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ORIGINAL ARTICLE

Siah-1S, a novel splice variant of Siah-1 (seven in absentia homolog), counteracts Siah-1-mediated downregulation of β-catenin

Y Mei[1], C Xie[1], W Xie[1], Z Wu[2] and M Wu[1]

[1]*Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, People's Republic of China and* [2]*Department of Pathology, Anhui Medical University, Hefei, Anhui, People's Republic of China*

Siah-1 (seven in absentia homolog) is known to cause indirect degradation of β-catenin through formation of a complex with Siah-interacting protein (SIP), Skp1 and Ebi. Here, we report the characterization of a novel splice variant of human Siah-1, designated Siah-1S, which is produced by an alternative splicing mechanism. The novel intron/exon junctions used to generate Siah-1S follow a non-conventional CT-AC rule. Siah-1S exhibits an even shorter half-life than Siah-1 and is able to catalyse self-ubiquitination that results in its subsequent degradation by proteasome. Siah-1S is shown to upregulate β-catenin-dependent Tcf/Lef transcriptional activation and antagonize Siah-1's potentiation effect on the apoptosis induced by etoposide in MCF-7 cells. Additionally, Siah-1S is found to interact with Siah-1 to form heterodimer or with itself to form homodimer. Unlike homodimer Siah-1 *Siah-1, neither Siah-1*Siah-1S nor Siah-1S*Siah-1S is able to bind to Siah-1-interacting protein, which may explain the underlying mechanism for Siah-1S's dominant negative effect on Siah-1. Importantly, results from *in vitro* soft agar assay demonstrated that Siah-1S displays a promotion effect on cells tumorigenicity.
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Keywords: Siah-1S; β-catenin; splice variant; self-ubiquitination; tumorigenesis

Introduction

The Drosophila SINA protein (seven in absentia) is required for R7 cell determination in the developing eye (Carthew and Rubin, 1990). In this process, SINA together with Phyllopod formed an E3 complex that interacts with Tramtrack, a potent repressor of neuronal cell fate, targeting it for degradation through ubiquitin–proteasome pathway. The destruction of Tramtrack is necessary for differentiation of R7 cells (Li *et al*., 1997; Tang *et al*., 1997). Recently, Ebi, an F-box protein, was reported to be involved in the degradation of Tramtrack, implying a potential functional link between SINA and Ebi (Dong *et al*., 1999). The human homologs of *sina* gene have been identified as two highly related *Sina*-like genes, *SIAH-1* and *SIAH-2* (Hu *et al*., 1997a), which encode Siah (seven in absentia homolog) proteins that contain an N-terminal RING domain that binds E2 proteins (Hu and Fearon, 1999; Lorick *et al*., 1999), and a C-terminal substrate-binding domain (SBD) that engaged in binding various substrate proteins and targeting them for proteasome-dependent degradation. The reported targets of Siah-mediated degradation include DCC (Hu *et al*., 1997b), Nco-R (Zhang *et al*., 1998), Kid (Germani *et al*., 2000), c-Myb (Tanikawa *et al*., 2000), OBF-1 (Boehm *et al*., 2001; Tiedt *et al*., 2001), Numb (Susini *et al*., 2001), Synaptophysin (Wheeler *et al*., 2002), TIEG-1 (Johnsen *et al*., 2002), Synphilin-1 (Nagano *et al*., 2003), CtIP (Germani *et al*., 2003), T-STAR (Venables *et al*., 2004), Polycystin (Kim *et al*., 2004), Af4 (Oliver *et al*., 2004), OGDHC-E2 (Habelhah *et al*., 2004), PHDs (Nakayama *et al*., 2004) and RINGO (Gutierrez *et al*., 2006). Interestingly, not all Siah binding proteins are targets for Siah-mediated degradation, for example, Siah does not appear to target Vav (Germani *et al*., 1999) or BAG1 (Matsuzawa *et al*., 1998) for degradation, suggesting that Siah may play some additional roles other than serving as an E3 ligase for substrate degradation.

Siah-1 was identified as a p53 inducible gene, which is upregulated during the physiological program of cell death (Amson *et al*., 1996; Nemani *et al*., 1996). Overexpression of Siah-1 was shown to induce cell-cycle arrest, tumor suppression and apoptosis (Matsuzawa *et al*., 1998; Bruzzoni-Giovanelli *et al*., 1999; Roperch *et al*., 1999; Tuynder *et al*., 2002). Recently, Siah-1 has been reported to be involved in a complex with Skp1, Ebi, Siah-interacting protein (SIP) and adenomatous polyposis coli protein to facilitate, in a p53-dependent manner, the degradation of β-catenin independent of its phosphorylation status (Liu *et al*., 2001; Matsuzawa and Reed, 2001). Overexpression of β-catenin is associated with enhanced cell proliferation and inhibition of

Correspondence: Dr M Wu, Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, People's Republic of China.
E-mail: wumian@ustc.edu.cn
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apoptosis ((Bienz and Clevers, 2000; Peifer and Polakis, 2000). So it is plausible that Siah-1-mediated β-catenin degradation may contribute, in part, to apoptosis and tumor suppression.

The crystal structure of a fragment of murine Siah-1a comprising the Cys-rich domain and the SBD revealed that Siah-1a may exist as dimmer and its SBD shares striking structural similarity of the TRAF-C region of TRAF (TNF-receptor associated factor) proteins (Polekhina *et al*., 2002). Subsequent study on Siah-1 structure indicated that SIP dimer binds across the saddle formed by an antiparallel orientation of identical β-sheets in each monomer and down into the two binding grooves formed by Siah-1 dimer (Matsuzawa *et al*., 2003; Santelli *et al*., 2005).

In this study, we identified a novel splice variant of Siah-1 that we designated as Siah-1S, which acts as a dominant-negative inhibitor of Siah-1 to upregulate β-catenin and Tcf/Lef transcriptional activity. Overexpression of Siah-1S was shown to diminish the potentiation effect of Siah-1 on etoposide induced apoptosis. We demonstrated that Siah-1S is able to interact with Siah-1 to form heterodimer or with itself to form homodimer. However, unlike Siah-1*Siah-1, neither Siah-1*Siah-1S nor Siah-1S*Siah-1S is able to bind to SIP, resulting in failure to degrade β-catenin, which may be the underlying mechanism for Siah-1S's dominant-negative effect. We also showed that similar to Siah-1, Siah-1S is self-degraded through ubiquitin–proteasome pathway. Importantly, Siah-1S is implicated in playing a role in tumorigenesis.

Results

Identification and isolation of Siah-1 splicing variant Siah-1S

When we amplified Siah-1 cDNA from the human lymph node cDNA library using primers P1 and P3 flanking the open reading frame (ORF) of Siah-1, an additional 755 bp PCR product was obtained in addition to the predicted 849 bp band encoding Siah-1. Subsequent sequencing determination of the lower molecular weight band indicated that it was identical to that of the Siah-1 cDNA except that a segment spanning nucleotides 576–669 were missing (Figure 1A,a). The missing of 94 bp fragment results in a frame shift mutation and produces an ORF of three amino-acids ended in a stop codon TAA (nucleotides 680–682) (Figure 1A,b). We named this alternative spliced transcript as Siah-1S (short). The ORF of *siah-1S* encoded a protein of 195 amino-acids as compared with Siah-1, which contains 282 amino acids (Figure 1A,c). To examine whether the Siah-1S naturally exists in the tissues and what the distribution pattern for the Siah-1S is, we examined the expression of Siah in four different cDNA libraries (human heart, lymph node, fetal brain and testis) by PCR method, and as shown in Figure 1B,a, both *siah-1* and *siah-1S* were detected in all the tissues examined, despite that siah-1S expression is relatively low in human lymph node and testis (lanes 2, 4). To confirm further the authenticity of Siah-1S transcript, reverse-transcription PCR was performed with template RNA from three different tumor tissue samples (breast, colorectal and esophagus cancers) using primer pair P2 and P3 (Figure 1A,a). As shown in Figure 1B,b, Siah-1S can be detected in all three tumor tissues. To detect the endogenous expression of Siah-1S, total proteins extracted from breast, kidney and esophagus cancer tissue samples were individually subjected to Western blotting using anti-Siah-1 antibody. As shown in Figure 1C, Siah-1S expression can be detected in all three tumor tissues, despite that the expression of Siah-1S is relatively low in breast cancer.

The splice junctions of Siah-1S does not conform to the conventional GT-AG rule

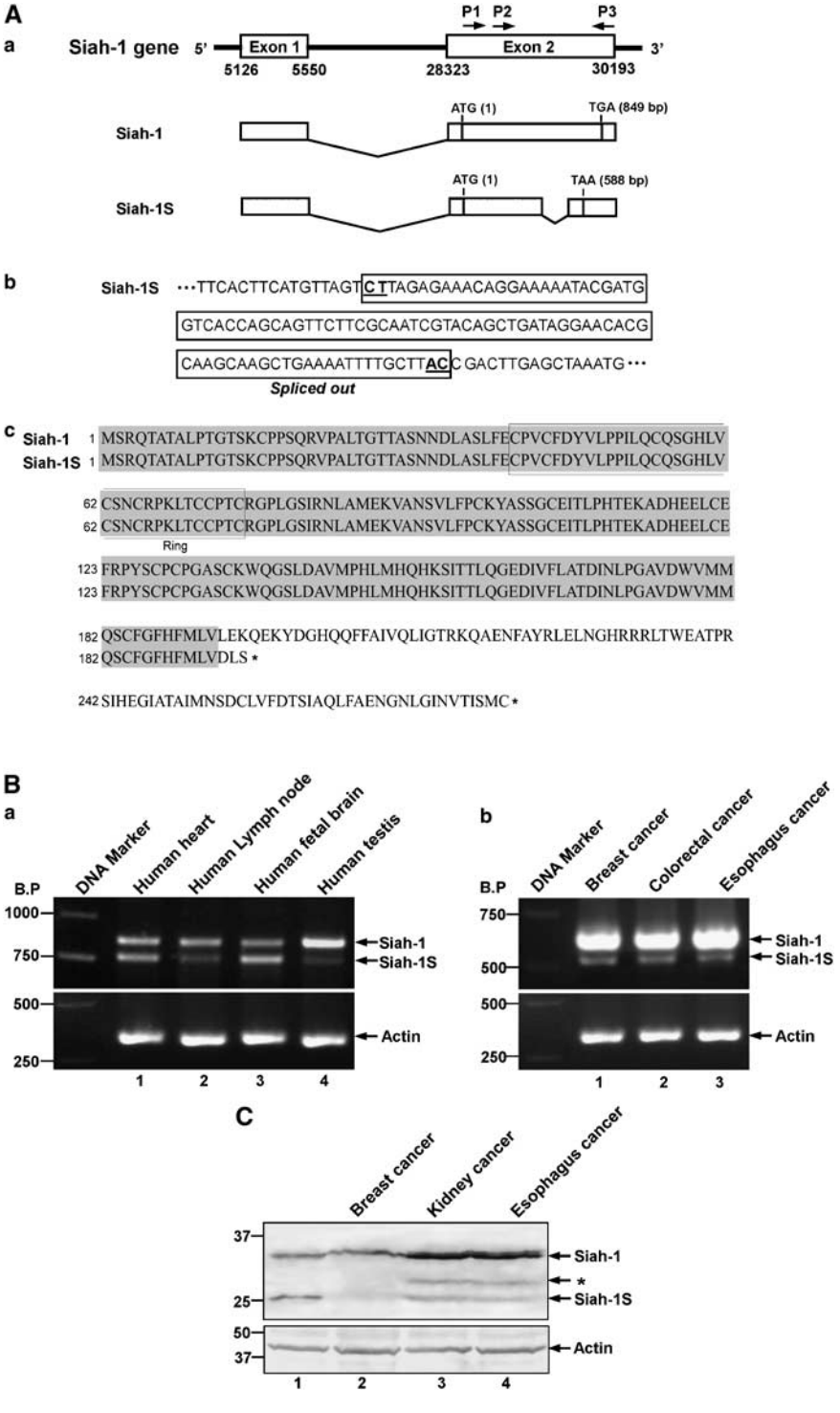
The human Siah-1 gene maps to chromosome 16q12 (GeneBank accession number AJ400626) and consists of two exons and one long intron ($\sim$23 kb) (Figure 1A,a). Careful inspection of the Siah-1S coding sequence with the genomic sequences revealed that CT and AC were utilized as the donor and acceptor splice sites respectively (Figure 1A,b), which does not conform to the consensus sequences for either major GT-AG or minor AT-AC introns. French *et al*. (1999) recently reported

Figure 1 Identification of a novel splicing variant of Siah-1. (**A**) (a) Organization of the Siah-1 and its splice variant genes. Exons are represented as open boxes, introns as thick lines. The size of exon or intron is indicated. The positions of the start and termination codons are indicated. Primers P1 (5′-ATGAGCCGTCAGACTGCT-3′), P2 (5′-ATTCGCAACTTGGCTATG-3′) and P3 (5′-TCAACACATGGAAATAGT-3′) used in PCR amplification are also arrowed. (b) The 94 nucleotides was alternatively spliced out to generate Siah-1S, the detailed sequence is boxed. The dinucleotides GT and AC are underlined. (c) Comparison of deduced amino-acid sequences of Siah-1 to Siah-1S. Identical residues are shaded. The ORF for Siah-1S predicts a protein of 195 amino-acids in length, whereas Siah-1 consists of 282 amino acids. The GeneBank accession number for Siah-1S is EF026094. (**B**) Tissue distribution of Siah-1S. (a), results from PCR amplification using four human tissue cDNA libraries as template (Lane 1, human heart; Lane 2, human lymph node; Lane 3, human fetal brain; Lane 4, human testis). The upper panel shows the PCR fragments of Siah-1 and Siah-1S by using primer pair P1 and P3 indicated in (A,a), β-actin was used as internal control shown in the lower panel. (b), reverse transcription-PCR amplification of Siah-1 and Siah-1S using primer pair P2 and P3 indicated in A,a. Siah-1S and Siah-1 cDNA fragments amplified from breast cancer, colorectal cancer and esophagus cancer tissue samples are indicated. β-actin was used as internal control. (**C**) Endogenous expression of Siah-1S. HEK293T cells were co-transfected with pCDNA3/Siah-1 and pCDNA3/Siah-1S, and the ectopic expressed untagged Siah-1 and Siah-1S were used as migration control. At 24 h post-transfection, cells were treated with MG132 for another 8 h. Cell lysates were then harvested and subjected to Western blot analysis using anti-Siah-1 antibody (Lane 1). Total cellular proteins (50 μg) extracted from breast cancer (Lane 2), Kidney cancer (Lane 3) and esophagus cancer (Lane 4) tissue samples were also subjected to Western blotting using anti-Siah-1 antibody. Endogenous Actin was used as loading control. Asterisk indicates the non-specific bands.

that gene *u83*, one of the human herpesvirus 6, was alternatively spliced through CT-AC rule. Taking these findings together, it is not inconceivable that CT-AC may represent a new minor class of intron.

Siah-1S is degraded through ubiquitin–proteasome pathway and subjected to self-ubiquitination

Siah-1 was reported to be a RING-finger E3 ligase and is involved in ubiquitin-mediated degradation of many target proteins, including Siah-1 itself (Hu and Fearon, 1999; Lorick *et al*., 1999). To address whether Siah-1S is also subjected to degradation through ubiquitin–proteasome pathway, the half-life of Siah-1 and Siah-1S were compared. Cycloheximide (CHX, 20 μg/ml) was added to HEK293T cells expressing Flag-Siah-1 and Flag-Siah-1S, respectively, for indicated periods of time before cell extracts were collected and analysed by Western blotting. As shown in Figure 2A, the protein



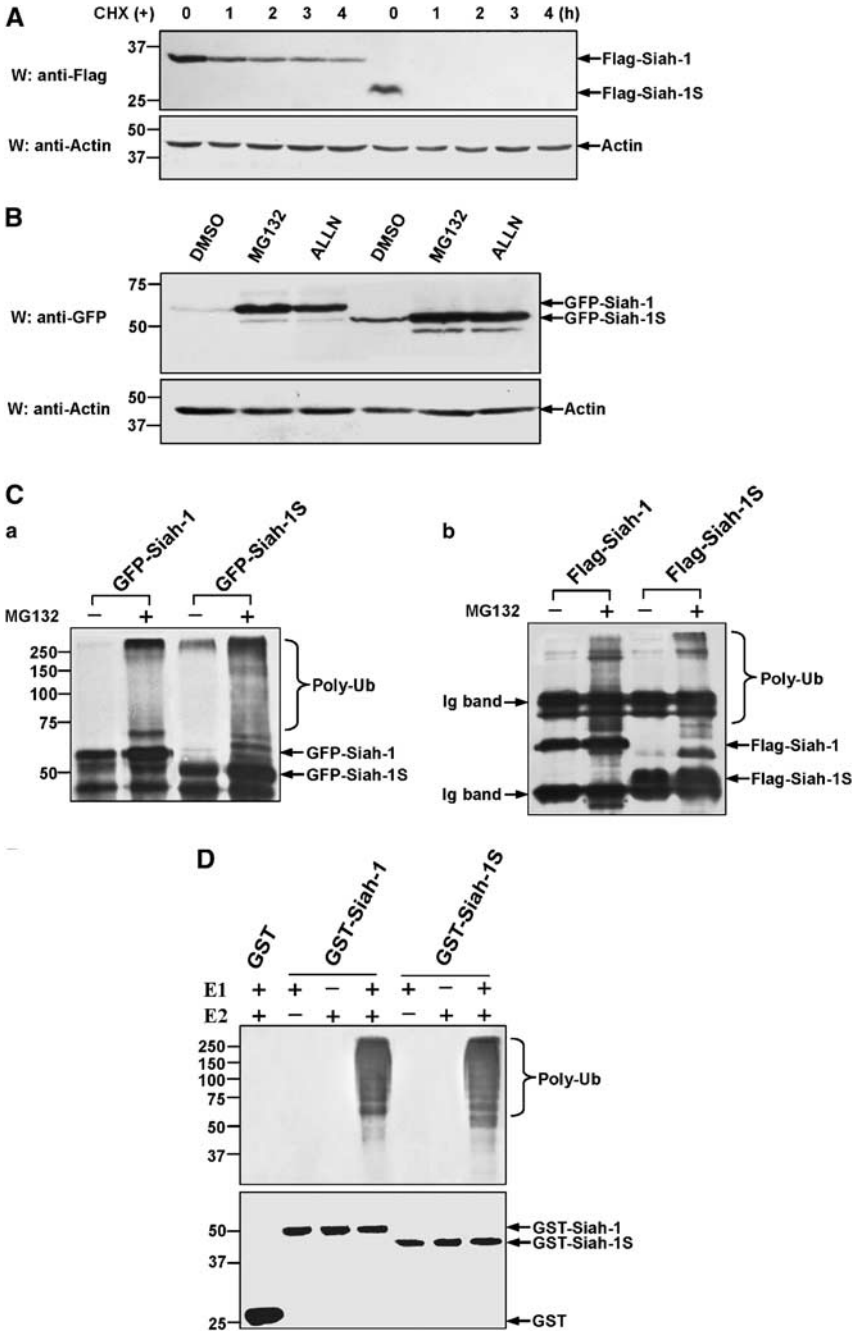


Figure 2 Self-ubiquitination of Siah-1 and Siah-1S. (**A**) Determining the stability of Siah-1 and Siah-1S. At 24 h post transfection of HEK293T cells with p3 × Flag/Siah-1 or p3 × Flag/Siah-1S, cells were treated with CHX (20 μg/ml) for indicated times (1, 2, 3 and 4 h) before cell extracts were collected. The protein levels of Siah-1 and Siah-1S were compared by Western blot analysis using anti-Flag antibody and endogenous Actin was used as loading control. (**B**) The degradation of Siah-1 and Siah-1S is subject to proteasome-mediated pathway. At 24 h after transfection of HEK293T cells with pEGFP-C1/Siah-1 or pEGFP-C1/Siah-1S, cells were incubated with or without proteasome inhibitor MG132 (20 mg/ml) or ALLN (100 μM) for another 8 h. The steady-state levels of Siah-1 and Siah-1S were then compared by Western blot analysis using anti-GFP antibody. (**C**) (a), HEK293T cells were transiently transfected with pEGFPC1/Siah-1 and pEGFPC1/Siah-1S individually. Twenty-four hours after transfection, cells were incubated with or without MG132 for another 20 h. Cell lysates were harvested and incubated with rabbit anti-GFP antibody bound to protein A/G-Sepharose. After 6 h incubation at 4°C, the immunoprecipitates were recovered by boiling beads in SDS sample buffer and analysed by Western blotting using mouse anti-GFP antibody. Polyubiquitylated GFP-Siah-1 and GFP-Siah-1S are indicated as Poly-Ub. (b), Similar to (a), HEK293T cells were transfected with either p3 × Flag/Siah-1 or p3 × Flag/Siah-1S and cell lysates were then incubated with anti-Flag antibody. The immunoprecipitates were subjected to Western blot analysis using anti-Flag antibody. Polyubiquitylated Flag-Siah-1 and Flag-Siah-1S are indicated as Poly-Ub. (**D**) Self-ubiquitination of Siah-1 and Siah-1S *in vitro*. Bacterially expressed and purified GST, GST-Siah-1 or GST-Siah-1S were subjected to an *in vitro* ubiquitination reaction in the presence of E1 and E2 singly or combinedly. The lower panel shows Ponceau S staining for same blot.

level of Siah-1S dropped far more rapidly than that of Siah-1, and Siah-1S was barely detected after 1 h treatment with CHX, indicating that Siah-1S has a shorter half-life than Siah-1. Next we examined whether the degradation of Siah-1 and Siah-1S was proteasome-dependent. As shown in Figure 2B, both the degradation of Siah-1 and Siah-1S were blocked in the presence of MG132 or ALLN, suggesting their degradation pathways are proteasome-dependent. To determine if ubiquitin is involved in the degradation pathway, an *in vivo* ubiquitination assay was performed. HEK293T cells expressing either GFP-Siah-1 or GFP-Siah-1S were treated with or without MG132 for 20 h, before they were collected. Cell lysates were immunoprecipitated with anti-GFP antibody, and immunoprecipitates were then subjected to Western blot analysis using anti-GFP antibody. As shown in Figure 2C,a, high-molecular-weight smear characteristic of polyubiquitiylated products were detected only in cells treated with MG132, but not in cells left untreated. Similar results were obtained when Siah-1 and Siah-1S were in their Flag-tagged forms (Figure 2C,b). These results indicate that both Siah-1 and Siah-1S are subjected to polyubiquitination modification under *in vivo* conditions. Since some E3 ligases possessing RING domain are auto-ubiquitinated, such as Mdm2, XIAP and c-IAP1 (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Yang *et al*., 2000), we investigated whether Siah-1 and Siah-1S can autoubiquitinate. To address this issue, an *in vitro* ubiquitination assay was performed. Bacterially expressed and purified GST, GST-Siah-1, or GST-Siah-1S was added to an *in vitro* ubiquitination reaction in the presence of E1 and/or E2. As shown in Figure 2D, both Siah-1 and Siah-1S are able to undergo self-ubiquitination. Taken together, we conclude that both Siah-1 and Siah-1S are able to be self-ubiquitinated, resulting in their subsequent degradation by proteasome.

Siah-1S upregulates β-catenin and Tcf/Lef reporter activity

Siah-1 was shown to participate in a pathway leading to β-catenin degradation independent of the phosphorylation status of β-catenin (Liu *et al*., 2001; Matsuzawa and Reed, 2001). To examine the effect of Siah-1S on β-catenin level, we performed transient transfection assays in HEK293T cells. As shown in Figure 3a, overexpression of Siah-1 markedly reduced levels of β-catenin (lane 2). In contrast, ectopic expression of Siah-1S did not induce degradation of β-catenin, rather, it enhanced levels of β-catenin (lane 3). Since β-catenin is known as a co-activator of transcription factor Tcf/Lef (Peifer and Polakis, 2000), we explored the effects of Siah-1 and Siah-1S on Tcf/Lef activity by a luciferase reporter assay. In this assay, a reporter plasmid with a mutated Tcf-binding site was used as negative control. As shown in Figure 3b, compared with control HEK293T cells, β-catenin expression exhibited a 15-fold induction of Tcf/Lef reporter activity. When Siah-1 was expressed, the Tcf/Lef transcriptional activity induced by β-catenin was reduced by almost half. In contrast, Siah-1S overexpression not only failed to suppress β-catenin-mediated activation of Tcf/Lef, but rather enhanced the Tcf/Lef reporter activity of about 1.7-fold (Figure 3b). Transactivation activity from the reporter plasmid containing the mutated Tcf/Lef-binding site (Tcf7mut) was not affected by overexpression of Siah-1 and Siah-1S either singly or combined in the presence or absence of β-catenin. These findings suggest that Siah-1S may act in a dominant negative manner to inhibit Siah-1's inhibitory effect on β-catenin-mediated activation of Tcf/Lef.

To determine whether the effect of Siah-1S on β-catenin and Tcf/Lef reporter activity is mediated through Siah-1, HEK293T cells transiently expressing GFP-β-catenin (0.2 μg) were transfected with either p3 × Flag/Siah-1(0.5 μg) or p3 × Flag/Siah-1(0.5 μg) plus p3 × Flag/Siah-1S (0.5 μg). As shown in Figure 3c, overexpression of Siah-1S is able to bring back the β-catenin level, which was suppressed by Siah-1. A reporter assay was again performed to validate the effect of Siah-1S on Siah-1's downregulation of Tcf/Lef promoter activity. As shown in Figure 3d, overexpression of β-catenin alone can induce Tcf/Lef transcriptional activity up to 10-fold. However, co-expression of Siah-1 and β-catenin reduced Tcf/Lef activity by almost half. If Siah-1S was additionally added, downregulated Tcf/Lef transcriptional activity owing to Siah-1 suppression can be completely restored (Figure 3d, column 4). To further determine whether upregulation of β-catenin is specifically owing to Siah-1S, HEK293T cells were co-transfected with fixed amount of pEGFP-C1/β-catenin (0.2 μg) and an increasing amount of p3 × Flag/Siah-1S (0, 0.15, 0.3, 0.45, 0.6 μg). As shown in Figure 3e, levels of β-catenin were concurrently increased with increasing expression of Siah-1S. Taken together, these data suggest that Siah-1S play a dominant negative role to upregulate β-catenin and Tcf/Lef reporter activity.

Siah-1S antagonizes the potentiation effect of Siah-1 on etoposide-induced apoptosis

To explore the functional significance of Siah-1 and Siah-1S in tumor cell survival, cell viability in response to anticancer drug etoposide was assessed. MCF-7 cells were transiently transfected with plasmid encoding either Siah-1 or Siah-1S, and cell death was measured 48 h after treatment with etoposide (25 μg/ml). As shown in Figure 4A,a and b, Siah-1 enhanced the cell death by about 50% compared to the control, however, Siah-1S was shown to antagonize etoposide-induced apoptosis. We have demonstrated that Siah-1S serves as a dominant negative inhibitor of Siah-1, so we next determined whether Siah-1S could affect Siah-1's potentiation effect on etoposide-induced apoptosis. MCF-7 cells were transfected with plasmids encoding either Siah-1 or Siah-1 plus Siah-1S, and cell death was measured 48 h after treatment with etoposide (25 μg/ml). As expected, Siah-1 sensitizes MCF-7 cells to etoposide-induced apoptosis, yet co-expression of Siah-1S and Siah-1 was shown to partly recover the reduced cell viability owing to Siah-1 expression (Figure 4B,a and b).

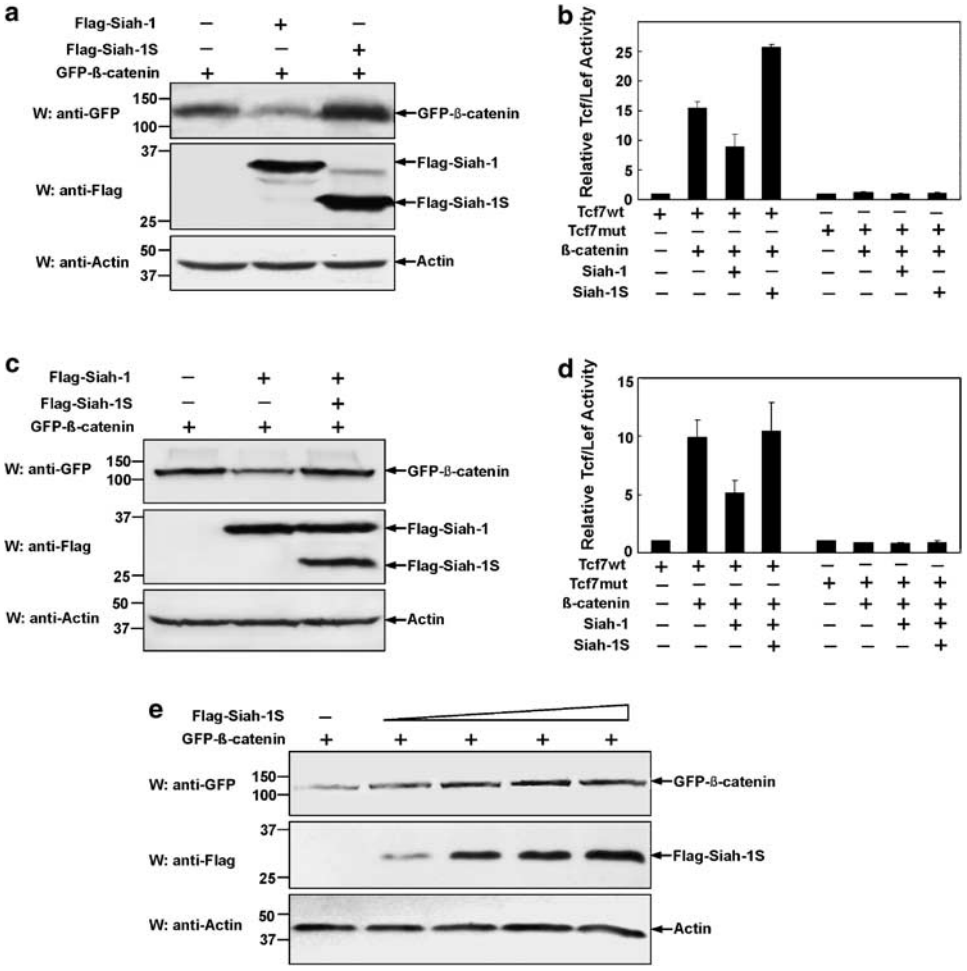


Figure 3 Effects of Siah-1 and Siah-1S on β-catenin and Tcf/Lef reporter activity. (**a**) HEK293T cells were transiently transfected with 0.2 μg of plasmid encoding GFP-β-catenin and Flag-Siah-1 (0.6 μg) or Flag-Siah-1S (0.6 μg) (total DNA amount normalized by empty vector). After 24 h, cell lysates were harvested and equal amount of total proteins were subjected to Western blot analysis using indicated antibodies with ECL-based detection. Endogenous Actin was used as loading control. (**b**) HEK293T cells were transfected with either pTcf7wt-Luc or pTcf7mut-Luc reporter plasmid, and both cells were further transfected with pEGFPC1/β-catenin, p3 × Flag/Siah-1 and p3 × Flag/Siah-1S singly or combinedly as indicated. *Renilla* luciferase plasmid pRL-CMV was also introduced into transfected cells as internal control. At 24 h after transfection, luciferase activity was measured and plotted after normalizing with respect to *Renilla* luciferase activity (mean ± s.d.). (**c**) HEK293T cells were transfected with plasmids GFP-β-catenin (0.2 μg), Flag-Siah-1 (0.5 μg) and Flag-Siah-1S (0.5 μg) singly or combinedly as indicated. Total DNA amount was normalized by the addition of empty p3 × Flag vector. Cell lysates preparation and Western blot analysis were performed according to those described in (**a**). (**d**) HEK293T cells were transiently transfected with either pTcf7wt-Luc or pTcf7mut-Luc reporter plasmid. Both cells were further transfected with pEGFPC1/β-catenin, p3 × Flag/Siah-1 and p3 × Flag/Siah-1S singly or combinedly as indicated. *Renilla* luciferase plasmid pRL-CMV was used as internal control. At 24 h after transfection, luciferase activity was measured and plotted after normalizing with respect to *Renilla* luciferase activity (mean ± s.d.). (**e**) HEK293T cells were transfected with pEGFPC1/β-catenin (0.2 μg) and increasing amount of p3 × Flag/Siah-1S (0, 0.15, 0.3, 0.45 and 0.6 μg). At 24 h post-transfection, cell lysates were analysed by Western blotting using anti-GFP antibody to compare β-catenin protein levels.

*Siah-1*Siah-1, but not Siah-1*Siah-1S or Siah-1S* Siah-1S, is able to interact with SIP*

SIP was shown to be a component of a novel ubiquitination pathway regulating β-catenin degradation through formation of a complex with Siah-1, Skp1 and Ebi (Matsuzawa and Reed, 2001). More recently, results from SIP knock-out mice showed that the degradation of β-catenin in response to DNA damages was significantly impaired in SIP$^{-/-}$ cells (Fukushima *et al*., 2006). To investigate whether Siah-1S can also interact with SIP, the immunoprecipitation assays were performed. HEK293T cells expressing Flag-Siah-1 or Flag-Siah-1S were individually transfected with either pEGFP-C1 (mock) or pEGFP-C1/SIP. Twenty-four-hour post-transfection, cells were treated with MG132 for another 8 h before cell lysates were harvested and subjected to co-immunoprecipitation. As shown in Figure 5A,a, Flag-Siah-1 could be immunoprecipitated with GFP-SIP, but not GFP alone, indicating that Siah-1 is able to bind SIP. However, Siah-1S failed to interact with SIP under the same experimental conditions as used in the co-immunoprecipitation study (Figure 5A,b).

Siah-1 was shown to form homodimer to produce three clefts or grooves where protein–protein interactions take

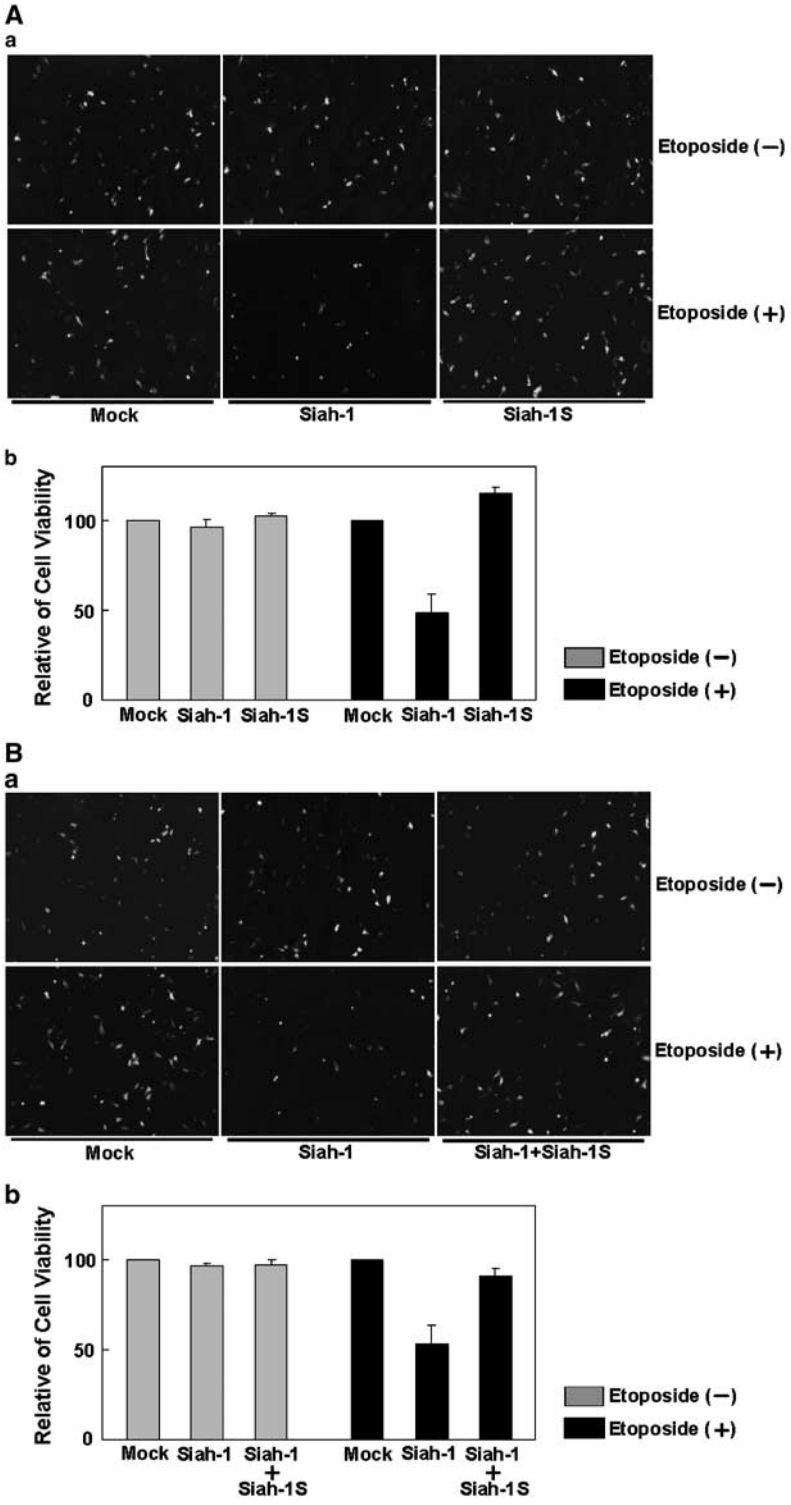


Figure 4 Siah-1S antagonizes Siah-1's potentiation effect on Etoposide-induced apoptosis in MCF-7 cells. (**A**) The effect of Siah-1 and Siah-1S on Etoposide induced apoptotic cell death. (a), MCF-7 cells expressing GFP were individually transfected with empty vector p3 × Flag (mock), p3 × Flag/Siah-1 or p3 × Flag/Siah-1S. At 24 h after transfection, cells were treated with etoposide (25 μg/ml) for another 48 h. (b), The viability of cells was measured by counting GFP-positive viable cells, and the relative viability was plotted. Values were shown as means ($\pm$s.d.). (**B**) Siah-1S diminishes Siah-1's potentiation effect on Etoposide induced cell death. (a), MCF-7 cells expressing GFP were transfected with mock vector, p3 × Flag/Siah-1 and p3 × Flag/Siah-1 plus p3 × Flag/Siah-1S separately. At 24 h later, cells were treated with etoposide (25 μg/ml) for another 48 h. (b), The viability of cells was measured by counting GFP-positive viable cells, and the relative viability was plotted. Values were shown as means ($\pm$s.d.).

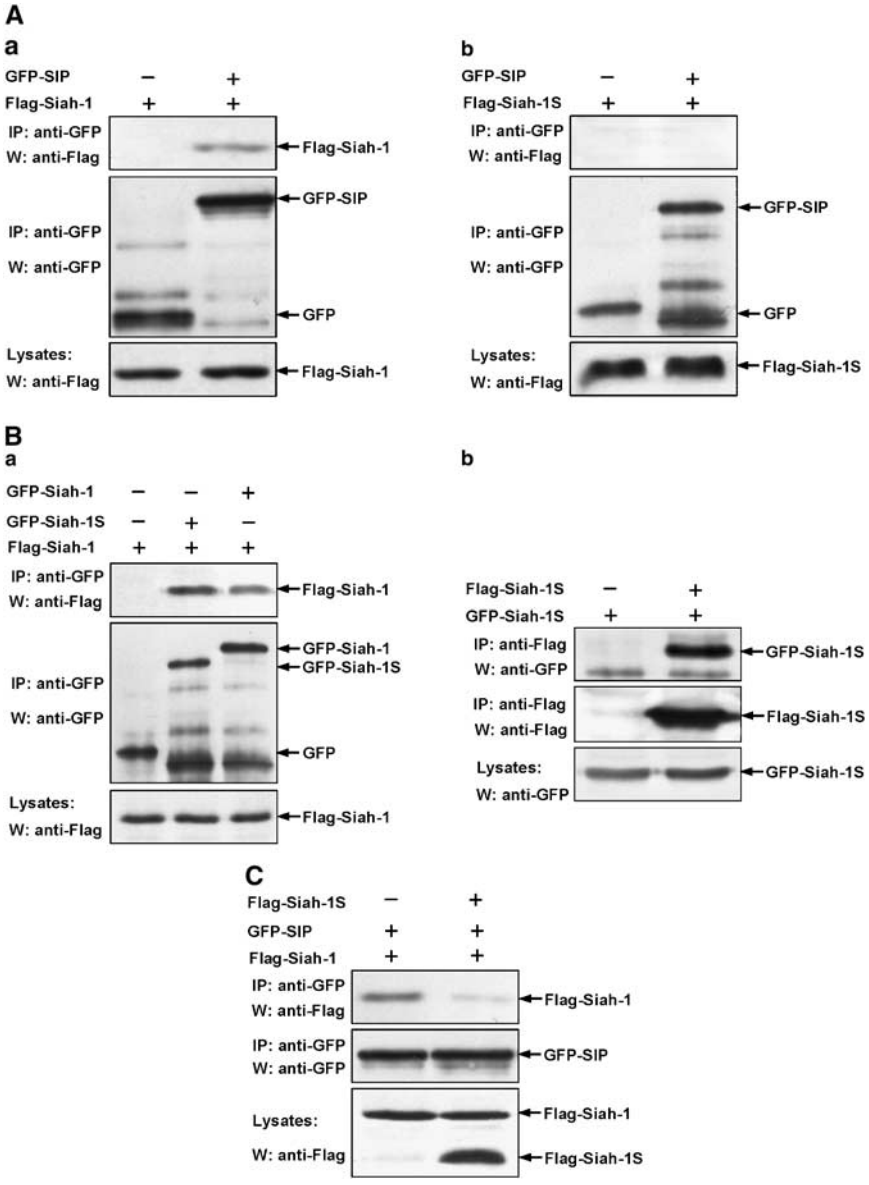


Figure 5 Heterodimer formed by Siah-1S and Siah-1 prevents Siah-1's binding to SIP. (**A**) Siah-1S loses the ability to bind to SIP. HEK293T cells expressing Flag-Siah-1 (a) or Flag-Siah-1S (b) were transfected with or without pEGFPC1/SIP. Twenty-four hours after transfection, cells were treated with MG132 (20 mg/ml) for another 8 h. Cell lysates were then immunoprecipitated with anti-GFP antibody bound to protein A/G-Sepharose. The immunoprecipitates were recovered in SDS sample buffer and subjected to Western blot analysis using anti-Flag antibody with ECL-based detection. (**B**) Siah-1S interacts with Siah-1 to form heterodimer or with itself to form homodimer. (a) HEK293T cells were transfected with p3 × Flag-Siah-1, pEGFPC1/Siah-1 and pEGFPC1/Siah-1S singly or combinedly as indicated. At 24 h after transfection, cells were treated with MG132 for another 8 h. Cell lysates were then immunoprecipitated with anti-GFP antibody. The immunoprecipitates were probed with anti-Flag antibody. (b), HEK293T cells expressing GFP-Siah-1S were transfected with or without p3 × Flag-Siah-1S. At 24 h post-transfection, cells were treated with MG132 for another 8 h, and cell lysates were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were probed with anti-GFP antibody. (**C**) HEK293T cells expressing GFP-SIP and Flag-Siah-1 were transfected with or without p3 × Flag/Siah-1S. At 24 h after transfection, cells were treated with MG132 for another 8 h. Cell lysates were harvested and immunoprecipitated with anti-GFP antibody. After recovering immune complexes and washing, the immunoprecipitates were analysed by Western blotting using anti-Flag antibody.

place (Polekhina *et al*., 2002; Matsuzawa *et al*., 2003). To test whether Siah-1S could also interact with Siah-1 to form a heterodimer, HEK293T cells expressing Flag-Siah-1 were transfected with pEGFP-C1, pEGFP-C1/Siah-1 or pEGFP-C1/Siah-1S separately. After treatment with MG132 for 8 h, cells were harvested and conducted to immunoprecipitation. As shown in Figure 5B,a, Flag-Siah-1 could be immunoprecipitated by GFP-Siah-1 or GFP-Siah-1S, but not GFP alone, demonstrating that Siah-1 may indeed physically interact with Siah-1 or Siah-1S in mammalian cells. To examine further whether Siah-1S was able to interact with itself to form homodimer, cell lysates from HEK293T cells expressing GFP-Siah-1S or GFP-Siah-1S plus Flag-Siah-1S were

subjected to co-immunoprecipitation. As shown in Figure 5B,b, Flag-Siah-1S was able to co-precipitate GFP-Siah-1S. Combined, these results showed that Siah-1 could interact with either Siah-1S to form Siah-1 *Siah-1S heterodimer or with itself to form homodimer Siah-1*Siah-1. Moreover, Siah-1S was able to interact with itself to form Siah-1S*Siah-1S homodimer.

Given that Siah-1S is able to interact with Siah-1 to form heterodimer, we investigated whether Siah-1S could interfere the interaction between Siah-1 and SIP. To address this issue, HEK293T cells transiently co-expressing Flag-Siah-1 and GFP-SIP were further transfected with or without p3 × Flag/Siah-1S. After treatment with MG132 for 8 h, cells were harvested and immunoprecipitation was undertaken. Compared to control, the presence of Siah-1S significantly reduced the amount of precipitated Flag-Siah-1 (Figure 5C, top panel). The middle panel in Figure 5C showed the input SIP was comparable. These findings suggest that Siah-1S may play a dominant negative effect on preventing Siah-1-mediated degradation of β-catenin by forming Siah-1*Siah-1S heterodimer, which is unable to associate with SIP.

Siah-1S has an opposite effect on cells tumorigenecity in vitro *reduced by Siah-1*

Siah-1 has been reported to suppress tumor formation (Roperch *et al*., 1999; Tuynder *et al*., 2002). Since Siah-1S acts as a dominant-negative inhibitor of Siah-1, we investigated the effect of Siah-1S on tumorigenecity of cells. To address this issue, *in vitro* soft agar assay was performed. A total of 5×10^3 MCF-7 cells stably expressing GFP, GFP-Siah-1, or GFP-Siah-1S were individually plated in six-well plates. After 3 weeks incubation at 37°C, cells were fixed with 70% ethanol and stained with trypan blue. As shown in Figure 6A and B, the number of colony-forming cells expressing GFP-Siah-1 was reduced by more than 70% compared with GFP-expressing cells. In contrast, the number of colony-forming cells expressing GFP-Siah-1S did not show any reduction, and rather, it increased by 40% compared with GFP-expressing cells. These results demonstrate that while Siah-1 shows ability to reduce cells tumorigenicity, its splice variant Siah-1S exhibits an opposite effect on promoting cells tumorigenicity *in vitro*.

Discussion

In this study, we have identified a naturally occurring alternatively spliced variant of *Siah-1* transcript, designated *Siah-1S*, which has a diametrically opposing action as compared with Siah-1. Siah-1S is produced by the deletion of 94 nucleotides (576–669 nt) from the full length Siah-1 cDNA, and as a result, Siah-1S has 87 amino-acid residues less than Siah-1. It is interesting to note that no consensus splice donor and splice acceptor sites were found to mediate this novel splicing, and moreover, this novel intron/exon junction sequence does not match either the major (GT-AG) or minor (AT-AC) class splice sites (Kramer, 1996), rather it utilizes CT-AC as donor and acceptor splice site dinucleotides. Coincidently, French *et al*. (1999) has recently reported that gene *u83* belonging to human Herpesvirus 6, also follows CT-AC rule, indicating that CT-AC may represent a new minor splicing class.

It has been known that many E3 ligases possessing RING domain are subjected to self-ubiquination (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Yang *et al*., 2000). Here, we demonstrated that compared with Siah-1, Siah-1S is even less stable and it also undergoes self-ubiquitination. β-catenin is a multifunctional cytoplasmic protein, which plays an important role in Wnt signaling pathway and in the maintenance of cell–cell adhesion (Provost and Rimm, 1999). β-Catenin accumulation results in its translocation into nucleus and stimulates Tcf/Lef-family transcription factors (Peifer and Polakis, 2000), which, in turn, induces expression of target genes important for cell proliferation (He *et al*., 1998; Tetsu and McCormick, 1999). In this study, we demonstrated that unlike Siah-1, Siah-1S could upregulate β-catenin level and Tcf/Lef transcriptional activity, providing a potential link between Siah-1S and β-catenin.

It has been reported that overexpression of Siah-1 mutant, which has lost the ability to bind SIP, was shown to have a dominant-negative effect on the regulation of β-catenin (Matsuzawa *et al*., 2003), and the underlying mechanism has not yet been fully elucidated. We showed that Siah-1S with the destruction of intact β-sheets is able to interact with Siah-1 to form heterodimer or with itself to form homodimer. Unlike Siah-1*Siah-1, neither Siah-1*Siah-1S nor Siah-1S *Siah-1S is able to bind to SIP, which could be owing to the possibility that the binding groove's structure in Siah-1*Siah-1S or Siah-1S*Siah-1S has been altered/destroyed. The failure of SIP in binding to Siah-1*Siah-1S or Siah-1S*Siah-1S may be the underlying mechanism for Siah-1S's dominant-negative effect. It is expected that with more Siah-1S, less functional homodimer Siah-1*Siah-1 will be formed, resulting in alleviating the destruction of β-catenin. These findings also suggest that Siah-1*Siah-1 interactions with SIP involve the entire concave surface, but not intermolecular contacts with just one monomer.

Siah-1 has been reported to induce apoptosis and suppress tumor formation (Matsuzawa *et al*., 1998; Roperch *et al*., 1999; Tuynder *et al*., 2002). Consistent with these reports, we demonstrated that overexpression of Siah-1 sensitizes etoposide-induced apoptosis and reduces cells tumorigenicity *in vitro*. In contrast, Siah-1S was shown to play an anti-apoptotic role and have ability to promote cells tumorigenicity *in vitro*, which may be partly because of the upregulation of oncogenic β-catenin and Tcf/Lef transcriptional activity by Siah-1S. Despite the oncogenic and apoptotic importance of Siah-1S, the physiological function of this variant has not yet been elucidated, for example, whether Siah-1S expression is associated with some particular human cancers, or how the Siah-1S expression is regulated. In conclusion, Siah-1S displays several distinctive properties, which are summarized in Table 1.

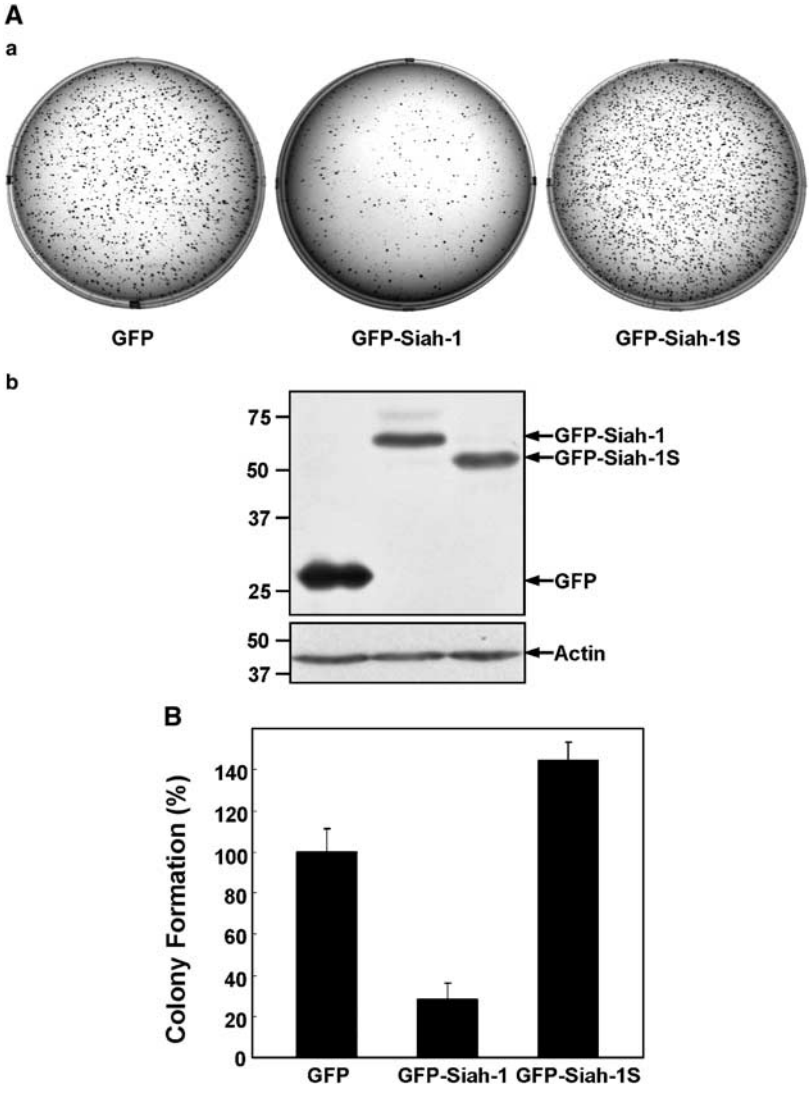


Figure 6 Effects of Siah-1 and Siah-1S on anchorage-independent growth of MCF-7 cells. (**A**) (a), MCF-7 cells stably expressing GFP, GFP-Siah-1, or GFP-Siah-1S were assayed for their ability to proliferate and to form colonies in soft agar. For the colony formation assay, 5×10^3 cells were used. After incubation for 3 weeks, the cells were fixed with 70% ethanol and stained with trypan blue. The relative protein levels of GFP, GFP-Siah-1 and GFP-Siah-1S from their respective stable cell lines were shown in (b). Endogenous Actin was used as loading control. (**B**) The colony number of the cells expressing GFP was arbitrarily set as 100, and the percentages of colony numbers present in the GFP-Siah-1 and Siah-1S plates were calculated and plotted. The mean numbers of colonies were from four independent experiments.

Table 1 Characteristics of Siah-1S

	Dimer formation	*Interact with SIP*	*Degrade β-catenin*	*Induce apoptosis*
Siah-1*Siah-1	Yes	Yes	Yes	Yes
Siah-1*Siah-1S	Yes	No	No	No
Siah-1S*Siah-1S	Yes	No	No	No

Materials and methods

Reagents and antibodies

The following antibodies were used in this study Monoclonal antibodies: anti-GFP (BD Biosciences, Palo Alto, CA, USA), anti-Flag (Sigma, St Louis, MO, USA) and anti-β-actin (Abcam, Cambridge, United Kingdom) and polyclonal antibody anti-GFP (BD Biosciences) and anti-Siah-1 (P-18) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). cycloheximide (CHX), etoposide, geneticin (G418) and ALLN (N-acetyl-leu-leu-norleucinal) were purchased from Sigma, and MG132 was purchased from Calbiochem (La Jolla, CA, USA).

Cell culture and transfection

Cell lines MCF-7 and HEK293T were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1 × nonessential amino-acid, 100 μg/ml penicillin, 1 × MEM sodium pyruvate, 100 μg/ml streptomycin (Invitrogen, Carsbad, CA, USA) at 37°C under an atmosphere of 5% CO_2 in air. Transfection of cells with various mammalian expression constructs by Lipofectamine™ 2000 (Invitrogen) was according to the methods provided by manufacturer's specification.

Establishing Siah-1 or Siah-1S stably expressing MCF-7 cell line was performed according to the method described by

Bruzzoni-Giovanelli *et al*. (1999). Briefly, pEGFPC1/Siah-1, pEGFPC1/Siah-1S and pEGFPC1 control vector was individually transfected into MCF-7 cells. Forty-eight hours after transfection, clones were selected in culture medium containing 1 mg/ml geneticin (G418) for 3 weeks. The positive clones were further confirmed by Western blotting.

Western blot analysis and immunoprecipitation
Western blotting were performed mainly as described by Mei *et al*. (2005) with following modifications. After incubated with first and second antibodies, blots were developed by ECL using Lumi-Phos WB (Pierce, Rockford, IL, USA). For immunoprecipitation, HEK293T Cells were transiently transfected with the indicated plasmids. After 24 h, cells were treated with 20 mg/ml MG132 for 8 h and lysed in a Triton-X-100-based lysis buffer (1% Triton-X-100, 150 mM NaCl, 20 mM HEPES, PH 7.4, 2 mM EDTA, 5 mM $MgCl_2$) supplemented with protease inhibitor cocktail and 20 mg/ml MG132 for 1 h on ice. The nuclear and cellular debris were cleared by centrifugation. The cytosolic lysate was incubated with indicated antibody bound to protein A/G-Sepharose. After incubation at 4°C overnight, the immunoprecipitates were washed five times in lysis buffer, and proteins were recovered by boiling the beads in sodium dodecyl sulfate (SDS) sample buffer and analysed using a Western blot.

Protein stability assay
HeLa cells in six-well plates were cultured to subconfluence and transiently transfected with indicated plasmids, followed by incubation at 37°C for 30 h. The cells were treated with 25 μg/ml CHX for indicated periods of time and were then harvested and analysed by Western blotting as described above.

Reporter assays
To measure Tcf/Lef transcriptional activity, HEK293T cells were transiently transfected with a reporter construct pTOP-FLASH or pFOP-FLASH (kindly provided by Dr Shu-ichi Matsuzawa) and various expression plasmids. *Renilla* luciferase reporter plasmid was included as internal control. Firely and *Renilla* luciferase activity were assayed using Dual-Luciferase Reporter Assay System according to manufacture's instructions (Promega corporation, WI, USA). Tcf/Lef reporter activities were normalized relative to *Renilla* luciferase activities and presented as means ($\pm$s.d.) of three independent experiments.

In vitro *ubiquitination assay*
Bacterially expressed and glutathione-beads-purified GST, GST-Siah-1 and GST-Siah-1S bound to glutathione beads were washed with ubiquitination buffer (50 mM Tris-HCL pH 8.0, 5 mM $MgCl_2$, 2 mM NaF, 1 mM dithiothreitol), and then subjected to *in vitro* ubiquitination reaction using ubiquitination buffer contained 50 nM E1, 500 nM UbcH5C (BostonBiochem, Cambridge, MA, USA), 25 uM Flag-Ubiquitin and 2 mM ATP (Sigma) for 1 h at 37°C. GST, GST-Siah-1 and GST-Siah-1S beads were washed with ubiquitination buffer for six times, and proteins were recovered by boiling the beads in 1 $\times$ SDS loading buffer and analysed by Western blotting using anti-flag antibody.

Cell death assay
Cell death assay were performed mainly as described previously (Iwai *et al*., 2004). Briefly, MCF-7 cells were grown in 24-well plates to 25% confluent and then transiently transfected with pEGFP-C1 and various indicated expression plasmids for 24 h, followed by treated with etoposide (25 μg/ml) or dimethylsulfoxide. After 48 h, GFP-positive cells were counted. The relative survival rate was normalized to the number of GFP-positive cells transfected with empty vector, and presented as means ($\pm$s.d.) of three independent experiments.

Colony formation in soft agar
MCF-7 cells stably expressing GFP, GFP-Siah-1 or GFP-siah-1S were individually suspended in DMEM containing 10% FBS and 0.3% Seaplaque low melting temperature agarose (BioWhittaker Molecular Applications, Rockland, ME, USA), and 1.5 ml containing 5×10^3 cells were plated in six-well plates over a 1.5 ml layer of solidified DMEM/10% FBS/0.6% agarose. Then plate was incubated at 37°C for 3 weeks. The cells were fixed with 70% ethanol and stained with trypan blue (Sigma).

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