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## CIB1 contributes to oncogenic signalling by Ras via modulating the subcellular localisation of sphingosine kinase 1

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### Abstract

CIB1 (calcium and integrin binding protein 1) is a small intracellular protein with numerous interacting partners, and hence has been implicated in various cellular functions. Recent studies have revealed emerging roles of CIB1 in regulating cancer cell survival and angiogenesis, although the mechanisms involved have remained largely undefined. In investigating the oncogenic function of CIB1, we initially found that CIB1 is widely up-regulated across a diverse range of cancers, with this up-regulation frequently correlating with oncogenic mutations of KRas. Consistent with this, we found that ectopic expression of oncogenic KRas and HRas in cells resulted in elevated CIB1 expression. We previously described the Ca<sup>2+</sup>-myristoyl switch function of CIB1, and its ability to facilitate agonist-induced plasma membrane localisation of sphingosine kinase 1 (SK1), a location where SK1 is known to elicit oncogenic signalling. Thus, we examined the role this may

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### Conflict of interest

L.V. Parise is a cofounder of Reveris Therapeutics.

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play in oncogenesis. Consistent with these findings, we demonstrated here that over-expression of CIB1 by itself is sufficient to drive localisation of SK1 to the plasma membrane and enhance the membrane associated enzymatic activity of SK1, as well as its oncogenic signalling. We subsequently demonstrated that elevated levels of CIB1 resulted in full neoplastic transformation, in a manner dependent on SK1. In agreement with our previous findings that SK1 is a downstream mediator of oncogenic signalling by Ras, we found that targeting CIB1 also inhibited neoplastic growth of cells induced by oncogenic Ras, suggesting an important pro-tumorigenic role for CIB1. Thus, we have demonstrated for the first time a role for CIB1 in neoplastic transformation, and revealed a novel mechanism facilitating oncogenic signalling by Ras and SK1.

## Keywords

CIB1; Ras; sphingosine kinase; plasma membrane localisation

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## Introduction

Calcium and integrin binding protein 1 (CIB1) is a  $\text{Ca}^{2+}$  binding protein first identified to interact with the  $\alpha_{\text{IIb}}\beta_3$  cytoplasmic domain of the platelet-specific  $\alpha_{\text{IIb}}\beta_3$  integrin<sup>1</sup>. In this  $\text{Ca}^{2+}$ -dependent complex CIB1 has been proposed to play a role in platelet activation and aggregation<sup>2, 3</sup>. Broad tissue expression of CIB1, however, suggests roles for this protein outside of platelets<sup>4</sup>. Indeed, CIB1 has been found to interact with a range of other proteins, which have been proposed to mediate a number of non-platelet functions for this protein<sup>2</sup>.

CIB1 is a myristoylated protein and can act as  $\text{Ca}^{2+}$ -myristoyl switch<sup>5</sup>. By analogy to recoverin and frequenin, the prototypical members of the  $\text{Ca}^{2+}$ -myristoyl switch protein family<sup>6</sup>, in the absence of bound  $\text{Ca}^{2+}$  the myristoyl group is thought to be sequestered into a hydrophobic pocket in the protein. Upon binding  $\text{Ca}^{2+}$  a conformation change causes the extrusion of the myristoyl group from its original sheltered groove and this confers dual effects; firstly to enable the target proteins to interact with the now available hydrophobic pocket, and secondly the extruded myristoyl group targets CIB1 and any newly bound interacting partner to the plasma membrane<sup>6</sup>. We have recently demonstrated that, via this mechanism, CIB1 is essential for the agonist-induced translocation of the cancer-associated lipid signalling enzyme sphingosine kinase 1 (SK1) from the cytoplasm to the plasma membrane<sup>5</sup>. Notably, this localisation of SK1 is known to elicit oncogenic signalling<sup>7</sup>, raising the possibility that CIB1 may contribute to oncogenesis through regulation of SK1.

A role for CIB1 in tumor progression has been previously inferred, with reports showing its elevated expression in liver, breast, and pancreatic cancers<sup>8-10</sup> and its involvement in enhancing cell survival, proliferation and tumour angiogenesis<sup>5, 9, 11-15</sup>. Recent studies have shown that depletion of CIB1 impaired triple negative breast cancer growth, leading to suggestions that CIB1 may represent a case of non-oncogene addiction in this form of cancer<sup>16</sup>. A direct role for CIB1 in inducing neoplastic transformation has, however, not been previously examined. Here, we show for the first time that CIB1 can directly induce full neoplastic transformation of cells in a manner highly dependent on SK1. We also show that CIB1 is up-regulated in a wide range of human cancers, and provide evidence indicating

this may be both a consequence of the common hyperactivation of the Ras-ERK pathway, and an important mediator of Ras-induced oncogenesis.

## Results and Discussion

### **CIB1 is widely over-expressed in human cancers and correlates with oncogenic Ras mutation**

To initially investigate the role of CIB1 in oncogenesis we examined its expression in human cancers using the public gene expression datasets in the Oncomine database<sup>17</sup>. CIB1 has been previously found to be elevated in some liver, breast and pancreatic cancers<sup>8–10</sup>, but a broad analysis of CIB1 expression in cancer has not been previously performed. Strikingly, we found that CIB1 was widely up-regulated in a broad array of cancer types, compared to their corresponding normal tissues (Figure 1A, S1). This includes a range of breast and ovarian cancer subtypes, lung adenocarcinoma, some leukaemias, and an array of gastrointestinal tumours. To confirm these findings we examined CIB1 protein expression in human tissues of several tumour types. Immunohistochemical (IHC) analysis of a panel of 35 human colorectal cancer tissues revealed significant upregulation of CIB1 in tumours compared to normal colorectal tissue matched from the same patients (Figure 1B). A similar elevation of CIB1 protein was also found in invasive ductal breast carcinoma and lung adenocarcinoma tissues by IHC (Figures 1C and S2A,B), and in acute myeloid leukaemia by immunoblot analysis of patient bone marrow biopsies (Figure 1D, S2C).

In light of this upregulation of CIB1 in multiple cancer types, we performed Kaplan Meier analysis on publically available cancer datasets<sup>18, 19</sup>. Similar to a previous report with liver cancer patients<sup>8</sup>, a significant correlation between elevated CIB1 expression and poorer prognosis was evident in patients with estrogen receptor negative breast cancer and lung adenocarcinoma (Figure S2D). The high expression of CIB1 in multiple cancers prompted us to examine a correlation between the presence of common oncogenes and CIB1 expression. Initial analysis of the Garnett human multicancer cell line gene expression dataset<sup>20</sup> found a highly significant correlation between high CIB1 expression and a number of oncogenic mutations, the most significant being oncogenic KRas mutations (Table S1). Activating mutations in the Ras genes (K-, H- and NRas) are found in around 16% of all human cancers, making it one of the most commonly mutated gene families in cancer<sup>21</sup>. This is particularly the case in colorectal cancer, where a third of tumours harbour oncogenic KRas mutations<sup>21</sup>. Since CIB1 was also up-regulated in colon cancer (Figure 1A,B), we then examined the public datasets detailing matched gene mutation and expression analysis for cancers of this type within The Cancer Genome Atlas (TCGA) resource. Strikingly, analysis of the colorectal adenocarcinoma datasets<sup>22</sup> showed a significant positive correlation between the presence of oncogenic KRas mutations and elevated CIB1 expression (Figure 1E). IHC analysis of CIB1 in human colorectal tissues with known KRas mutation status also confirmed a similar correlation at the protein level (Figure 1F). By further mining the Oncomine database, we also found higher CIB1 expression in KRas mutated patients of lung adenocarcinoma and acute myeloid leukaemia (AML), where KRas mutation is less common (Figure 1G). These observations together suggested a potential role for Ras mutation to mediate enhanced CIB1 expression. Thus, we next directly examined the

effect of expression of oncogenic Ras on endogenous CIB1 expression in HEK293 cells, which endogenously possess only wildtype Ras<sup>23</sup>. Notably, ectopic expression of oncogenic KRas or HRas in these cells resulted in a significant upregulation of CIB1 mRNA and protein compared to vector control cells (Figure 1H, I), to a comparable extent to that seen in the human cancer analysis (Figure 1A–G).

Although the mechanisms of transcriptional regulation of CIB1 remain unknown, our findings suggested that transcription factors downstream of the Ras/MAPK pathway are likely to be involved. Indeed, analysis using the UCSC Genome Browser<sup>24, 25</sup> revealed binding sites in the 5' proximal region of the *CIB1* gene for c-Fos and c-Myc, two common Ras effectors<sup>26, 27</sup>, supporting this hypothesis.

### CIB1 induces oncogenic signalling by SK1

Since we had established up-regulation of CIB1 expression in multiple human cancers we next investigated the oncogenic potential of CIB1 by initially examining the effect of its over-expression on cell proliferation. Consistent with previous studies that have shown reduction in cell survival and proliferation in response to RNAi knockdown or genetic ablation of CIB1 expression<sup>5, 13, 15, 16, 28</sup>, over-expression of CIB1 resulted in a significant increase in the cellular growth rate in both low and higher serum culture conditions, and also supported some serum-independent cell growth, compared to vector control cells (Figure 2A).

While previous studies had implicated a partial role for PAK1 in CIB1-mediated cancer cell survival<sup>16</sup>, the main mediator(s) of this effect remained unclear. Since CIB1 has an established role in agonist-induced translocation of SK1 from the cytoplasm to the plasma membrane<sup>5</sup>, and this localisation of SK1 is known to enhance cell proliferation and survival<sup>7</sup>, we investigated whether CIB1 over-expression itself could induce plasma membrane localisation of SK1. Fluorescence microscopy showed that overexpression of CIB1 induced an increase in SK1 protein localised to the plasma membrane (Figures 2B,C, S3A), which was supported by elevated plasma membrane-associated SK1 catalytic activity (Figure 2D), and enhanced intracellular and extracellular S1P generation (Figure 2E) in CIB1 overexpressing cells. This CIB1-induced elevation of S1P was blocked by inhibition of SK1 with the isoform-selective SK1 inhibitor, SK1-I<sup>29</sup> (Figure S3B), confirming this effect was dependent on SK1. Notably, consistent with the importance of the Ca<sup>2+</sup>-myristoyl switch function of CIB1<sup>5</sup>, this altered localisation of SK1 was dependent on CIB1 myristoylation since introduction of an alanine at the myristoylation site (CIB1<sup>G2A</sup>) to block CIB1 myristoylation completely prevented the redistribution of SK1 to the plasma membrane (Figure 2B–D, S3A) and subsequent elevation of intracellular and extracellular S1P (Figure 2E). Importantly, we confirmed that this non-myristoylated CIB1<sup>G2A</sup> variant retained its Ca<sup>2+</sup>-dependent interaction with SK1 (Figure S3C).

Our previous studies demonstrated an important role for phosphorylation of SK1 at Ser225 in translocation of SK1 from the cytoplasm to the plasma membrane in response to cell agonists like tumour necrosis factor- $\alpha$ <sup>7</sup>. Other studies by Obeid and colleagues<sup>30</sup>, however, showed sustained activation of the Ras/MAPK pathway through oncogenic KRas expression could induce plasma membrane localisation of SK1 in a manner independent of SK1

phosphorylation at Ser225. The mechanisms facilitating this sustained plasma membrane localisation, however, were not defined. Notably, our observations that CIB1 is up-regulated by Ras (Figure 1H,I), together with our data demonstrating that CIB1 over-expression alone can drive plasma membrane localisation of SK1 (Figure 2B,C), now provides a likely mechanism for these previous observations. To interrogate this, we examined the effect of oncogenic Ras on the localisation of SK1 and the non-phosphorylatable SK1<sup>S225A</sup> in wildtype and CIB1<sup>-/-</sup> mouse embryonic fibroblasts (MEFs). Consistent with the previous findings<sup>30</sup>, in wildtype MEFs oncogenic Ras induced plasma membrane localisation of both SK1 and SK1<sup>S225A</sup> (Figures 2F,G, S3D). In CIB1<sup>-/-</sup> MEFs, however, this oncogenic Ras-induced relocalisation of SK1 was not observed (Figure 2F,G, S3D), consistent with a critical role for CIB1 in this process. Notably, the structural basis for the S225 phosphorylation-independent association of SK1 with CIB1 has been recently proposed by Adams *et al.*<sup>31</sup> to potentially involve flexibility in helix- $\alpha$ 8 of SK1, which we have previously implicated in CIB1 binding<sup>5</sup>.

Since both CIB1 and SK1 appear elevated in a wide range of cancers, we next investigated whether these two genes display co-ordinate regulation in human cancers. Gene expression analysis, however, showed that SK1 was rarely elevated in the cancer cohorts where significantly increased CIB1 expression was observed (Figure S4A). We also found no clear association between CIB1 and SK1 expression in lung adenocarcinoma patients with KRas mutations (Figure S4B). Furthermore, ectopic expression of CIB1 in HEK293 cells showed no effect on endogenous SK1 mRNA nor protein (Figure S4C,D), further suggesting that CIB1 is more likely to enhance SK1 signalling through altering its sub-cellular localisation, rather than expression.

### **CIB1 induces neoplastic transformation in a SK1-dependent manner**

Next we investigated whether expression of CIB1 had the ability to induce neoplastic transformation of cells. Focus formation assays with NIH3T3 fibroblasts (Figure 3A) showed that cells over-expressing CIB1 induced numerous foci, indicating loss of contact inhibition in these cells consistent with neoplastic transformation. In stark contrast, cells expressing the non-myristoylated CIB1<sup>G2A</sup> did not generate foci, again demonstrating the importance of this modification for the oncogenic function of CIB1. To confirm that the CIB1 over-expressing cells had undergone full neoplastic transformation we next performed subcutaneous xenografts of the cells into the flanks of NOD/SCID mice. Consistent with the *in vitro* focus formation assays, tumours formed in all five mice injected with cells over-expressing CIB1, while no tumours were evident in either vector or CIB1<sup>G2A</sup> expressing cells (Figure 3B). Thus, these findings indicate CIB1 can directly induce full neoplastic transformation in a manner dependent on its myristoylation, again consistent with the importance of the Ca<sup>2+</sup>-myristoyl switch function of this protein.

To determine the dependence on SK1 for the CIB1-induced neoplastic transformation we examined the effect of a range of SK inhibitors on neoplastic growth of the CIB1 over-expressing cells. Strikingly, the SK1-selective inhibitor SK1-I, as well as the pan-SK inhibitors MP-A08<sup>32</sup> and SKI-II<sup>33</sup> all effectively blocked CIB1-induced neoplastic growth in both focus formation (Figure 3C) and soft agar colony formation assays (Figure S5A),

consistent with an important role for SK1 in this process. To further confirm the role of SK1 we next examined the ability of CIB1 to induce neoplastic transformation in immortalised SK1 knockout (SK1<sup>-/-</sup>) MEFs. Consistent with our observations in NIH3T3 cells, CIB1 induced neoplastic transformation in MEFs from wildtype mice, as assessed by focus formation assays (Figure 3D). No such neoplastic growth was observed, however, in MEFs from SK1<sup>-/-</sup> mice clearly showing a requirement for SK1 in CIB-induced neoplastic transformation. Together these findings, for the first time, define a molecular mechanism for the role of CIB1 in cancer through its regulation of SK1.

### Ablating CIB1 expression attenuates Ras-induced neoplastic transformation

SK1 has been previously shown to have a role in oncogenic signalling by Ras since chemical inhibitors of SK or a dominant-negative SK1 attenuated Ras-induced neoplastic transformation<sup>34, 35</sup>. To further validate these findings we examined the ability of Ras to induce neoplastic transformation of immortalised SK1<sup>-/-</sup> MEFs. Focus formation assays showed that oncogenic Ras failed to induce neoplastic transformation in these cells lacking SK1, consistent with an essential role for SK1 in oncogenic signalling by Ras (Figure 4A).

To investigate whether the CIB1-mediated SK1 translocation is specifically involved in oncogenesis by Ras, we examined immortalised CIB1<sup>-/-</sup> MEFs, where we had shown deficiency in Ras-induced plasma membrane localisation of SK1 (Figure 2F,G). Compared to wildtype MEFs, significant attenuation of Ras-induced neoplastic transformation was observed in these CIB1<sup>-/-</sup> cells (Figure 4B, S5B). To further validate these findings, we also examined the effect of the non-myristoylated CIB1<sup>G2A</sup>, which we found to block localisation of SK1 to the plasma membrane (Figure 2B, C)<sup>5</sup>, on Ras-induced transformation of NIH3T3 cells. As expected, oncogenic Ras induced efficient neoplastic transformation of these cells, as assessed by focus formation and colony formation assays (Figures 4C and S5C). Consistent with our earlier findings, over-expression of wildtype CIB1 alone also induced neoplastic transformation, which was not further enhanced by oncogenic Ras. Cells expressing CIB1<sup>G2A</sup> were, however, remarkably resistant to Ras induced neoplastic transformation in both focus formation (Figure 4C) and colony formation assays (Figure S5C). Notably, while previous studies have suggested that loss of CIB1 expression attenuates ERK1/2 activation<sup>15, 16, 36</sup>, our analysis of this pathway found no effect on the levels of phospho-ERK1/2 in the basal state or in response to oncogenic Ras by the overexpression of either wildtype or CIB1<sup>G2A</sup> (Figure 4D). Thus, these observations are consistent with the notion that the effects of attenuated Ras-induced neoplastic signalling by CIB1<sup>G2A</sup> occur downstream of ERK1/2, at the level of SK1.

### Conclusions

A major novel finding of this study is the identification of CIB1 as an important effector of oncogenic signalling by Ras through the enhancement of subcellular localisation of SK1 to the plasma membrane. An oncogenic role for SK1 has been well-established in a variety of cancers<sup>37-39</sup>, and we have previously elucidated that plasma membrane localisation of the enzyme is critical for its oncogenic signalling function<sup>7</sup>. Notably, in these previous studies we described a mechanism whereby phosphorylation of SK1 induces its plasma membrane localisation and subsequent oncogenic signalling<sup>7, 40, 41</sup>. The current studies, however,



demonstrate that an alternative, phosphorylation-independent mechanism driving plasma membrane localisation and subsequent oncogenic signalling by SK1 can also exist involving CIB1. The fact that this regulation of SK1 is mediated by enhanced expression of CIB1 in response to oncogenic Ras mutations, combined with the high frequency of these Ras mutation in human cancer<sup>21</sup>, suggest that these findings may have broad implications for many cancers. Thus, overall, these findings indicate that CIB1 represents a promising target for modulating Ras and SK1 signalling outcomes in cancer, warranting further investigation of the CIB1-SK1 interaction for the development of novel cancer therapeutics.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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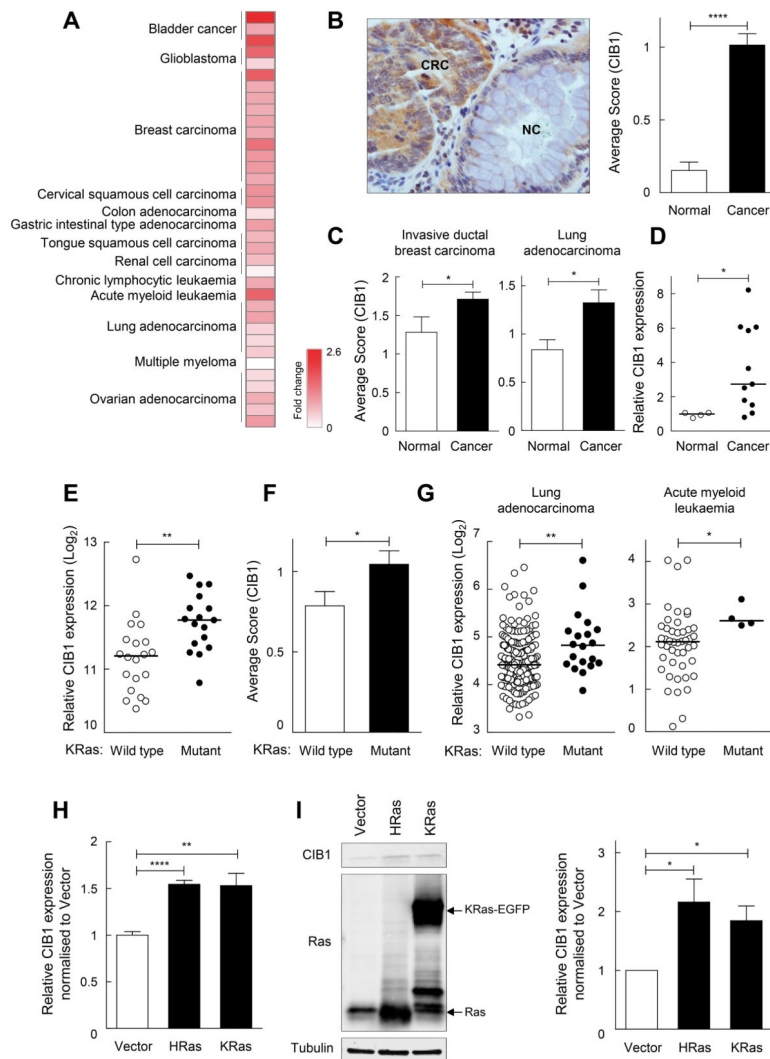
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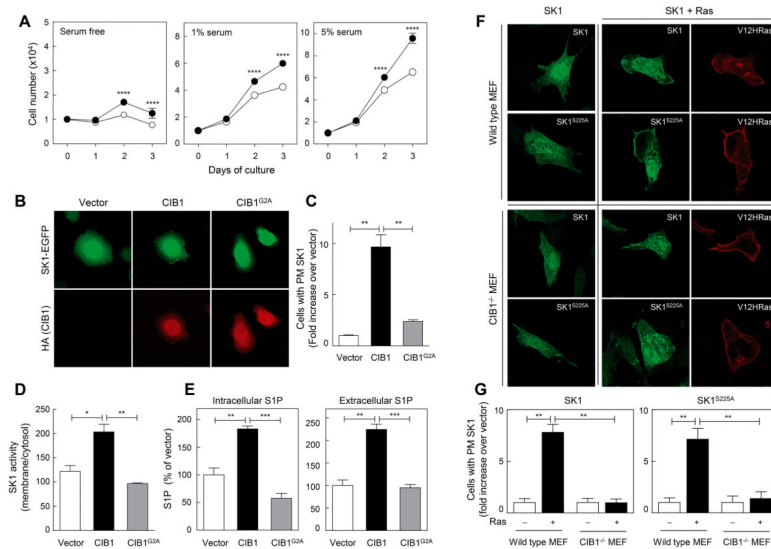


**Figure 1. CIB1 expression is elevated in multiple cancers and is associated with oncogenic Ras mutation**

(A) Gene expression datasets from the Oncomine database<sup>17</sup> were mined for CIB1 expression in normal compared to cancer tissues. The heat map shows all the datasets with significant elevated levels of CIB1 in cancer tissues ( $p < 0.0001$ ). Each row represents a cancer subtype from an individual dataset. Further detail is presented in Figure S1. (B) Paired histology sections from tumours excised from 35 colorectal cancer patients were assessed for levels of CIB1 protein by immunohistochemistry analysis. Custom made tissue microarrays (TMAs) were used with no less than two representative cores of tumour tissue and matched non-neoplastic colonic mucosa being inserted into the recipient TMA block. Sections (4  $\mu$ m) of paraffin wax-embedded tissue were mounted on coated slides, dewaxed, and rehydrated using standard techniques. Antigen retrieval was performed with the microwave using 10 mM citrate buffer (pH 6), by bringing it to a boil on high heat, and then simmering for 15 min. After cooling to 40°C, the sections were incubated for 60 min at room temperature with mouse anti-human primary CIB1 monoclonal antibody (1:250 dilution; R&D Systems). The ADVANCE™ kit (Dako) employing DAB as the detection

system was used. Immunostaining was performed on the DAKO Autostainer Plus platform. Counterstaining was performed using Mayer's hematoxylin. Samples were scored as either strongly positive (++), weakly positive (+) if heterogeneous staining was detected in cell cytoplasm, or negative if there was no staining present. A negative control was performed by omitting the primary antibody. \*\*\*\* $p < 0.0001$ . NC: normal crypt; CRC: colorectal cancer. Samples were obtained with informed consent from patients diagnosed with colorectal adenocarcinoma according to institutional guidelines and studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee. (C) Commercial breast and lung TMAs containing two representative cores of tumour or normal tissues (Protein Biotechnologies) were used to examine the level of CIB1 expression by IHC analysis. Results show average scores of CIB1 staining in normal breast (n = 16), invasive ductal breast carcinoma (n = 69), normal lung (n = 20), and lung adenocarcinoma (n = 31) tissues. Only tissues with valid staining in both duplicate cores were scored. \* $p < 0.05$ . (D) Diagnostic bone marrow or apheresis product samples were obtained with informed consent from patients diagnosed with AML or health volunteers according to institutional guidelines. Studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee. Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation and resuspended in IMDM containing 0.5% foetal calf serum (FCS). Morphological analysis revealed >80% AML blasts post centrifugation. Cell lysates from bone marrows of 11 AML patients and 4 healthy donors were resolved by SDS-PAGE and immunoblot analysis using anti-CIB1 and anti-actin antibodies (both from Millipore) as previously described<sup>42</sup>. Densitometry of the CIB1 and tubulin bands was quantified using the Image Studio™ Lite software (LI-COR). \* $p < 0.05$ . (E) TCGA colorectal cancer (COADREAD) dataset were mined for the level of CIB1 expression in cancerous tissues with or without KRas mutations. CIB1 expression data (exon expression, Illumina HiSeq) and COADREAD clinical data were obtained from the UCSC Cancer Genome browser (<https://genome-cancer.ucsc.edu/proj/site/hgHeatmap>; dataset ID: TCGA\_COADREAD\_exp\_HiSeqV2\_exon). \* $p < 0.05$ . (F) Custom made TMAs containing 31 colorectal cancerous tissues with wild type KRas and 30 with known KRas somatic mutation were examined for level of CIB1 expression by IHC. To screen for mutations of the *KRas* gene, DNA was prepared from cancer tissue macro-dissected from formalin fixed, paraffin embedded (FFPE) slides using the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA) and a multiplex assay was performed to screen for mutations in codons 12 and 13 of the *KRAS* gene<sup>43</sup>. Only tissues with valid staining in both duplicate cores were scored. \* $p < 0.05$ . (G) Oncomine datasets were mined for the level of CIB1 expression in cancerous tissues with or without KRas mutations. Oncomine data for lung adenocarcinoma (Okayama lung) and acute myeloid leukaemia (Raponi leukaemia) are shown. \* $p < 0.05$ ; \*\* $p < 0.01$ . (H) HEK293 cells were transiently transfected with oncogenic HRas<sup>G12V</sup> or GFP tagged KRas<sup>G12V</sup>. The gene expression of CIB1 was measured by quantitative RT-PCR using SYBR green PCR master mix (QIAGEN) and the primers AGTACCAGGACTTGACGTTCC (forward) and CTTGAGCTCTGGAAGGCTGA (reverse). The PCR thermocycling conditions were 50 °C for 2 min, 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 33 s, and 72 °C for 33 s. The housekeeping gene GAPDH was measured by primers ACCCAGAAGACTGTGGATGG (forward) and CAGTGAGCTTCCCGTTCAG (reverse). The relative gene expression of CIB1 normalised to relative gene expression GAPDH was analysed using the comparative

quantitation method using the Rotor-Gene Q Series software. Data shows mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$ ; \*\*\*\*  $p < 0.0001$ . (I) Lysates of HEK293 cells ectopically expressing HRas<sup>G12V</sup> or GFP tagged KRas<sup>G12V</sup> were analysed by immunoblot using anti-CIB1, anti-Ras (both from Millipore), and anti-tubulin antibodies (Abcam). Densitometry of the CIB1 and tubulin bands was performed using the Image Studio™ Lite software (LI-COR). Data shows mean  $\pm$  SEM of relative CIB1 expression normalised to vector control from three independent experiments. \* $p < 0.05$ . pCX<sup>bla</sup> (Vector) and pCX<sup>bla</sup>-HRas<sup>G12V</sup> plasmids were generated as follows. The blasticidin (bla) resistance gene was amplified from pLenti6/TR (Invitrogen) with the following oligonucleotides 5'-TGCATCTCAATTAGTCAGCAA-3' and 5'-TAGAATTCTTCGAATTAGCCCTCCCACACATAAC-3', digested with *Xma*I and *Eco*RI and cloned into pGEM4Z (Promega). The *Xma*I/*Bst*BI cassette from pGEM4Z-bla was used to replace the neomycin (neo) resistance gene in pCX<sup>neo</sup>-IRES-EGFP digested with *Xma*I/*Bst*BI to produce pCX<sup>bla</sup>-IRES-EGFP. Digestion of pCX<sup>bla</sup>-IRES-EGFP with *Sac*I/*Xho*I and subsequent blunting, re-ligation produced pCX<sup>bla</sup>. HRas<sup>G12V</sup> was PCR amplified from pSG5V12Ras, a kind gift from J. Downward<sup>44</sup>, with the following oligonucleotides 5'-TAGAATTCGCCACC ATGACCGAATACAAGCTTGTTG-3' and 5'-TAGAATTCTCAGGAGAGCA CAACTTGC-3', digested with *Eco*RI and cloned into pCX<sup>bla</sup>. Sequencing verified integrity and orientation of all the cDNA's. GFP-KRas1b<sup>G12V</sup> was a kind gift from R. Parton<sup>45</sup>. HEK293 cells were originally obtained from American Type Culture Collection (ATCC), cultured for fewer than 6 months after resuscitation as previously described<sup>5</sup> and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Statistical analysis was performed with non-parametric two-tailed Mann-Whitney tests for the data shown in panels B–G and two tailed unpaired t tests for data in panels H and I.



**Figure 2. Over-expression of CIB1 promotes translocation of SK1 to the plasma membrane** (A) Proliferation of NIH3T3 cells stably transfected with CIB1 (●) or vector control (○) in serum free or low serum conditions (1% and 5% FCS) were measured in quadruplicate by MTS assays (Promega) according to manufacturer's protocol. Data shows mean  $\pm$  SD; representative of three independent experiments. \*\*\*\* $p$ <0.0001. (B) HeLa cells over-expressing HA-tagged CIB1, CIB1<sup>G2A</sup> (Ref. 5), or vector control were stimulated with phorbol 12-myristate 13-acetate and visualised by fluorescence microscopy, as previously described<sup>5</sup>. SK1 was visualised via its EGFP tag (green)<sup>40</sup> and CIB1 via immunofluorescence staining of its HA epitope tag (red) using anti-HA antibodies (Cell Signaling). Over 350 cells were visualised (B) and quantitated (C) from each of three independent experiments. \*\* $p$ <0.01. (D) Lysates of NIH3T3 cells over-expressing CIB1, CIB1<sup>G2A</sup>, or vector control were subjected to sub-cellular fractionation as previously described<sup>40</sup>. SK1 in the membrane and cytosolic fractions were assessed by SK1 activity assays under conditions largely specific for SK1<sup>46</sup>, and expressed as a ratio of membrane to cytosol. Total SK1 activity in whole cell lysates showed no significant difference between groups. Data shows mean  $\pm$  SEM;  $n=3$ . \* $p$ <0.05, \*\* $p$ <0.01. (E) S1P generation in NIH3T3 cells over-expressing CIB1, CIB1<sup>G2A</sup>, or vector control was assessed by metabolically labelling the cells with <sup>3</sup>H-sphingosine, as described previously<sup>32</sup>. Radiolabelled intracellular and extracellular S1P were measured by scintillation counting. Data shows mean  $\pm$  SEM;  $n=3$ , \*\* $p$ <0.01; \*\*\* $p$ <0.001. (F) Wild type or CIB1<sup>-/-</sup> MEFs were transfected with vectors encoding EGFP-tagged SK1 or SK1<sup>S225A</sup> (Ref. 40) and effects on subcellular localisation assessed by confocal microscopy with or without co-transfection with a vector encoding oncogenic HRas<sup>G12V</sup>. Localisation of SK1 and SK1<sup>S225A</sup> were visualised by their EGFP fusions (green) and Ras was visualised via anti-Ras immunofluorescence staining (red). Due to low transfection efficiency of MEF cells, between 100–200 cells were visualised (F) and quantitated (G) from each of three independent experiments. \*\* $p$ <0.01. HeLa and NIH3T3 cells were obtained from the ATCC, cultured for fewer than 6 months after resuscitation, and transfected as previously described<sup>5, 7</sup>, MEFs were generated from wild type, SK1<sup>-/-</sup> and CIB1<sup>-/-</sup> mouse<sup>11, 47</sup>

embryos at 14.5 days *post coitum*. The cells were cultured in a humidified atmosphere with 10% CO<sub>2</sub>. Passage one MEFs were immortalized by transfection with a plasmid encoding the SV40 large T antigen using Nucleofection (Lonza), with stable transfectants selected by zeocin resistance. Statistical analysis was performed with two-way analysis of variance (ANOVA) for the data in panel A and two tailed unpaired t tests for the data in panels C–E and G.

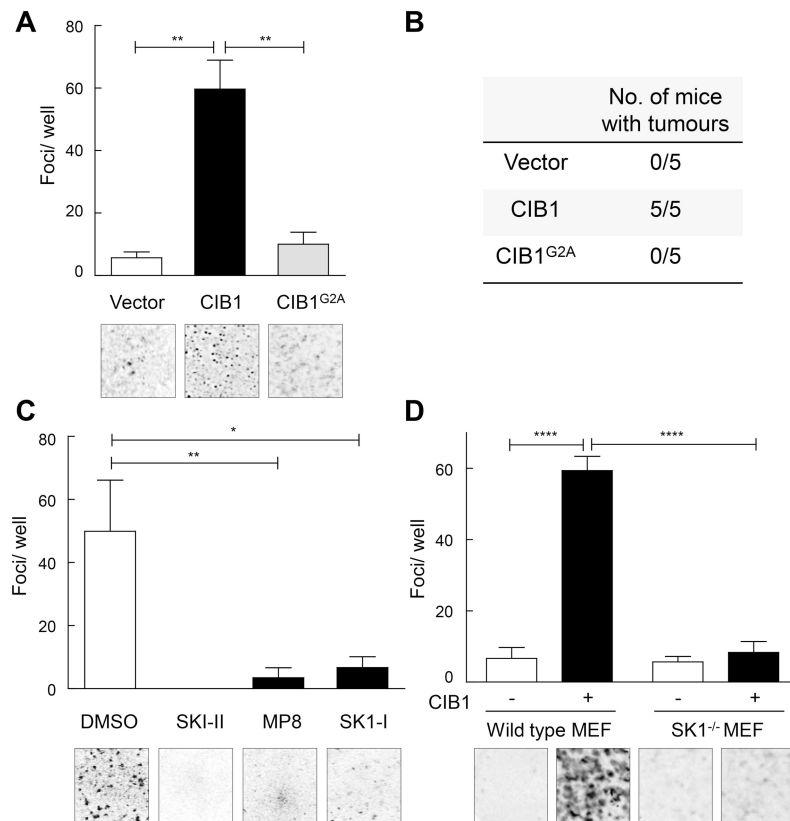
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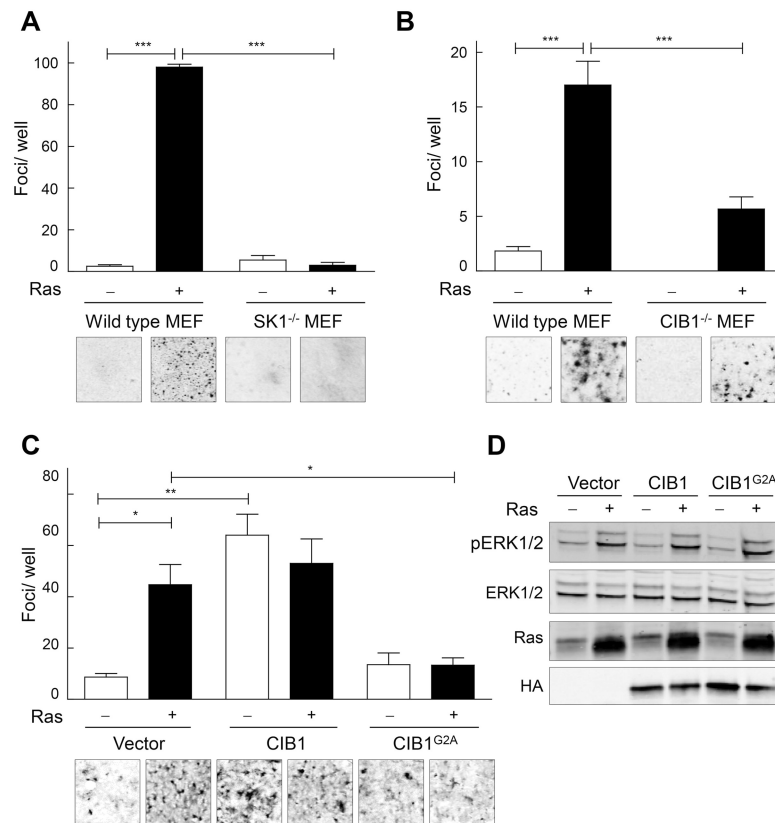




### Figure 3. CIB1 promotes neoplastic transformation in a manner dependent on SK1

(A) NIH3T3 cells stably transfected with CIB1, CIB1<sup>G2A</sup> or vector control were assessed for neoplastic growth by focus formation assays, as described previously<sup>35</sup>. Data shows mean  $\pm$  SD; representative of three independent experiments.  $**p < 0.01$ . Cells expressing CIB1<sup>G2A</sup> remained viable but did not form obvious foci. (B) NIH3T3 cells stably transfected with vectors encoding CIB1, CIB1<sup>G2A</sup> or empty vector control were assessed for their ability to form tumours when subcutaneously injected into the flank of NOD/SCID mice. Seven week old female NOD-SCID mice were obtained from the Animal Resources Centre, Western Australia, and housed in sterile micro-isolator cages and provided with sterile food and water and treated following the conditions approved by the Institutional Animal Ethics Committee. Stably transfected NIH3T3 cells ( $1 \times 10^6$ /mouse) resuspended in sterile PBS were subcutaneously injected into the flanks of five mice/cell line. Mice were monitored daily for tumour growth, humanely killed 3 weeks post-injection, and tumours excised, weighed, and then analysed by immunoblot with HA antibodies to confirm CIB1 expression. (C) NIH3T3 cells stably transfected with CIB1 were assessed for neoplastic growth by focus formation assays in the presence of vehicle alone or SK1 inhibitors SKI-II (5 $\mu$ M), MP-A08 (20 $\mu$ M) or SK1-I (5  $\mu$ M). Data shows mean  $\pm$  SD; representative of three independent experiments.  $*p < 0.05$ ;  $**p < 0.01$ . (D) Wild type or SK1<sup>-/-</sup> MEFs over-expressing CIB1 were assessed for neoplastic transformation by focus formation assays. Data shows mean  $\pm$  SD; representative of three independent experiments.  $****p < 0.0001$ . To generate cell lines stably expressing CIB1, pCX<sup>neo</sup>-IRES-EGFP and pCX<sup>neo</sup>-CIB1(HA)-IRES-EGFP were generated as described below. pCX-EGFP was a kind gift from M. Okabe<sup>48</sup>. EGFP was

replaced with a polylinker following digestion with *EcoRI* and ligation of annealed kinased oligonucleotides 5'-AATTCGGTACCGAG CTCGCTAGCGCGGCCGCCTCGAGC-3' and 5'-AATTGCTCGAGGCGGCCG CGCTAGCGAGCTCGGTACCG-3' to produce pCX4. A blunted *SaI* and *EcoRI* cassette from pCX4 encompassing the chicken  $\beta$ -actin promoter was used to replace the CMV promoter of pcDNA3-IRES-EGFP following blunted *BglII* and *EcoRI* digestion to produce pCX<sup>neo</sup>-IRES-EGFP. pCX<sup>neo</sup>-CIB1(HA)-IRES-EGFP was produced by cloning with *EcoRI*. NIH3T3 cells transfected with these CIB1 constructs were selected for G418 resistance and then sorted for high GFP expression using a Becton Dickinson FACS Aria (BD Biosciences). Statistical analysis was performed with two tailed unpaired t tests.



#### Figure 4. CIB1 is an effector of oncogenic Ras signalling

(A) Wild type or SK1<sup>-/-</sup> MEFs were stably transfected with pSG5V12Ras (for expression of HRas<sup>G12V</sup>) or vector control and assessed for neoplastic transformation by focus formation assays as described above. (B) Wild type and CIB1<sup>-/-</sup> MEFs were stably transfected with pCX<sup>bla</sup>-HRas<sup>G12V</sup>, which was sub-cloned from pSG5V12Ras as described above. Due to the potent expression of HRas<sup>G12V</sup> by pCX<sup>bla</sup>-HRas<sup>G12V</sup> which contains a more potent Kozak sequence, the stable transfectants were diluted 1:1000 with their corresponding parental MEF lines to enable visualisation and quantification of oncogenic Ras-induced foci. For (A) data shows mean  $\pm$  SD; representative of three independent experiments. \*\*\* $p$ <0.001; and (B) data shows mean  $\pm$  SEM;  $n$  = 6. \*\*\* $p$ <0.001. (C) NIH3T3 cells stably expressing CIB1, CIB1<sup>G2A</sup>, or vector control were transiently transfected with HRas<sup>G12V</sup> and applied to focus formation assays. Data shows mean  $\pm$  SEM;  $n$  = 3. \* $p$ <0.05, \*\* $p$ <0.01. (D) NIH3T3 cells over-expressing CIB1, CIB1<sup>G2A</sup> were co-transfected with or without HRas<sup>G12V</sup>. The level of pERK1/2, ERK1/2, Ras, and HA tagged CIB1 in these cells were analysed by immunoblotting using antibodies from Cell Signaling, Promega, Millipore and Sigma-Aldrich, respectively. Results are representative of three independent experiments. Statistical analysis was performed with two tailed unpaired t tests.