 ligase is shown in Figure 2. Substantial mono-ubiquitination of β-catenin is observed within 10 minutes of activation of the reaction and both the extent of ubiquitination and the number of added ubiquitins increase over time (lanes 2-6). No ubiquitination was observed in reactions lacking ubiquitin, Siah-1 or ATP (lanes 7-9). Siah-1 robustly auto-ubiquitinates but as shown in lane 11, the auto-ubiquitination reaction in the absence of β-catenin does not result in the distinctive ladder that is observed in the presence of the substrate (cf. lanes 6 vs. 11). Immunoblotting with β-catenin antibody confirms that the species observed by coomassie staining correspond to modified β-catenin substrate (Fig. 2, bottom panel). The specific pattern of two strong bands of ubiquitinated β-catenin seen in Figure 1 can arise from multiple mono-ubiquitination events. In order to confirm that β-catenin is polyubiquitinated we performed the in vitro ubiquitination reaction using K(0)-ubiquitin (Fig. S2B). This reaction shows a substantial overall decrease in the ladder of K(0)-ubiquitin β-catenin compared to the ladder observed with wt-ubiquitin, confirming that β-catenin is indeed poly-ubiquitinated.

The specificity of Siah-1 for β-catenin in the in vitro ubiquitination assay was confirmed using a substrate Skp1 that does not bind to Siah-1. Figure S2A shows that Skp1 is not ubiquitinated. To further demonstrate that Siah-1 ubiquitination requires physical interaction, the assay was performed using SIP, which is known to directly interact with Siah-1 (18). As anticipated, SIP is efficiently poly-ubiquitinated by Siah-1 (Fig. S2B). These results show that Siah-1 acting on its own as an E3 ligase specifically poly-ubiquitinates β-catenin in vitro, in a phosphorylation-independent manner.

Siah-1 forms K11 ubiquitin chains on β-catenin. In order to determine the specific ubiquitin chain assembled on β-catenin by Siah-1, the ubiquitinated substrate was characterized by tandem mass spectrometry. The products from the standard in vitro ubiquitination reactions were resolved on an SDS-PAGE gel and the second band, labeled ** (Fig. 2, lane 6) was excised, subjected to in-gel trypsin digest, followed by liquid chromatography-mass spectrometry (LC-MS/MS). The strategy involved identifying ubiquitin peptides from the trypsin proteolysis that contain lysine residues with the mass addition of 114.04 Da from the covalently attached ubiquitin signature peptide (-GG) that remains attached after trypsin cleavage. A single modified peptide sequence, K6TLTGK11, from ubiquitin was identified indicating a Lys11 linked ubiquitin chain on β-catenin. A number of studies have already shown that formation of Lys11 ubiquitin chains can target the substrates for degradation in the cell (25,26). These data indicate that Siah-
molecules is below the detection limit and only the amount of highly poly-ubiquitinated β-catenin from the SCF(TBL1) is able to validate the observations. Observed between experiments, which serves to purify stocks. A high reproducibility was obtained, including using proteins from different species. Since the differences between the time courses are modest, the experiments were repeated several times.

In order to obtain further insights into these observations, we compared Siah-1 in vitro ubiquitination assays performed under identical conditions for β-catenin alone and pre-incubated with SCF(TBL1) (Fig. 3). In order to specifically monitor ubiquitination of β-catenin, the reactions were detected by Western blot with β-catenin antibody, as reported previously (27). We note that although species with long poly-ubiquitin chains are indeed generated in the reaction (Fig. 2), the amount of highly poly-ubiquitinated β-catenin molecules is below the detection limit and only the first few bands are observed using this approach.

Interestingly, Siah-1 alone appeared to ubiquitinate β-catenin at a faster rate than the SCF(TBL1) complex. Figure 3 presents a time-course of β-catenin ubiquitination by Siah-1 in the absence (lanes 2-6) and presence (lanes 7-11) of SCF(TBL1). The data show that free β-catenin is depleted by 60 min in the absence of SCF(TBL1), whereas a significant amount of non-ubiquitinated β-catenin still remains when TBL1 is present in the reaction (Fig. 3, lanes 6 vs. lane 11). Furthermore, mono-ubiquitination of β-catenin by Siah-1 alone is observed at the 5 minute point, and two additional bands of ubiquitinated β-catenin also appear at 10 and 15 min (lanes 2-4). In contrast, only one band of ubiquitinated β-catenin is observed at 15 min after pre-incubation with SCF(TBL1) (lane 9). This clearly shows that the formation of ubiquitin chains on β-catenin is delayed in the presence of all SCF(TBL1) proteins. Since the differences between the time courses are modest, the experiments were repeated several times, including using proteins from different purification stocks. A high reproducibility was observed between experiments, which serves to validate the observations.

To further investigate if a single component from the SCF(TBL1) is able to attenuate Siah-1-dependent ubiquitination of β-catenin, the effect of pre-incubating SIP, Skp1 or TBL1 with β-catenin was tested. Lanes 12-16 in Figure 3 show that the addition of TBL1 alone has an effect similar to the SCF(TBL1) complex (lanes 7-11). Together, these results show that TBL1 inhibits the in vitro poly-ubiquitination of β-catenin by Siah-1.

TBL1 inhibits Siah-1-mediated β-catenin degradation in cells. It has previously been shown that expression of Siah-1 correlates with reduced levels of β-catenin in cells (7-9, 28). Overexpression of Siah-1 in the absence of a Wnt ligand reduced levels of β-catenin and lowered the induction of Tcf/Lef-target genes (7,8). It has also been shown that Wnt activation stimulates interaction of TBL1 and TBLR1 with β-catenin (17). In the presence of a Wnt ligand, expression of Siah-1 also decreased the amount of expressed Tcf/Lef-target genes, therefore promoting degradation of β-catenin, but the effect was not as substantial as in the absence of a Wnt ligand (7).

To determine the effect of TBL1/TBLR1 on β-catenin degradation during Wnt signaling we examined the levels of β-catenin in cells. It is important to note that the endogenous level of Siah-1 in cells is extremely low and does not rise to appreciable levels until p53 is activated. Thus, endogenous Siah-1 cannot ordinarily be observed on western blots. In essence, because Siah-1 is not prevalent in quiescent cells, the "normal" condition when Siah-1 is actively involved in regulating β-catenin corresponds only when there is stress to the cells so that Siah-1 is upregulated through activation of p53. Consequently, the standard and now well-accepted approach to study the effects of Siah-1 is via overexpression (7,8, 12-15). In order to study the roles of non-canonical pathways for β-catenin degradation, the basal phosphorylation-dependent poly-ubiquitination of β-catenin by the SCFcomplex must be down regulated. The standard protocol for this is to use LiCl to inhibit GSK-3β kinase, which suppresses the phosphorylation of β-catenin and therefore its ability to be recognized and degraded by the SCFcomplex (3,17).

In our experiments, small interfering RNA (siRNA) was used to knock down the expression of TBL1 and TBLR1 in HEK293T cells, and LiCl is added after transfection of Siah-1 to monitor the effect on degradation of β-catenin. The amount of nuclear and cytoplasmic β-catenin was detected over a period of time by Western blot analysis. More than 80% reduction of TBL1 and TBLR1 expression was achieved in 293T cells by the siRNA (Fig. 4A, bottom panels). Expression of Siah-1 leads to a significant decrease in the level of nuclear β-catenin over the time course of Wnt...
induction when TBL1 and TBLR1 are knocked down. In contrast, the presence of TBL1 and TBLR1 is seen to protect β-catenin from Siah-1-mediated degradation in the nucleus (Fig. 4A). The same effect was observed with cytoplasmic β-catenin, where a significant decrease in the level of β-catenin is seen when TBL1 and TBLR1 are knocked down (Fig. 4B).

In order to ensure that the level of transfected Siah-1 is comparable to previously published data and to the amount of protein expressed upon activation of p53 during DNA damage, we measured the level of Siah-1 after transfection and upon addition of adriamycin (7,8,29). The data confirms that our transfection protocol does not result in gross overexpression of Siah-1, and in fact the level of Siah-1 is similar to the level observed upon activation of p53 (Fig. S5).

Taken together, our results indicate that upon Wnt signaling, TBL1 and TBLR1 serve to protect β-catenin from Siah-1-induced degradation.

Siah-1 ubiquitates β-catenin at lysines outside the TBL1 binding site. The core of β-catenin is an armadillo (arm) repeat domain, which has an elongated α-helical structure that facilitates the interaction of the majority of β-catenin substrates (19,24). The interaction site of TBL1 with β-catenin was previously mapped to the N-terminal region of this (arm) domain, specifically residues 134-467 (17). In order to test if TBL1 directly blocks access to the lysine residues targeted for ubiquitination by Siah-1, mass spectrometry was used to identify the ubiquitination sites on β-catenin (30,31). The standard in vitro ubiquitination reaction was carried out and the reaction products were run on an SDS-PAGE gel (Fig. 5A). Bands 1 and 2 were excised, digested by trypsin and analyzed by LC-MS/MS. Lys666 or Lys671 at the C-terminal (Ct) domain of β-catenin (Ct: residues 665-781) were identified as the predominant sites of ubiquitination (Fig. 6A and 6B).

Efficient poly-ubiquitination and degradation of β-catenin during genotoxic stress is critical to preventing constitutive cell proliferation and preserving genomic stability. Upon UV-induced DNA damage, β-catenin is targeted for poly-ubiquitination and subsequent proteasomal degradation by a p53-induced mechanism that does not require phosphorylation of the substrate (7,8). The critical protein in this pathway is Siah-1, which mediates an efficient depletion of β-catenin, thereby down regulating transcription of Wnt target genes. Siah-1-mediated degradation of β-catenin was initially demonstrated through the formation of an SCF-like complex (SCF(TBL1)), comprised of Siah-1, SIP, Skp1 and TBL1 (8). We established an in vitro ubiquitination assay with reconstituted SCF(TBL1) to investigate the mechanism of action of this complex, but interestingly we found that Siah-1 alone functions as an E3 ligase that is able to directly bind and poly-ubiquitinate β-catenin in vitro (Fig. 2 and 6C).

In addition to demonstrating that poly-ubiquitination of β-catenin can occur through a
direct interaction with Siah-1, we established that Siah-1/UbcH5 assembles Lys11 ubiquitin chains at a novel β-catenin ubiquitination site. We have also found that during Wnt signaling TBL1, a transcriptional co-activator of β-catenin, plays a role in protecting β-catenin from Siah-1-mediated poly-ubiquitination and proteasomal degradation. TBL1 and Siah-1 were found to bind β-catenin at the (arm) domain. The absence of poly-ubiquitination of TBL1 by Siah-1 in the in vitro ubiquitination assay (Fig. S2A) rules out the possibility of TBL1 competing with β-catenin as a substrate for Siah-1. While inhibition of β-catenin ubiquitination via an allosteric binding effect cannot be ruled out, our results support a model in which TBL1 protection of β-catenin from poly-ubiquitination by Siah-1 is due to direct competition for the Siah-1 binding site.

The function of TBL1 as transcriptional co-activator and co-repressor of β-catenin. TBL1 appears to serve two roles in regulating the activity of β-catenin. Besides the initially identified role of TBL1 in recruiting β-catenin to the SCF(TBL1) complex, it has also been shown to function as a transcriptional co-activator of β-catenin in recruiting it to the promoter site of Wnt-target genes (left side Fig. 7) (17). Our results indicated that TBL1 can inhibit the poly-ubiquitination of β-catenin by Siah-1 in vitro (Fig. 3) and stabilize β-catenin in cells by protecting it from Siah-1-mediated ubiquitination and proteasomal degradation (Fig. 4).

We note that the in vitro ubiquitination of β-catenin by Siah-1 is not an extremely efficient reaction. Liu and coworkers showed that APC is required to observe Siah-1-mediated degradation of β-catenin in cells and that APC directly interacts with Siah-1 and with β-catenin (7,34). Our data indicates that Siah-1 binding to β-catenin is very dynamic and weak (Fig. 6C). It is likely that APC serves as a molecular bridge for Siah-1 and β-catenin, thus stabilizing the complex to increase the efficiency of ubiquitin chain formation in cells. The situation in cells would also be different because TBL1 recruits β-catenin to the Tcf/Lef transcription factors, which in effect protects β-catenin from access by Siah-1. This interpretation is supported by the inhibitory effect of TBL1 in the cell based experiments (Fig. 4). Furthermore, our data indicates that TBL1 (TBLR1) forms a complex with β-catenin in the cytoplasm and nucleus, since β-catenin can be protected from Siah-1 induced degradation in both cellular compartments (Fig. 4). Together, our results suggest a model in which down regulation of Wnt target genes during DNA damage involves binding of β-catenin by Siah-1 and poly-ubiquitination of TBL1-unbound, non-phosphorylated β-catenin (Fig. 7). Once a complex between β-catenin and TBL1 (TBR1) is formed upon Wnt stimulation, it binds the Wnt-target gene promoter and transcription factors such as Tef, therefore limiting the ability of Siah-1 to access and poly-ubiquitinate β-catenin. We propose that the activation of Wnt-target genes during genotoxic stress and the regulation of cellular processes such as cell proliferation and apoptosis depend on the balance of Siah-1-mediated degradation of β-catenin versus TBL1 (TBLR1)-facilitated protection and activation of β-catenin targeted genes.

The role of SCF(TBL1) components SIP and Skp1 on β-catenin poly-ubiquitination and degradation. Our results leave open the question as to whether Siah-1 is sufficient for the recruitment and poly-ubiquitination of β-catenin in cells or additional components from the SCF(TBL1) complex are necessary. Siah-1 has a substrate binding domain for recruiting substrates and a RING domain for binding the E2-ubiquitin complex. It has been shown to act alone in poly-ubiquitinating many substrates such as DCC, NCoR and synphilin-1 (12,13,15). Our data also indicates that Siah-1 can function as a simple E3 ligase for poly-ubiquitination of β-catenin. If Siah-1 functions on its own, what is the role of SIP? One possibility is that SIP, like DCC and NCoR, is simply another target of Siah-1 for poly-ubiquitination and degradation. In vitro ubiquitination reactions show an efficient poly-ubiquitination of SIP in the presence or absence of Skp1, TBL1 and β-catenin (Fig. S2B). It has been reported that over-expression of SIP mutants that cannot interact with Siah-1 prevent the efficient degradation of β-catenin in HEK293T cells and the level of SIP is essential for β-catenin degradation in gastric and renal cancer cells (35-37). However, overexpression of only Siah-1 is sufficient for β-catenin degradation in cells and the in vitro ubiquitination of β-catenin is not affected by the addition of SIP (Fig. 4 and S3B) (7,9). It is possible that the interaction between SIP and Siah-1 is important for the proper localization of Siah-1 or for protection of Siah-1 from auto-ubiquitination and degradation.

The role of Skp1 as an adaptor is to provide an anchor in SCF ligases between Cullins, in our case SIP, and the F Box–protein TBL1. Interaction between SIP and Skp1 has been demonstrated by affinity chromatography and NMR chemical shift perturbation experiments (18). However, evidence for an effective interaction between the F Box domain of TBL1 and Skp1 is lacking. A wealth of biochemical and structural data have established that the binding between Skp1 and F Box domains from Skp2 or β-TrCP is
very strong with a half-life of the Skp1-Skp2 complex longer than 9 hours and the surface area of the core interface of more than 2000Å² (38,39). TBL1 has a putative F Box domain that appears to have a very low affinity for Skp1. Previous studies using the *Drosophila* homologue of TBL1, Ebi have been unsuccessful to detect an interaction between Ebi and Skp1 (40). We have also been unable to detect this interaction by pull down experiments with purified proteins, co-expression of Skp1 and TBL1 followed by pull downs and negative gel shift assays. Using the very sensitive NMR chemical shift perturbation assay, we found an extremely weak (K_D ~mM) interaction between Skp1 and TBL1 (1-170) (data not shown). Thus, it is unclear how Skp1 is able to play the role of the adaptor in the SCF(TBL1) complex. Although additional experiments are required to systematically evaluate if the SCF(TBL1) does indeed form in vivo as postulated, the role of Siah-1 in degradation of β-catenin and the modulation of this activity by TBL1 are clear.

The SCF(TBL1) complex closely resembles the *Drosophila* Sina/Phyl/Ebi complex. In *Drosophila*, the transcriptional repressor Ttk88 is recruited and poly-ubiquitinated by the Sina (Siah-1 homologue)/Phyllopod (Phyl) complex for proteasomal degradation (41). Phyl functions as an adaptor of Sina in recruiting different substrates for poly-ubiquitination during *Drosophila* neurogenesis (42). Both Phyl and mammalian SIP contain a Siah-binding motif or a degron sequence that has been crystallized in complex with Siah-SBD, demonstrating an almost identical interaction (43). Ttk88 can directly bind to Sina and Phyl, but it also requires Ebi (a homologue of TBL1) for an efficient poly-ubiquitination and degradation of Ttk88. In contrast to TBL1, which has been shown to directly bind β-catenin, Ebi has been shown to have a strong affinity for Sina and Phyl, but weak and indirect interaction with the substrate Ttk88 (40). The similarity between mammalian and *Drosophila* Siah/Sina-mediated ubiquitin complexes suggests the formation of a Siah-1-mediated E3 ligase that does not resemble the conventional SCF complex. Siah-1 and Sina can directly bind and recruit the substrate for poly-ubiquitination, but they also require the assistance of the adaptor protein SIP/Phyl. Skp1 has been demonstrated to be dispensable to the function of the complex by us and others (40). Most interestingly Ebi does not interact directly with the Ttk88 substrate, but with Sina and Phyl, whereas TBL1 appears to play a dual role in facilitating both activation and degradation of protein substrates.

**Tight regulation of β-catenin by multiple E3 ligases.** The existence of multiple ubiquitination pathways leading to degradation implies the physiological importance of tightly regulating β-catenin. Besides the major E3 ligases SCF^{β-TrCP} and the p53-induced Siah-1, poly-ubiquitination of β-catenin is also initiated by a recently identified single subunit E3 ligase, Jade-1 (44). Interestingly, membrane-associated β-catenin is regulated by a different set of E3 ligases, such as Hakai and the muscle specific Ozz E3 ubiquitin ligase (45,46). Thus the mechanism of recognition, recruitment and poly-ubiquitination of β-catenin by different E3 ligases as well as the signaling event initiating the degradation of the substrate appears to vary substantially. The activation of the SCF^{β-TrCP} depends on the presence or absence of a Wnt ligand, whereas Siah-1 mediated β-catenin degradation is induced by activation of p53 during genotoxic stress. In the SCF^{β-TrCP} complex, β-TrCP binds the phosphorylated N-terminal domain of β-catenin leading to the attachment of ubiquitin chains on Lys19 and Lys49 (47,48). Here, we show that Siah-1 binds the non-phosphorylated β-catenin, leading to the attachment of ubiquitin chains at the very C-terminal region of the (arm) domain on Lys666 and Lys671. Notably, this coincides with the binding surface of most transcription co-activator complexes such as p300/CPB and TRRAP/TIP60 histone acetyltransferases (HATs) and the PAF1 complex (49). Thus poly-ubiquitination of β-catenin by Siah-1 could prevent the transcription of many Wnt target genes. Mutations in β-catenin can lead to inefficient degradation and therefore greater accumulation in the nucleus. The corresponding over activation of Tcf/Lef genes by β-catenin is believed to be responsible for the initiation and progression of many types of cancer (3). Understanding the uniqueness and precise mechanism of action of each E3 ubiquitin ligase targeting β-catenin for degradation and the molecular basis for defective activity of mutants is an important goal for understanding the accumulation of β-catenin in the cell and its relationship to oncogenesis.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Reconstitution of the SCF(TBL1)-like E3 ligase in vitro. (A) Recombinantly expressed and purified proteins: β-catenin, His6-MBP-Siah-1, His6-TBL1, His6-SIP, His6-Skp1, His6-UbcH5a and ubiquitin (BostonBiochem) visualized by a Coomassie-stained SDS-PAGE gel. (B) in vitro ubiquitination of FL-β-catenin with E1, E2-UbcH5a, ubiquitin, Siah-1, SIP, Skp1, and TBL1. Upon activation with ATP the samples were incubated for 60 minutes at 30 °C. The reactions were stopped by the addition of SDS-Loading buffer and boiling for 15 minutes. The samples were resolved on a 4-12% gradient SDS-PAGE gel and visualized by Coomassie staining. Full scale Coomassie stained SDS-PAGE gel is shown in Supplementary materials Figure S2A.

Figure 2. In vitro ubiquitination of β-catenin by Siah-1 E3 ubiquitin ligase. The in vitro ubiquitination of β-catenin by Siah-1 was reconstituted in a 20 µl reaction using E1 (BostonBiochem), ubiquitin, E2-UbcH5a, His6-MBP-Siah-1 (0.1 µM) and β-catenin-FL (0.3 µM). After the addition of ATP the samples were incubated at 30 °C for different time periods. The reactions were terminated with SDS-Loading buffer and boiling for 15 minutes. ¼ of the sample volume from each of the reactions was resolved on a 4-12% gradient SDS-PAGE gel and visualized by Coomassie staining (top panel). The same ubiquitination reaction was detected on a Western blot with β-catenin antibody (Cell Signaling) using the remaining ¼ of the reaction (bottom panel).

Figure 3. TBL1 inhibits Siah-1-mediated poly-ubiquitination of β-catenin. The in vitro ubiquitination assay was performed with E1, E2, ubiquitin and Siah-1. The substrate β-catenin was pre-incubated with SCF(TBL1) or TBL1 for 30 minutes at RT before the rest of the ubiquitination components were added. The reactions were activated by the addition of ATP and incubated at 30 °C for different time periods. The reactions were resolved on an SDS-PAGE gel and detected on a Western blot.
with β-catenin antibody (Cell Signaling). Full scale Coomassie stained SDS-PAGE gel is shown in Figure S4.

**Figure 4.** TBL1/R1 protects β-catenin from Siah-1-mediated poly-ubiquitination and subsequent degradation in vivo. TBL1 and TBLR1 were knocked down in 293T cells with siRNA. 24 hours later the cells were transfected with Siah-1 and treated with 20 mM LiCl. (A) β-catenin levels were detected in the nuclear fraction by immunoblotting. The panels on the left are with transient expression of Siah-1 and on the right are without expression of Siah-1. The levels of transfected Siah-1 in the whole cell extract is shown in the bottom two panels. (B) Detection of β-catenin levels in the cytoplasmic fraction of the cell.

**Figure 5.** Siah-1 poly-ubiquitinates β-catenin on Lysines 666 and 671. (A) The in vitro ubiquitination assay was performed using recombinantly produced proteins as explained above. The reactions were resolved on an SDS-PAGE and stained with Coomassie blue. Bands 1 and 2 from lane 2 were excised from the gel, subjected to trypsin digestion and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS). (B) Summary of the peptides identified from β-catenin and ubiquitin in each of the analyzed bands shown in A. The specific Lysine residues modified with the –GG signature peptide from ubiquitin is shown in bold. (C) Schematic presentation of β-catenin domains indicating the position of Lys666 and Lys671 ubiquitinated by Siah-1 and previously mapped TBL1 and Siah-1 binding sites.

**Figure 6.** The armadillo (arm) repeat domain of β-catenin is necessary and sufficient for binding and poly-ubiquitination by Siah-1. (A) Schematic diagram of β-catenin single and tandem domains tested for Siah-1 poly-ubiquitination in (B) by in vitro ubiquitination assay and a summary of the ubiquitination results for each of the constructs tested. (B) The in vitro ubiquitination assay was performed with purified recombinant β-catenin FL, (Nt+arm), (arm+Ct), (Ct) and (Nt) domain as a substrate in addition to the ubiquitination machinery: E1, E2, Siah-1, ubiquitin and ATP. The ubiquitination reaction is visualized by Coomassie blue. (C) 15N-1H NMR chemical shift perturbation assay confirming the interaction between Siah-1 and β-catenin. Overlay of TROSY-HSQC spectra of 15N-labeled Siah-SBD (90-282) acquired in the absence (gray) and presence (red) of β-catenin-FL. The experiment was collected at pH 8 and 30 °C with 15N-Siah-SBD at 122 µM, and β-catenin added to 91.5 µM.

**Figure 7.** Model of the mechanism of TBL1-mediated activation and Siah-1-induced poly-ubiquitination of β-catenin. (A) During Wnt activation, the degradation of β-catenin is inhibited leading to its accumulation in the cytoplasm and translocation to the nucleus. TBL1/TBLR1 form a complex with β-catenin and mutually co-localize to the promoter site of Wnt-target genes, stimulating their transcription. (B) Upon DNA damage during Wnt signaling, Siah-1 targets free β-catenin in the cytoplasm and nucleus for poly-ubiquitination and proteasomal degradation, resulting in down regulation of Wnt-target gene transcription.
Figure 1.

A. 

B. 

| FL-β-catenin | E1/UbcH5a | Ubiquitin | Siah-1 | TBL1 | Skp1 | SIP | Skp1 | Ubch5a | ATP | Time (min) |
|--------------|-----------|-----------|--------|------|------|-----|------|--------|-----|------------|
| +            | -         | -         | +      | -    | +    | -   | +    | -      | +   | 60         |
| +            | +         | -         | +      | -    | +    | +   | +    | -      | +   | 60         |
| -            | -         | +         | +      | -    | +    | +   | +    | -      | +   | 60         |
| -            | -         | -         | +      | -    | +    | +   | +    | -      | +   | 60         |
| +            | -         | +         | +      | -    | +    | +   | +    | -      | +   | 60         |
| +            | -         | +         | +      | -    | +    | +   | +    | -      | +   | 60         |
| -            | -         | +         | +      | -    | +    | +   | +    | -      | +   | 60         |
| -            | -         | -         | +      | -    | +    | +   | +    | -      | +   | 60         |

Lane 1 2 3 4 5 6 7 8 9

188kDa 98 62 49 38 28 17 14 6 3
**Figure 2.**

| FL-β-catenin | + | + | + | + | + | + | + | - |
| E1/UbcH5a    | + | + | + | + | + | + | + | - | + |
| ubiquitin    | + | + | + | + | + | - | + | - | + |
| Siah-1       | + | + | + | + | + | - | + | - | + |
| ATP          | + | + | + | + | + | - | - | - | + |
| Time (min)   | 0 | 10 | 15 | 30 | 45 | 60 | 60 | 60 | 60 |

Lane #: 1 2 3 4 5 6 7 8 9 10 11

---

**Commassie stained SDS-PAGE gel**

- β-catenin
- β-catenin-(ub)_n
- β-catenin-(ub)_n

**WB: β-catenin Ab**

- β-catenin
- β-catenin
- β-catenin

188 kDa

98

62
Figure 3.

| FL-β-catenin | + | + | + | + | + | + | + | + | + | + |
|--------------|---|---|---|---|---|---|---|---|---|---|
| E1/UbcH5a   | + | + | + | + | + | + | + | + | + | + |
| ubiquitin    | + | + | + | + | + | + | + | + | + | + |
| Siah-1      | + | + | + | + | + | + | + | + | + | + |
| TBL1        | - | - | - | - | - | + | + | + | + | + |
| SIP, Skp1   | - | - | - | - | - | + | + | + | + | + |
| ATP         | - | + | + | + | + | + | + | + | + | + |
| Time (min)  | 5 | 10| 15| 30| 60| 5 | 10| 15| 30| 60|

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

WB: β-catenin Ab
Figure 4.

### A. siRNA Control TBL1/TBLR1 siRNA Control TBL1/TBLR1

| LiCl (min) | 0 | 100 | 200 | 300 | 0 | 100 | 200 | 300 |
|------------|---|-----|-----|-----|---|-----|-----|-----|
| Siah-1     | + | +   | +   | +   | + | +   | +   | +   |

| 100 kD     | 75 |
|------------|----|
| 37         |    |

| 50         |    |
|------------|----|
| 50         |    |

| 50         |    |
|------------|----|
| 37         |    |

### B. siRNA Control TBL1/TBLR1 siRNA Control TBL1/TBLR1

| LiCl (min) | 0 | 100 | 200 | 300 | 0 | 100 | 200 | 300 |
|------------|---|-----|-----|-----|---|-----|-----|-----|
| Siah-1     | + | +   | +   | +   | + | +   | +   | +   |

| 100 kD     | 75 |
|------------|----|

| 50         |    |
|------------|----|

| 50         |    |
|------------|----|

| 50         |    |
|------------|----|
| 37         |    |
Figure 5.

A. FL-β-catenin + +
E1/UbcH5a - +
ubiquitin - +
Siah-1 - +
ATP - +
Time (min) 90 90
Lane # 1 2

B.

| Protein | Residues | Peptide sequence | Bands |
|---------|----------|-----------------|-------|
| β-catenin | 662-671 | R.MSEDK666PQDYK.K | 1, 2 |
| β-catenin | 666-672 | R.MSEDK666PQDYK671K.R | 1, 2 |

C.

N-term

| Peptide sequence | Bands |
|-----------------|-------|
| 133 TBL1 footprint | 467 |
| 133 Siah-1 footprint | 671 |
Figure 6.

A. Domains

| Domain | N-term | Armadillo | C-term | Ubiquitination | Construct |
|--------|--------|-----------|--------|---------------|-----------|
| Nt-Arm-Ct | 1 133 |           | 671 781 | Yes           | 1-781     |
| Nt-Arm-Ct | 1 133 |           | 671 781 | No            | 1-133     |
| Nt-Arm-Ct | 1 133 | 134 671 | 781    | Yes           | 1-671     |
| Nt-Arm-Ct | 1 133 | 134 671 | 781    | Yes           | 134-671   |
| Nt-Arm-Ct | 1 133 | 671 781 | N.D. 671-781 | Yes | 134-781     |

B. β-catenin

|     | FL | (Nt+arm) | (arm+Ct) | (arm) | (Nt) |
|-----|----|----------|----------|-------|------|
| E1/UbCH5 | -  + | -  + | -  + | -  + | -  + |
| ubiquitin | -  + | -  + | -  + | -  + | -  + |
| Siah-1 | -  + | -  + | -  + | -  + | -  + |
| ATP | -  + | -  + | -  + | -  + | -  + |
| Lane # | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 | 98 62 49 38 28 17 14 |
Figure 7.
Direct ubiquitination of β-catenin by siah-1 and regulation by the exchange factor TBL1
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Hee-Jung Choi, William I. Weis, Cun-Yu Wang and Walter J. Chazin
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Additions and Corrections

Direct ubiquitination of β-catenin by Siah-1 and regulation by the exchange factor TBL1
Yoana N. Dimitrova, Jiong Li, Young-Tae Lee, Jessica Rios-Esteves, David B. Friedman, Hee-Jung Choi, William I. Weis, Cun-Yu Wang, and Walter J. Chazin
The Acknowledgment section was inadvertently deleted from the article. It should read as follows:

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Gene deletion of 7,8-linoleate diol synthase of the rice blast fungus. STUDIES ON PATHOGENICITY, STEREOCHEMISTRY, AND OXYGENATION MECHANISMS.
Fredrik Jernerén, Ane Sesma, Marina Franceschetti, Mats Hamberg, and Ernst H. Oliw
Dr. Franceschetti’s name was misspelled. The correct spelling is shown above.

Agonist-selective dynamic compartmentalization of human mu opioid receptor as revealed by resolutive FRAP analysis.
Aude Saulière-Nzeh Ndong, Claire Millot, Maithé Corbani, Serge Mazères, André Lopez, and Laurence Salomé
Dr. Saulière-Nzeh Ndong’s name was misprinted. The author’s name is shown correctly above.

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