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Fused kinase is stabilized by Cdc37/Hsp90 and enhances Gli protein levels

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Abstract

Serine/threonine kinase Fused (Fu) is an essential component of Hedgehog (Hh) signaling in *Drosophila*, but the biochemical functions of Fu remain unclear. Here, we have investigated proteins co-precipitated with mammalian Fu and identified a kinase-specific chaperone complex, Cdc37/Hsp90, as a novel-binding partner of Fu. Inhibition of Hsp90 function by geldanamycin (GA) induces rapid degradation of Fu through a ubiquitin-proteasome pathway. We next show that co-expression of Fu with transcription factors Gli1 and Gli2 significantly increases their protein levels and luciferase reporter activities, which are blocked by GA. These increases can be ascribed to Fu-mediated stabilization of Gli because co-expression of Fu prolongs half-life of Gli1 and reduces polyubiquitination of Gli1. Finally, we show that GA inhibits proliferation of PC3, a Hh signaling-activated prostate cancer cell line. This growth inhibition is partially rescued by expression of ectopic Gli1, suggesting that Fu may contribute to enhance Hh signaling activity in cancer cells.

Keywords: Cdc37; Hsp90; Chaperone; Geldanamycin; Hedgehog; Fused; Ubiquitin; Gli

Hedgehog (Hh) signaling plays important roles in embryonic patterning by regulating cell differentiation and proliferation [1], and it also regulates stem cell maintenance and proliferation in adult [2]. On the other hand, deregulation of mammalian hedgehog signaling pathway is involved in the development of cancers, especially basal cell carcinoma and medulloblastoma [2,3].

Hh signaling is initiated by binding of Hh to its receptor Patched (Ptc), which, in the absence of Hh, inhibits the activity of the transmembrane protein Smoothened (Smo). Binding of Hh releases the inhibition of Smo, and this induces activation of Gli transcription factors via an unknown mechanism through a cytoplasmic protein complex composed of Gli, the serine/threonine kinase Fused (Fu), the kinesin-related protein Costal-2 (Cos2), and Suppressor of fused (Sufu) [1].

Fu is required for high levels of Hh signaling at the anterior/posterior boundary in *Drosophila* embryo and imaginal discs [4–6]. In zebrafish myotome, knockdown of Fu by morpholino oligonucleotides results in the absence of muscle pioneers which require maximum Hh signaling for differentiation [7]. Therefore, it is expected that Fu also positively regulates Gli activity in mammals. However, recent studies have suggested that Fu is not required at least during mouse embryogenesis [8,9] and the biochemical function of Fu remains unknown.

Here, we have identified Cdc37 and Hsp90, which together form a kinase-specific chaperone complex, as novel-binding proteins of Fu. We show that Cdc37/Hsp90 regulates the stability of Fu. In addition, we suggest that Fu stabilizes Gli protein and thus enhances Gli-mediated transcription by preventing polyubiquitination of Gli.

Materials and methods

Materials. Geldanamycin (GA) was purchased from Wako and Alexis. 4,5,6,7-tetrabromobenzotriazole (TBB) and MG132 were from Calbiochem. Chloroquine was from Sigma. Cyclopamine was from Tronto Research Chemicals.

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For immunoprecipitation, anti-FLAG M2 antibody-conjugated beads were from Sigma and protein G–Sepharose was from Pierce. Normal rabbit IgG was from Santa Cruz.

For Western blotting, anti-myc, anti-HA, and anti-Omni probe antibodies were all from Santa Cruz. Anti-FLAG antibody was from Sigma. Anti-Cdc37 antibody was from Becton–Dickinson. Anti-ERK1/2 antibody was from Cell Signaling. Anti-actin antibody was from Chemicon.

Plasmids. Human Fu cDNA (IMAGE clone ID: 5271185) was inserted into pEF-BOS-FLAG vector. The deletion mutants of Fu, Fu-K (amino acids 1–304) and Fu-ΔK (amino acids 306–1315) were inserted into pCMV2-FLAG vectors. Human Cdc37 cDNA was amplified by reverse-transcription polymerase chain reaction (RT-PCR) and inserted into pEF-BOS-myc vector. HA-tagged wild-type ubiquitin and 0K mutant ubiquitin in which all lysine residues are replaced with arginine were inserted into pcDNA3.1 vectors. Omni-probe-tagged mouse Gli1 and Gli2 vectors and Gli-dependent luciferase reporter plasmid were kindly provided by Dr. H. Sasaki (RIKEN, CDB). 6× myc-tagged mouse Gli1 was inserted into pcDNA3.1. *Renilla* luciferase reporter pRL-TK was purchased from Promega. pTK-Hyg was from Clontech.

Stable transfections. NIH3T3 cells were cultured in DMEM with 10% calf serum (CS) and co-transfected with pEF-BOS-FLAG-Fu or pEF-BOS-GFP (negative control) and pTK-hyg in a ratio of 20:1 by using Lipofectamine (Invitrogen). FLAG-Fu expressing clones were selected with hygromycin.

PC3 cells (obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University) were cultured in RPMI-1640 with 10% fetal bovine serum and transfected with pcDNA3.1-6× myc-Gli1. 6× myc-Gli1 expressing clones were selected with G418.

Antibody production. Hexahistidine (6× His)-tagged Fu-K (amino acids 1–304) were expressed in *Escherichia coli* and were purified with Ni–NTA Sepharose (Qiagen). Anti-Fu antibody was raised by immunizing rabbits with 6× His-Fu-K.

Pulse-chase experiment. NIH3T3 cells stably expressing FLAG-Fu were incubated in cysteine/methionine-free DMEM (Invitrogen). Thirty minutes later, cells were pulse-labeled with 50 μCi/ml Redivue Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham) in cysteine/methyonine-free DMEM with 10% CS for 12 h. Cells were washed with PBS and incubated in normal DMEM with 10% CS in the presence or absence of 0.5 μM GA for the indicated times. Cell lysates were immunoprecipitated with anti-FLAG M2 beads and separated by SDS–PAGE. Gels were dried, exposed to an imaging plate, and the relative radioactivity of FLAG-Fu was quantified with the BAS2000 imaging analyzer (Fujifilm).

C3H10T1/2 cells were transfected with FLAG-Gli1 with or without Fu. 20 h after transfection, cells were pulse-labeled with [^{35}S]-methionine/cysteine (Perkin-Elmer) for 3 h and chased for the indicated minutes. FLAG-Gli1 was immunoprecipitated with anti-FLAG antibody and visualized as described above.

Luciferase assay. C3H10T1/2 cells were cultured in DMEM with 10% fetal bovine serum in 3.5 cm dishes and transfections were performed with the indicated combination of 0.4 μg Gli-dependent luciferase reporter plasmid, 75 ng pRL-TK, 0.4 μg pcDNA3.1-His-Gli1, pcDNA3.1-His-Gli2, and 1.6 μg pEF-BOS-FLAG-Fu vector using Lipofectamine. After 5 h incubation with the lipofection reagents, cells were treated with DMSO or 0.5 μM GA in DMEM with 10% FBS for 18 h. Reporter activity was measured by Dual-Luciferase assay system (Promega). Experiment was repeated three times.

Results

Identification of Cdc37 and Hsp90 as novel-binding proteins of Fu

To identify novel proteins that interact with Fu, we generated an NIH3T3 cell line stably expressing FLAG-tagged Fu (NIH-FLAG-Fu). Cell lysates were prepared, and Fu proteins were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were separated by SDS–PAGE and visualized by silver staining (Fig. 1A). A band with a molecular mass of ~40 kDa (Fig. 1A, arrow A) was the major specific band in immunoprecipitates from NIH-FLAG-Fu cell lysates, but this band was not present from the control lysate. By liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the protein was identified to be Cdc37. Cdc37 is known to interact with signaling protein kinases and functions as a co-chaperone of molecular chaperone Hsp90 [10]. We generated antibody specific to Fu (Fig. 1B) and observed co-immunoprecipitation of Cdc37 from COS7 cell lysates (Fig. 1C). Co-immunoprecipitation of Hsp90 from NIH-FLAG-Fu cell lysates was also confirmed by Western blotting (Fig. 1D). These results suggest that Fu forms a complex with Cdc37/Hsp90 *in vivo*.

Fu consists of the N-terminal kinase domain and the C-terminal domain, the function of which is not well characterized. We found that both endogenous Hsp90 and myc-Cdc37 associated with the kinase domain (amino acids 1–304) but not with the C-terminal non-catalytic domain of Fu (amino acids 306–1315, Fig. 1E). This result is consistent with previously reported client kinases of Cdc37/Hsp90 [10].

Cdc37/Hsp90 regulates stability of Fu

Cdc37/Hsp90 regulates the stability of client protein kinases such as Cdk4, Raf1, and Akt [10]. To examine whether Cdc37/Hsp90 also stabilizes Fu, we tested the effect of GA, a specific inhibitor of Hsp90, on the stability of Fu. We found that GA treatment rapidly decreased endogenous Fu levels in HEK293 cells (Fig. 2A, left). In contrast, ERK1/2 levels, which is not a client kinase of Cdc37/Hsp90, were unchanged after GA treatment. Recently, it was reported that casein kinase 2 (CK2)-mediated phosphorylation of Cdc37 on Ser13 is essential for stable interactions between Cdc37 and its client kinases [11]. To investigate the importance of Cdc37 for stability of Fu, we treated HEK293 cells with TBB, a specific inhibitor of CK2. TBB treatment significantly reduced Fu levels in a time-dependent manner, whereas ERK1/2 levels were not changed (Fig. 2A, right). These results suggest that Cdc37 and Hsp90 are required for Fu stability.

To examine the mechanism of the reduction of Fu protein levels in response to GA, we measured half-life of FLAG-Fu in the presence or absence of GA by pulse-chase analysis. FLAG-Fu was much more unstable in GA-treated cells than in DMSO-treated cells (Fig. 2B, upper). Measurement of the radioactivity of FLAG-Fu revealed that the half-life of FLAG-Fu was markedly shortened from ~4 h to ~30 min after treatment with GA (Fig. 2B, lower). This result indicates that inhibition of Hsp90 function promotes rapid degradation of Fu.

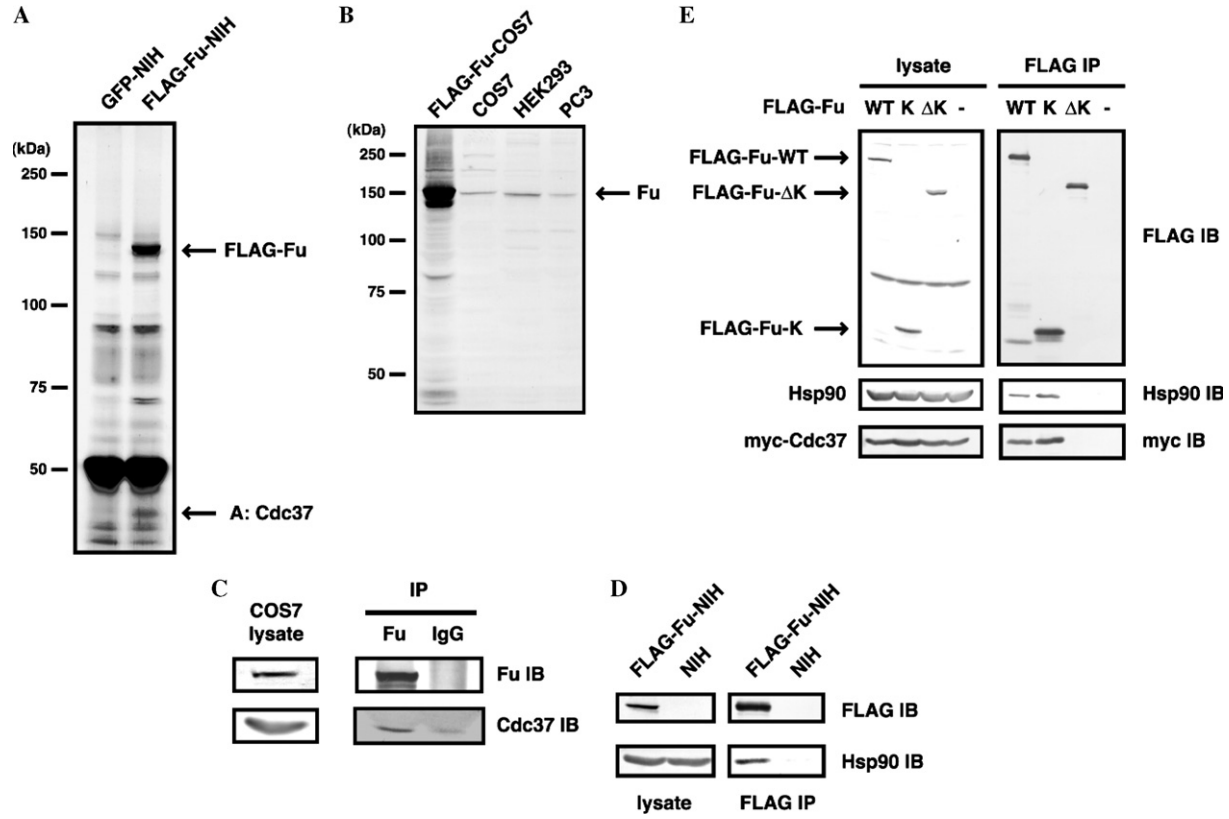

Fig. 1. Cdc37/Hsp90 interacts with Fu. (A) Identification of Cdc37 as a novel-binding protein of Fu. (B) Cell lysates from FLAG-Fu overexpressing COS7, COS7, HEK293, and PC3 cells were immunoblotted with anti-Fu antibody. (C) Endogenous Fu forms complex with Cdc37 in COS7 cells. (D) Co-immunoprecipitation of Hsp90 with FLAG-Fu from NIH-FLAG-Fu cell lysates. (E) Cdc37 and Hsp90 bind to kinase domain of Fu.

Fu degradation is mediated by ubiquitin-proteasome pathway

Recent studies showed that several other Cdc37/Hsp90 client kinases are degraded by proteasomes following GA treatment [10]. Therefore, we examined the effects of proteasome inhibitor MG132 on GA-induced degradation of Fu. MG132 significantly prevented GA-induced degradation of Fu in HEK293 cells (Fig. 2C). In contrast, lysosome inhibitor chloroquine was ineffective. These results indicate that the degradation of Fu induced by GA is dependent on proteasomes.

Ubiquitination is important to facilitate the degradation of targeted proteins by the proteasome. To examine whether Fu is also ubiquitinated, we transfected COS7 cells with vectors expressing FLAG-Fu and HA-tagged wild-type ubiquitin (HA-Ub-WT) or the 0K mutant of ubiquitin (HA-Ub-0K) in which all lysines were replaced with arginine to avoid further ubiquitination. After transfection, cells were treated with a combination of MG132 and GA. We observed a smear signal above the proper molecular weight of Fu when cells expressing HA-Ub-WT and FLAG-Fu were treated with MG132 (Fig. 2D). This smear signal presumably represents polyubiquitinated Fu because no significant signal was obtained when cells expressing HA-Ub-WT alone or HA-Ub-0K and FLAG-Fu were treated with MG132. These results suggest that Fu is polyubiquitinated before degradation through the proteasome-dependent pathway.

Fu enhances Gli stability and reporter activity by suppressing polyubiquitination of Gli

Fu is a positive regulator of the transcription factor Ci in *Drosophila* [4,5], but the function of Fu in mammalian Hh signaling is not well understood. To examine the effect of Fu on the activities of Gli1 and Gli2, mammalian homologs of Ci, we performed luciferase assay using the Hh-responsive cell line C3H10T1/2. Whereas Gli1 and Gli2 alone induced ~28-fold and ~4.8-fold increases in luciferase reporter activities, respectively, co-expression of Fu further increased reporter activities to ~100-fold and ~9.8-fold increases, respectively (Fig. 3A, left). We found that protein levels of both Gli1 and Gli2 were significantly increased by co-expression with Fu, whereas levels of both proteins were almost undetectable when Gli1 and Gli2 were expressed alone (Fig. 3A, right). Furthermore, the effects of Fu on reporter activities and protein levels of both Gli1 and Gli2 were abrogated when Fu degradation was induced by GA treatment (Fig. 3A). Therefore, it is possible that Fu stabilized Gli1 and Gli2 and thereby enhanced protein levels and reporter activities of them.

To examine whether Fu stabilizes Gli, we analyzed degradation kinetics of Gli1 in the presence or absence of Fu (Fig. 3B). Pulse-chase analysis showed that Gli1 was rapidly degraded with a half-life of ~90 min when it was expressed alone. In contrast, co-expression of Gli1 with

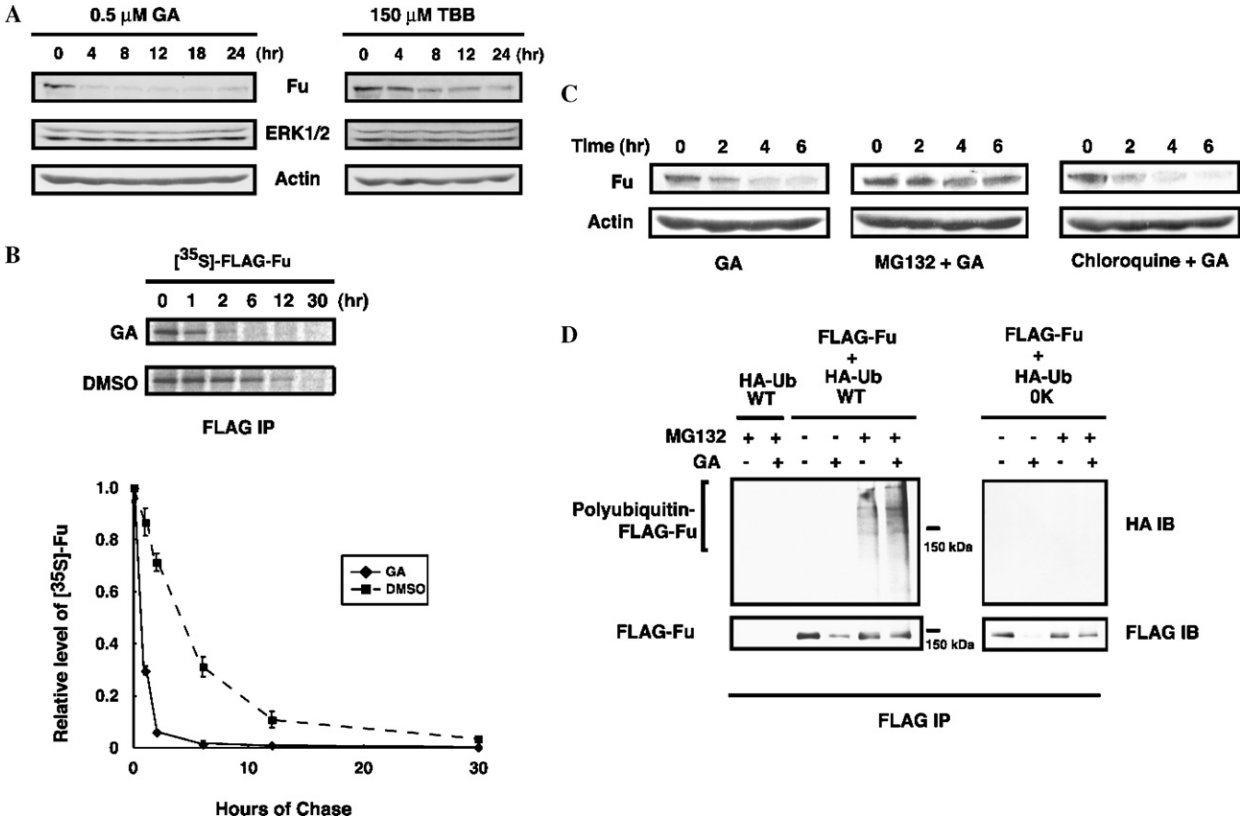

Fig. 2. Cdc37/Hsp90 regulates Fu stability. (A) HEK293 cells were treated with 0.5 μM GA (left) or 150 μM TBB (right) for the indicated times. The amounts of Fu, ERK1/2 (negative control), and actin were analyzed by Western blotting. (B) The amount of [^{35}S]-FLAG-Fu in the presence or absence of GA after chase (upper). Relative level of [^{35}S]-FLAG-Fu was quantified and represented graphically (lower). Each data point represents means ± SD of three independent experiments. (C) HEK293 cells were pretreated with DMSO, 10 μM MG132, or 100 μM chloroquine for 30 min and further incubated in the presence of 0.5 μM GA for the indicated times. The amount of Fu was analyzed by Western blotting. (D) COS7 cells were transfected with indicated plasmids. Cells were pretreated with DMSO or 5 μM MG132 for 30 min and further incubated in the presence or absence of 0.5 μM GA for 4 h. Polyubiquitination of FLAG-Fu was analyzed by immunoprecipitation of whole cell lysates with anti-FLAG antibody, followed by immunoblotting with anti-HA antibody. Probable polyubiquitin signal and molecular weight markers are indicated.

Fu prolonged half-life of Gli1 because ~80% of [^{35}S]-Gli1 was present even after 120 min of chase. This result indicates that Gli1 degradation was prevented by Fu. Because Gli1 is degraded through the ubiquitin-proteasome pathway [12], we next tested whether Fu suppresses polyubiquitination of Gli1. C3H10T1/2 cells were transfected with 6× myc-Gli1 and FLAG-ubiquitin with or without non-tagged Fu and were treated with MG132. In order to evaluate the levels of polyubiquitination of Gli1, the amount of Gli1-expressing plasmid was adjusted to obtain similar expression level of Gli1 with/without Fu (Fig. 3C). We found that co-expression of Fu with Gli1 reduced the levels of polyubiquitinated Gli1 compared to expression of Gli1 alone at 4 h of MG132 treatment. These results suggest that Fu stabilizes Gli1 by suppressing Gli1 polyubiquitination and thus enhances Gli1 transcriptional activity.

Fu may contribute to the growth of PC3 prostate cancer cells

It has been reported that in prostate cancer cell line PC3, Hh signaling is highly activated and cell growth is inhibited by Smoothened inhibitor cyclopamine [13,14]. Therefore, we finally examined whether Fu contributes to the growth of PC3 by stabilizing Gli. After PC3 cells were treated with DMSO, GA, or cyclopamine, protein levels of Fu were analyzed (Fig. 4A), and the effect on cell proliferation was evaluated by BrdU incorporation assay (Fig. 4B). As reported previously [14], cyclopamine treatment significantly decreased numbers of BrdU-positive cells to 57% compared to DMSO treatment, suggesting that the growth of PC3 cells is dependent, at least in part, on Hh signaling. GA treatment induced degradation of Fu (Fig. 4A) and also inhibited cell proliferation to 17% compared to DMSO treatment (Fig. 4B).

We next generated PC3 cells stably expressing 6× myc-Gli1 (PC3-Gli1). In these PC3-Gli1 cells, ectopic myc-Gli1 is overexpressed, and therefore, protein level of myc-Gli1 was not affected significantly by GA treatment (Fig. 4A). When PC3-Gli1 cells were treated with cyclopamine, a significant decrease in numbers of BrdU-positive cells was not observed (Fig. 4B). This result indicates that the anti-proliferative effect of cyclopamine on PC3 cells was bypassed by ectopic Gli1. We next treated PC3-Gli1 cells with GA and found partial rescue of the growth inhibition (Fig. 4B). Therefore, these results suggest that Hh signaling is down-regulated by GA and Fu may play

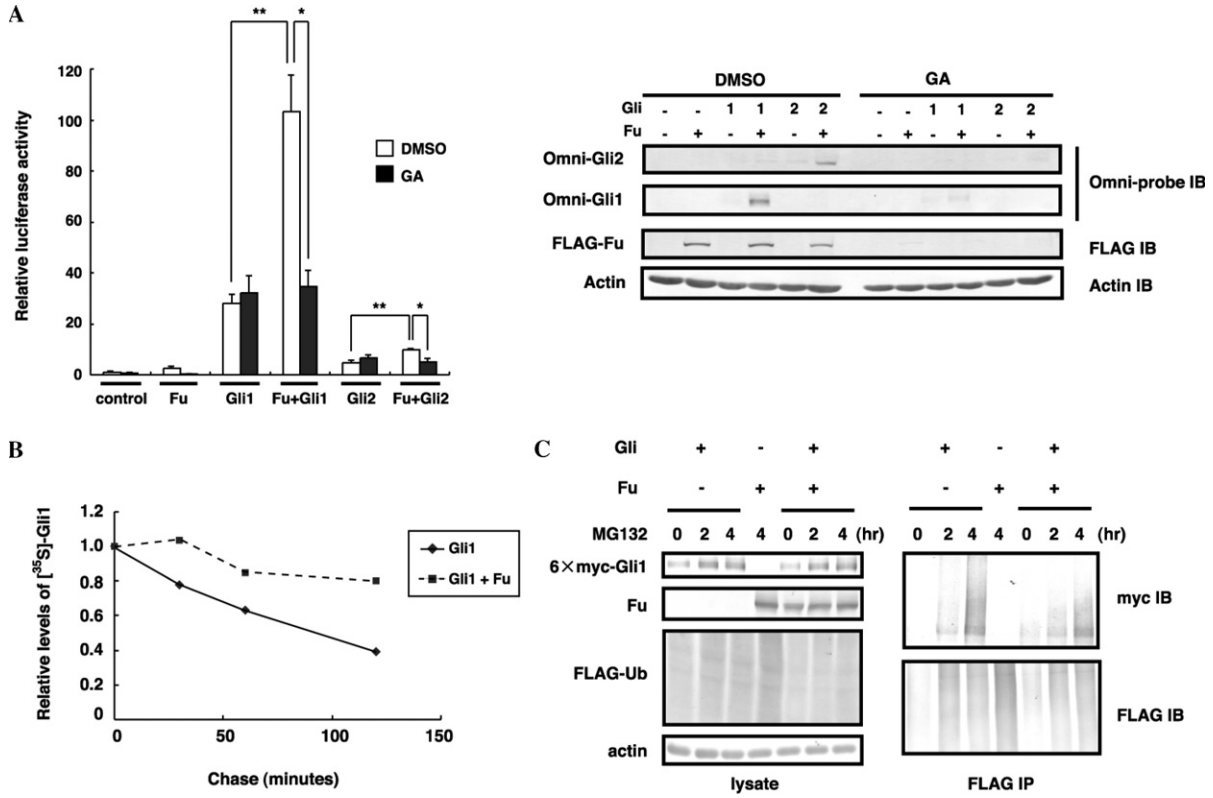

Fig. 3. Fu increases reporter activities and protein levels of Gli1 and Gli2. (A) Fu increases Gli1 and Gli2 reporter activities (left) and protein levels (right), which are abrogated by GA treatment. Each error bar represents the SD of three independent experiments. $^{*}P < 0.03$, $^{**}P < 0.01$ in Student's t test. (B) The amount of [^{35}S]-FLAG-Gli1 in the absence or presence of Fu. Relative levels of [^{35}S]-FLAG-Gli1 were quantified by densitometry (lower). (C) C3H10T1/2 cells were transfected with the indicated plasmids. 20 h after transfection, cells were treated with 10 μM MG132 for the indicated hours. Polyubiquitination of Gli1 was analyzed by immunoprecipitation of whole cell lysates with anti-FLAG antibody, followed by immunoblotting with anti-myc antibody.

important role in the growth of PC3 cells by stabilizing Gli.

Discussion

In the present study, we identified Cdc37 and Hsp90 as novel binding proteins of Fused kinase in mammalian cells. We showed that Cdc37/Hsp90 is important for Fu stability, but the interaction between Fu and Hsp90 appears to be weak because we could detect co-immunoprecipitation of Hsp90 with Fu by Western blotting but could not detect by silver staining (Fig. 1A and D). These results suggest that the interaction between Fu and Hsp90 is dynamic. Indeed, it has been reported that Hsp90 is mainly required for maturation of newly synthesized client kinases such as the src-family kinase Lck and IKK [15,16]. Therefore, Hsp90 might bind transiently to mature client kinases, including Fu, only when they take an unusual conformation and dissociate once these kinases are refolded properly.

We showed that Fu enhanced protein levels and reporter activities of both Gli1 and Gli2 (Fig. 3). In contrast, previous studies showed that Fu has a little or no effect on Gli1 reporter activity [17,18]. These conflicting results might be ascribed to the amount of Fu expressed in each experiment. Because the transcriptional activity of Gli1 itself is very strong, it may be difficult to observe the effect of Fu when only a small amount of Fu is expressed. Indeed, we found that the effects of Fu on expression levels and reporter activities of Gli1 and Gli2 correlated with the amount of Fu plasmid used (data not shown). Therefore, our results may reflect a situation in which Fu is strongly expressed, such as that in ependymal cells (discussed below). Another possibility is that the effect of Fu on Gli1 depends on the cell lines used. We used Hh-responsive C3H10T1/2 cells, but Osterlund et al. [18] used HEK293 cells, in which stability and activity of Gli1 may be differentially regulated compared to C3H10T1/2 cells.

Fu-knockout mice showed postnatal growth defects characterized by a communicating form of hydrocephalus and died within 2 weeks of birth [8,9]. Merchant et al. [9] reported that the cause of the hydrocephalus in Fu-knockout mice appeared to be the overproduction of cerebral spinal fluid (CSF) and that *Fu* is expressed at high levels in the choroid plexus and ependymal cells that are involved in CSF production and have been speculated to be neural stem cells [19]. Hh signaling regulates stem cell properties and interestingly, it has been reported that expression of *Shh* is very weak in the dorsal

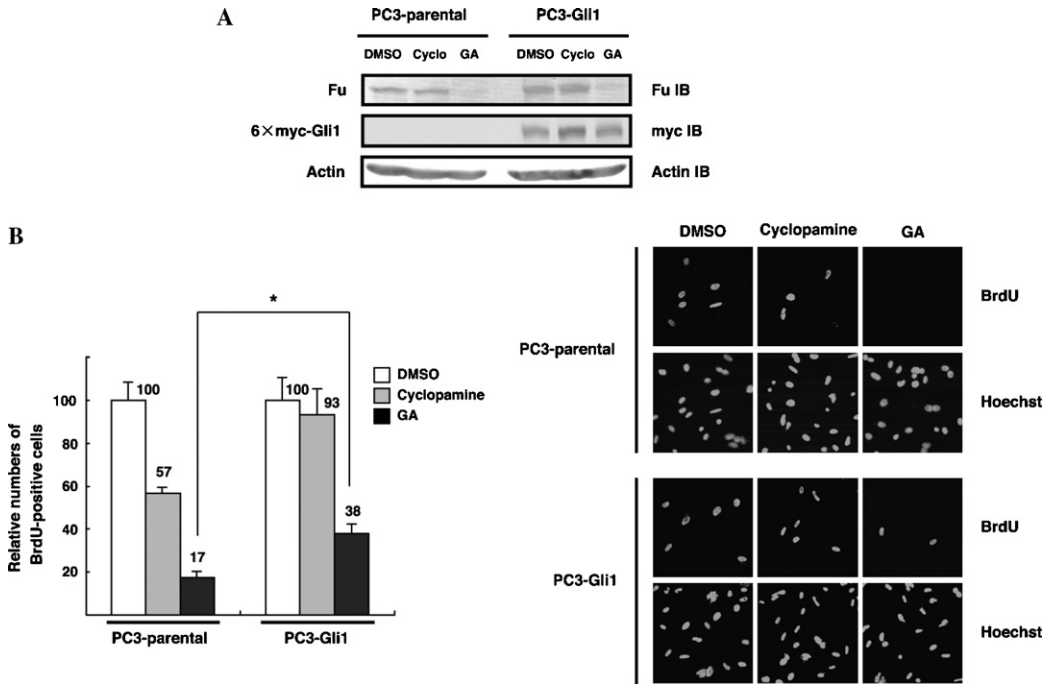

Fig. 4. GA inhibits PC3 cell proliferation probably through the degradation of Fu. Parental PC3 cells and PC3-Gli1 cells were treated with DMSO, 0.5 μM GA, or 10 μM cyclopamine in RPMI-1640 with 2.5% FBS for 22 h and further incubated in the presence of BrdU for 2 h. (A) Expression of Fu and 6× myc-Gli1 was examined by Western blotting. (B) Numbers of BrdU-positive cells treated with each reagent relative to controls treated with DMSO were determined by counting ~500 cells under fluorescence microscopy (left). Each error bar represents the SD of three independent experiments. $^{*}P < 0.02$ in Student's t test. Representative microscopic fields of each sample are also shown. All nuclei were stained with Hoechst dye (right).

brain, where ependymal cells exist, from E14.5 to E17.5 [20]. Therefore, Fu may play important roles in the maintenance of stem cells under low Hh conditions by enhancing Gli stability during the late embryonic and postnatal stages.

Although Fu seems to be no longer essential in Hh signaling at least during embryogenesis, our present study suggests that Fu could enhance Hh signaling activity by stabilizing Gli proteins (Fig. 3). It has been reported that Gli1 is also dispensable for mouse embryogenesis like Fu [21], but it is important for proliferation of tumor cells [22]. Furthermore, removal of destruction signals in Gli1 enhances Gli1 stability and accelerates tumor formation in transgenic mice [12]. We therefore speculate that stabilization of Gli1 or Gli2 by Fu may be involved in the tumorigenesis caused by abnormally activated Hh signaling. In support of this idea, we showed that GA treatment induced Fu degradation and suppressed proliferation of PC3 prostate cancer cells (Fig. 4). A previous study suggested that Hsp90 inhibitor caused growth arrest of prostate cancer cells by degrading androgen receptor, HER2, and Akt [23]. However, considering that the growth of these cells is largely dependent on sustained Hh signaling [13,14] and that the inhibitory effect of GA was partially but significantly rescued by ectopic Gli1 (Fig. 4B), Fu might also contribute to the proliferation of PC3 cells by stabilizing Gli1 and/or Gli2. Further studies of Fu are needed to clarify the involvement of Fu in development and human cancers.

Acknowledgments

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