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# The involvement of Gab1 and PI 3-kinase in β1 integrin signaling in keratinocytes

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Abbreviations used: MAPK, mitogen-activated protein kinase; SCC, squamous cell carcinoma; PI3K, phosphatidylinositol 3-kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

## Abstract

The control of the stem cell compartment in epidermis is closely linked to the regulation of keratinocyte proliferation and differentiation. β1 integrins are expressed 2-fold higher by stem cells than transit-amplifying cells. Signaling from these β1 integrins is critical for the regulation of the epidermal stem cell compartment. To clarify the functional relevance of this differential expression of β1 integrins, we established HaCaT cells with high β1 integrin expression by repeated flow cytometric sorting of this population from the parental cell line. In these obtained cells expressing β1 integrins by 5-fold, MAPK activation was markedly increased. Regarding the upstream of MAPK, Gab1 phosphorylation was also higher with high β1 integrin expression, while Shc phosphorylation was not altered. In addition, enhanced phosphatidylinositol 3-kinase activation was also observed. These observations suggest that Gab1 and phosphatidylinositol 3-kinase play pivotal roles in the β1 integrin-mediated regulation of the epidermal stem cell compartment.

Keywords: HaCaT cell, keratinocyte, stem cell, β1 integrin, Gab1, phosphatidylinositol 3-kinase, mitogen-activated protein kinase

## Introduction

The epidermis is a stratified, rapidly renewing tissue in which terminally differentiated cells are continuously lost from the skin surface and replaced by proliferation in the innermost or basal layer adjacent to the basement membrane. Cell regeneration is thought to be achieved by a hierarchy of proliferative cells within the basal layer consisting of stem cells and transient amplifying cells [1]. In the early stages of epidermal differentiation, basal cells detach from the basement membrane and migrate into the suprabasal layers, where they progress through an intricate series of stage-specific morphological and biochemical changes involving the synthesis of the differentiation-specific keratins K1 and K10, as well as involcrin, filagrin, and loricrin. Hence, the adhesive properties of basal keratinocytes are closely linked to the regulation of keratinocyte proliferation and differentiation. The anchorage of keratinocytes to extracellular matrix negatively regulates their differentiation [2; 3].

Epidermal cells adhere to the basement membrane via adhesion molecules known as integrins, which comprise a superfamily of heterodimeric receptors that consist of one α and one β subunit. The two subunits collaborate to bind to ligands, which are extracellular matrix proteins or counter-receptors of the Ig superfamily [4]. Watt and colleagues have established that a major cell-surface marker that distinguishes epidermal stem cells from transit-amplifying cells is β1 integrins, which are expressed 2-fold higher on stem cells than transit-amplifying cells [5; 6]. Exit from the stem cell compartment is accompanied by a decrease in surface β1 integrin levels [7].

Whereas β1 integrins have been proved to be a useful epidermal stem cell marker, the functional relevance of this increased cell-surface density of β1 integrins is on the way to the resolution. High expression of β1 integrins helps to maintain the patterned distribution of stem cells, since stem cells are less motile than transit-amplifying cells and thus tend to remain clustered within the epidermal basal

layer [8]. Integrins can also mediate signals provided by neighboring cells and the extracellular milieu [4], and β1 integrin-mediated signaling maintains the epidermal stem cell compartment via mitogen-activated protein kinase (MAPK) activation [9]. In addition to the epidermal stem cells, Janes et al. suggests that the variation of the integrin expression influence the development of the squamous cell carcinoma (SCC) [10]. Whereas Khavari and colleagues have demonstrated the involvement of Gab1 in Ras/MAPK activation from epidermal growth factor receptor [11], their roles in β1 integrin-mediated signaling remained unclear.

Here, we have described further roles of β1 integrin expression for the signaling transduction pathway in keratinocytes. We obtained HaCaT cells showing high expression levels of β1 integrins, by a repetition of selecting and culturing cells expressing relatively high levels of β1 integrins. HaCaT cells with high β1 integrin expression showed increased activation of the MAPK cascade. Those accompanied augmented phosphorylation of Gab1, but not of Shc. Also, those cells showed enhanced phosphatidylinositol 3-kinase (PI3K) activation and the amplified association between PI3K and β1 integrin. Thus, increased β1 integrin expression, which is the marker of the epidermal stem cells, regulates the MAPK signal transduction via Gab1 and PI3K signal transduction in keratinocytes.

## Materials & Methods

### Cells and immunofluorescence analysis

A human keratinocyte cell line, HaCaT, was kindly provided by Dr Toshio Kuroki (Institute of Molecular Oncology, Showa University, Tokyo, Japan). The HaCaT cells were cultured in DMEM supplemented with 2mM glutamine, 10% FCS and 15 mM HEPES (Invitrogen, Carlsbad, CA).

HaCaT cells were grown to confluency and dispersed by trypsin treatment. Cells were washed and stained at 4 ˚C using mouse anti-human β1 integrin antibody (HUTS-4, Chemicon, Temecula, CA) for 20 min, followed by labeling with FITC-conjugated secondary antibody (Southern Biotech, Birmingham, AL). After the washing, cells expressing relatively high β1 integrins were sorted on an Epics Altra flow cytometer (Beckman Coulter, Miami, FL) and reseeded into next cultures. These operations were repeated six times. Acquired cells were confirmed to express high β1 integrins by the immunofluorescence analysis and used for the experiment. HaCaT cells with median levels of β1 integrin expression were also sorted 6 times and used as controls. Immunofluorescence analysis was performed as described [12]. Briefly, cells were stained as described above and then analyzed on an Epics Altra flow cytometer with fluorescence intensity shown as 50% log density plots. Unreactive isotype-matched antibodies (Beckman Coulter) were used as controls for background staining.

### Immunoprecipitations and Western Blot Analysis

Immunoprecipitations and immunoblotting were performed as described previously [13]. Cells were lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 50mM Tris-HCl (pH 8.0), 1mM Na orthovanadate, 2mM EDTA, 50mM NaF, and protease inhibitors. Then, the cell lysates were precleared twice by incubation with appropriate control antibodies plus protein G-Sepharose beads (Amersham Pharmacia Biotech, Buckinghamshire, UK), followed by incubation with protein G-beads plus antibodies to

proteins of interest for 2 hours at 4˚C. The antibodies used for immunoprecipitation included anti-ERK2, anti-Gab1, anti-Shc (Santa Cruz Biotech, Santa Cruz, CA), and anti-PI3K antibodies (Upstate Biotech, Lake Placid, NY). Immunoprecipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes for immunoblotting. These membranes were incubated with HRP-conjugated anti-phosphotyrosine antibody (4G10, Upstate Biotech) to detect protein tyrosine phosphorylation, or were incubated with Anti-ACTIVE$^{(R)}$MAPK antibodies (Promega, Madison, WI), followed by incubation with HRP-conjugated anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The blots were developed using an enhanced chemiluminescence kit (Pierce, Rockford, IL) and detected using Fluoro-S imager (Bio-Rad Laboratories, Hercules, CA). The blots were stripped and reprobed with antibodies specific for the protein of interest to verify equivalent amounts of protein in each lane. Band intensity was quantified using Quantity One software (Bio-Rad Laboratories). For the inhibition of PI3K activity, Cells were cultured with or without 20μM LY294002 (xxxxxxxx) for 30min before the lysis of cells.

**PI3K activity assay**

PI3K activity was measured in vitro using a competitive ELISA (Echelon Biosciences Inc., Salt Lake City, UT). PI3K was immunoprecipitated from an equal amount of protein lysate from HaCaT cells, followed by incubation with phosphatidylinositol (4, 5)-biphosphate [PI(4, 5)$P_2$] substrate (100 pmol) with ATP for 2 hr at room temperature. The supernatant was then incubated with a PI(3, 4, 5)$P_3$ detector protein for 1 hr at room temperature, and the reaction mixtures were transferred to PI(3, 4, 5)$P_3$-coated detection plates for 1 hr at room temperature. After washing, secondary detection reagent and developing solution (supplied with the kit) were added. PI(3, 4, 5)$P_3$ detector protein binding to the plate was determined by measuring the absorbance at

450 nm.

### Statistical Analysis.

All data are shown as mean values ± SEM. Comparisons between groups were made using the *t* test.

## Results

### Generation of HaCaT cells expressing high levels of β1 integrins

HaCaT cells expressing high levels of β1 integrins were generated by repeated sorting and culture of cells expressing relatively high β1 integrin levels. The population of HaCaT cells of which β1 integrin expression was in the highest 3-5% was collected using flow cytometric cell sorter and this population was passaged. When cultured, these cells expressed higher levels of β1 integrins than their parental cells, and the sorting was repeated. After repeated 6 times, acquired cells (β1 integrin$^{high}$ cells) were used for the experiment. HaCaT cells with median levels of β1 integrin expression were also sorted 6 times and used as controls (β1 integrin$^{normal}$ cells). Expressions of high β1 integrin levels were confirmed by flow cytometry. β1 integrin$^{high}$ cells showed higher expression of β1 integrins by approximately 5-fold compared with β1 integrin$^{normal}$ cells (Fig. 1). β1 integrin$^{normal}$ cells expressed β1 integrins at the identical levels as the original cells (data not shown). In addition, these β1 integrin$^{high}$ and β1 integrin$^{normal}$ cells expressed all the other examined surface proteins including epidermal growth factor receptor, and intracellular signaling molecules at identical levels (data not shown). Thus, this system was considered to be suitable for assessing how β1 integrin levels influence signaling outcomes since β1 integrin expression was considered physiological in these cells.

### Increased ERK activity in β1 integrin$^{high}$ cells

The direct signaling event mediated by integrins is the activation of the MAPK cascade [9; 11; 14]. Therefore, ERK activation were first determined in β1 integrin$^{high}$ cells and β1 integrin$^{normal}$ cells. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After those were probed with anti-phosphoERK antibodies, band intensity was quantified. Total protein amount of ERK in β1 integrin$^{high}$ cells was equivalent to that in β1 integrin$^{normal}$ cells (data not shown). However, β1 integrin$^{high}$

cells showed 80% increase of ERK activity compared to β1 integrin$^{normal}$ cells (182.5 ± 5.6%; Fig. 2). ERK activity in β1 integrin$^{normal}$ cells was identical to that in the original HaCaT cells (data not shown). We also examined the activation of the EGF receptor, and β1 integrin$^{high}$ cells, β1 integrin$^{normal}$ cells and original HaCaT cells had the same tyrosine phosphorylation levels of the EGF receptor (data not shown). Thus, the elevation of β1 integrin expression in HaCaT cells amplifies MAPK signal transduction pathway similarly to the previous study using the normal keratinocyte [9].

## Increased Gab1 activation in β1 integrin$^{high}$ cells

While β1 integrins control MAPK cascade and regulate the proliferation and differentiation in keratinocytes [9], the upstream of Ras in β1 integrin signaling cascade remains unresolved. In other integrins, the stimulation from α6β4 integrins in keratinocytes causes the Ras/MAPK activation via Shc [15]. In the case of the stimulation from epidermal growth factor receptor, Gab1 facilitates Ras/MAPK signaling [11]. Therefore, we examined whether the activation of Shc or Gab1 was located upstream of the MAPK activation in β1 integrin$^{high}$ cells. Proteins immunoprecipitated from cell lysates with anti-Shc or anti-Gab1 antibody-conjugated beads were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with phosphotyrosine-specific monoclonal antibodies. β1 integrin density did not influence the expression of Gab1 or Shc since total amount of Shc and Gab1 was not altered between β1 integrin$^{high}$ cells and β1 integrin$^{normal}$ cells (Fig. 3). Shc was equally tyrosine phosphorylated in β1 integrin$^{high}$ cells compared with β1 integrin$^{normal}$ cells (Fig. 3A). By contrast, Gab1 tyrosine phosphorylation was markedly stronger in β1 integrin$^{high}$ cells than in β1 integrin$^{normal}$ cells (Fig. 3B). Thus, β1 integrin upregulation resulted in enhanced tyrosine phsophorylation of Gab1.

## Enhanced PI3K activity and association with β1 integrins in β1 integrin$^{high}$ cells

One of the signaling pathways that protects cells from anoikis is activation of AKT through upstream activation of PI3K [16]. PI3K is a central molecule which regulates cell survival pathways, gene expression and cell metabolism, and cytoskeletal rearrangements [17]. In addition, PI3K could serve as a downstream factor mediating signals from activation of β1 integrins in keratinocytes [18]. Therefore, we investigated the relationship between the increased expression of β1 integrins and PI3K activity. Assessed by competitive ELISA, PI3K activity was significantly enhanced in β1 integrin$^{high}$ cells (156.8 ± 10.6%) compared with β1 integrin$^{normal}$ cells (Fig. 4A). PI3K activity in β1 integrin$^{normal}$ cells was identical to that in original HaCaT cells (data not shown). PI3K expression levels were not altered in β1 integrin$^{high}$ cells compared with β1 integrin$^{normal}$ cells or the original HaCaT cells (data not shown).

Then, to clarify further the relation of increased β1 integrins to the enhanced PI3K activity, we examined the association between β1 integrins and PI3K. The lysates from β1 integrin$^{high}$ cells and β1 integrin$^{normal}$ cells were immunoprecipitated by anti-PI3K antibody and the coprecipitations of β1 integrins were determined by western blot analysis using anti-β1 integrin antibody (Fig. 4B). β1 integrin coprecipitates were augmented in β1 integrin$^{high}$ cells (216.5 ± 35.1%) compared to β1 integrin$^{normal}$ cells. Hence, amplified β1 integrin expression causes augmented PI3K association and leads to enhanced PI3K activity.

Effects of inhibited PI3K on Gab1 and ERK activities

Increase of β1 integrin density led to the increased Gab 1 and ERK activities and enhanced PI3K activity. Since increase of β1 integrins also caused enhanced PI3K association, PI3K seems to be an upstream factor of Gab1 and ERK. Therefore, we tested whether a PI3K inhibitor, LY294002, can block the increased activities of Gab1 and ERK induced by increased β1 integrins (Fig. 5). Cells were cultured with or without 20 μM LY294002 for 30 min before the lysis of cells. LY294002 blocked the increase of

the Gab1 phosphorylation in β1 integrin$^{high}$ cells(Fig. 5A). The increase of ERK activity were also blocked by LY294002 (Fig.5B). Therefore, Gab1 and ERK are the downstream factor of PI3K in β1 integrin$^{high}$ cells, suggesting that increased β1 integrin enhanced the Gab1 and ERK activity via PI3K.

## Discussion

Epidermal stem cells express 2-fold higher β1 integrins than transit-amplifying cells [5; 6] and suprabasal keratinocytes do not normally express integrins [5; 19]. While the introduction of a dominant-negative β1 integrin mutant into cultured human keratinocytes reduces MAPK activation and stimulates the exit from the stem cell compartment [9], the further detailed mechanisms for the regulation by the increased β1 integrins remains unresolved. The current study supports this previous observation about the functional role of highly expressed β1 integrins in keratnocytes. β1 integrin$^{high}$ cells showed increased ERK activity (Fig. 2). Then, this increased activity was accompanied with amplified Gab1 tyrosine phosphorylation (Fig. 3B), while Shc tyrosine phosphorylation showed no apparent change in β1 integrin$^{high}$ cells (Fig. 3A). Furthermore, β1 integrin$^{high}$ cells also showed enhanced PI3K activity (Fig. 4A) and augmented PI3K association with β1 integrins (Fig. 4B). Then, the inhibition of PI3K led to the inhibition of increased Gab1 and ERK activities (Fig. 5), suggesting that Gab1 and ERK is the downstream factor of PI3K. PI3K is also reported as the downstream factor of Ras [20]. Thus, PI3K may have dual roles in this pathway by facilitating Gab1 phosphoryltion and by serving as downstream effector of Ras (Fig. 6). This intrinsic loop may sustain the activity of this signaling pathway, which may be important of epidermal stem cell fate. These changes in β1 integrin$^{high}$ cells suggests the further detailed functional relevance of increased expression of β1 integrins in epidermal stem cells [5; 6] (Fig. 6).

When sections of SCCs are examined by immunohistochemistry, considerable variation in integrin expression is observed and associated with the prognosis. An increase in surface integrin levels by only several folds is unlikely to be detected by conventional immunohistochemical staining and thought to exist more frequently in SCCs. Then, it have been supposed that these small changes could profoundly influence the proliferation and diffentiation [10]. Our observations of enhanced PI3K and MAPK

activity in β1 integrin$^{high}$ HaCaT cells also support this hypothesis and suggest that β1 integrins and/or those downstream molecules are reasonable target molecules for the treatment of SCC.

It has been suggested that Ras acts within epidermis to support proliferative capacity and oppose terminal differentiation [11; 21]. While Ras/MAPK activation in β1 integrin-mediated signal transduction is independent of FAK [9], little is known about the upstream of Ras/MAPK activation except FAK. In α6β4 integrins, Shc mediates the Ras/MAPK activation [15]. In the signal transduction from growth factor, MAPK activation can proceed normally in Shc$^{-/-}$ cells [22] and is impaired in Gab1$^{-/-}$ cells [23; 24]. Gab1 overexpression prolongs the persistence of active Ras/MAPK in response to epidermal growth factor stimulation [11]. Our observations of increased tyrosine phosphorylation of Gab1, but not Shc, in β1 integrin$^{high}$ cells, are consonant with these prior observations about the epidermal growth factor stimulation. The difference of the signal transduction pathway between α6β4 integrins and β1 integrins may make intricate regulation of differentiation and proliferation by these integrins possible.

Relationship between Gab1 and Ras/MAPK signal transduction pathway and PI3K remains unresolved. The lipid product of PI3K, phosphatidylinositol-3, 4, 5-trisphosphate, recruits a subset of signaling proteins with pleckstrin homology domains including Gab1 to the membrane, where they are activated [17].This is consistent with the result suggesting that Gab1 and ERK is the downstream factor of PI3K (Fig. 5). Besides, PI3K is one of the major downstream effector cascades of Ras [20]. Nevertheless, both Gab1/Ras/MAPK and PI3K formed the intricate signal transduction pathway from β1 integrins (Fig. 6). In β1 integrin$^{normal}$ cells, the inhibition of Gab1 and ERK phosphorylation by LY294002 was limited compared with the inhibition in β1 integrin$^{high}$ cells (Fig. 5). It implies that various pathways or surface molecules play roles for the Gab1 and ERK phosphorylation and the engagement of PI3K and β1 integrin is partial in β1 integrin$^{normal}$ cells. Then, the marked inhibition in

β1 integrin$^{high}$ cells suggests the critical role of PI3K in the activation of Gab1 and ERK by the increase of β1 integrins. Whereas we used HaCaT cells for acquisition of enough abundant and uniform cells to determine the precise extent of tyrosine phosphorylation of signaling molecules, further investigations in primary keratinocytes may be needed for the confirmation of the role in vivo.

In conclusion, the results of this study have provided a novel mechanism of signaling alteration by increased expression of β1 integrins, which leads to increased MAPK activation via Gab1 and enhanced PI3K activity via augmented association of β1 integrins and PI3K, indicating the functional relevance in keratinocyte stem cells or malignant cells. Especially, tonic signaling from high surface density of β1 integrins may be important to establish constitutive activation in keratinocyte stem cells. These signal transduction pathways may be worth intense explorations for understanding the regulation of the stem cell compartment as well as malignancies of the keratinocyte.

## Acknowledgments

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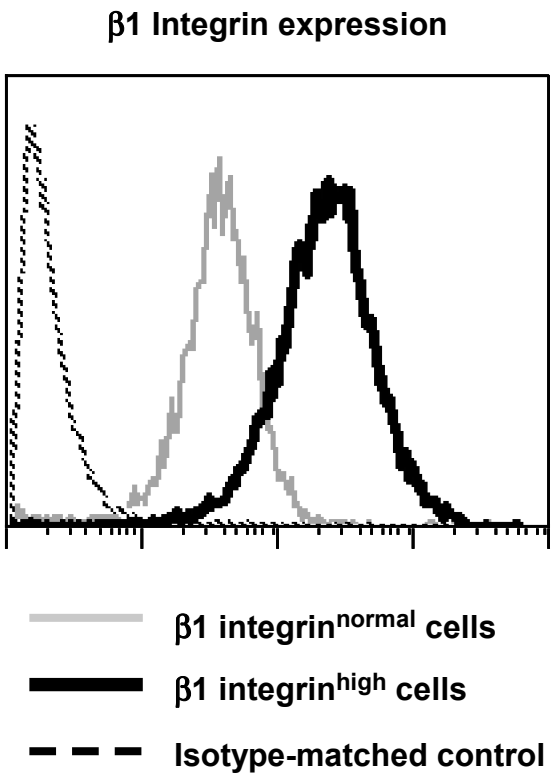



**Figure 1 Kuwano et al.**

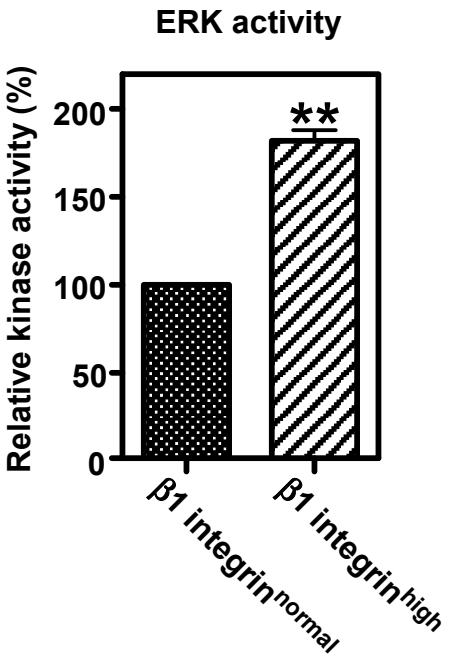
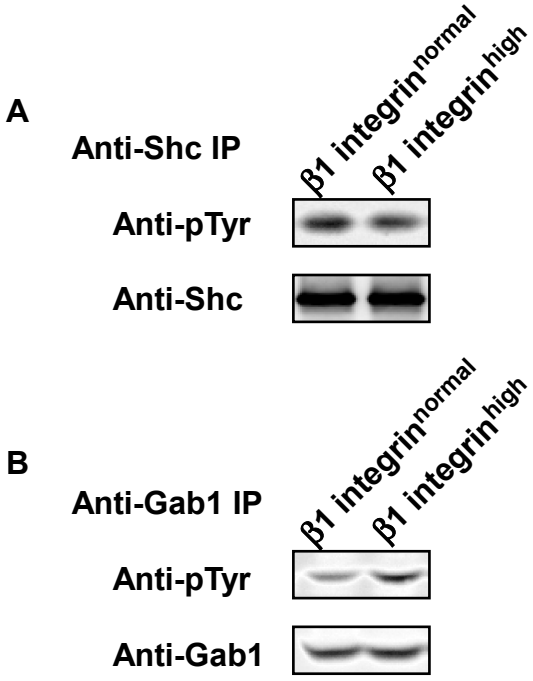



**Figure 2 Kuwano et al.**

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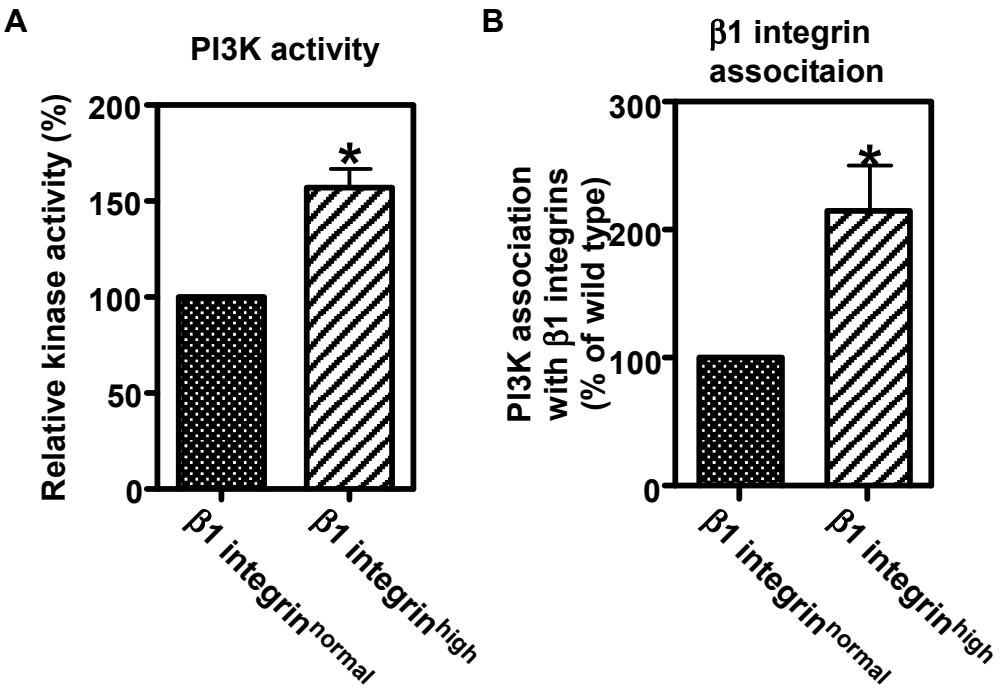


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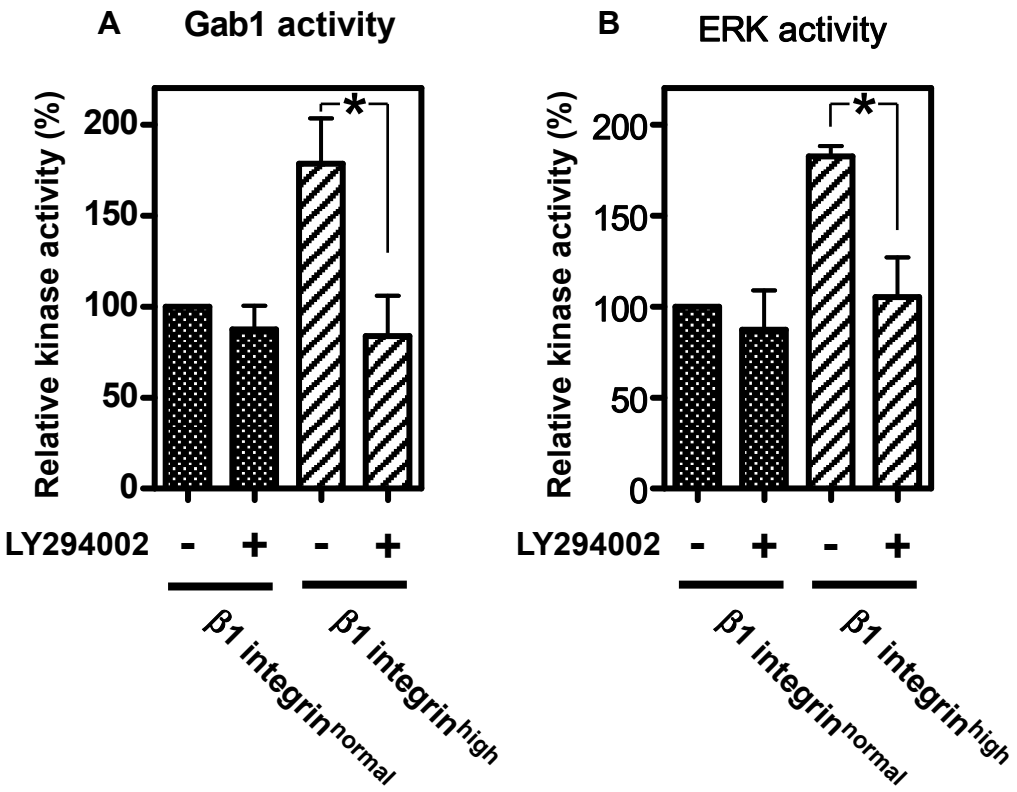


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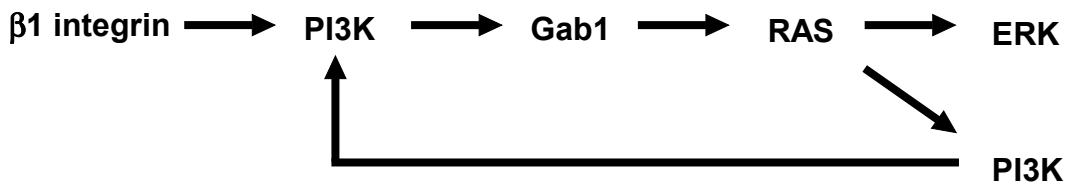


**Figure 6 Kuwano et al.**