H-Ras is degraded by Wnt/β-catenin signaling via β-TrCP-mediated polyubiquitylation

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Accepted 21 November 2008
Journal of Cell Science 122, 842-848 Published by The Company of Biologists 2009
doi:10.1242/jcs.040493

Summary

Ras is an important proto-protein that is regulated primarily by GDP/GTP exchange. Here, we report a novel regulatory mechanism whereby turnover of both endogenous and overexpressed H-Ras protein is controlled by β-TrCP-mediated ubiquitylation, proteasomal degradation and the Wnt/β-catenin signaling pathway. The interaction of H-Ras with the WD40 domain of β-TrCP targeted H-Ras for polyubiquitylation and degradation. This process was stimulated by Axin or adenomatous polyposis coli (Apc), and was inhibited by Wnt3a. Ras-mediated cellular transformation was also inhibited by the expression of β-TrCP and/or Axin. In vivo regulation of Ras stability by Wnt/β-catenin signaling was determined via measurements of the status of Ras in the intestines of mice stimulated with recombinant Wnt3a by intravenous tail vein injection. The regulation of Ras stability by Wnt/β-catenin signaling provides a mechanical basis for crosstalk between the Wnt/β-catenin and the Ras-ERK pathways involved in transformation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/6/842/DC1

Key words: Ras, β-TrCP, Polyubiquitylation, Tumor, Wnt

Journal of Cell Science

Introduction

The Ras proteins are small guanine nucleotide-binding proteins whose activity is modulated by GDP/GTP exchange (Vetter and Wittinghofer, 2001). Aberrant activation of Ras by non-hydrolyzable GTP-binding mutations is a major cause of human cancers (Bos, 1989). The H-, N- and K-Ras isoforms share high sequence similarities and exhibit diversity mainly in the C-terminal hypervariable regions, which are responsible for functional specificities (Wennerberg et al., 2005). Ras activity is regulated by several mechanisms, including guanine nucleotide exchange by upstream signaling (Vetter and Wittinghofer, 2001) as well as lipid-based modifications, which are required for membrane translocation and functionality (Schubbert et al., 2007). Ras protein trafficking is also affected by mono- and di-ubiquitylation (Jura et al., 2006); however, the mechanisms that control such ubiquitylation and the physiological outcome of Ras ubiquitylation are not known.

In this study, we report a novel polyubiquitylation-dependent H-Ras degradation mechanism that is regulated by Wnt/β-catenin signaling. We found that the F-box protein β-transducin repeat-containing protein (β-TrCP), which functions as a substrate recognition subunit of the SCF-β-TrCP E3 ubiquitin (Ub) ligase (Willems et al., 2004), mediates H-Ras degradation. We also found that Axin and adenomatous polyposis coli (Apc), the negative regulators of Wnt/β-catenin signaling, increased degradation and polyubiquitylation of H-Ras by enhancing the β-TrCP-H-Ras interaction. The regulation of H-Ras stability by Wnt/β-catenin signaling controls cellular proliferation and transformation (Park et al., 2006; Jeon et al., 2007). β-TrCP and Axin synergistically affected cellular transformation, suggesting that these proteins are crucial signaling intermediates facilitating Wnt/β-catenin control of H-Ras stability and cellular transformation. Finally, in vivo Ras regulation by Wnt/β-catenin signaling was further revealed by measuring Ras regulation in the intestines of intravenously Wnt3a-injected mice.

In summary, Wnt/β-catenin signaling regulates Ras protein stability, which in turn directly affects transformation. In addition, the regulation of Ras stability by Wnt/β-catenin signaling is controlled by β-TrCP-mediated polyubiquitylation and degradation via the proteasomal machinery. The regulation of Ras protein stability via polyubiquitylation is therefore one of multiple Ras regulatory mechanisms, including GTP/GDP exchange and lipid modification, that regulate Ras stability, activity, sub-cellular localization and cellular transformation.

Results

Both endogenous and overexpressed H-Ras proteins are regulated by polyubiquitylation and proteasomal degradation

Our previous observations that Axin and Apc destabilize Ras (Park et al., 2006; Jeon et al., 2007) led us to investigate a mechanism for Ras degradation. The half-lives of both Ras and β-catenin were ~9 hours in HEK293 cells in which new protein synthesis was inhibited (Fig. 1A, upper panel). The half-life of Ras was significantly prolonged in the presence of the proteasomal inhibitor *N*-acetyl-Leu-Leu-Nle-CHO (ALLN) (Fig. 1A, upper panel). The endogenous Ras and β-catenin proteins were increased by ALLN treatment in a time-dependent manner (Fig. 1A, lower panel). Both overexpressed (Fig. 1B) and endogenous (Fig. 1C) H-Ras were polyubiquitylated, and these proteins accumulated in ALLN-treated cells as β-catenin did. Interestingly, many components of the proteasomal protein degradation machinery, as well as ubiquitin, were identified as H-Ras-interacting proteins by proteomic analysis of proteins co-immunoprecipitated with H-Ras in cell extracts obtained form ALLN-treated HEK293 cells (supplementary material

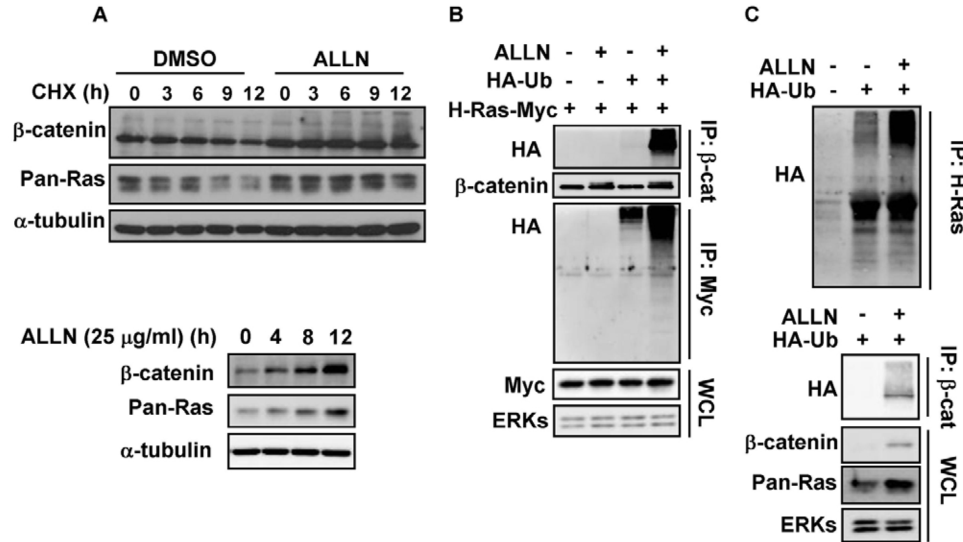

Fig. 1. Both endogenous and overexpressed Ras are polyubiquitylated and degraded by the proteasome. (A) Upper panel: HEK293 cells were treated with cycloheximide (CHX) for the indicated times (0, 3, 6, 9 or 12 hours) with or without ALLN. Lower panel: HEK293 cells were treated with ALLN for 0, 4, 8 or 12 hours. Whole cell lysates (WCLs) were subjected to immunoblotting using indicated antibodies. (B) HEK293 cells were transfected with plasmids expressing H-Ras-Myc and HA-Ub. At 24 hours after transfection, cells were treated with ALLN for 12 hours before harvest. After lysis, immunoprecipitation was performed with anti-β-catenin or anti-Myc antibody. The ubiquitylation of either H-Ras or β-catenin was detected by immunoblotting with anti-HA antibody. WCLs were analyzed by immunoblotting for indicated proteins. (C) HEK293 cells were transfected with plasmid expressing HA-Ub. At 24 hours after transfection, cells were treated with ALLN for 12 hours. After lysis, immunoprecipitation was performed with anti-H-Ras or anti-β-catenin antibody. The polyubiquitylation of H-Ras or β-catenin was detected by anti-HA antibody. β-Catenin, Pan-Ras and ERK proteins were detected in WCLs.

Fig. S1). These results demonstrate that polyubiquitylated H-Ras is subject to proteasomal degradation.

β-TrCP regulates the polyubiquitylation and stability of both overexpressed and endogenous H-Ras

The F-box protein β-TrCP was tested as a potential candidate for mediating Ras recognition of the E3 ligase complex owing to the involvement of β-catenin in the regulation of Ras stability (Jeon et al., 2007). Endogenous Ras protein was decreased by β-TrCP overexpression; however, this decrease was recovered following co-treatment with ALLN (Fig. 2A, left panel). β-TrCP overexpression produced a dose-dependent decrease in ectopic H-Ras expression, whereas the effect of β-TrCP knockdown by siRNA yielded the opposite trend (Fig. 2A, right panel). The decreases in the active

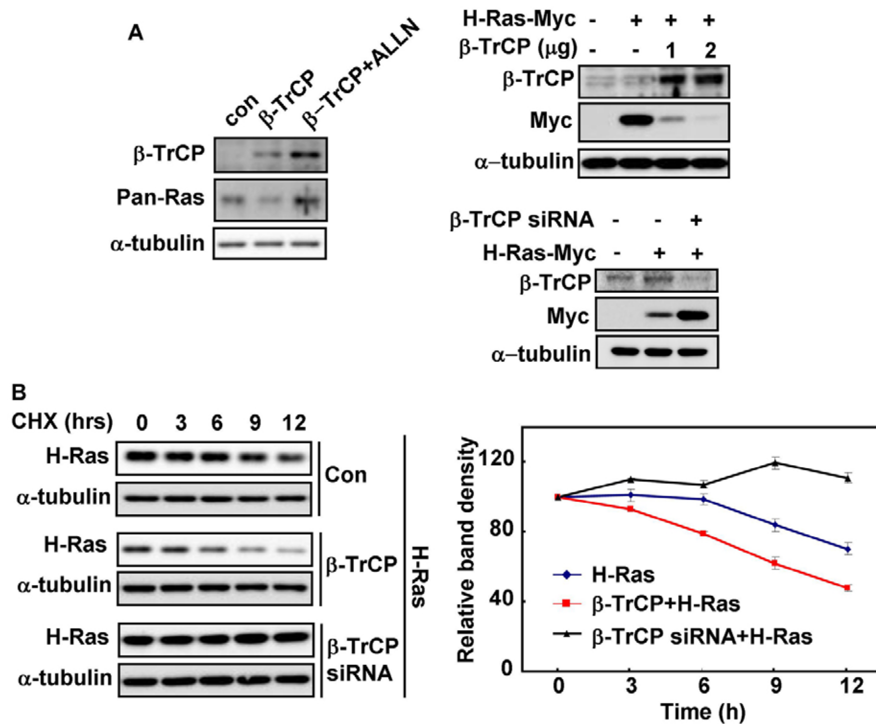

Fig. 2. The β-TrCP degrades H-Ras. (A) HEK293 cells were transfected with H-Ras-Myc or Flag-β-TrCP expression plasmids or β-TrCP siRNA. Where indicated, cells were treated with ALLN for 12 hours before harvest. At 36 hours after transfection, cells were harvested and whole-cell lysates (WCLs) were subjected to immunoblotting. (B) HEK293 cells were transfected with H-Ras-Myc or Flag-β-TrCP expression plasmids or β-TrCP siRNA. At 24 hours after transfection, cells were treated with CHX for the indicated times (0, 3, 6, 9 and 12 hours) and harvested. WCLs were subjected to immunoblotting. Quantification of band densities is shown in the right panel. Error bars indicate standard deviation.

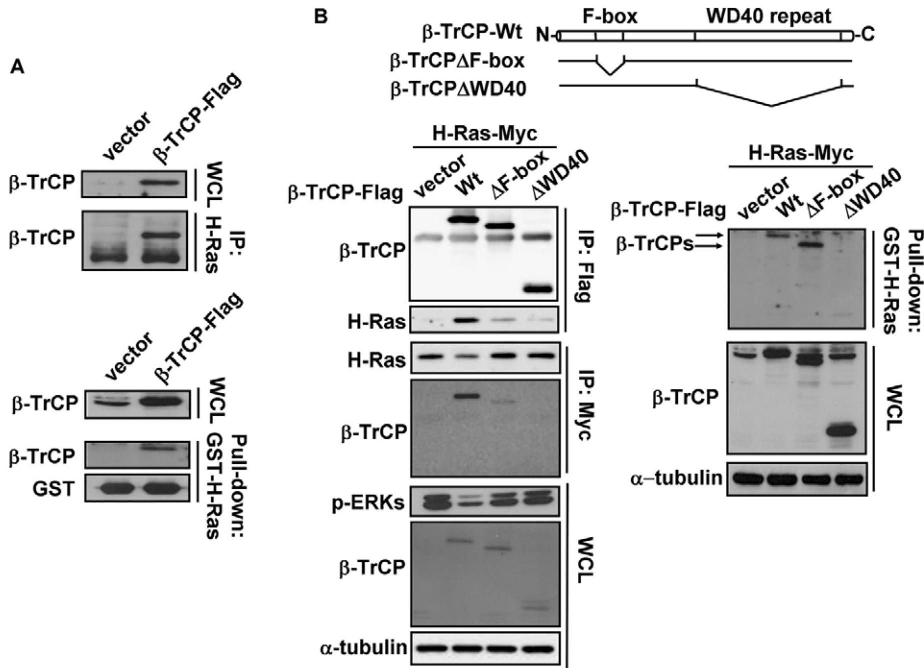

Fig. 3. β-TrCP interacts with H-Ras via its WD40 domain. (A) HEK293 cells were transfected with either vector or Flag-β-TrCP for 36 hours. Immunoprecipitation or pull-down analyses were performed with anti-H-Ras antibody or recombinant GST-H-Ras protein. β-TrCP and GST-H-Ras were detected by immunoblot analyses. (B) Schematic representation of the primary structure of the wild-type and mutant β-TrCP proteins is shown in the upper panel. Lower panels: HEK293 cells were transfected with vector or Flag-β-TrCP (Wt, ΔF-box or ΔWD40) plasmids together with H-Ras-Myc expression plasmid. Cell lysates were immunoprecipitated with anti-Flag or anti-Myc resin (left), or pulled down with GST-H-Ras recombinant protein (right) followed by immunodetection. β-TrCP, H-Ras, p-ERKs and α-tubulin were detected in the whole-cell lysates (WCLs) by western blotting.

form of H-Ras/endogenous Pan-Ras stability and ERK activity by β-TrCP overexpression were also observed in other cell types such as Chang and SW480 cells (supplementary material Fig. S2). H-Ras mRNA levels were not reduced by β-TrCP overexpression (data not shown), which suggested that H-Ras regulation by β-TrCP occurred at the level of protein stability. The protein turnover rate of H-Ras was decreased by overexpression of β-TrCP and was increased by knockdown of β-TrCP ($t_{1/2}$ values were 3-6 hours and greater than 12 hours, respectively) (Fig. 2B). An interaction between β-TrCP and H-Ras was detectable by co-immunoprecipitation and in vitro GST pull-down analyses (Fig. 3A), but an interaction between H-Ras and a β-TrCP homolog, FBW8, was not detected (supplementary material Fig. S3). The β-TrCP-H-Ras interaction was further characterized by co-immunoprecipitation and pull-down analyses using β-TrCP-deletion mutants missing either the F-box (ΔF-box-β-TrCP) that is required for Skp1 binding (Feldman et al., 1997) or the WD40 domains (ΔWD40-β-TrCP) that are required for substrate binding (Fig. 3B). H-Ras protein interacted with wild-type (Wt) β-TrCP, but this interaction was barely detectable with ΔWD40-β-TrCP (Fig. 3B, lower left panel). Furthermore, the loss of H-Ras-binding capacity with ΔWD40-β-TrCP was clearly confirmed by in vitro pull-down analyses using GST-H-Ras (Fig. 3B, lower right panel). The ΔF-box-β-TrCP mutant bound to H-Ras but with reduced affinity in co-immunoprecipitation experiments (Fig. 3B, lower left panel); however, we observed good binding affinity of the ΔF-box-β-TrCP mutant with H-Ras in the GST-H-Ras pull-down analysis, indicating that the F-box is not required for H-Ras binding to β-TrCP (Fig. 3B, lower right panel). Although Wt-β-TrCP expression reduced H-Ras protein levels, this degradation was not observed in cells transfected with either the ΔF-box-β-TrCP or ΔWD40-β-TrCP mutant (Fig. 3B, lower left panel). The ERK activities in whole-cell lysates, which were detected by phosphorylation of ERKs in the activating residues (p-ERKs), were proportional to the levels of Ras (Fig. 3B, lower left panel). Both endogenous and overexpressed H-Ras proteins were polyubiquitylated by β-TrCP overexpression (Fig. 4A,B). β-TrCP-mediated destabilization of H-Ras was inhibited following ALLN treatment, and we detected an increased accumulation of ubiquitylated H-Ras in cells overexpressing β-TrCP (Fig. 4A,B). Consistent with this result, knockdown of β-TrCP expression significantly decreased H-Ras ubiquitylation (Fig. 4C). Moreover, H-Ras ubiquitylation was not enhanced by ΔF-box-β-TrCP overexpression (Fig. 4D). In summary, β-TrCP mediates the polyubiquitin-dependent degradation of H-Ras.

Ubiquitylation and degradation of H-Ras are regulated by Wnt/β-catenin signaling via modulation of binding affinity of β-TrCP with H-Ras

We have previously observed Ras destabilization by Axin (Jeon et al., 2007) and Apc (Park et al., 2006), which are negative regulators of the Wnt/β-catenin pathway. Axin overexpression reduced Ras levels and ERK activities in multiple cell types (Jeon et al., 2007). Moreover, Axin regulates Ras proteins regardless of the mutational status of Ras (Jeon et al., 2007) (supplementary material Fig. S4). Here, we demonstrate that overexpression of Axin or Apc increased polyubiquitylation and subsequently decreased the protein levels of both endogenous and overexpressed H-Ras (Fig. 5A). By contrast, a decrease of polyubiquitylation and a concomitant increase in the H-Ras protein level were observed following siRNA-mediated knockdown of Axin (supplementary material Fig. S5). Moreover, co-expression of Axin or Apc with β-TrCP further enhanced H-Ras ubiquitylation via enhancement of the interaction between β-TrCP and H-Ras (Fig. 5B). However, the effect of Axin on the enhancement of the binding affinity of β-TrCP and H-Ras was more significant than that of Apc, and this difference correlated with the degree of H-Ras ubiquitylation (Fig. 5B). The important role of β-TrCP in the regulation of Ras ubiquitylation by the negative Wnt/β-catenin signaling was confirmed by blockade of the Apc-mediated ubiquitylation of H-Ras by β-TrCP siRNA (Fig. 5C).

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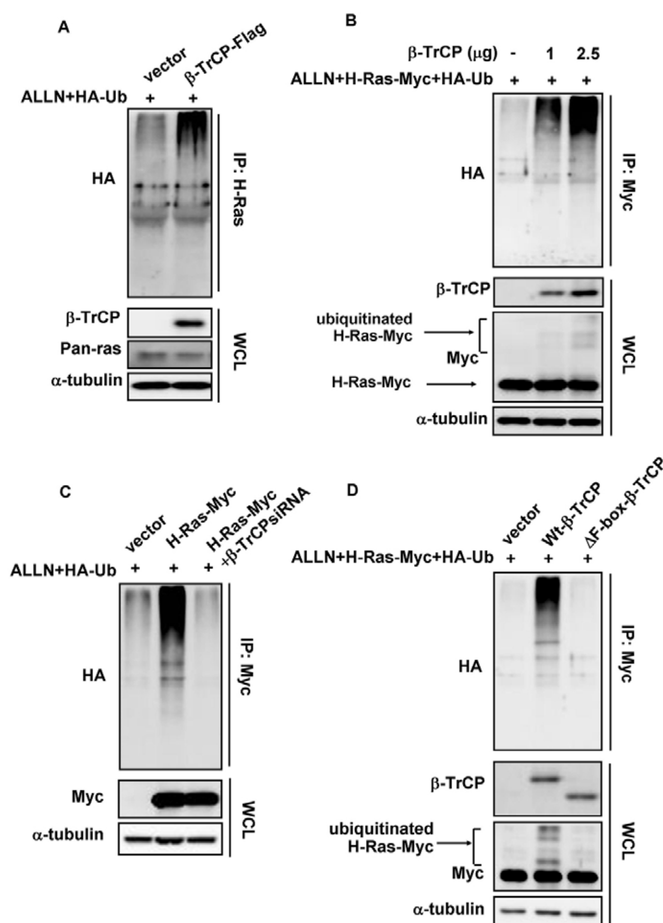

Fig. 4. β-TrCP polyubiquitylates H-Ras. (A-D) HEK293 cells were transfected with vector, H-Ras-Myc, Flag-β-TrCP (Wt or ΔF-box) and HA-Ub expression plasmids and/or β-TrCP siRNA. At 24 hours after transfection, cells were treated with ALLN, and lysates were immunoprecipitated with anti-H-Ras antibody or anti-Myc resin followed by immunoblotting. β-TrCP, Pan-Ras, Myc-H-Ras and α-tubulin were detected in whole-cell lysates (WCLs) by immunoblotting.

To confirm that Wnt/β-catenin signaling stabilizes H-Ras by reducing its ubiquitylation, we assessed the effects of cell exposure to extracellular recombinant Wnt3a. As expected, polyubiquitylation of H-Ras was reduced by Wnt3a treatment but not by epidermal growth factor (EGF) treatment (Fig. 5D). In addition, consistent with our model, Wnt3a treatment increased the total amount of H-Ras protein (Fig. 5E). The binding affinity of β-TrCP to H-Ras, however, was significantly reduced by Wnt3a treatment (Fig. 5E). Two lines of evidence demonstrate that Wnt/β-catenin signaling can inhibit the polyubiquitylation-mediated degradation of H-Ras. First, Wnt3a treatment decreased the ubiquitylation and increased the level of H-Ras protein (Fig. 5D,E). Second, Axin and Apc enhanced β-TrCP-mediated ubiquitylation and degradation of H-Ras (Fig. 5B). A closer examination of the mechanism behind these effects revealed that Wnt/β-catenin signaling modulated the affinity of β-TrCP for H-Ras. Specifically, β-TrCP-H-Ras binding affinity was downregulated by Wnt3a and upregulated by Axin or Apc (Fig. 5B,E). Overall, Wnt/β-catenin signaling modulated the binding of β-TrCP to H-Ras, thereby regulating ubiquitylation and proteasomal degradation of H-Ras.

β-TrCP inhibits H-Ras-mediated cellular transformation

Apc and Axin regulate EGF- and Ras-induced cellular proliferation by regulating Ras protein stability (Park et al., 2006; Jeon et al., 2007). To determine whether β-TrCP-mediated destabilization of H-Ras inhibits cellular transformation, we assessed the effects of β-TrCP on anchorage-independent cell growth by measuring foci formation. Foci formation of DLD-1 colorectal cancer cells following transfection of Wt-*H-Ras* was significantly reduced by β-TrCP expression (Fig. 6A). Inhibition of foci formation by β-TrCP was further enhanced by Axin co-expression (Fig. 6B). β-TrCP and Axin synergistically affected cellular transformation via polyubiquitylation and destabilization of H-Ras, suggesting that these proteins are crucial signaling intermediates that facilitate Wnt/β-catenin control of H-Ras stability and cellular transformation.

Ras levels are upregulated in the intestines of mice intravenously injected with recombinant Wnt3a

To characterize the in vivo Ras regulation by Wnt/β-catenin signaling, 12-week-old FVB mice were intravenously injected with PBS or Wnt3a for 12 hours. β-Catenin and Pan-Ras, as well as p-ERK, were concomitantly increased in the colon tissues of mice treated with Wnt3a (Fig. 7A). The intensity of the polyubiquitylation of Ras was low, although the Pan-Ras protein levels were significantly higher in the colonic tissue stimulated with Wnt3a (Fig. 7B). Both β-catenin and Pan-Ras were localized at the membrane, and its level was upregulated and colocalized at the nucleus and cytosol, as well as at the membrane of the villi of the Wnt3a-stimulated colon tissues when visualized by immunofluorescence staining (Fig. 7C-J).

Discussion

Ras regulates cell proliferation via multiple crucial signaling pathways, including the ERK and PI3 kinase-Akt pathways (Katz and McCormick, 1997). Ras is also a well-characterized proto-oncogene, and its various mutations contribute to transformation and tumorigenesis in a wide variety of human cancers (Herrmann, 2003; Hingorani and Tuveson, 2003; Repasky et al., 2004). The direct regulation of Ras activity itself, however, is not fully understood. Ras activity is known to be regulated by GDP/GTP exchange as well as by lipid modifications that are required for membrane localization (Schubbert et al., 2007). The regulation of Ras protein stability via polyubiquitylation is a newly discovered Ras regulatory mechanism involved in the regulation of cellular transformation.

We demonstrated that H-Ras is targeted for polyubiquitylation by β-TrCP and then subsequently degraded by the proteasomal degradation machinery. Two main lines of evidence indicate that Wnt/β-catenin signaling regulates the polyubiquitylation-mediated degradation of H-Ras. First, Wnt3a treatment decreased H-Ras ubiquitylation and, second, Axin and Apc enhanced β-TrCP-mediated ubiquitylation and degradation of H-Ras. Closer examination of the mechanism behind these effects revealed that Wnt/β-catenin signaling reduced the affinity of β-TrCP for H-Ras. Specifically, β-TrCP-H-Ras binding affinity was downregulated by Wnt3a and upregulated by Axin/Apc. The role of Wnt/β-catenin signaling in the modulation of affinity of β-TrCP binding to H-Ras was supported by a strong correlation between the levels of ubiquitylated H-Ras and β-TrCP that interacted with H-Ras. The weak effect of Apc on the β-TrCP-H-Ras binding affinity compared with that of Axin is correlated with the degrees of H-Ras polyubiquitylation enhancement by β-TrCP. Therefore, Wnt/β-

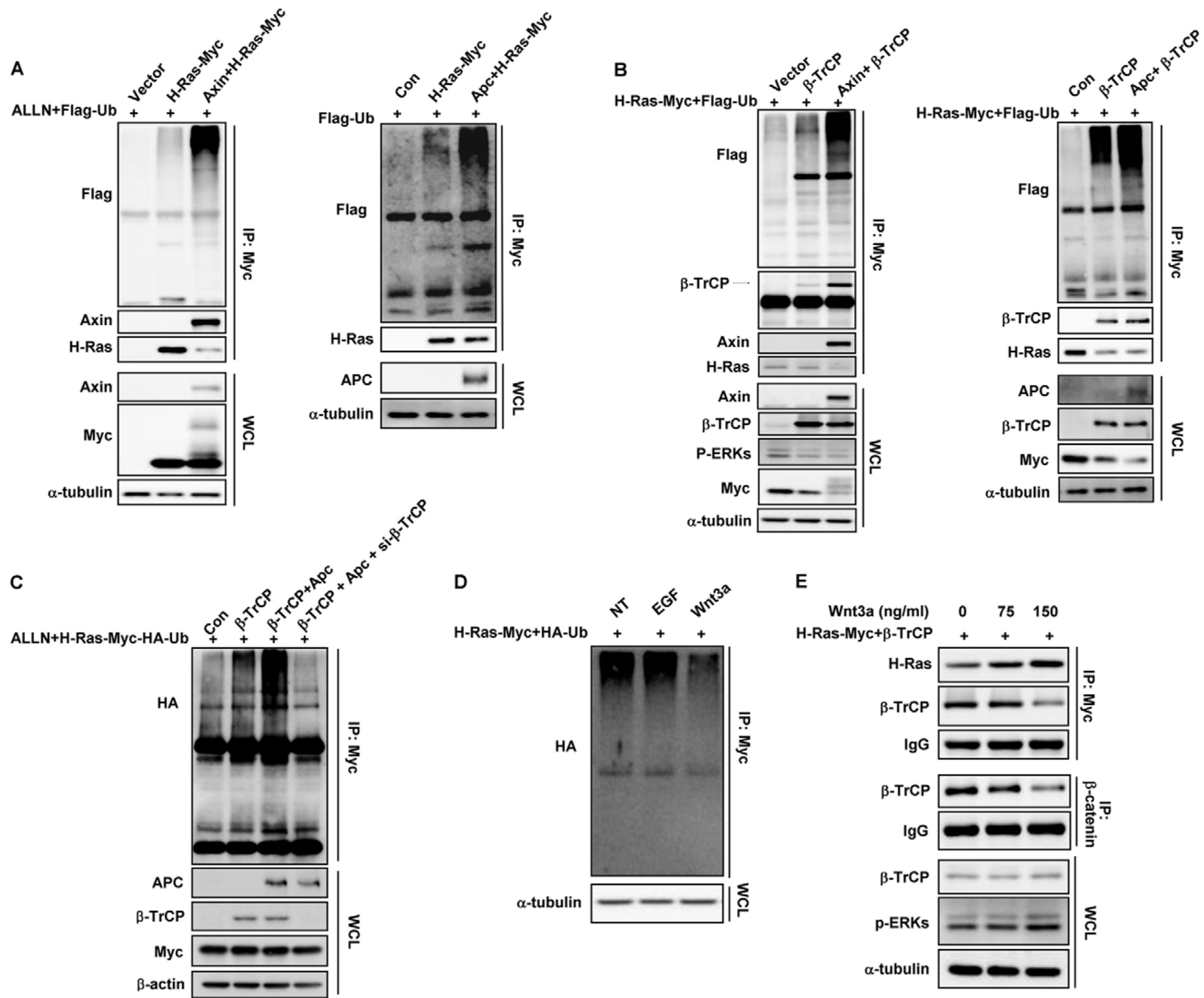

Fig. 5. Wnt/β-catenin signaling regulates polyubiquitylation and stability of H-Ras via modulation of β-TrCP binding affinity with H-Ras. (A,B) HEK293 cells were transfected with vector, H-Ras-Myc, Flag-β-TrCP, Axin, Apc, Flag-Ub and/or HA-Ub expression plasmids for 36 hours. Where indicated, transfected cells were treated with ALLN for 12 hours before harvest. Cell lysates were immunoprecipitated with anti-Myc resin or Pan-Ras antibody (for monitoring endogenous Ras). The immunoprecipitated lysates (IPLs) and whole-cell lysates (WCLs) were analyzed with indicated antibodies. (C-E) HEK293 cells were transfected with combination of vector, H-Ras-Myc, Flag-β-TrCP, HA-Ub and Apc expression plasmids and/or β-TrCP siRNA. At 24 hours after transfection, cells were treated with ALLN for 12 hours. Where required, cells were treated with Wnt3a (100 ng/ml), EGF (20 ng/ml) or different concentration of Wnt3a (0, 75 or 150 ng/ml) for 2 hours before harvest. Cell lysates were immunoprecipitated with anti-Myc resin or β-catenin antibody. IPLs or WCLs were analyzed by immunoblotting with indicated antibodies.

catenin signaling inhibited the binding of β-TrCP to H-Ras, thereby reducing ubiquitylation and proteasomal degradation of H-Ras. Overall, we identified a novel mechanism for the regulation of H-Ras protein stability by Wnt/β-catenin signaling.

In addition, in vivo Ras regulation via Wnt/β-catenin signaling was further supported by simultaneous increases in the levels of β-catenin and Pan-Ras in colon tissues of mice that received intravenous injections of recombinant Wnt3a. The polyubiquitylated Pan-Ras was reduced in these mice, although the protein level of Pan-Ras was significantly upregulated in the tissue stimulated with Wnt3a, confirming in vivo regulation of Ras stability and polyubiquitylation by the Wnt/β-catenin signaling pathway. We did not distinguish between the different Ras isoforms and their regulation by Wnt/β-catenin signaling in vivo owing to the absence of specific antibodies.

However, we observed degradation of K-Ras or N-Ras proteins by overexpression of β-TrCP (supplementary material Fig. S6). Therefore, K-Ras and N-Ras as well as H-Ras are subjected to regulations at the level of protein stability, though the mechanisms remain to be characterized.

The regulation of H-Ras stability by Wnt/β-catenin signaling is directly related to the proliferation and transformation of cells, as shown by suppression of the H-Ras-induced cellular transformation by overexpression of Axin or Apc (Park et al., 2006; Jeon et al., 2007). In this study, we provided evidence for a role of β-TrCP in the suppression of H-Ras-induced cellular transformation by Axin, as shown by synergistic effects of β-TrCP and Axin on anchorage-independent growth of cells. Both the Wnt/β-catenin and Ras-ERK pathways are implicated in the development of human cancers, including hepatocellular carcinoma (HCC) (de La Coste et al., 1998;

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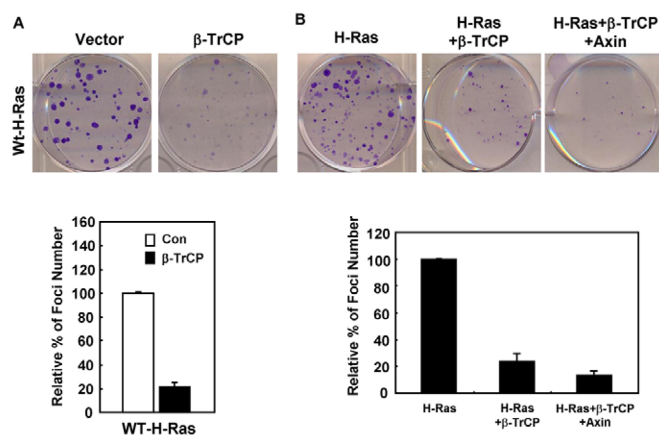

Fig. 6. The regulation of H-Ras stability via β-TrCP correlates with cellular transformation. (A,B) DLD-1 cells were transfected with vector and/or plasmids expressing H-Ras-Myc, Flag-β-TrCP and/or Axin. The transfected cells were selected with 800 μg/ml of G418 for 20 days and stained with 0.5% crystal violet in 20% ethanol, and the foci were photographed. The relative percentages of foci numbers were quantified. Error bars indicate standard deviation.

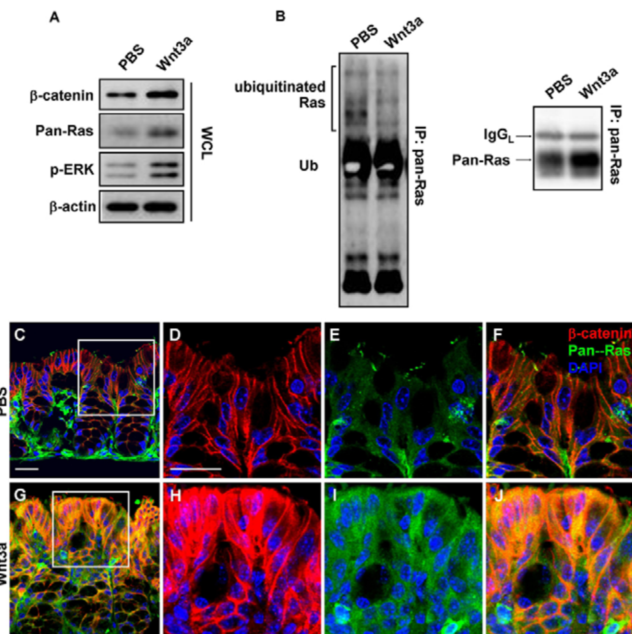

Fig. 7. In vivo regulation of Ras stability and ERK activity by Wnt/β-catenin signaling in mouse. The 12-week-old FVB mice were intravenously injected with PBS or Wnt3a for 12 hours, and then the colon tissues were harvested and frozen for biochemical analysis/fixed by paraformaldehyde as described in the Materials and Methods. (A,B) The whole-cell lysates (WCLs) were immunoprecipitated with anti-Pan-Ras antibody, and WCLs and immunoprecipitated lysates were immunoblotted with anti-β-catenin, anti-Pan-Ras, anti-β-actin, anti-p-ERK or anti-Ub antibody. (C-J) The immunofluorescence staining was performed as described in the Materials and Methods. Immunofluorescence staining patterns of β-catenin and Pan-Ras in the colon tissue from PBS- and Wnt3a-injected mouse were monitored by confocal microscope. The images in D-F and H-J are the higher magnification images of the boxed areas in C and G. Scale bars: 20 μm.

Wong et al., 2001; Taniguchi et al., 2002) and colorectal cancer (CRC) (Polakis, 2000; Downward, 2003; Fang and Richardson, 2005); however, the relationship between these two pathways in tumorigenesis is poorly understood. Mutations of *Axin* or *β-catenin* have also been frequently found in human HCCs (de La Coste et al., 1998; Wong et al., 2001; Taniguchi et al., 2002). About 50-70% of all HCCs examined exhibited an abnormal β-catenin accumulation in the cytoplasm and nucleus, although the mechanism for HCC development due to *Axin* and *β-catenin* mutations was unclear. We observed significant correlations between the levels of β-catenin, Ras and ERK pathway components (Raf-1, MEK and ERK) in highly proliferative samples from humans with HCC (data not shown). These results suggest crosstalk or synergism between the ERK and Wnt/β-catenin pathways that contributes to cellular transformation.

In support of this model, H-Ras mutations alone are not sufficient for the development of either hepatocyte dysplasia or tumors, whereas mutations of both β-catenin and H-Ras together resulted in a 100% incidence of HCC in murine models (Harada et al., 2004). Furthermore, inactivating *Apc* and activating *ras* mutations produce a synergistic effect that is sufficient to transform normal colonic epithelial cells and render them tumorigenic (D'Abaco et al., 1996). Individuals exhibiting *ras* mutations are resistant to tumor development in the presence of normal *Apc*, suggesting that the ability of *Apc* to suppress tumorigenesis induced by *ras* mutations (Kinzler and Vogelstein, 1996). The regulation of Ras stability by Wnt/β-catenin signaling provides a potential mechanistic basis for the synergism between the Wnt/β-catenin and Ras-ERK pathways that results in tumorigenesis. In addition, both wild-type and non-GTP-hydrolysable mutant Ras proteins were reduced by overexpression of Axin or Apc. These observations offer an important clue for the tumor-resistant phenotype of the *ras* mutated mice that retain wild-type components of the Wnt/β-catenin pathway (Harada et al., 2004). Therefore, Ras protein destabilization via Wnt/β-catenin signaling potentially acts as a safeguard against tumorigenesis that could be caused by aberrant activation of Ras or hyperstimulation of upstream Ras activators such as the EGF receptor.

Materials and Methods

Plasmids

Human H-Ras cDNA was amplified from human embryonic kidney 293 (HEK293) cells using RT-PCR and the product was then cloned into the *Eco*RI/*Hin*dIII sites of pcDNA3.1-myc (Invitrogen, Carlsbad, CA) and into the *Bam*HI/*Xho*I sites of pGEX4T1 (Amersham Biosciences, Piscataway, NJ). The pMT3-Ras-L61, pCS2-MT-Axin, pSUPER-Axin siRNA and pCMV-APC vectors have been described in previous studies (Park et al., 2006; Jeon et al., 2007). The pcDNA3.1-Flag-β-TrCP and pcDNA3.1-Flag-FBW8 constructs were generated as previously reported (Min et al., 2003), and pEF-HA-Ub was obtained from Zhijian J. Chen (University of Texas Southwestern Medical Center, Dallas, TX). Both pCS4-3xHA-Ub and pCS4-3xFlag-Ub were obtained from Dae-Won Kim (Yonsei University, Seoul, Korea). The deletion mutants (ΔF-box and ΔWD40) of β-TrCP were generated by PCR-based mutagenesis (Stratagene, La Jolla, CA). All constructs and mutations were confirmed by nucleotide sequencing.

Cell culture, transfection, drug treatment and siRNA-mediated gene silencing

HEK293, L929 mouse fibroblast cells and Chang human normal hepatocytes were maintained in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS). DLD-1 and SW480 human colon cancer cells were maintained in RPMI1640 (Gibco) containing 10% FBS. Cells were plated at a density of 7.5×10^5 cells per 10 cm diameter dish and transfected with the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. As required, the following drugs and recombinant proteins were applied: *N*-acetyl-Leu-Leu-Nle-CHO (ALLN, 25 or 50 μg/ml), cycloheximide (CHX, 50 μg/ml), epidermal growth factor (EGF, 20 ng/ml) and Wnt3a (100 ng/ml). The Wnt3a recombinant protein was purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO). HEK293 cells were plated at a density of 7.5×10^5 per 10 cm diameter dish and transfected with siRNA using the Lipofectamine Plus Reagent (Invitrogen). The siRNA sequences for β-TrCP (NM_033637) were 5′-CACAUAAACUCGUAUCUUA-3′ and 5′-GCGTTGTA-

TTCGATTTGAT-3′. The GFP siRNA (5′-GUUCAGCGUGUCCGGCGAGTT-3′) was used as a negative control. All siRNAs were synthesized by Samchully (Korea).

Immunoprecipitation, in vivo ubiquitylation assay and immunoblotting

Cells were lysed with RadioImmunoPrecipitation Assay (RIPA) buffer (Upstate Biotechnology, Lake Placid, NY). For in vivo ubiquitylation assays, 10 mM N-ethylmaleimide (NEM) (Sigma) was added to the RIPA buffer. The prepared lysates were immunoprecipitated with anti-Myc resin (A7470, Sigma), anti-Flag resin (A2220, Sigma), anti-Myc antibody (Cell Signaling, Beverly, MA), anti-H-Ras antibody (Oncogene Research Product, San Diego, CA), anti-Pan-Ras antibody (Upstate Biotechnology) or anti-β-catenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 12 hours. Each experiment using this assay was performed at least three times.

Immunoblotting was performed with the following primary antibodies: anti-Pan-Ras monoclonal (clone Ras10) and anti-Axin (Upstate Biotechnology). Anti-α-tubulin and anti-H-Ras antibodies were purchased from Oncogene Research Products, and anti-β-TrCP antibody was purchased from Zymed (South San Francisco, CA). Anti-HA, anti-GST, anti-ubiquitin (P4D1), anti-β-catenin, anti-APC, anti-ERK and anti-p-ERK antibodies were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-mouse (Cell Signaling) and anti-rabbit (Bio-Rad, Herculus, CA) antibodies were used as secondary antibodies.

In vitro GST pull-down assay

Glutathione S-transferase (GST)-H-Ras fusion protein was expressed in *Escherichia coli* BL21. After purification with glutathione beads, GST-H-Ras was used for the GST pull-down assay. The pull-down assay was carried out in RIPA buffer with purified GST-H-Ras fusion protein (10 μg), lysates overexpressing Wt-β-TrCP or mutant β-TrCP (500 μg) and 40 μl GSH-conjugated agarose beads (30% slurry) for 2 hours at 4°C. Immunoblotting was performed with anti-GST (Santa-Cruz) or anti-β-TrCP antibody.

Foci formation assay

DLD-1 cells were seeded at a density of 1×10^5 cells per 35 mm diameter well and transfected with pcDNA3.1-H-Ras-Myc, pcDNA3.1-Flag-β-TrCP and pCS2-MT-Axin in triplicate. The transfected cells were selected in RPMI containing 800 μg/ml G418, which was added every 4 days. After 20 days, the cells were stained with 0.5% crystal violet in 20% ethanol. The numbers of foci were counted by Image Pro 5.1 (MediaCybernetics, Bethesda, MD).

Animals and tissue preparation

The mice were given a standard maintenance and diet from Dae Han Bio Link (Korea). The 12-week-old male FVB mice were injected intravenously (tail vein) with PBS or Wnt3a (30 μg/kg dissolved in PBS) four times (at 30 minutes, 2 hours, 8 hours, and 12 hours) as described previously (Shibazaki et al., 1998). The animal studies were approved and performed under guidelines by the Institute of Health Guidelines for the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine (Seoul, Korea). Half of each tissue sample was immediately frozen in liquid nitrogen and stored at –70°C for immunoblotting. The remaining half was fixed in 10% neutral buffered formalin for histological analysis.

Immunofluorescence analyses

For immunofluorescence staining, 10 μm cryosections were treated with 0.1% Triton X-100 for 15 minutes and rinsed with PBS. The slides were incubated in 3% hydrogen peroxide at room temperature for 15 minutes to block endogenous peroxidase activity. Sections were then blocked in 5% BSA for 30 minutes and incubated with primary antibody overnight at 4°C. All incubations were carried out in humid chambers. The concentrations of primary antibodies used for staining are as follows: anti-β-catenin (BD transduction laboratory, San Jose, CA: 1:50) and Pan-Ras (Santa Cruz; 1:20). The primary antibody was omitted in negative control experiments, which showed very low background staining. The sections were then incubated with Alexa Fluor 488- or Alexa Fluor 555-conjugated IgG secondary antibody (Molecular Probes, Eugene, OR; 1:200) at room temperature for 1 hour, counterstained with DAPI (Boehringer Mannheim, Mannheim, Germany) and mounted in Gel/Mount media. The fluorescent signals were visualized on confocal microscope at excitation wavelengths of 488 nm (Alexa Fluor 488), 543 nm (Alexa Fluor 555) and 405 nm (DAPI).

Identification of proteasome complex interacting with H-Ras

HEK293 cells were plated (7.5×10^5/10 cm plate) and then transfected with pcDNA3.1-Wt-H-Ras-Myc and pEF-HA-Ub. The cells were treated with ALLN for 12 hours before harvest and then lysed with RIPA buffer. Twenty mg of lysates were immunoprecipitated with anti-Myc resin, resolved by SDS-PAGE and stained with Coomassie Brilliant Blue 250. Six sections of band areas were subjected to LC/MS-MS analyses (Drews et al., 2007) to identify the proteasome complex components.

We thank Natalie G. Ahn at the University of Colorado/HHMI for her critical comments about the manuscript. This work was supported by a National Research Laboratory Grant from the Korea Science and Engineering Foundation (KOSEF) funded by the Korean government (MEST) (2005-01564; 2006-02681; R112000078010020; 2007-000-10004-0). S.-E.K., J.-Y.Y. and W.-J.J. were supported by a BK21 scholarship from the Ministry of Education and Human Resources Development.

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