

# Induction of $\beta$ -catenin by the suppression of signal regulatory protein $\alpha 1$ in K562 cells

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Received December 14, 2010; Accepted February 4, 2011

DOI: 10.3892/ijmm.2011.630

**Abstract.** The signal regulatory protein (SIRP)  $\alpha 1$  is a cell surface receptor expressed predominantly in monocytes, granulocytes, dendritic cells, as well as hematopoietic stem cells. In contrast, SIRP $\alpha 1$  expression is significantly reduced in the majority of myeloid malignancies. SIRP $\alpha 1$  is a negative regulator of signaling and its reduced expression is considered to play a role in the pathogenesis of these diseases through aberrant signaling. To identify SIRP $\alpha 1$  downstream target genes, we established SIRP  $\alpha 1$ -knockdown chronic myeloid leukemia K562 (K562SIRP $\alpha 1$ KD) cells expressing reduced levels of SIRP $\alpha 1$  by stably transfecting SIRP $\alpha 1$  siRNAs. Microarray analysis demonstrated that several genes, including  $\beta$ -catenin, were significantly induced in K562SIRP $\alpha 1$ KD cells. Real-time PCR and Western blot analyses, confirmed the induction of this gene. Phosphorylation of Ser9 of glycogen synthesis kinase (GSK)  $-3\beta$ , results in the inactivation of GSK- $3\beta$ , leading to the induction of  $\beta$ -catenin. We found significant phosphorylation of extracellular signal-regulated kinase (ERK), Akt, as well as of GSK- $3\beta$ -Ser9, which may play a role in the up-regulation of  $\beta$ -catenin in K562SIRP $\alpha 1$ KD cells. To our knowledge, this is a first report demonstrating the relationships between SIRP $\alpha 1$  and  $\beta$ -catenin in leukemia cells.

## Introduction

The signal regulatory proteins (SIRPs) comprise a family of cell surface receptors that regulate signal transduction through tyrosine kinase receptors by interacting with Src homology 2 domain-containing protein tyrosine phosphatases (SHP) -1 and SHP-2 (1). SIRPs are also termed Src homology 2 domain-containing phosphatase substrate-1 (SHPS-1),

brain immunoglobulin-like molecule with a tyrosine-based activation motif (BIT), p84, and macrophage fusion receptor (MFR). SIRPs are transmembrane glycoproteins consisting of a large extracellular region with 3 immunoglobulin-like domains, a single hydrophobic transmembrane region, and a cytoplasmic tail containing 2 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (1). In humans, there are at least 15 members designated as SIRPs. They consist of two subtypes distinguished by the presence or absence of a cytoplasmic domain containing ITIMs. SIRP $\alpha 1$  is the most characterized member of the human SIRP family. Its overexpression leads to a reduced responsiveness to tyrosine kinase ligands, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin (1). Therefore, SIRP $\alpha 1$  is considered as a negative regulator of signaling.

SIRP $\alpha 1$  is expressed predominantly in monocytes, granulocytes, dendritic cells, and their precursors, as well as in bone marrow hematopoietic stem cells (2). In contrast, SIRP $\alpha 1$  expression is absent or significantly reduced in the majority of myeloid blasts from patients with acute myeloid leukemia (AML) or chronic myeloid leukemia (CML) (2). The data suggest that the down-regulation of SIRP $\alpha 1$  plays a role in the aberrant signal transduction, contributing to the pathogenesis of hematological malignancies.

In the present study, we first cloned cell lines expressing reduced levels of SIRP $\alpha 1$  by stable transfection of SIRP $\alpha 1$  short inhibitory RNAs (siRNAs) and tried to identify the target genes regulated by SIRP $\alpha 1$ . By performing microarray analysis between SIRP $\alpha 1$ -knockdown cells and parental cells, we found that  $\beta$ -catenin was significantly induced in SIRP $\alpha 1$ -knockdown cells. Furthermore, we revealed aberrant signaling in these cells.

## Materials and methods

**Cell culture and generation of SIRP $\alpha 1$ -knockdown cells.** K562 cells were grown in RPMI (Gibco-BRL, Rockville, MD) containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. To generate SIRP $\alpha 1$ -knockdown cells, SIRP $\alpha 1$  siRNA expression vectors and their control vector were transfected into K562 cells by electroporation. Two different SIRP $\alpha 1$  siRNA expression vectors (pcPURU6bicassette), designed by the manufac-

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**Key words:** signal regulatory protein  $\alpha 1$ , hematological malignancies,  $\beta$ -catenin

turer (Takara, Otsu, Japan), were employed for this study to obtain optimal effects. The target sequences of these siRNA expression vectors were, GACACAATGATATCACAT and GCCTCAACCGTTACAGAGA. In addition, we employed the pcPURU6biSTOP vector (Takara) as a control. The electroporation procedure was performed as previously described (3,4). K562 clones stably transfected with the SIRPα1 siRNA vectors were isolated by limiting dilution by selection with 1 mg/ml puromycin for approximately 3–4 weeks. The clones were isolated and maintained for further analysis.

**Microarray analysis.** Total cellular RNA was isolated from parental and SIRPα1-knockdown cells using an RNA mini purification kit (Qiagen, Miami, FL) according to the manufacturer's protocol. The samples were then sent to the manufacturer and analyzed. A human 35K array (Operon Biotechnologies, Huntsville, AL) involving 35,000 human genes was employed for this study.

**mRNA expression analysis.** Total RNA was extracted from transgenic cells by the Isogen reagent (Nippon Gene, Tokyo, Japan) and reverse transcription was performed with Superscript II (Invitrogen). Quantitative real-time PCR was performed using the Quantitect SYBR-Green PCR reagent (Qiagen) according to the manufacturer's protocol and an Opticon mini real-time PCR instrument (Bio-Rad, Hercules, CA) as previously described (5). The sequences of the primers were: β-catenin: forward, 5'-TGCACATCAGGATACCC AGC-3' and reverse, 5'-GCAAGTTCACAGAGGACCCC-3'; Aven: forward, 5'-GATTTTCAGTGTCTCCTTAG-3' and reverse, 5'-CCTTGCCATCATCAGTTCTC-3' (3); interferon-inducible transmembrane protein 1 (IFITM1): forward, 5'-TGACAAGGAGGAACATGAG-3' and reverse, 5'-CTGT TACAGAGCCGAATACC-3' (4); insulin-like growth factor (IGF)-1A: forward, 5'-GTGGATGCTCTTCAGTTCGTGT GTG-3' and reverse, 5'-TG GCATGTCACTCTTCACTCC-3' (5); glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5'-GAAGGTGAAGGTCGGAGT-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3' (6). The thermal cycling conditions were: step 1, 95°C for 15 min; step 2, 95°C for 30 sec; step 3, 55°C for 30 sec; step 4, 72°C for 30 sec. Steps 2–4 were repeated for 35 cycles. The copy number of each sample was calculated as previously described (7).

**Surface marker expression analysis by flow cytometry.** For flow cytometry analysis, the cells were incubated with PE-conjugated mouse anti-human CD172a (BioLegend, San Diego, CA) or PE mouse IgG (BioLegend), lysed with FACS lysis solution (Becton-Dickinson, Mountain View, CA) and applied to a Beckman Coulter Epics XL flow cytometer (Beckman Coulter, Switzerland), as described previously (8,9).

**Western blot analysis.** First, the cells were collected and lysed in buffer A (10 mM Hepes, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM DTT, 1X protease inhibitor cocktail) (Roche, Indianapolis, IN) for 10 min on ice. After 1,300 x g centrifugation for 10 min, the resulting supernatants were collected as cytosol extracts. The pellets were washed with buffer B (20 mM Hepes, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>,

1X protease inhibitor cocktail), suspended and incubated on ice for 10 min, centrifuged at 8,000 x g for 15 min and the supernatants were collected, representing the nuclear extract. Proteins of 20–30 μg were separated by SDS-PAGE, transferred to Hybond-P PVDF membranes (GE Healthcare, Piscataway, NJ) and immunoblotted as described previously (10). To examine the expression of β-catenin, mouse monoclonal anti-β-catenin (BD Biosciences, San Jose, CA) and rabbit polyclonal anti-RNA polymerase II (pol-II) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used. To examine the signaling, anti-phospho-ERK, anti-phospho-GSK-3β (Ser9) and anti-β-catenin rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-total-ERK mouse monoclonal was obtained from BD Biosciences.

## Results

**Establishment of SIRPα1-knockdown K562 cells.** RNA interference using siRNAs has been widely explored for the suppression of cellular mRNA levels to investigate the functions of specific genes. In the present study, we used a plasmid vector-based siRNA expression system to silence SIRPα1 expression. We first generated human chronic myeloid leukemia K562 cells stably expressing SIRPα1 siRNAs as well as control siRNA vector-transfected clones as controls. K562 cells were selected for this study because of their relatively high transfection efficiency among hematopoietic cell lines. Among the >30 lines isolated and analyzed, clone 2-6 exhibited sufficient suppression of SIRPα1 mRNA and protein expressions (Fig. 1). In addition, clone 1-2 had a modest level of SIRPα1 suppression compared with the vector-transfected clones vec 4, vec 5 and vec 6 (Fig. 1). Thereafter, clones 2-6 and 1-2 were used to assess the effects of SIRPα1 suppression, and were designated as K562SIRPα1-knockdown (K562SIRPα1KD) cells.

**Microarray analysis of SIRPα1-knockdown cells.** To identify SIRPα1 downstream target genes, microarray analysis were carried out in 2-6 cells and vec 6 cells (controls) using a human 35K array containing 35,000 human genes. The microarray analysis identified several candidate SIRPα1 target genes. Among the 35,000 genes examined, 155 genes were up-regulated by ≥2.5-fold in 2-6 cells compared with vec 6 cells. The top 100 up-regulated genes are shown in Table I. A further 21 genes were down-regulated in 2-6 cells by ≥2.5-fold compared with vec 6 cells (Table II). These results suggest that a large portion of genes were up-regulated by SIRPα1 suppression. In this study, we focused on the genes with fully characterized functions that are known to play roles in hematopoiesis or oncogenesis, to clarify the roles of SIRPα1 in leukemogenesis. On this basis, we selected IGF-1A (5), IFITM1 (4), Aven (3) and β-catenin (11), as candidates for SIRPα1 target genes for further analysis (Table I, bold type).

**Induction of β-catenin by the suppression of SIRPα1 in K562 cells.** Next, we evaluated the expression levels of these genes by quantitative real-time PCR. IGF-1A, IFITM1 and Aven expression in K562SIRPα1KD cells (1-2, 2-6) was not higher than that in all of the control cell lines (Fig. 2A and C).

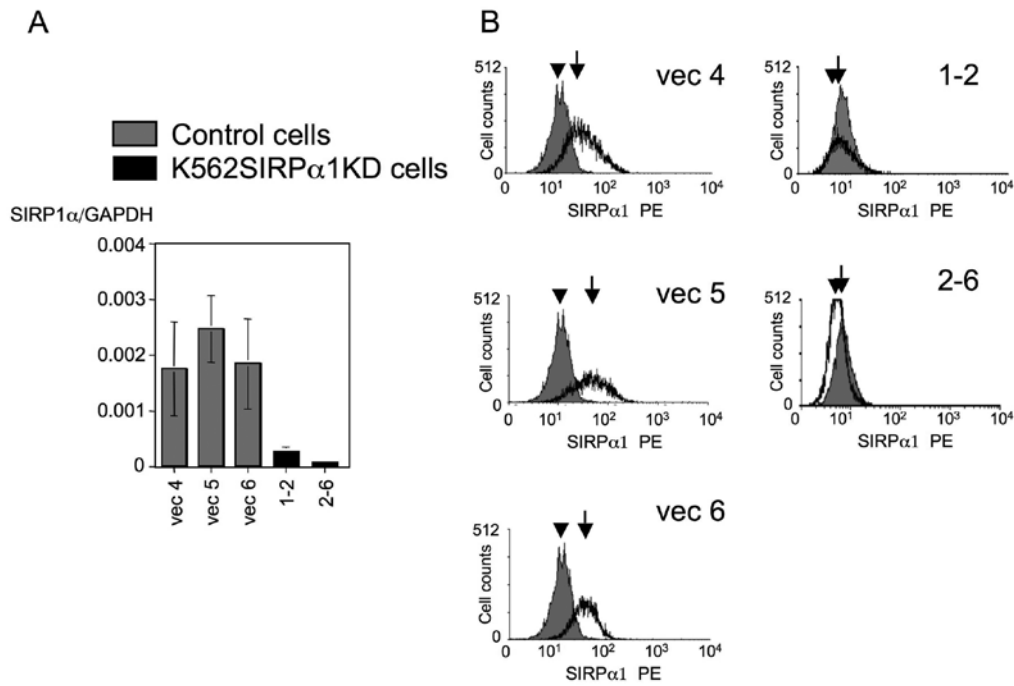


Figure 1. Establishment and characterization of signal regulatory protein (SIRP)  $\alpha$ 1-knockdown cells. (A) Expression of SIRP $\alpha$ 1 in K562SIRP $\alpha$ 1KD (1-2 and 2-6) and control (vec 4, vec 5 and vec 6) cells were examined by quantitative real-time PCR. (B) Flow cytometry analysis of SIRP $\alpha$ 1 expression in K562SIRP $\alpha$ 1KD cells. Arrows indicate the SIRP $\alpha$ 1 expression while arrowheads indicate control PE-IgG.

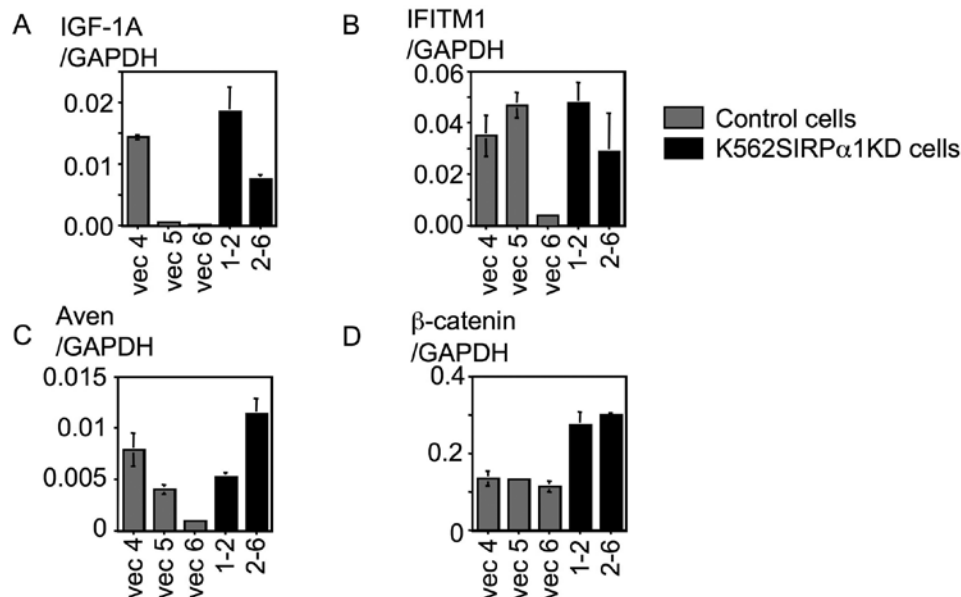


Figure 2. Expression levels of IGF-1A (A), IFITM1 (B), Aven (C) and  $\beta$ -catenin (D) in K562SIRP $\alpha$ 1KD and control cells evaluated by quantitative real-time PCR. Each gene transcript level was adjusted by the expression of GAPDH and the relative levels are shown. The data presented were obtained from three independent PCR reactions and reproducibility was confirmed by independent real-time PCR from different batches of cDNA.

Among these candidate target genes analyzed, induction of  $\beta$ -catenin (Fig. 2D) in K562SIRP $\alpha$ 1KD cells was significantly higher than that in all of the controls (vec 4, vec 5 and vec 6), suggesting that this gene is a candidate SIRP $\alpha$ 1 target gene. Moreover, the protein expression was verified. As accumulation of nuclear  $\beta$ -catenin is a hallmark of the Wnt/ $\beta$ -catenin pathway activation (12), we examined nuclear  $\beta$ -catenin expression. The expression of nuclear  $\beta$ -catenin was signifi-

cantly induced in K562SIRP $\alpha$ 1KD cells (Fig. 3). These results further support the notion that  $\beta$ -catenin is a *bona fide* target of SIRP $\alpha$ 1 suppression in K562 cells.

*Down-regulation of SIRP $\alpha$ 1 results in the constitutive phosphorylation of downstream signaling pathway components.* SIRP $\alpha$ 1 is a negative regulator of signal transduction pathway in hematopoietic cells. Therefore, we investigated whether

Table I. Top 100 genes up-regulated by the reduced expression of SIRPα1.

GenBank Acc. no.	Gene name	2-6 cells	vec 6 cells	Fold (2-6/vec 6)
NM_004114	Fibroblast growth factor 13 (FGF-13)	2886.048383	189.534326	15.227049
NM_001004441	Cytosolic phosphoprotein DP58	109.89096	15.72243	6.989439
NM_016356	Doublecortin domain-containing protein 2 (RU2S protein)	71.414144	11.361267	6.285755
NM_001005517.1	Olfactory receptor, family 5, subfamily K, member 4	71.444194	13.359442	5.347843
NM_001014447.1	Carboxypeptidase Z precursor (EC 3.4.17.-)	527.009034	103.87235	5.073622
NM_016081.3	Palladin	247.182321	49.262066	5.017701
NM_005397	Podocalyxin-like protein 1 precursor	932.635219	186.880069	4.990555
NM_002825	Pleiotrophin precursor (Heparin-binding growth-associated molecule)	686.704318	143.668824	4.779773
NM_017870	Transmembrane protein 132A isoform a	715.503749	149.823429	4.775647
NM_014208	Dentin sialophosphoprotein precursor	77.029946	16.303536	4.724739
<b>NM_000618</b>	<b>Insulin-like growth factor IA precursor (IGF-IA) (Somatomedin C)</b>	<b>1018.304811</b>	<b>225.444</b>	<b>4.516886</b>
NM_004791	Integrin, β-like 1 (with EGF-like repeat domains)	288.313109	65.562363	4.39754
NM_080923	Leukocyte common antigen precursor (EC 3.1.3.48)	85.932371	20.248899	4.243805
NM_139178	Alkylated repair protein alkB homolog 3 (Prostate cancer antigen 1)	313.916694	76.363342	4.11083
XR_015734.1	Similar to poly (A) binding protein, cytoplasmic 1 (LOC730694)	50.62764	12.366117	4.094061
XM_046570	Thrombospondin type-1 domain-containing protein 7B precursor	77.198251	19.537378	3.951311
NM_001003802	SWI/SNF-related matrix-associated actin-dependent regulator	430.425242	109.8625	3.917854
NM_007124.2	Utrophin (Dystrophin-related protein 1)	402.041209	102.79723	3.911012
NM_016081.3	Palladin	239.848937	61.354264	3.909246
NM_005950	Metallothionein-1G (MT-1G)	958.952109	251.840839	3.80777
NM_053002	Mediator of RNA polymerase II transcription, subunit 12 homolog	54.071448	14.22992	3.799842
NM_006198	Brain-specific polypeptide PEP-19 (Brain-specific antigen PCP-4)	64.073629	17.35297	3.692372
NM_003711	Lipid phosphate phosphohydrolase 1 (EC 3.1.3.4)	198.697225	53.938171	3.683796
XM_371843	40S ribosomal protein S27a	53.964192	14.692522	3.672902
NM_013243	Secretogranin-3 precursor (Secretogranin III)	101.750072	27.795954	3.660607
NM_001005242	Plakophilin-2. [Source:Uniprot/SWISSPROT;Acc:Q99959]	174.429702	47.732964	3.654282
NM_024603	C1orf165 protein. [Source:Uniprot/SPTREMBL;Acc:Q96A62]	79.177826	21.73915	3.642177
NM_001040666.1	Six-transmembrane epithelial antigen of prostate 2	101.697841	28.123405	3.616128
NM_001738	Carbonic anhydrase 1 (EC 4.2.1.1) (Carbonic anhydrase I)	6414.058686	1778.230467	3.60699
NM_015184	Phospholipase C-like 2 [Source:RefSeq_peptide;Acc:NP_055999]	804.370328	223.582882	3.597638
NM_014271	X-linked interleukin-1 receptor accessory protein-like 1 precursor	93.280764	25.935829	3.596599
NM_030925	Calcium-binding protein 39-like (Mo25-like protein) (Antigen MLAA-34)	70.914507	19.894027	3.564613
NM_078487	Cyclin-dependent kinase 4 inhibitor B (p14-INK4b)	454.500252	130.690858	3.477674
NM_198520	Putative uncharacterized protein C12orf63	63.881177	18.475531	3.45761
NM_198529	EF-hand calcium binding domain 5 isoform 2	100.882984	29.189642	3.456123
NM_173651	FSIP2 protein (Fragment). [Source:Uniprot/SPTREMBL;Acc:Q0IJ58]	72.986471	21.129759	3.454203
NM_006317	Brain acid soluble protein 1 (BASP1 protein)	106.385213	31.055775	3.425618
XM_372726	Zinc finger protein 793	66.372516	19.433129	3.415431
NM_001351	Deleted in azoospermia-like (DAZ-like autosomal)	159.327806	46.693028	3.41224
XM_049952	CDNA: FLJ23529 fis, clone LNG06042	86.193571	25.540171	3.374824
NM_005996	T-box transcription factor TBX3 (T-box protein 3)	540.746573	162.293936	3.331896
NM_006681	Neuromedin-U precursor	8730.889857	2623.113082	3.328446
XM_495872	Tripartite motif-containing protein 48 (RING finger protein 101)	77.279335	23.429972	3.298311
XR_019222.1	T-cell receptor α V gene segment	80.366181	24.368193	3.297995
NM_033123	Phospholipase C, ζ 1	59.025773	17.975733	3.283636
NM_003069	Probable global transcription activator SNF2L1 (EC 3.6.1.-)	1000.813672	305.064819	3.280659
NM_173808.2	Neuronal growth regulator 1 precursor	75.430598	23.012862	3.277758
NM_019590.2	KIAA1217 (KIAA1217), mRNA	75.35322	23.083556	3.264368
NM_005079	Tumor protein D52 (N8 protein)	473.368385	145.857438	3.245418
NM_001037499.1	β-defensin 114 precursor (Defensin, β 114)	79.644312	24.55774	3.243145
NM_001003894	Testis-specific chromodomain protein Y 2	54.577744	16.835353	3.241853

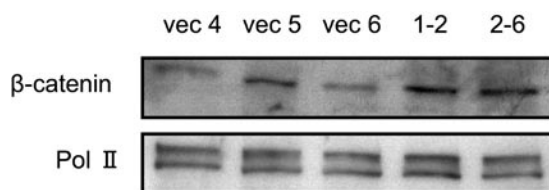
Table I. Continued.

GenBank Acc. no.	Gene name	2-6 cells	vec 6 cells	Fold (2-6/vec 6)
NM_001627	CD166 antigen precursor (Activated leukocyte-cell adhesion molecule)	96.370399	30.061435	3.205782
NM_003638	Integrin $\alpha$ -8 precursor	101.727362	31.961668	3.182793
NM_001010849	Homeobox and leucine zipper protein Homez	85.686462	27.115651	3.160037
NM_001010899	Putative uncharacterized protein C1 or f120	84.55642	26.85426	3.148715
XM_495806	Cubilin precursor (Intrinsic factor-cobalamin receptor)	94.588903	30.077584	3.14483
NM_006863.1	Leukocyte immunoglobulin-like receptor 6	59.037768	18.86086	3.130174
NM_138812	DPY30 domain-containing protein 1	65.889606	21.081296	3.125501
NM_030806	Uncharacterized protein C1orf21	67.263809	21.631143	3.109582
NM_016109	Angiopoietin-related protein 4 precursor (Angiopoietin-like 4)	242.690102	78.81203	3.079354
NM_014936	Ectonucleotide pyrophosphatase/phosphodiesterase 4	66.80234	21.82649	3.060608
NM_014344	Four jointed box 1 ( <i>Drosophila</i> ) (FJX1)	409.372877	133.974268	3.055608
XM_378901	Filaggrin	55.540496	18.310712	3.033224
XR_018723.1	Zinc finger protein 532	84.544161	27.91241	3.028909
NM_001039349.1	EGF-containing fibulin-like extracellular matrix protein 1 precursor	81.825645	27.047837	3.02522
NM_005235	Receptor tyrosine-protein kinase erbB-4 precursor (EC 2.7.10.1)	61.011904	20.221253	3.017217
NM_014178	Syntaxin-binding protein 6 (Amisyn)	320.260653	106.244934	3.014362
<b>NM_003641</b>	<b>Interferon-induced transmembrane protein 1 (IFITM1)</b>	<b>2913.517684</b>	<b>968.963981</b>	<b>3.006838</b>
NM_022103	Zinc finger protein 667. [Source:Uniprot/SWISSPROT;Acc:Q5HYK9]	63.016295	21.061444	2.992022
NM_005103	Fasciculation and elongation protein $\zeta$ 1 (Zygin-1) (Zygin I)	567.054207	189.723385	2.988847
NM_016413	Carboxypeptidase B2 precursor (EC 3.4.17.20)	87.418119	29.460729	2.967276
NM_178507	OAF homolog [Source:RefSeq_peptide;Acc:NP_848602]	1459.239286	492.139811	2.965091
NM_004105	EGF-containing fibulin-like extracellular matrix protein 1 precursor	289.087712	98.005855	2.949698
NM_001079691.1	CG018 protein. [Source:Uniprot/SPTREMBL;Acc:Q8WTU5]	47.394301	16.081872	2.947064
NM_173581	Galactosidase, $\beta$ 1 like 3	75.654935	25.685351	2.945451
NM_015150	Raftlin (Raft-linking protein) (Cell migration-inducing gene 2 protein)	74.796985	25.499121	2.933316
<b>NM_020371</b>	<b>Cell death regulator Aven</b>	<b>815.785271</b>	<b>278.559101</b>	<b>2.92859</b>
NM_032279	ATPase type 13A4 [Source:RefSeq_peptide;Acc:NP_115655]	80.886985	27.695794	2.920551
NM_058184	Uncharacterized protein C21 or f42	85.525321	29.286907	2.920258
NM_004666	Pantetheinase precursor (EC 3.5.1.92) (Pantetheine hydrolase)	79.671281	27.419836	2.905607
NM_014491	Forkhead box protein P2 (CAG repeat protein 44)	83.339812	28.71173	2.90264
NM_170737	ATP-sensitive inward rectifier potassium channel 15	57.870689	20.053033	2.885882
NM_198718	Prostaglandin E2 receptor, EP3 subtype (Prostanoid EP3 receptor)	88.348509	30.743942	2.873688
NR_001291.1	HBII-52 small nucleolar RNA [Source:RFAM;Acc:RF00105]	79.260679	27.682666	2.863188
NM_001005499	Olfactory receptor, family 6, subfamily C, member 70	55.592152	19.566889	2.841134
NM_024022	Transmembrane protease, serine 3 (EC 3.4.21.-)	274.334735	96.72902	2.836116
NM_001333	Cathepsin L2 precursor (EC 3.4.22.43) (Cathepsin V) (Cathepsin U)	1309.318959	462.206131	2.83276
NR_001293.1	Small nuclear ribonucleoprotein-associated protein N (snRNP-N)	67.913486	23.984582	2.831548
NM_001079858.1	G-protein coupled receptor 64 precursor (Epididymis-specific protein 6)	82.293592	29.066496	2.831218
<b>NM_001904</b>	<b>Catenin <math>\beta</math>-1 (<math>\beta</math>-catenin)</b>	<b>3410.18856</b>	<b>1207.549842</b>	<b>2.824056</b>
NM_005020	Calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1C	98.036868	34.743659	2.82172
NM_006113	Protein vav-3	60.930996	21.610505	2.819508
NM_002838	Leukocyte common antigen precursor (EC 3.1.3.48) (L-CA)	519.624944	184.425135	2.817539
NM_001005365	Prostate, ovary, testis-expressed protein on chromosome 8	76.074205	27.035439	2.81387
NM_032646	Tweety 2 isoform 1 [Source:RefSeq_peptide;Acc:NP_116035]	269.503648	96.804551	2.783998
NM_001001290	Solute carrier family 2, facilitated glucose transporter member 9	72.721164	26.121595	2.783948
NM_021193	Homeobox protein Hox-D12 (Hox-4H)	84.678609	30.417265	2.783899
NM_005656	Transmembrane protease, serine 2 precursor (EC 3.4.21.-)	57.38994	20.638692	2.780697
NM_016108	Androgen-induced protein 1 (AIG-1)	604.895167	217.826946	2.776953
NM_025103	Intraflagellar transport 74 homolog	72.518015	26.215263	2.766252

Bold type indicates genes selected for further analysis.

Table II. Genes down-regulated more than 2.5 fold by the reduced expression of SIRP $\alpha$ 1<sup>a</sup>.

GenBank Acc. no.	Gene name	2-6 cells	vec 6 cells	Fold (2-6/vec 6)
NM_006287	Tissue factor pathway inhibitor precursor (TFPI)	3911.396491	25922.939	0.150886
NM_006998	Secretagoin. [Source:Uniprot/SWISSPROT;Acc:O76038]	251.690093	946.217219	0.265996
NM_017780	Chromodomain-helicase-DNA-binding protein 7 (EC 3.6.1.-)	25.927877	93.21762	0.278144
NM_001657	Amphiregulin precursor (AR) (Colorectum cell-derived growth factor)	242.6557	863.262479	0.281091
NM_001003927	EVI2A protein precursor	71.228953	253.122942	0.281401
NM_000700	Annexin A1 (Annexin I) (Lipocortin I) (Calpactin II)	208.778862	723.423551	0.288598
NM_172127	Calcium/calmodulin-dependent protein kinase type II $\delta$ chain	66.778141	210.50114	0.317234
NM_004245	Protein-glutamine $\gamma$ -glutamyltransferase 5 (EC 2.3.2.13)	180.978597	560.88993	0.322663
NM_001781	Early activation antigen CD69 (Early T-cell activation antigen p60)	1150.994635	3507.226992	0.328178
NM_018283	Probable 7,8-dihydro-8-oxoguanine triphosphatase NUDT15	31.493252	93.591466	0.336497
NM_006667	Membrane-associated progesterone receptor component 1 (mPR)	22.459972	65.323707	0.343826
NM_001995	Long-chain-fatty-acid-CoA ligase 1 (EC 6.2.1.3)	183.960162	534.919363	0.343903
NM_024501	Homeobox protein Hox-D1	41.86217	121.032208	0.345876
NM_004388	Di-N-acetylchitobiase precursor (EC 3.2.1.-)	21.069894	60.187009	0.350074
NM_002220	Inositol-trisphosphate 3-kinase A (EC 2.7.1.127)	1380.039093	3774.819762	0.365591
NM_005337	Nck-associated protein 1-like (Membrane-associated protein HEM-1)	294.448658	786.115236	0.374562
NM_004415	Desmoplakin (DP) (250/210 kDa paraneoplastic pemphigus antigen)	113.991935	301.60958	0.377945
NM_002246	Potassium channel subfamily K member 3	217.624294	564.105003	0.385787
NM_006138	Membrane-spanning 4-domains subfamily A member 3	4758.214986	12255.18655	0.388261
NM_012098	Angiopoietin-related protein 2 precursor (Angiopoietin-like 2)	500.691514	1284.749678	0.389719
NM_002194	Inositol polyphosphate 1-phosphatase (EC 3.1.3.57) (IPPase) (IPP)	228.162913	584.964267	0.390046
NM_080792 <sup>a</sup>	Signal-regulatory protein $\alpha$ -1	141.492509	294.449431	0.480532

Figure 3. Nuclear  $\beta$ -catenin expression is increased in K562SIRP $\alpha$ 1KD cells. Equal amounts of soluble nuclear proteins were loaded in each lane and immunoblotted for  $\beta$ -catenin and nuclear RNA-polymerase II (Pol II).

the reduction of SIRP $\alpha$ 1 expression affected downstream signal transduction pathways in these cells. Therefore, we first examined the activation of ERK and Akt phosphorylation by Western blotting. As shown in Fig. 4A, slight phosphorylation of ERK was reproducibly observed in K562SIRP $\alpha$ 1KD cells compared to their control, in two independent experiments. In addition, Fig. 4B demonstrates significant phosphorylation of Akt in K562SIRP $\alpha$ 1KD cells. Phosphorylation of Ser9 of GSK-3 $\beta$ , results in the inactivation of this protein, leading to the up-regulation of  $\beta$ -catenin (13). Therefore, we next evaluated the phosphorylation status of GSK-3 $\beta$ . As we predicted, Ser9 of GSK-3 $\beta$  was phosphorylated in K562SIRP $\alpha$ 1KD cells (Fig. 4C). Collectively, the results illustrate that down-regulation of SIRP $\alpha$ 1 results in the aberrant phosphorylation of several signaling pathway components, such as ERK, Akt and GSK-3 $\beta$ . These may play a role in the induction of  $\beta$ -catenin in K562SIRP $\alpha$ 1KD cells.

## Discussion

Qin *et al* have reported that overexpression of SIRP $\alpha$ 1 by stably transfected SIRP $\alpha$ 1 plasmid into the SK-Hep1 liver cancer cell line results in a decrease in the expression of  $\beta$ -catenin and cyclin D (14). The authors concluded that SIRP $\alpha$ 1 may block the cell cycle in liver cancer, inhibit cell proliferation and promote cell apoptosis by decreasing the expression of cyclin D1 and  $\beta$ -catenin. Although the mechanisms of the  $\beta$ -catenin suppression by the overexpression of SIRP $\alpha$ 1 remain unsolved, their findings are quite consistent with our current study that SIRP $\alpha$ 1 negatively regulates the expression of  $\beta$ -catenin.

The Ras-ERK-mitogen-activated protein kinase (MAPK) pathway is frequently activated in hematological malignancies (15,16). In addition, phosphoinositide 3-kinase (PI3K)/Akt signaling is frequently activated in blasts of AML patients and strongly contributes to the proliferation, survival and drug resistance of these cells (17). Although genetic alterations affecting the functions of transcription factors that regulate myeloid maturation play important roles in leukemogenesis (18,19), inappropriate MAPK, as well as PI3K activation may also play a role in leukemic transformation. Our current study revealed the contribution of the down-regulation of SIRP $\alpha$ 1 to the constitutive activation of this aberrant signaling in leukemia cells. In addition, we examined the effect of specific signaling inhibitors (i.e. the MEK inhibitor, U0126 and the PI3K inhibitor, LY294002) on K562SIRP $\alpha$ 1KD cells and

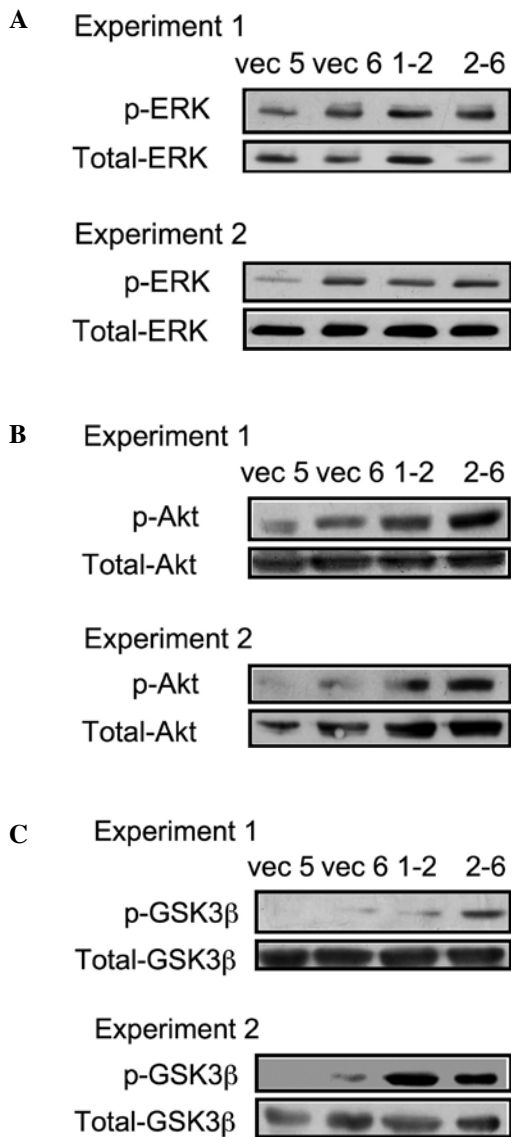


Figure 4. Down-regulation of signal regulatory protein  $\alpha 1$  results in the constitutive phosphorylation of downstream signaling pathway components. The phosphorylation levels of extracellular signal-regulated kinase (ERK) (A), Akt (B) and glycogen synthesis kinase (GSK) - $\beta$  (C) were examined by Western blot analysis. The results of two independent experiments are shown in the upper (Experiment 1) and lower (Experiment 2) panels. The membranes were first probed with an anti-phospho-ERK (A), anti-phospho-Akt (B) and anti-phospho-GSK- $\beta$  (Ser9) (C) rabbit polyclonal antibodies. Next, the membranes were stripped and reprobed with anti-total-ERK (A), Akt (B) and GSK- $\beta$  (C) mouse monoclonal antibodies, respectively, to verify equal protein loading.

found that they do not apparently affect the expression of  $\beta$ -catenin, or the phosphorylation GSK- $\beta$  (data not shown). These results suggest that the induction of  $\beta$ -catenin induced by SIRP $\alpha 1$  suppression, does not occur through a single pathway, but is rather due to complex aberrant signaling.

The bipartite transcription factor  $\beta$ -catenin/TCF has been recognized as the major effector of the Wnt signaling pathway for more than a decade. It is known that activation of the Wnt/ $\beta$ -catenin signaling pathway through loss-of-function mutations in the adenomatous polyposis coli (APC) protein and axin, or gain-of-function mutations in  $\beta$ -catenin are linked to various human cancers including colorectal carcinomas and

melanomas (20). However, until recently the role of  $\beta$ -catenin in normal and malignant hematopoiesis compared to that in solid tumors has not been evaluated extensively. Muller-Tidow *et al* were the first to identify a role of the Wnt/ $\beta$ -catenin pathway in the pathogenesis of AML (21). Activating mutations of the fms-like tyrosine kinase (FLT) 3, one of the commonest mutations in AML, was found to induce leukemogenic effects (22). Subsequently, constitutive activation of the Wnt/ $\beta$ -catenin signaling pathway has been documented in a significant portion of AML cases (11). Ysebaert *et al* reported that  $\beta$ -catenin was expressed in 61% of the 82 AML cases they examined (23). In a survival analysis, they demonstrated that  $\beta$ -catenin appeared as a new independent prognostic factor predicting poor event-free survival and shortened overall survival (23). A similar report was recently published by another group, in which analysis of a cohort of 59 AML patients, demonstrated that high expression of  $\beta$ -catenin is related to shortened overall survival (24).

To our knowledge, this is a first report demonstrating the relationship between SIRP $\alpha 1$  and  $\beta$ -catenin in leukemia cells. As  $\beta$ -catenin expression is related to the adverse prognosis, our study may shed light on the importance of SIRP $\alpha 1$  expression, which is relatively easy to examine by flow cytometry, in predicting the prognosis of patients with hematological malignancies.

#### Acknowledgements

We thank Dr Masako Nakahara for establishing the K562SIRP $\alpha 1$ KD cell line. We also thank Ms. Yuko Iseki for assistance with the microarray studies, the Western blot analysis and Ms. Saki Honta for assistance with the real-time PCR analysis. This study was supported in part by the Takeda Science Foundation, a foundation from the Kitasato University School of Allied Health Sciences (Grant-in-Aid for Research Project, nos. 2009-1001, 2010-1004).

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