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# Patched1 interacts with cyclin B1 to regulate cell cycle progression

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The initiation of mitosis requires the activation of M-phase promoting factor (MPF). MPF activation and its subcellular localization are dependent on the phosphorylation state of its components, *cdc2* and cyclin B1. In a two-hybrid screen using a bait protein to mimic phosphorylated cyclin B1, we identified a novel interaction between cyclin B1 and *patched1* (*ptc1*), a tumor suppressor associated with basal cell carcinoma (BCC). *Ptc1* interacted specifically with constitutively phosphorylated cyclin B1 derivatives and was able to alter their normal subcellular localization. Furthermore, addition of the *ptc1* ligand, sonic hedgehog (*shh*), disrupts this interaction and allows cyclin B1 to localize to the nucleus. Expression of *ptc1* in 293T cells was inhibitory to cell proliferation; this inhibition could be relieved by coexpression of a cyclin B1 derivative that constitutively localizes to the nucleus and that could not interact with *ptc1* due to phosphorylation-site mutations to Ala. In addition, we demonstrate that endogenous *ptc1* and endogenous cyclin B1 interact *in vivo*. The findings reported here demonstrate that *ptc1* participates in determining the subcellular localization of cyclin B1 and suggest a link between the tumor suppressor activity of *ptc1* and the regulation of cell division. Thus, we propose that *ptc1* participates in a G<sub>2</sub>/M checkpoint by regulating the localization of MPF.

**Keywords:** basal cell carcinoma/cytoplasmic retention signal/G<sub>2</sub>/M checkpoint/M-phase promoting factor/nevoid basal cell carcinoma syndrome

## Introduction

Entry into mitosis requires the activation of M-phase promoting factor (MPF), a universal cell cycle regulatory complex. At late G<sub>2</sub>, MPF translocates to the nucleus where it is responsible for the phosphorylation of many nuclear substrates required for mitotic progression (Peter *et al.*, 1990; Yamashiro *et al.*, 1991; Verde *et al.*, 1992). The MPF complex consists of two proteins that are regulated separately (Pondaven *et al.*, 1989; Ducommun *et al.*, 1991; Li *et al.*, 1995; Borgne *et al.*, 1999). The Ser/Thr kinase *cdc2*, which is ubiquitously expressed

throughout the cell cycle, undergoes activation at the G<sub>2</sub>/M transition (Ducommun *et al.*, 1991; Krek and Nigg, 1991; Borgne and Meijer, 1996) as a result of dephosphorylation of Thr14 and Tyr15 and phosphorylation of Thr161 (Solomon *et al.*, 1990; Krek and Nigg, 1991; Norbury *et al.*, 1991). In contrast, cyclin B1 is temporally regulated and does not begin to accumulate until S phase. At G<sub>2</sub> phase, cyclin B1 protein reaches maximal levels and begins to enter the nucleus in a phosphorylation-dependent manner (Hagting *et al.*, 1998, 1999; Yang and Kornbluth, 1999).

An important aspect of cyclin B1 regulation involves the cytoplasmic retention signal (CRS) domain. Initially, the CRS domain was described as mediating the cytoplasmic retention of cyclin B1 (Pines and Hunter, 1994), but more recently it has been demonstrated that a phosphorylation-dependent nuclear export signal (NES) resides within the CRS (Toyoshima *et al.*, 1998; Yang *et al.*, 1998). When the NES of cyclin B1 is active, it facilitates rapid export of unphosphorylated cyclin B1 from the nucleus (Yang *et al.*, 1998; Yang and Kornbluth, 1999). Phosphorylation of serine residues within the CRS domain, beginning in late G<sub>2</sub>, disrupts the NES function and allows for the nuclear accumulation of MPF to initiate the onset of mitosis (Yang *et al.*, 1998).

Previous studies in our laboratory identified four major sites of serine phosphorylation within the CRS of *Xenopus* cyclin B1 (residues S94, S96, S101 and S113) (Li *et al.*, 1995). In addition, mutation of these four Ser residues to Ala, mimicking an unphosphorylated state, substantially decreased MPF activity in *Xenopus* oocyte maturation assays. Conversely, substitution of Glu residues to mimic the phosphorylated state greatly increased MPF activity in the oocyte system (Li *et al.*, 1997). This study suggested that the localization of cyclin B1, and of active MPF, is regulated by phosphorylation within the CRS domain. Recently, it was demonstrated that UV-induced DNA damage prevents cyclin B1 from entering the nucleus or, alternatively, causes cyclin B1 to shuttle rapidly from the nucleus to the cytoplasm, thereby delaying mitosis until the damage can be repaired (Jin *et al.*, 1998; Toyoshima *et al.*, 1998). Thus, the regulation of cyclin B1 localization is a key factor involved in a G<sub>2</sub>/M checkpoint control. In an effort to identify biologically relevant partners that may regulate the trafficking and localization of phosphorylated cyclin B1, we employed a yeast two-hybrid approach.

Surprisingly, this search for cyclin B1-interacting proteins identified *ptc1*, a 12-pass integral membrane protein recently characterized as the receptor for sonic hedgehog (*shh*), an important developmental morphogen (Chen and Struhl, 1996; Marigo *et al.*, 1996; Stone *et al.*, 1996; Goodrich and Scott, 1998). *Ptc1* is a tumor suppressor frequently mutated in association with nevoid basal cell carcinoma syndrome (NBCCS) and basal cell

carcinoma (BCC) (Chidambaram *et al.*, 1996; Gailani *et al.*, 1996; Hahn *et al.*, 1996b; Unden *et al.*, 1996). NBCCS is acquired through autosomal dominant inheritance and predisposes patients to BCC and associated medical problems (Gorlin, 1995). Despite the undisputed role of *ptc1* in BCC development, the mechanism of *ptc1* anti-proliferative and/or tumor suppressor function has not been clearly defined. In this report, we describe the novel interaction between cyclin B1 and *ptc1*, demonstrate that cyclin B1 localization is mediated by *ptc1*, and show that MPF activity can be regulated by *ptc1*. Our data support the existence of a G<sub>2</sub>/M checkpoint where MPF is sequestered by *ptc1* to prevent mitotic progression.

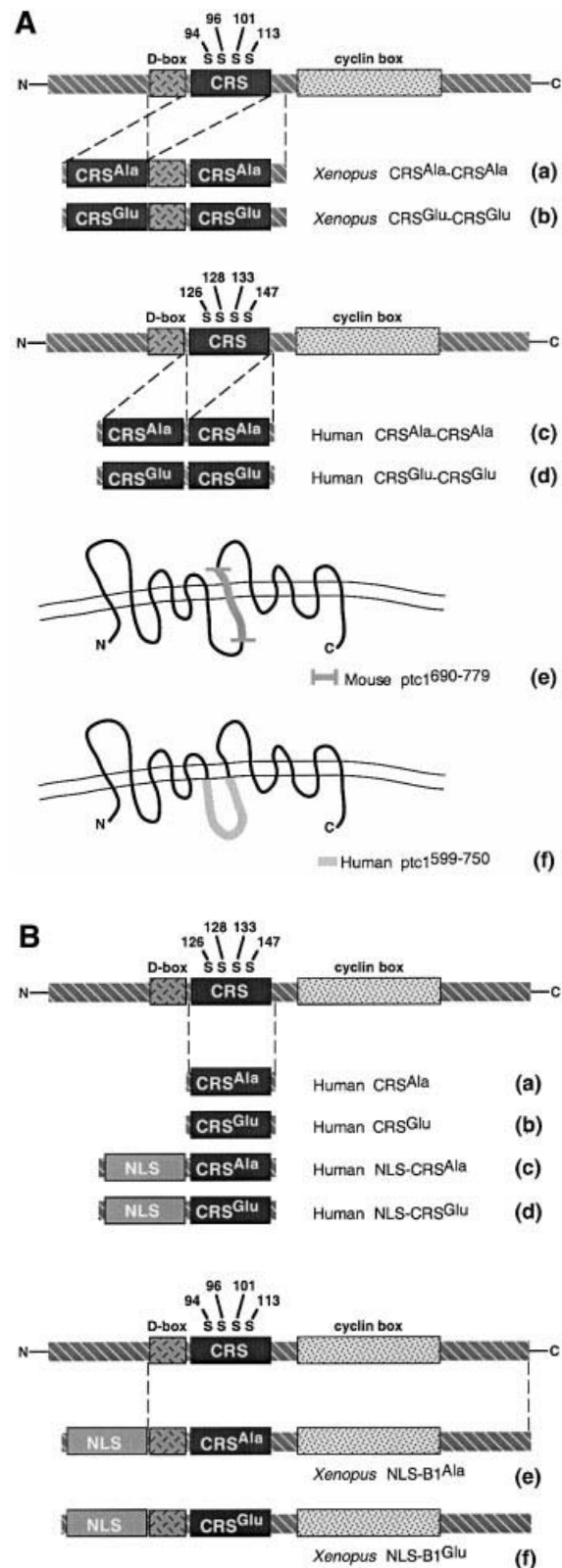
## Results

### Isolation of *ptc1* from a yeast two-hybrid screen

In this study, we exploited a *Xenopus* cyclin B1 mutant with the four Ser phosphorylation sites in the CRS domain mutated to Glu, designed to mimic phosphoserine residues. Similarly, we utilized a mutant with the four Ser residues mutated to Ala to mimic an unphosphorylated state (Li *et al.*, 1997). These derivatives contain a tandem repeat of the CRS domain (CRS<sup>Ala</sup>–CRS<sup>Ala</sup> and CRS<sup>Glu</sup>–CRS<sup>Glu</sup>) (Figure 1A, a and b), based on previous observations that a minimum of 100 amino acids for the bait protein will enhance potential two-hybrid interactions (Kong *et al.*, 2000). Employing a yeast two-hybrid screen, a partial cDNA clone encoding *ptc1* (residues 690–779) was identified in a mouse embryonic cDNA library as positively interacting with the CRS<sup>Glu</sup>–CRS<sup>Glu</sup> construct (Table I). This *ptc1* cDNA clone did not interact with the CRS<sup>Ala</sup>–CRS<sup>Ala</sup> construct. The 90-amino acid-fragment of *ptc1* identified from the screen represented a portion of the large intracellular loop (residues 690–736), a transmembrane segment (residues 737–755), and a portion of the second large extracellular loop (residues 756–779) (Figure 1A, e).

*Ptc1* contains two large extracellular loops involved in binding shh and one large intracellular loop (Chen and Struhl, 1996; Marigo *et al.*, 1996; Stone *et al.*, 1996). A biologically relevant interaction of cyclin B1 with *ptc1* would most likely involve the large intracellular loop, but not the transmembrane or extracellular domains, which should be inaccessible to cyclin B1. To confirm this, a synthetic gene encoding only the large intracellular loop of human *ptc1* (consisting of residues 599–750) was assayed

in the yeast two-hybrid system with the mutant CRS–CRS domains of cyclin B1 (Figure 1A, f). For both His<sup>−</sup> minimal media and β-gal liquid assays, this derivative of *ptc1* exhibited interaction with only the CRS<sup>Glu</sup>–CRS<sup>Glu</sup> construct (Table I and data not shown). In addition, we



**Fig. 1.** (A) Schematic representation of cyclin B1 and patched1 constructs used in the yeast two-hybrid screen. (a–d) Cyclin B1 constructs containing a tandem repeat of the CRS domain. Ser residues (S) of the CRS domain were mutated to Ala or Glu to mimic the unphosphorylated or phosphorylated state of cyclin B1, respectively. (a and b) Constructs representing the *Xenopus laevis* CRS–CRS mutants with the destruction box (D-box) domain of cyclin B1 included. (c and d) Constructs representing the human CRS–CRS mutants. (e) Mouse *ptc1* clone isolated from the two-hybrid screen. (f) Human *ptc1* construct synthesized for further testing in the yeast two-hybrid system. (B) Schematic representation of cyclin B1 constructs used in mammalian cell studies. (a and b) Constructs representing the human CRS mutants. (c and d) Human CRS mutants with an appended NLS of *Xenopus* nucleoplasmin at the N-terminus. (e and f) Full-length *Xenopus* cyclin B1 derivatives with an appended NLS are represented.

**Table I.** Yeast two-hybrid interactions

Bait protein	Interacting protein	
	Mouse ptc1 <sup>(690-779)</sup>	Human ptc1 <sup>(599-750)</sup>
<i>Xenopus</i> CRS <sup>Ala</sup> -CRS <sup>Ala</sup>	–	–
<i>Xenopus</i> CRS <sup>Glu</sup> -CRS <sup>Glu</sup>	+	+
Human CRS <sup>Ala</sup> -CRS <sup>Ala</sup>	ND	–
Human CRS <sup>Glu</sup> -CRS <sup>Glu</sup>	ND	+
Lamin	–	–

ND, not determined.

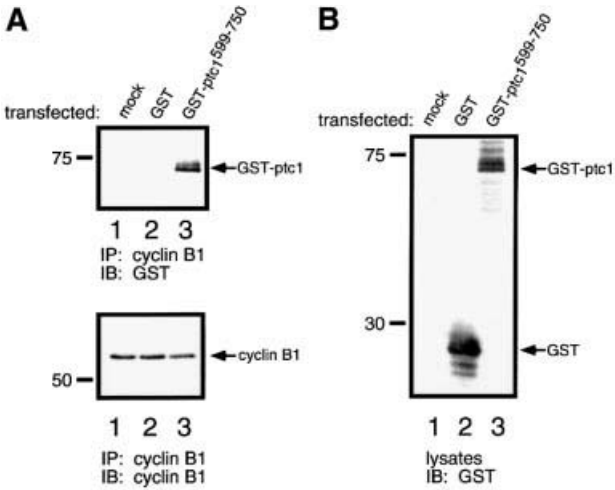
examined whether the CRS domain of human cyclin B1 would interact with human ptc1 (Figure 1A, c and d). As before, we found that the large intracellular loop of human ptc1 interacts specifically with the human CRS<sup>Glu</sup>-CRS<sup>Glu</sup> construct (Table I). Thus, both the mouse ptc1 fragment and the human ptc1 intracellular loop were dependent upon the phosphorylation state of the CRS domain of cyclin B1 for binding. These results confirm that the large intracellular loop of ptc1 has the potential to interact with the phosphorylated CRS domain of cyclin B1 and, furthermore, that the intracellular portion of the original 90-amino-acid fragment of ptc1 isolated from the screen, corresponding to human ptc1 residues 704–750, is sufficient for this interaction.

**The large intracellular loop of ptc1 and cyclin B1 interact *in vivo***

To determine whether the intracellular loop of ptc1 can bind to endogenous full-length cyclin B1, we transfected a glutathione *S*-transferase (GST)–ptc1 intracellular loop fusion construct specific for mammalian expression (GST–ptc1<sup>599-750</sup>) into 293T cells. Mock-transfected cells were used as a negative control; in addition, a construct encoding GST protein alone was transfected into 293T cells as a negative control. Cell lysates were immunoprecipitated with cyclin B1 antiserum and interacting GST fusions were visualized by immunoblotting with GST antiserum (Figure 2A, upper panel). The GST–ptc1<sup>599-750</sup> fusion protein was found to bind specifically to endogenous cyclin B1 (Figure 2A, lane 3). As a control, the lower panel of Figure 2A demonstrates the presence of cyclin B1 in all samples as determined by immunoblotting. In addition, expression of the GST fusion proteins was examined in lysates by immunoblotting (Figure 2B, lanes 2 and 3). These results confirm that the large intracellular loop of ptc1 binds to full-length cyclin B1 *in vivo*.

**Association between endogenous cyclin B1 and endogenous ptc1**

The ptc1 transcript can be found in early embryonic tissues and in adult tissues as well, including brain, lung, kidney and liver (Goodrich *et al.*, 1996; Hahn *et al.*, 1996a). Therefore, to demonstrate an interaction between endogenous ptc1 and endogenous cyclin B1, we used human embryonic kidney 293T cells in a coimmunoprecipitation assay. Immunoprecipitation of endogenous ptc1 using a ptc1 antiserum, followed by immunoblotting using antiserum against cyclin B1, reveals the presence of cyclin B1



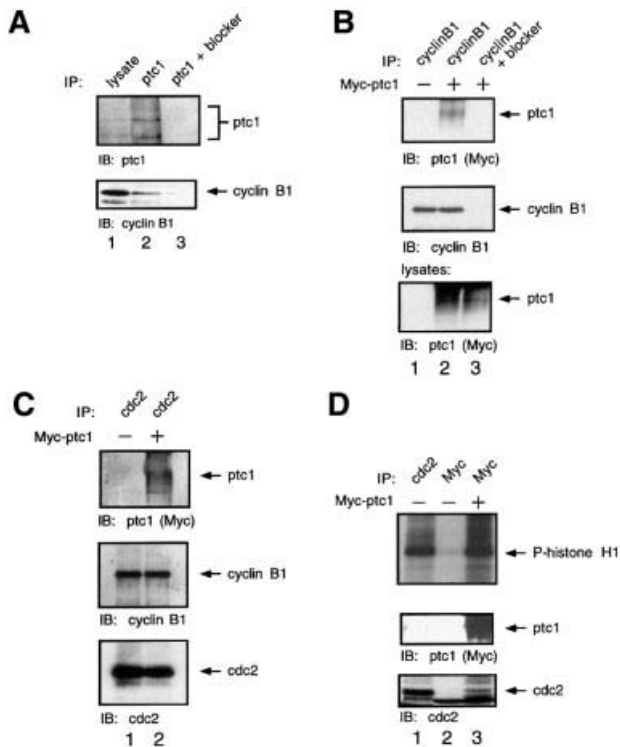
**Fig. 2.** Patched1 intracellular loop binds endogenous cyclin B1. (A) Mammalian GST fusion genes encoding either GST alone (GST) or GST fused to the intracellular loop of ptc1 (GST–ptc1<sup>599-750</sup>) were transfected into 293T cells and immunoprecipitated with cyclin B1 antibody. Samples were analyzed by 10% SDS–PAGE and immunoblotted with mAb GST (upper panel) followed by mAb cyclin B1 (lower panel). (B) Expression of GST fusion proteins in lysates.

associated with ptc1 (Figure 3A, lower panel, lane 2). As a negative control and to demonstrate antibody specificity, lane 3 shows an anti-ptc1 immunoprecipitate in which a cognate ptc1 blocking peptide was pre-incubated with the antiserum. As a positive control, lane 1 demonstrates the presence of cyclin B1 and ptc1 in cell lysates (Figure 3A, lower and upper panels). These results indicate that endogenous ptc1 and endogenous cyclin B1 interact and can be recovered by coimmunoprecipitation.

We then wished to examine whether exogenously expressed full-length ptc1 can also bind endogenous cyclin B1. Therefore, Myc–ptc1-transfected 293T cells were immunoprecipitated with cyclin B1 antiserum. As demonstrated in Figure 3B, cyclin B1 immunoprecipitates reveal an association with ptc1 (upper panel, lane 2). This interaction is specific, as addition of cyclin B1 blocking peptide abolishes the association (Figure 3B, upper panel, lane 3). As controls, the middle panel demonstrates the expression of cyclin B1 in these samples (Figure 3B, lanes 1 and 2), and the expression of Myc–ptc1 protein was confirmed by immunoblotting cell lysates (Figure 3B, lower panel, lanes 2 and 3). These results demonstrate that exogenously expressed ptc1 binds endogenous cyclin B1 *in vivo*.

**Ptc1 binds to an activated MPF complex**

To investigate whether ptc1 associates with cyclin B1 complexed to cdc2, we immunoprecipitated endogenous cdc2 from Myc–ptc1-transfected cells. As demonstrated in Figure 3C, cdc2 immunoprecipitates reveal an association with ptc1 (upper panel, lane 2). The middle panel demonstrates the expression of cyclin B1 complexed to cdc2 immunoprecipitates (Figure 3C, lanes 1 and 2). The lower panel shows the expression of cdc2 (Figure 3C, lanes 1 and 2). These results suggest that ptc1, cyclin B1 and cdc2 can be recovered in a complex *in vivo*. Alternatively, ptc1–cyclin B1 and ptc1–cdc2 complexes may exist independently.



**Fig. 3.** Patched1 interacts with cyclin B1. (A) Endogenous ptc1 and endogenous cyclin B1 interact in 293T cells. Lysates were immunoprecipitated as indicated. Upper panel, immunoblot of ptc1 shows ptc1 in the lysate and immunoprecipitate. Lane 3 indicates the use of ptc1 blocking peptide to discriminate antibody specificity. Lower panel, immunoblot of cyclin B1 showing cyclin B1 in lysate and ptc1 immunoprecipitate, but not the peptide blocked lane. (B) Patched1 binds endogenous cyclin B1. 293T cells were transfected with Myc-ptc1, immunoprecipitated with cyclin B1 antibody and immunoblotted as indicated. Lane 3 indicates the use of cyclin B1 blocking peptide to discriminate antibody specificity. (C) Patched1, cyclin B1 and cdc2 form a complex. 293T cells were transfected with Myc-ptc1, immunoprecipitated with cdc2 antibody and immunoblotted as indicated. (D) Active MPF complex associates with patched1. 293T cells were transfected with Myc-ptc1 as indicated. Upper panel, lysates were immunoprecipitated and assayed for histone H1 kinase activity. Middle panel, anti-Myc immunoblot displaying expression of Myc-ptc1. Lower panel, anti-cdc2 immunoblot displaying the presence of cdc2 in lane 1 (positive control) and lane 3 (ptc1-transfected cells). The lower band represents the IgG band from mouse Myc antiserum as detected by mouse cdc2 antiserum.

One of several nuclear substrates for MPF is histone H1 protein (Pondaven *et al.*, 1989). Therefore, to determine whether ptc1 can interact with an active MPF complex, we examined histone H1 kinase activity associated with ptc1-transfected cells. As demonstrated in Figure 3D, histone H1 kinase activity was readily detected in association with immunoprecipitated Myc-ptc1 (upper panel, lane 3). As a positive control, cdc2 immunoprecipitates from mock-transfected cells also demonstrate the phosphorylation of histone H1 proteins (Figure 3D, upper panel, lane 1). As a negative control for histone H1 activity, mock-transfected cells were immunoprecipitated with Myc antiserum (Figure 3D, upper panel, lane 2). To confirm the presence of Myc-ptc1, immunoprecipitates were immunoblotted with Myc antiserum (Figure 3D, middle panel, lane 3). We also immunoblotted with cdc2 antiserum and confirmed that cdc2 is associated with Myc-ptc1 (Figure 3D, lower panel, lane 3). These data indicate that ptc1 interacts with

active MPF, but the presence of cdc2 in ptc1 immunoprecipitates does not preclude the possibility that other cdk's may also be associated with ptc1.

### **Ptc1 colocalizes with a cyclin B1 derivative that mimics phosphorylation of the CRS**

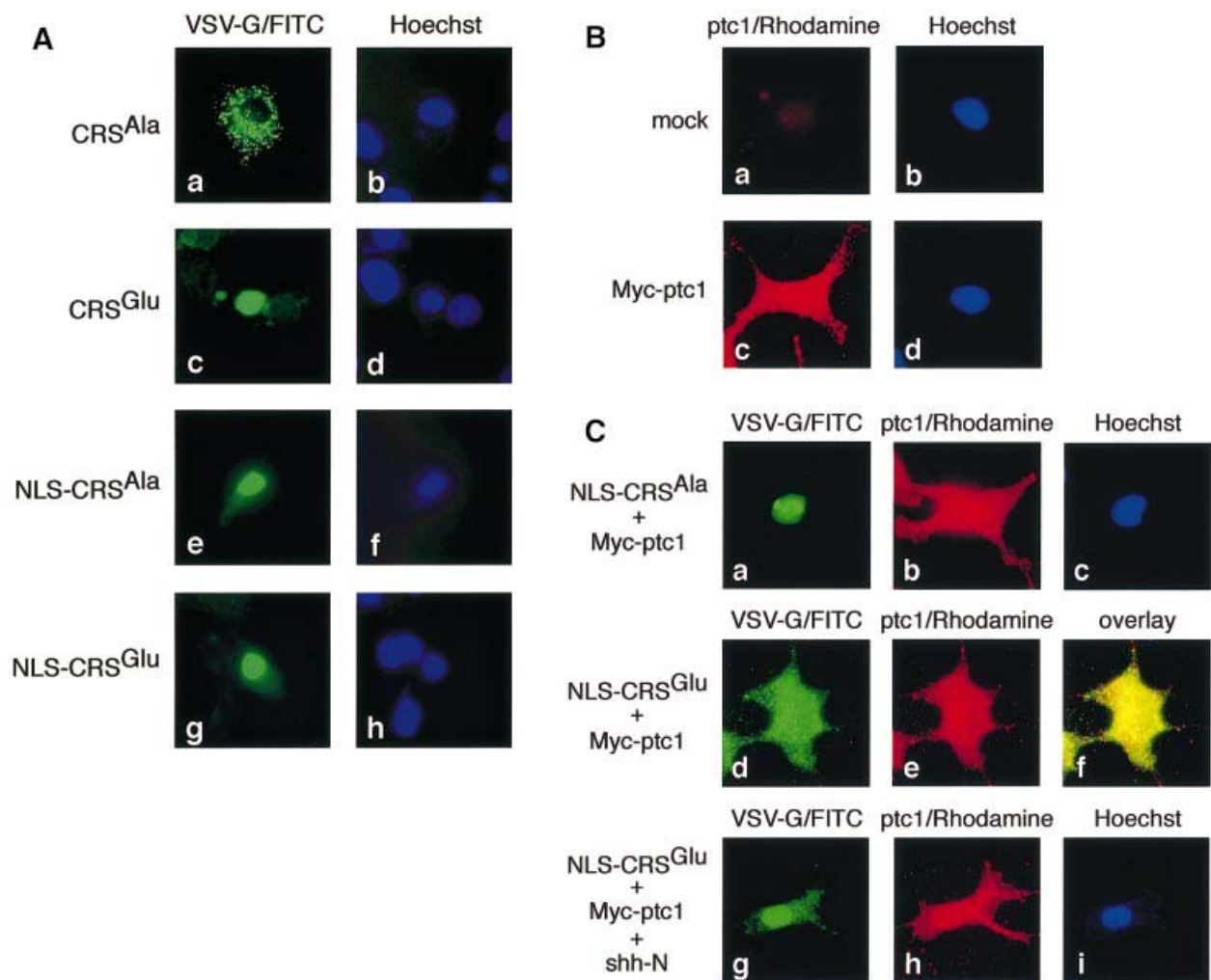
As published previously, the localization of cyclin B1 is dependent on the phosphorylation state of the CRS domain (Li *et al.*, 1997). Extending these results, we constructed derivatives of the human cyclin B1 CRS domain (Figure 1B, a and b) and utilized indirect immunofluorescence studies to examine their subcellular localization. These experiments reveal that CRS<sup>Ala</sup> localizes exclusively to the cytoplasm, while CRS<sup>Glu</sup> is able to enter the nucleus (Figure 4A, a and c, respectively). Both of these constructs, when modified by appending a nuclear localization signal (NLS) from *Xenopus* nucleoplasmin, NLS-CRS<sup>Ala</sup> and NLS-CRS<sup>Glu</sup> (Figure 1B, c and d), localize constitutively to the nucleus (Figure 4A, e and g). To visualize Myc-tagged ptc1, ptc1 antiserum was used (Figure 4B, c). The punctate membrane staining of ptc1, as demonstrated in Figure 4B, is consistent with previous reports describing an atypical membrane localization for ptc1 (Stone *et al.*, 1996; Carpenter *et al.*, 1998).

Since endogenous ptc1 expression levels in COS-1 cells are low, we chose to cotransfect Myc-tagged ptc1 with either NLS-CRS construct to determine whether full-length ptc1 could alter their subcellular localization. NLS-CRS<sup>Ala</sup> remained nuclear in the presence of Myc-ptc1 (Figure 4C, a, b and c). In contrast, NLS-CRS<sup>Glu</sup> exhibited altered localization to the membrane when coexpressed with Myc-ptc1 (Figure 4C, d, e and f). Thus, these data suggest that ptc1 has the ability to recruit phosphorylated cyclin B1.

### **Ptc1 mediates the localization of a phosphorylated cyclin B1 derivative**

To confirm that ptc1 can alter the localization of cyclin B1, cellular fractionation studies were performed to examine whether full-length ptc1 affects the localization of full-length cyclin B1. The *Xenopus* cyclin B1 derivative used in this study contained the four Ser phosphorylation sites in the CRS domain mutated to Glu with an appended NLS to target this protein to the nucleus (NLS-B1<sup>Glu</sup>) (Figure 1B, f). Cellular fractionation of NLS-B1<sup>Glu</sup>-transfected cells reveals nearly complete nuclear localization of this cyclin B1 derivative (Figure 5A, upper panel, lane 1). In contrast, cotransfection of NLS-B1<sup>Glu</sup> with Myc-ptc1 results in predominantly cytoplasmic/plasma membrane localization of this nuclear-targeted cyclin B1 protein (Figure 5A, upper panel, lane 4). These results suggest that ptc1 inhibits phosphorylated cyclin B1 from entering the nucleus, and confirm the previous colocalization data discussed above (Figure 4C, d, e and f).

Since ptc1 is the receptor for sonic hedgehog (shh) and therefore an integral component of the hedgehog signaling pathway (Chen and Struhl, 1996; Goodrich and Scott, 1998), cells expressing both ptc1 and NLS-B1<sup>Glu</sup> constructs were exposed to shh-N to determine whether this morphogen modulates the interaction between cyclin B1 and ptc1. The shh-N construct used in this study contains the first 197 amino acids of human shh protein, which have been shown to be sufficient for its interaction with ptc1



**Fig. 4.** Patched1 colocalizes with the CRS<sup>Glu</sup> domain of cyclin B1 at the cell membrane. (A) Localization of human CRS and NLS-CRS constructs in COS-1 cells detected by immunofluorescence. (a) CRS<sup>Ala</sup> construct expressed in cytoplasm. (c) CRS<sup>Glu</sup> construct expressed in nucleus. (e and g) NLS-CRS<sup>Ala</sup> and NLS-CRS<sup>Glu</sup> constructs expressed in nucleus. (b, d, f and h) Nuclei detected with Hoechst dye. (B) Localization of Myc-tagged ptc1 in COS-1 cells detected by immunofluorescence. (a) Mock-transfected cell. (c) Myc-ptc1 expressed at membrane. (b and d) Nuclei detected with Hoechst dye. (C) Altered localization of NLS-CRS<sup>Glu</sup> due to cotransfection of ptc1. (a) NLS-CRS<sup>Ala</sup> remains nuclear in the presence of ptc1 expression (b). In contrast (d), NLS-CRS<sup>Glu</sup> associates with the cell membrane due to ptc1 expression (e). (f) NLS-CRS<sup>Glu</sup> and ptc1 colocalization. (g, h and i) Sonic hedgehog restores nuclear accumulation of NLS-CRS<sup>Glu</sup>. (g) NLS-CRS<sup>Glu</sup> retains nuclear localization in the presence of ptc1 (h) with shh-N exposure. (c and i) Nuclei detected with Hoechst dye.

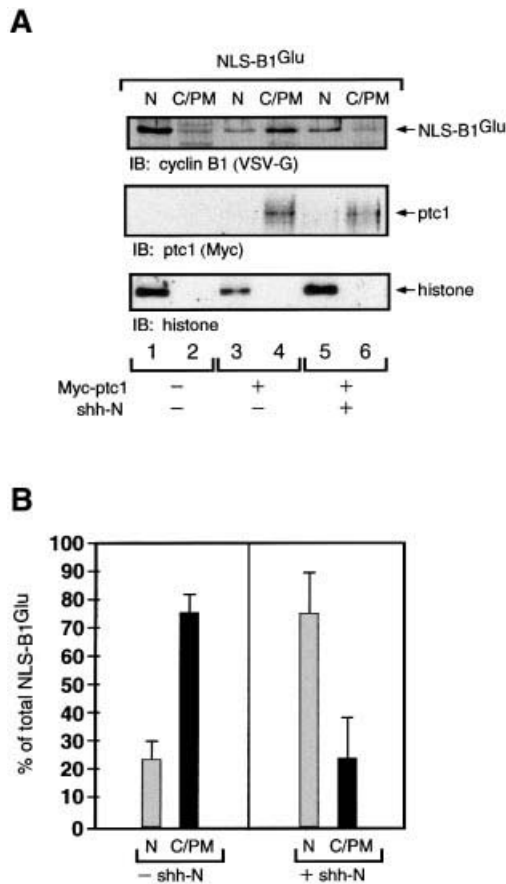
(Lee *et al.*, 1994; Marigo *et al.*, 1996; Stone *et al.*, 1996). Addition of shh-N to cotransfected cells altered the localization pattern of NLS-B1<sup>Glu</sup>, demonstrating an increase in nuclear accumulation of this cyclin B1 derivative (Figure 5A, lane 5). This suggests that shh-N inhibits the interaction between ptc1 and NLS-B1<sup>Glu</sup>, allowing cyclin B1 translocation to the nucleus. As a control, immunoblotting with Myc antiserum shows that ptc1 is expressed solely in the cytoplasmic/plasma membrane fraction (Figure 5A, middle panel, lanes 4 and 6). The lower panel indicates that the nuclear fractions are intact as demonstrated by nuclear histone protein expression (Figure 5A, lanes 1, 3 and 5). Figure 5B presents quantitation of localization data based on three independent cellular fractionation experiments, and demonstrates that 76% of NLS-B1<sup>Glu</sup> is retained in the cytoplasm when ptc1 is present. With the addition of shh-N, only 24% of NLS-B1<sup>Glu</sup> is retained in the cytoplasm. These results confirm by biochemical fractionation that ptc1 mediates

the localization of phosphorylated cyclin B1 to the cytoplasmic/plasma membrane fraction of the cell, and that this localization can be altered by cellular exposure to shh.

In addition, to confirm the apparent subcellular redistribution of phosphorylated cyclin B1 due to shh exposure, we performed indirect immunofluorescence. Shh-N was added to COS-1 cells cotransfected with Myc-ptc1 and NLS-CRS<sup>Glu</sup>. As demonstrated in Figure 4C, shh-N addition restores the nuclear accumulation of NLS-CRS<sup>Glu</sup> in cells overexpressing ptc1 (compare Figure 4C, g and d).

#### **Ptc1 expression regulates cell proliferation**

To examine the effects of ptc1 and cyclin B1 expression on cell cycle progression, cell proliferation assays were performed. The overexpression of ptc1 alone prevents cell growth, consistent with an anti-proliferative function (Figure 6A). Cells transfected with NLS-B1<sup>Ala</sup> or NLS-



**Fig. 5.** Cyclin B1 localization mediated by patched1 expression and shh exposure. (A) Upper panel, expression of NLS-B1<sup>Glu</sup> protein in cellular fractions. Middle panel, cytoplasmic/plasma membrane (C/PM) expression of ptc1 in cotransfected cells. Lower panel, nuclear (N) expression of endogenous histone proteins. (B) Quantitation of data presented in lanes 3–6 of (A) representing the average of three independent cellular fractionation experiments. The standard deviation is shown.

B1<sup>Glu</sup> alone exhibited intermediate levels of proliferation, compared with mock-transfected cells (Figure 6A). These NLS-appended derivatives were used to ensure that even the non-phosphorylated derivative (NLS-B1<sup>Ala</sup>) would still exhibit biological activity, due to the appended NLS (Li *et al.*, 1997). When ptc1 was coexpressed together with either NLS-B1<sup>Ala</sup> or NLS-B1<sup>Glu</sup>, we found that NLS-B1<sup>Ala</sup>, but not NLS-B1<sup>Glu</sup>, was able to restore cellular proliferation (Figure 6A). As a control for protein expression levels in all the samples, Figure 6B presents immunoblotting of cell lysates to detect Myc-ptc1 (upper panel) or cyclin B1 derivatives (lower panel).

Overexpression of mitotic cyclins increases cell cycle progression from G<sub>2</sub> to M, yet also leads to an increase in mitotic arrest (Murray *et al.*, 1989). We interpret the intermediate levels of proliferation observed as a reflection of these two conflicting roles. The mitotic index for the various cell populations is presented (Figure 6C). This bar graph demonstrates that the overexpression of NLS-B1<sup>Ala</sup>, NLS-B1<sup>Glu</sup> and coexpression of NLS-B1<sup>Ala</sup> with ptc1 leads to an increase in the mitotic index for these samples even though their relative rates of proliferation are intermediate compared with mock-transfected cells. The

mitotic index of cells expressing ptc1, or ptc1 plus NLS-B1<sup>Glu</sup>, is significantly decreased, reflecting fewer cells in M phase. Taken together, these results strongly suggest that cell division is inhibited by the interaction of ptc1 with the derivative of cyclin B1 that mimics phosphorylation. The inhibitory effects of ptc1 on cell proliferation can be overcome by coexpression of NLS-B1<sup>Ala</sup>, which is unable to interact with ptc1.

In addition, we investigated the physiological effect of shh addition on cell cycle progression. We found that cellular exposure to shh-N increased the cell number in all samples examined (Figure 6D). These data indicate that endogenous ptc1 can be affected by addition of shh-N. Also, cells overexpressing ptc1 respond similarly to exogenous shh-N, resulting in the promotion of cell growth. These results confirm that ptc1 inhibits cell division by interaction with phosphorylated cyclin B1, and that this inhibition can be relieved by shh cellular exposure.

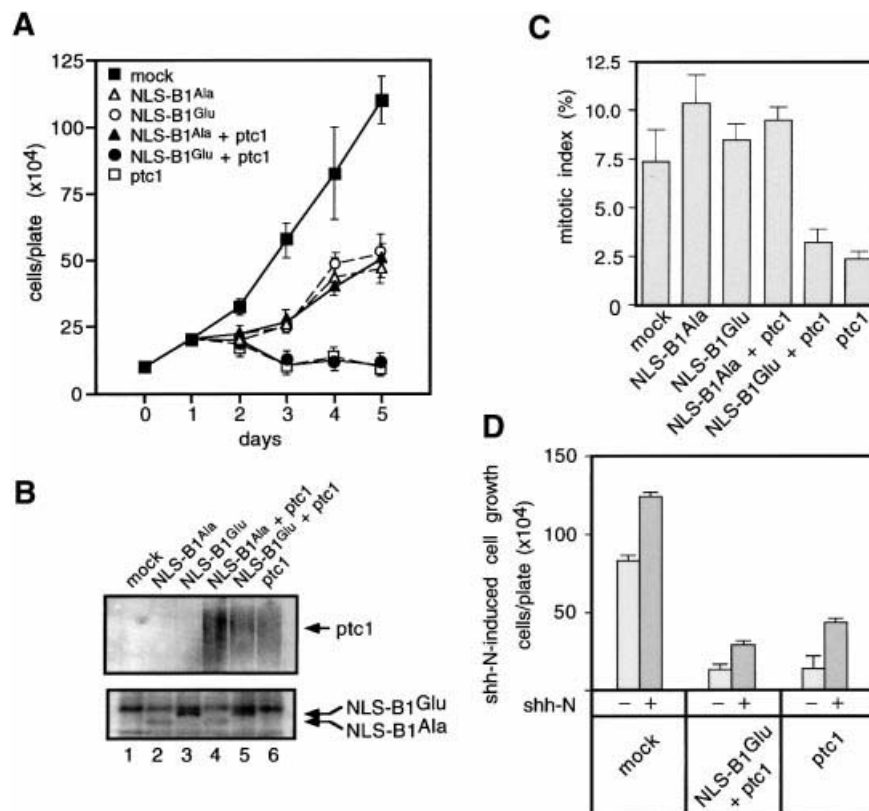
## Discussion

We have demonstrated a novel interaction between cyclin B1, the regulatory subunit of MPF and the tumor suppressor ptc1. Specifically, we show that the large intracellular loop of ptc1, containing amino acid residues 599–750, is sufficient for the interaction with cyclin B1. We also show that ptc1 is able to mediate the localization of phosphorylated cyclin B1. In addition, fractionation studies show that NLS-B1<sup>Glu</sup> is expressed primarily in the cytoplasmic/plasma membrane fraction when ptc1 is present, indicating that ptc1 mediates the localization of phosphorylated cyclin B1. Taken together with the immunofluorescence data, these results confirm that ptc1 interacts with the phosphorylated CRS domain of cyclin B1 at the cell periphery, and also demonstrate a novel function for ptc1 that involves the release of phosphorylated cyclin B1 in response to shh stimulation. The presence of cdc2 as a constituent of a cyclin B1-ptc1 complex is demonstrated by histone H1 kinase and immunoprecipitation assays.

One model to account for these results is presented in Figure 7. In this model, an MPF-ptc1 complex inhibits cell growth. In this manner, ptc1 may function as a tumor suppressor at the G<sub>2</sub>/M transition by binding phosphorylated cyclin B1, thereby delaying the nuclear events normally mediated by MPF activation. This model of regulation also suggests that stimulation of ptc1 by shh elicits the release of cyclin B1, allowing translocation of MPF to the nucleus and resumption of the cell cycle.

The ptc1 gene in vertebrates is essential for normal embryonic development and also functions as a tumor suppressor (Gailani *et al.*, 1996; Hahn *et al.*, 1996a; Johnson *et al.*, 1996). ptc1 inactivation has been found to underlie NBCCS and BCC development (Chidambaram *et al.*, 1996; Hahn *et al.*, 1996b; Uden *et al.*, 1997). NBCCS is an autosomal dominant syndrome characterized by developmental disorders and predisposition to BCC (Gorlin, 1995). BCC is the most common type of human cancer with 750 000 estimated cases per year in the United States alone (Gailani and Bale, 1997). ptc1 can form a complex with smoothened (smo), a 7-pass transmembrane protein and signaling component of the hedgehog pathway





**Fig. 6.** Anti-proliferative function of the cyclin B1-ptc1 complex. **(A)** Growth curve of 293T cells transfected with the indicated constructs. There is a decrease in cell proliferation with cotransfection of ptc1 and NLS-B1<sup>Glu</sup>. Error bars represent the standard error of the mean. **(B)** Upper panel, expression of Myc-tagged ptc1 in cell lysates obtained from the last day of the growth curve. Lower panel, expression of VSV-G-tagged cyclin B1 derivatives in cell lysates. **(C)** Decrease in mitotic index in cells cotransfected with ptc1 and NLS-B1<sup>Glu</sup>. The percentage of cells showing a mitotic phenotype from three independent transfections with the standard deviation is shown. **(D)** Increase in cell proliferation with cellular exposure to shh-N. The average value of two experiments is shown.

(Stone *et al.*, 1996; Murone *et al.*, 1999). Previous findings have suggested that constitutive activation of smo, due to the aberrant inactivation of ptc1, can lead to the oncogenic effects of BCC (Stone *et al.*, 1996). Yet the mechanism of ptc1 as a tumor suppressor and the role of smo activation remain unclear. In this study we have identified a novel downstream component of the shh-ptc1 signaling pathway, which may represent a key factor in the mechanism by which shh and ptc1 control mitogenic effects.

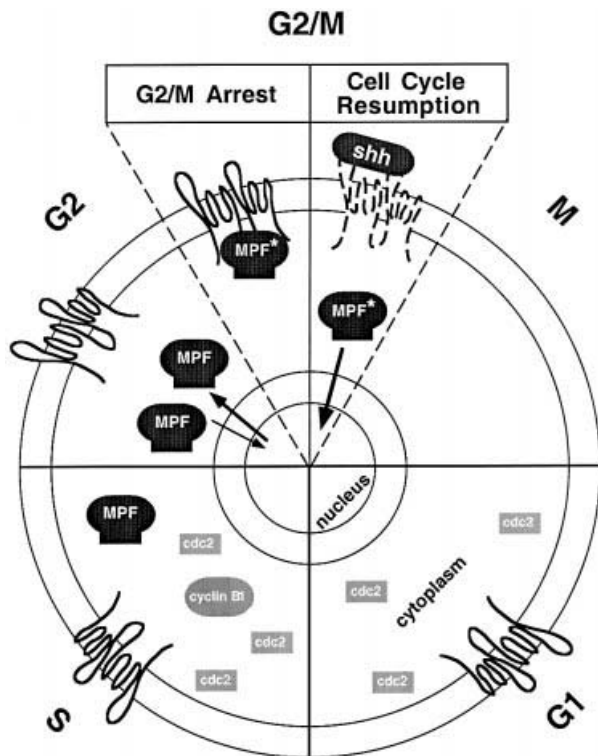
Several known tumor suppressors (p53, pRb, p16) act in the nucleus as negative regulators of cell division by interaction with cyclin D-cdk4/cdk6 and/or cyclin E-cdk2 complexes (Weinberg, 1995; Dean, 1997). ptc1 was one of the first characterized tumor suppressors localized at the cell membrane. Two recent reports have characterized other areas of ptc1 localization. Denef *et al.* (2000) have demonstrated that ptc1 is internalized and degraded after shh has bound to the two large extracellular loops. Incardona *et al.* (2000) found ptc1 as populating endosomal vesicles, as well as the cell surface. In our hands, ptc1 shows a punctate distribution in the plasma membrane as seen by immunofluorescence studies, consistent with previous reports indicating atypical membrane staining for ptc1 (Stone *et al.*, 1996; Carpenter *et al.*, 1998).

Further investigation will be required to understand fully the significance of the interaction between cyclin B1

and ptc1 described here. Our results demonstrate that ptc1 binds cyclin B1 in a phosphorylation-dependent manner. Since cyclin B1 becomes phosphorylated only during late G<sub>2</sub> (Borgne *et al.*, 1999), ptc1 can presumably only bind cyclin B1 at the G<sub>2</sub> phase or G<sub>2</sub>/M transition of the cell cycle. Our laboratory recently reported that when cyclin F interacts with cyclin B1, the complex localizes to the nucleus (Kong *et al.*, 2000). It is possible that in response to different cellular signals phosphorylated cyclin B1 is either sequestered by ptc1 or translocated to the nucleus by cyclin F, although this remains to be examined. However, none of these reports excludes the possibility of ptc1 interacting with cyclin B1.

Another issue for future investigation should include the effect of the interaction of active MPF with ptc1 with respect to the shh-ptc1 pathway or other signaling pathways. A recent report implies that smo may be phosphorylated indirectly by ptc1 and that this event would occur after shh has bound to ptc1 (Denef *et al.*, 2000). Since we have shown that ptc1 interacts directly with MPF, it is a distinct possibility that MPF represents the kinase responsible for phosphorylation and activation of smo. The association between ptc1 and cyclin B1 may fulfill at least two purposes: (i) ptc1 binding of MPF may mediate the anti-proliferative effects of ptc1; and (ii) cyclin B1 localization to the membrane may allow for the phosphorylation of ptc1 or smo.





**Fig. 7.** Model of patched1 regulation of MPF. G<sub>1</sub> phase, cdc2 is present in abundance throughout the cell cycle. S phase, cyclin B1 is synthesized and begins to accumulate in the cytoplasm by late S phase. Cyclin B1 binds to cdc2 via a region termed the cyclin box. G<sub>2</sub> phase, before forming an active MPF complex, cyclin B1-cdc2 enters and rapidly exits the nucleus due to a NES located within the CRS domain. G<sub>2</sub>/M transition, cyclin B1 becomes phosphorylated, which creates a fully active MPF complex (MPF\*). ptc1 binds to phosphorylated cyclin B1 and sequesters an active MPF complex in pre-mitotic cells which inhibits the translocation of MPF to the nucleus. Cellular exposure to shh promotes the degradation of ptc1, which in turn facilitates the release of cyclin B1. MPF is now available for nuclear import.

In summary, the evidence presented here provides a link between ptc1 signaling and cell cycle progression. However, further work is warranted to assess the entire contribution of MPF activity in the shh-ptc1 pathway and to elucidate the biological conditions required for the regulation of MPF by ptc1.

## Materials and methods

### Construction of cyclin B1, ptc1 and shh derivatives

The lexA-CRS-CRS (*Xenopus*) fusion proteins for the yeast screen and the lexA-CRS-CRS (human) fusion proteins for the His<sup>-</sup> minimal media assays were constructed as described previously (Kong *et al.*, 2000). To construct the fusion protein containing the intracellular loop of human patched1 for the yeast assays, oligonucleotides encoding residues 599–750 were synthesized and incorporated in-frame to the transactivation domain of pVP16.

To construct cyclin B1 derivatives for immunofluorescence studies, oligonucleotides encoding the human CRS domain consisting of residues 110–160 were synthesized and incorporated into the pcDNA3 vector.

To construct NLS-CRS derivatives, a DNA fragment encoding the human CRS domain, consisting of residues 110–160, was ligated in-frame to oligonucleotides encoding the NLS of *Xenopus* nucleoplasmin and then inserted into the pcDNA3 vector.

The full-length cyclin B1 derivatives, NLS-B1<sup>Ala</sup> and NLS-B1<sup>Glu</sup>, were constructed as described previously (Li *et al.*, 1997). All cyclin B1

constructs used in this study were tagged at the C-terminus with an epitope (GLEVIVVPHSLPFML) derived from glycoprotein G of vesicular stomatitis virus (VSV-G), recognized by mAb P5D4 (Kreis and Lodish, 1986).

To construct the GST-ptc1 derivative (GST-ptc1<sup>599–750</sup>), a DNA fragment encoding the large intracellular loop of human ptc1 consisting of residues 599–750 was ligated in-frame to the mammalian expression vector pEBG.

To construct an epitope-tagged and biologically active derivative of human shh protein (Marigo *et al.* 1996; Stone *et al.* 1996), oligonucleotides encoding residues 1–197 were synthesized and incorporated in-frame to DNA encoding the FLAG-epitope tag (DYKDDDDK) at the C-terminus and inserted into the pcDNA3 vector.

### Yeast two-hybrid screen and assays

The *Saccharomyces cerevisiae* strain L40 generated by Dr Stan Hollenberg was transformed with derivatives of pBTM116 (constructed by Dr Paul Bartel and Dr Stan Fields). The derivatives constructed for this work encoded lexA-CRS<sup>Ala</sup>-CRS<sup>Ala</sup> (*Xenopus*) or lexA-CRS<sup>Glu</sup>-CRS<sup>Glu</sup> (*Xenopus*) fusion proteins; these were screened against a 9.5 d.p.c. mouse embryonic cDNA library encoding fusion proteins with the transactivation domain of pVP16, kindly provided by Dr Stan Hollenberg (Vojtek and Hollenberg, 1995). The two-hybrid screen and His<sup>-</sup> minimal media assays were performed as described previously (Kong *et al.*, 2000). The ability to activate the lacZ reporter was also checked by filter assay as described (Vojtek *et al.*, 1993).

### Immunoprecipitation assays

Two days after transfection (except for endogenous immunoprecipitations), cells were harvested and lysed with 1.0% NP-40 buffer (Kong *et al.*, 2000). Protein content was standardized as determined by the Bradford assay. Lysates were pre-cleared with protein A-Sepharose beads and then incubated with the indicated antibodies overnight at 4°C. Addition of protein A-Sepharose beads with incubation overnight at 4°C followed. Samples were washed with 0.5% NP-40 buffer and analyzed by SDS-PAGE, transferred to nitrocellulose membrane and detected by ECL. Antibody + blocker samples were treated as described by the manufacturer (Santa Cruz). In brief, antibody (2 µg) was incubated with blocking peptide (10 µg) in 1.0% NP-40 buffer for 2 h at room temperature, and then addition of lysate sample with further incubation overnight at 4°C. For the endogenous immunoprecipitation assay, ptc1 was immunoprecipitated from 1 mg of protein per sample. The lysate sample represents 60 µg of total cell lysate.

### Histone H1 kinase assay

For detection of phosphorylation of histone H1 *in vitro* by MPF, 293T cells were transfected with plasmids encoding Myc-tagged ptc1 and proteins were immunoprecipitated with anti-Myc (9E10) antibody after lysis in 1.0% NP-40 buffer. Protein content was standardized as determined by the Bradford assay. Active MPF was obtained by immunoprecipitation with anti-cdc2 antibody (Santa Cruz). The immunoprecipitates were incubated at 30°C for 20 min in 50 µl of kinase reaction buffer as described previously (Li *et al.*, 1995). The reaction was terminated by addition of SDS sample buffer. Samples were analyzed by 10% SDS-PAGE.

### Immunofluorescence

Two days after transfection using the calcium phosphate coprecipitation method, COS-1 cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100, 0.2 M glycine and 2.5% fetal bovine serum in phosphate-buffered saline (PBS). Triple-label immunofluorescence was performed as follows: (i) VSV-G-tagged cyclin B1 derivatives were detected with mAb P5D4 and FITC-conjugated goat anti-mouse antiserum; (ii) patched1 expression was visualized using goat antibody pAb patched1 (Santa Cruz) and rhodamine-conjugated rabbit anti-goat antiserum; and (iii) nuclei were stained with Hoechst 33342. Using a modification of techniques described elsewhere (Stone *et al.*, 1996), COS-1 cells were exposed to shh-N-conditioned media from shh-N-expressing cells at 24 h after transfection and allowed to recover for 24 h.

### Cellular fractionation

Fractionation samples prepared as described elsewhere (Greenwood and Johnson, 1995) were standardized for protein content by the Bradford assay and then analyzed by 10% SDS-PAGE and transferred to nitrocellulose membrane. VSV-G-tagged NLS-B1<sup>Glu</sup>, Myc-tagged ptc1 and endogenous histone proteins were detected by ECL (Amersham)

using mAb P5D4, mAb 9E10 (Santa Cruz) and mAb 052 (Chemicon), respectively. Quantitation of fractionation data is presented as a percentage of NLS-B1<sup>Glu</sup> protein and was performed using NIH Image software. Using a modification of techniques described elsewhere (Stone *et al.*, 1996), 293T cells were exposed to shh-N-conditioned media from shh-N-expressing cells at 48 h after transfection and then allowed to recover for 24 h. All samples were harvested at the same time (72 h after transfection).

#### Cell proliferation assay

293T cells were seeded at a density of  $1.0 \times 10^5$  cells/plate (60 mm) and transfected with 2.5 µg of cyclin B1 DNA and/or 5.0 µg of ptc1 DNA. Cells were trypsinized and counted every 24 h after transfection (day 1). The mean values of three plates are represented ( $\pm$ SEM). Cell lysate samples from the last day were analyzed by 10% SDS-PAGE for protein expression by ECL after transfer to nitrocellulose membrane. For shh-N-induced growth, 293T cells were seeded at a density of  $1.0 \times 10^5$  cells/plate (60 mm) and transfected with 5.0 µg of cyclin B1 DNA and/or 5.0 µg of ptc1 DNA. Appropriate samples were exposed to shh-N-conditioned media at 24 and 48 h after transfection. At 72 h after transfection, samples were counted. The average of two plates per sample is represented.

#### Mitotic index assay

Transfected 293T cells were split onto collagen-coated coverslips and allowed to recover for 24 h. Cells were fixed and permeabilized as described previously. Nuclei were stained with Hoechst 33342. Five hundred cells of each sample from three independent transfections were counted (total cells counted per sample = 1500). Results shown represent the average percentage ( $\pm$ SD) of cells displaying a mitotic phenotype over the total number of cells counted.

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