

Figure 4 Model for closure of the DA in normal and EP₄-deficient mice. As the fetus approaches term, intracellular cAMP levels in the cells of the DA are maintained largely by the binding of PGE₂ to the EP₄ receptors and stimulation of adenylyl cyclase through G_s. To maintain normal levels of cAMP, other pathways that maintain cAMP levels are downregulated. At birth, a dramatic decrease in circulating levels of PGE₂ occurs. This results in a drop in intracellular cAMP, which in turn triggers the series of events leading to remodelling of the DA. In the absence of the EP₄ receptor, the cells in the DA maintain intracellular cAMP levels independently of PGE₂. As a result, these cells fail to respond to the loss of this pathway at birth. Indomethacin mimics the events that occur at birth by inhibiting synthesis of PGE₂. Black circles, PGE₂; question marks indicate that the sources of cAMP have not been defined. Indo, indomethacin.

DA response to PGE₂ and indomethacin. At E18.5, pregnant mice received a single intravenous dose of indomethacin (10 µg kg⁻¹ in bicarbonate buffer, pH 7.4). At the time indicated, the mice were killed, fetuses removed and immediately placed on ice, and the DA examined. PGE₂ was prepared in ethanol and diluted to 1 µg µl⁻¹ in sterile PBS. Neonates received a single subcutaneous injection of 15 µl; controls received vehicle. Pups were returned to their mother until the time indicated.

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Complementation of dominant suppression implicates CD98 in integrin activation

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The integrin family of adhesion receptors are involved in cell growth, migration and tumour metastasis¹. Integrins are heterodimeric proteins composed of an α and a β subunit, each with a large extracellular, a single transmembrane, and a short cytoplasmic domain. The dynamic regulation of integrin affinity for ligands in response to cellular signals is central to integrin function². This process is energy dependent and is mediated through integrin cytoplasmic domains³. However, the cellular machinery regulating integrin affinity remains poorly understood. Here we describe a genetic strategy to disentangle integrin signalling pathways. Dominant suppression occurs when overexpression of isolated integrin β_1 cytoplasmic domains blocks integrin activation. Proteins involved in integrin signalling were identified by their capacity to complement dominant suppression in an expression cloning scheme. CD98, an early T-cell activation antigen that associates with functional integrins⁴, was found to regulate integrin activation. Furthermore, antibody-mediated crosslinking of CD98 stimulated β_1 integrin-dependent cell adhesion. These data indicate that CD98 is involved in regulating integrin affinity, and validate an unbiased genetic approach to analysing integrin signalling pathways.

Strategies based on genetic selection have proven to be powerful in the dissection of a number of different signal-transduction pathways. We therefore designed a new genetic cloning strategy, based on changes in integrin affinity, to identify potential modulators of integrin activation. This strategy used a Chinese hamster ovary (CHO) cell line ($\alpha\beta\gamma$) stably expressing a chimaeric integrin ($\alpha_{IIB}\alpha_{6A}\beta_3\beta_1$) that contains the extracellular and transmembrane domains of $\alpha_{IIB}\beta_3$ fused to cytoplasmic domains of $\alpha_{6A}\beta_1$ (ref. 5). This chimaeric integrin has the ligand-binding properties of $\alpha_{IIB}\beta_3$ and is activated through the $\alpha_{6A}\beta_1$ cytoplasmic domains. In $\alpha\beta\gamma$ cells this chimaeric integrin is constitutively active, as measured by

the binding of a ligand mimetic monoclonal antibody, PAC1, which recognizes only the activated form of the chimaeric integrin⁶.

Overexpression of isolated integrin β_1 cytoplasmic domains, in the form of a Tac- β_1 chimaera, results in cell-autonomous, concentration-dependent suppression of integrin signalling^{7,8}. Expression of Tac- β_1 in fibroblast cell lines interferes with cell spreading, migration, fibronectin matrix assembly, and integrin activation^{7,8}. This dominant suppression is structurally specific as neither chimaeric molecules with integrin α -cytoplasmic domains nor certain β -cytoplasmic domain variants have inhibitory activity⁷. Excess free integrin β_1 cytoplasmic domains may cause dominant suppression by titration of essential intracellular proteins that are responsible for

integrin activation (Fig. 1a). We hoped that our strategy allowed us to rescue the dominant suppression caused by Tac- β_1 expression by overexpressing these essential intracellular proteins (Fig. 1a).

To identify proteins that complement Tac- β_1 suppression, $\alpha\beta$ Py cells were co-transfected with a CHO-cell cDNA expression library and Tac- β_1 . Fluorescence-activated cell sorting (FACS) was used to isolate cells in which the chimaeric integrin was in an activated form despite the presence of Tac- β_1 (Fig. 1b). Transfected cDNAs, enriched in this way for complementing activity, were then recovered from the sorted cell populations. Six separate FACS screens resulted in the isolation of 1,200 cDNAs, which were then grouped into 75 pools each of 16 cDNAs. Each pool of cDNAs was screened

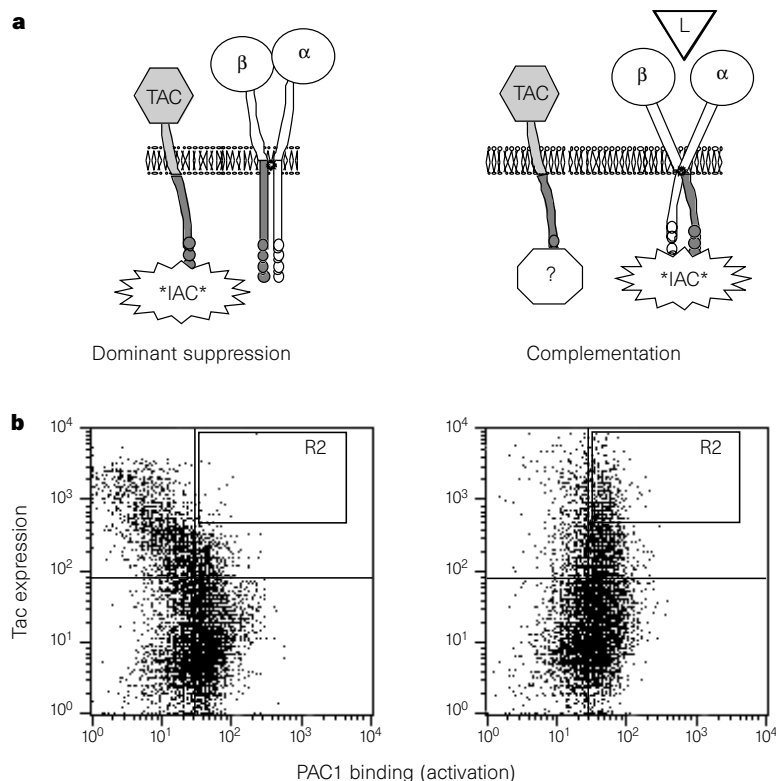


Figure 1 Complementation of dominant suppression and its effect on ligand binding. **a**, Complementation of dominant suppression. Schematic representation of the rationale behind the expression cloning strategy. Intracellular proteins that regulate integrin affinity, shown here as the integrin activation complex (IAC), interact with the cytoplasmic domains of integrins resulting in an increase in ligand binding affinity. Dominant suppression of integrin affinity by Tac- β_1 may be caused by the titration of proteins within the IAC (left). We hypothesized that overexpression of components of IAC, or proteins that block binding to the β cytoplasmic domains of the chimaeric molecule, result in an increase in integrin affinity in the presence of Tac- β_1 (right). **b**, Initial enrichment. $\alpha\beta$ Py cells were transiently transfected with Tac- β_1 and a cDNA expression library. After 48 h, cells were collected and stained for Tac expression (ordinate) and PAC1 binding (abscissa)⁷. Fluorescence-activated cell sorting (FACS) was used to isolate those cells in which the chimaeric integrin is able to bind to PAC1 in the presence of high levels of Tac- β_1 (R2). Shown here are representative dot plots obtained with cells that have been transfected with either Tac- α_5 or Tac- β_1 and analysed by two-colour flow cytometry⁶. A cell in which complementation of dominant suppression has occurred will appear in the upper right-hand quadrant (R2) in the Tac- β_1 transfected cells.

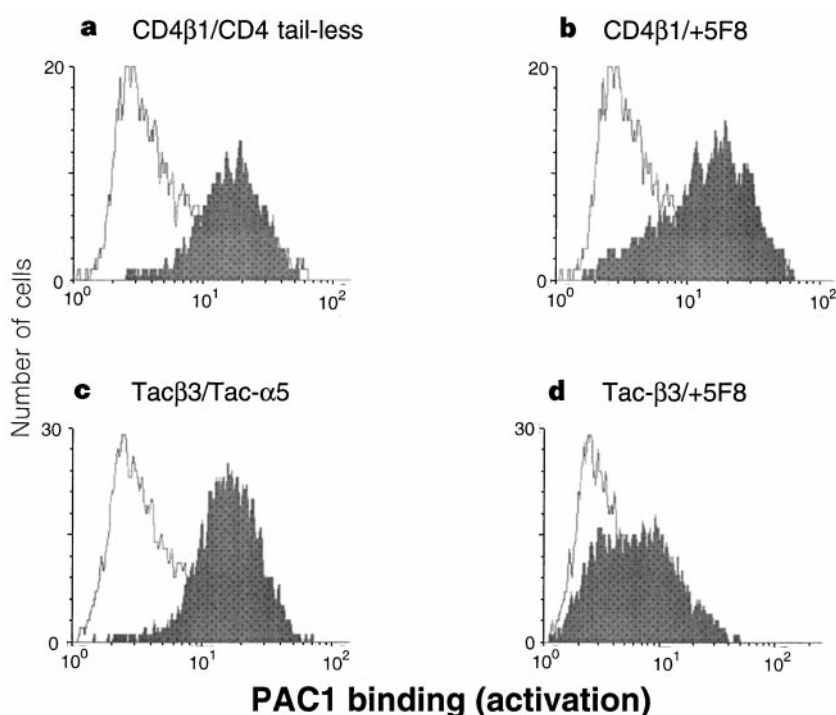


Figure 2 Cytoplasmic domain specificity of complementation of dominant suppression. Data represent PAC1 binding to the CD4- (a, b) or Tac- (c, d) positive subset of transfected $\alpha\beta$ Py cells. **a**, PAC1 binding to $\alpha\beta$ Py cells transfected with CD4- β_1 (ref. 21) (open graph) or with a CD4 construct lacking a cytoplasmic domain (shaded). **b**, Co-transfection with cDNA clone 5F8 and CD4- β_1 is indicated by the shaded graph. PAC1 binding is restored in CD4- β_1 -positive cells co-transfected with 5F8 (shaded). Transfection with 5F8 did not reduce CD4 expression (data not shown). **c**, **d**, PAC1 binding to $\alpha\beta$ Py cells transfected with Tac chimaeras. Tac- β_3 transfection is indicated by the open graphs. **c**, The shaded graph indicates binding to cells transfected with a non-inhibitory chimaera, Tac- α_5 . **d**, Co-transfection of clone 5F8 with Tac- β_3 restores PAC1 binding (shaded).

for complementing activity by transfecting the pooled cDNAs into $\alpha\beta$ py cells along the Tac- β_1 . cDNAs whose expression caused an increase in PAC1 binding even in the presence of high levels of Tac- β_1 were identified as positives. Tac- β_1 expression levels were monitored in all experiments by staining with an anti-Tac antibody (7G7B6) to eliminate those cDNAs with complementation of dominant suppression that resulted from reduced expression of Tac- β_1 . One out of 75 pools contained cDNA that complemented dominant suppression. This pool was divided into four groups of four cDNAs and rescreened as described above, and only one pool

contained a positive cDNA. In this smaller pool, only a single cDNA, 5F8, was able to reverse Tac- β_1 suppression.

The CHO-cell cDNA library was rescreened in four additional experiments that resulted in the enrichment of 1,059 more cDNA clones. Three additional cDNAs identical to 5F8 were isolated from these clones. Consequently, in this cDNA library, 5F8 was unique in complementing suppression. This library has a reported minimal complexity of 1.8×10^7 independent clones. Thus the effect of 5F8 appears to be highly specific. Transfection of cDNAs reported to interact with or regulate integrins, including α -actinin, vinculin, paxillin, integrin-linked kinase, and β_3 -endonexin, had no effect on Tac- β_1 suppression of integrin activation (data not shown). This result further supports the idea that 5F8 complementation of dominant suppression is very specific.

To determine if the rescue by 5F8 is dependent on the cytoplasmic or extracellular domain of Tac- β_1 , we assessed the ability of 5F8 to complement suppression initiated by a β_1 tail and transmembrane domain joined to the extracellular domain of CD4 (Fig. 2). As with Tac- β_1 , co-transfection of 5F8 with CD4- β_1 resulted in a reversal of dominant suppression, indicating that the rescue was independent of the extracellular domain of the suppressive chimaera. The isolated β_3 cytoplasmic domain is similar to the β_1 cytoplasmic domain and can also initiate dominant suppression^{7,8}. We therefore investigated whether rescue by 5F8 is specific to the β_1 cytoplasmic domain. We found that 5F8 also reversed Tac- β_3 suppression (Fig. 2). Thus 5F8 can complement suppression initiated by either β_1 or β_3 cytoplasmic domains.

The 5F8 sequence has been deposited in Genbank (accession no. U93712) and contains 1,902 base pairs, with an open reading frame encoding a polypeptide of 533 amino acids. In addition, it contains a poly(A) tract and polyadenylation signal. Analysis of the predicted topology of the encoded protein using Tmpred⁹ suggested that it possesses at least one transmembrane domain with the amino terminus inside the cell (type II transmembrane protein). Furthermore, a BLAST¹⁰ database search showed that the amino-acid sequence encoded by 5F8 is 72% identical to that of the heavy chain of the 4F2 antigen, CD98 (ref. 11). Overexpression of human CD98 also reversed dominant suppression, and so we conclude that we have isolated its hamster homologue (Fig. 3a).

CD98 was originally identified as an early T-cell activation antigen. It is part of a heterodimer of relative molecular mass 120,000 (M_r 120K), consisting of a heavy chain of approximately 80K that bears CD98 epitopes, and a light chain of 40K (ref. 12). It is a membrane protein that specifically associates with β_1 integrins⁴. Indeed, it has been shown that CD98 co-precipitated with functional $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins⁴. Furthermore, co-precipitation with $\alpha_4\beta_1$ was only observed when $\alpha_4\beta_1$ was activated in the presence of 2 mM Mn^{2+} . The light chain is poorly characterized, and its cDNA has yet to be cloned. Thus we cannot assess the role of the light chain in the effect of CD98 on integrin activation.

To determine whether the rescue of dominant suppression is a function of other integrin-associated membrane proteins, we overexpressed membrane proteins previously implicated in integrin function: CD9 (ref. 13), CD47 (integrin-associated protein)¹⁴, and urokinase plasminogen activator receptor (uPAR)¹⁵; in each case, protein expression was confirmed by flow cytometry (data not shown). Expression of these proteins failed to reverse dominant suppression by Tac- β_1 (Fig. 3a). Furthermore, overexpression of CD98 or the other membrane-associated proteins in the absence of Tac- β_1 did not increase the affinity of already activated integrins (Fig. 3b). Thus the rescue of Tac- β_1 suppression by CD98 is not an artefact of overexpression of membrane proteins.

The $\alpha_3\beta_1$ integrin-mediated adhesion of the small cell lung-cancer cell line (SCLC) H345 to laminin and fibronectin can be regulated dynamically¹⁶. We therefore tested whether CD98 might be involved in the regulation of integrins in SCLC adhesion. An anti-CD98 monoclonal antibody (4F2) markedly enhanced single-

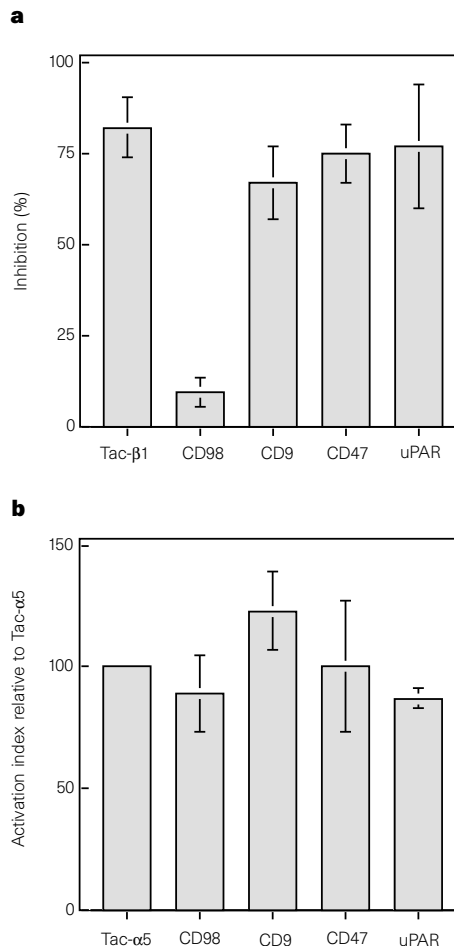


Figure 3 Human CD98 complements dominant suppression, whereas several other membrane proteins do not. $\alpha\beta$ Py cells were transfected with 2 μ g of either Tac- β_1 (**a**) or Tac- α_5 (**b**). They were simultaneously transfected with 4 μ g of cDNAs encoding either human CD98 (ref. 11), CD9 (ref. 22), CD47 (ref. 14) or uPAR. After 48 h, cells were collected and analysed for PAC1 binding to the Tac-positive subset of cells. To obtain quantitative estimates of integrin activation we calculated a numerical activation index defined as $100 (F_o - F_R) / (F_{LIBS6} - F_R)$, as previously described²⁵, where F_o is median fluorescence intensity of PAC1 binding; F_R is the background fluorescence intensity of PAC1 binding in the presence of a competitive inhibitor (1 μ M Ro43-5054), and F_{LIBS6} is the maximal fluorescence intensity in the presence of 2 μ M anti-LIBS6, an activating monoclonal antibody. The mean \pm s.d. of three independent experiments for each membrane protein is shown. **a**, Specificity of CD98 complementation. Percentage inhibition was defined as $100(AI_0 - AI) / AI_0$, where AI_0 is the activation index in Tac- α_5 transfected cells. AI is the activation index in Tac- β_1 transfected cells. The co-transfected membrane protein is indicated below each column. **b**, Effect of membrane proteins in the absence of dominant suppression. Cells were transfected with Tac- α_5 alone or the indicated membrane protein. Data are expressed as relative activation = $100(AI_{MP} / AI_{\alpha_5})$, where AI_{MP} is the activation index in the presence of the membrane protein and AI_{α_5} is the activation index with Tac- α_5 alone. The activation index with Tac- α_5 alone was 60%.

cell adhesion of H345 cells to both laminin and fibronectin (Fig. 4). Addition of the function blocking anti- β_1 antibody (P5D2) or 2 mM EDTA abrogated this effect. Control antibodies for 4F2 (D57, anti- $\alpha_{IIb}\beta_3$) and for P5D2 (P41, anti- β_3) had no effect. In addition, B6H12, a monoclonal antibody against CD47 (ref. 17), did not enhance adhesion to fibronectin or laminin (results not shown). To assess the role of antibody-mediated CD98 crosslinking in enhancing integrin function, we examined the effects of monovalent Fab fragments of 4F2 (Fab-4F2) on SCLC cell adhesion. Fab-4F2 did not enhance SCLC cell adhesion to laminin or fibronectin (Fig. 4). The binding of the Fab fragments was confirmed by their ability to block the enhanced adhesion caused by the intact antibody. Consequently, clustering of CD98 is required for 4F2-induced upregulation of β_1 integrin function.

Our genetic complementation strategy has led to the identification of CD98 as a protein involved in integrin activation. Indeed, CD98 specifically associates with β_1 integrins, and that association may be activation dependent, as CD98 is preferentially immunoprecipitated with activated integrins⁴. In addition, CD98 lacking a cytoplasmic domain fails to complement dominant suppression (C.A.F., B. Lewis & M.H.G., unpublished results). This result indicates that the association between integrins and CD98 may be mediated by the cytoplasmic domains of both proteins. It is likely that endogenous hamster CD98 is expressed in CHO cells, as 5F8 was isolated from a cDNA library derived from CHO-cell mRNA and CD98 is expressed in many tissue-culture cell lines¹¹. Cross-linking CD98 with 4F2 antibody stimulated β_1 -dependent adhe-

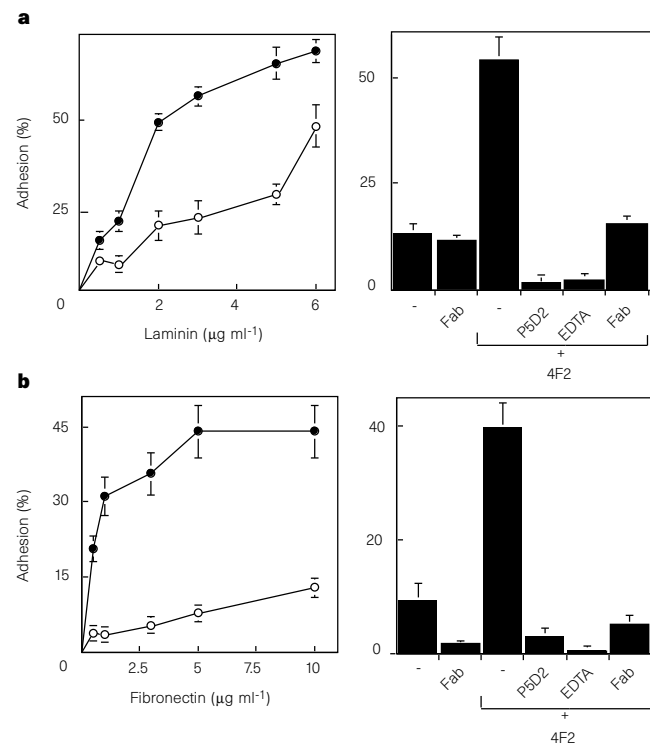


Figure 4 The affect of a 4F2 monoclonal antibody on adhesion of the small cell lung-cancer cell line (SCLC) H345 to extracellular matrix. Left, attachment of SCLC cells to 96-well tissue-culture plates coated with increasing concentrations of laminin (**a**) or fibronectin (**b**) in the presence (filled circles) or absence (open circles) of 20 $\mu\text{g ml}^{-1}$ 4F2 monoclonal antibody. Right, addition of 4F2-Fab (100 $\mu\text{g ml}^{-1}$) to SCLC cells does not increase adhesion to plates coated with 3 $\mu\text{g ml}^{-1}$ laminin (**a**) or 10 $\mu\text{g ml}^{-1}$ fibronectin (**b**); addition of P5D2 (a β_1 -integrin function-blocking antibody) or 2 mM EDTA also block the increase of adhesion of SCLC cells to plates coated with either 3 $\mu\text{g ml}^{-1}$ laminin (**a**) or 10 $\mu\text{g ml}^{-1}$ fibronectin (**b**). Results show the mean percentage adhesion above background (which was consistently <5%) compared with poly-L-lysine (taken as 100%) of 4–6 independent experiments in duplicate/triplicate – s.e.m.

sion. Thus the rescue of dominant suppression by overexpression of CD98 in $\alpha\beta\text{py}$ cells may work because overexpression of CD98 may favour clustering. Further work will be required to establish relationships between CD98 clustering, its association with integrins, and the identity of CD98 ligands that might cluster CD98.

Our finding that CD98 is involved in integrin function explains the observation that CD98-mediated membrane fusion of cells transfected with HIV gp160 is integrin dependent^{18,19}. Further, when T cells are activated, CD98 expression increases before an increase in β_1 integrin-mediated adhesion²⁰. Therefore changes in integrin function in these two cell types could depend upon CD98 regulation. Collectively, these data indicate that CD98 may be an important regulator of integrin function in many contexts. □

Methods

cDNA constructs and antibodies. Expression plasmids encoding the extracellular domain of murine CD4 fused to the human β_1 transmembrane and cytoplasmic domains and CD4 tailless, which lacks the β_1 cytoplasmic domain, were a gift from M. Lukashev²¹. Human 4F2 antigen (CD98) cDNA was provided by J. M. Leiden¹¹, and was subcloned into pcDNA1 as an EcoRI fragment. The pCDM8 construct encoding CD9 has been described²². The pRc/RSV plasmid, pIAP45, encoding CD47, and B6H12, the anti-CD47 antibody, were gifts from F. Lindberg and E. Brown¹⁴. cDNA encoding uPAR, in pcDNA1, pCuPAR1, was provided by L. Miles. The hybridoma cell line 4F2(C13) was purchased from American Type Culture Collection (ATCC). Ascites were produced in pristane-primed BALB/c mice. Fab fragments were prepared by papain digestion of purified 4F2 IgG (2 mg ml⁻¹) for 5 h at 37 °C. Digestion was terminated by the addition of iodoacetamide. Fab fragments were purified on Protein-A sepharose columns. Fab fragments were characterized by SDS-PAGE and exhibited characteristic mobilities.

Expression cloning. A cDNA library, made from poly(A)⁺ mRNA from CHO-K1 cells, directionally cloned into the NotI site of the mammalian expression vector, pcDNA1, was purchased from Invitrogen (San Diego, CA). The library is reported to contain 1.8 × 10⁷ primary recombinants. A CHO cell line that expresses the polyoma large T antigen and an activated recombinant chimaeric $\alpha_{IIb}\beta_3$ integrin ($\alpha\beta\text{py}$ cells) was constructed as described⁵. $\alpha\beta\text{py}$ cells were transfected with the Tac- β_1 (Tac- β_1 , Tac- β_3 and the non-inhibitory Tac- α_5 chimaeras were gifts from S. LaFlamme and K. Yamada⁸; 4 μg per plate) using lipofectamine (Gibco, BRL), according to the manufacturer's instructions. The transfection efficiency ranged from 30% to 50%, as determined by the binding of the anti-Tac antibody (7G7B6)⁷. After 48 h incubation, cells were collected and stained as described⁶, and cells positive for both PAC1 and Tac were isolated by fluorescence-activated cell sorting (FACSTAR, Becton Dickinson). Plasmid DNA was extracted²³ and digested with DpnI to remove plasmids that were not replicated in transfected cells, and used to transform *Escherichia coli* MC1061/P3. Individual colonies were grown, and the bacteria were then pooled into groups of 16 for plasmid purification (Qiagen, Chatsworth, CA). Groups of cDNAs were transfected into the $\alpha\beta\text{py}$ cell line along with Tac- β_1 and the transfectants were screened by flow cytometry as described above. Pools containing cDNAs that altered PAC1 binding were further screened in smaller pools of four, and then the positive pools were screened as individual colonies.

Adhesion assay. Laminin and fibronectin (Calbiochem) were used as substrates in serum-free adhesion assays. Cells of the small cell lung-cancer cell line H345 (ATCC) were maintained in RPMI and 10% serum and for experimental purposes were passaged into serum-free medium RPMI 1640 containing SITA (Sigma). Cells (1–2 × 10⁶ per ml) were washed twice in RPMI 3.5 d post-passage and disaggregated into single cells. Cells in RPMI (50 μl) were added to 96-well tissue-culture plates (Costar) coated with extracellular matrix proteins blocked with 1 mg ml⁻¹ BSA. Cells were allowed to attach for 45 min at 37 °C. Cell attachment was determined by crystal violet staining. The attachment of H345 cells to wells coated with 25 mg ml⁻¹ poly-L-lysine and fixed with 20% glutaraldehyde before aspiration was defined as 100% adhesion²⁴.

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Inhibitory and activating functions for MAPK Kss1 in the *S. cerevisiae* filamentous-growth signalling pathway

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Mitogen-activated protein kinase (MAPK) cascades are conserved signalling modules that regulate responses to diverse extracellular stimuli, developmental cues and environmental stresses (reviewed in refs 1–3). A MAPK is phosphorylated and activated by a MAPK kinase (MAPKK), which is activated by an upstream protein kinase, such as Raf, Mos or a MAPKK kinase. Ste7, a MAPKK in the yeast *Saccharomyces cerevisiae*, is required for two developmental pathways: mating⁴ and invasive (filamentous) growth^{5,6}. Kss1 and Fus3, the MAPK targets of Ste7, are required for mating^{7,8}, but their role in invasive growth has been unclear.

Because no other *S. cerevisiae* MAPK has been shown to function in invasive growth, it was proposed^{5,6,9} that Ste7 may have non-MAPK targets. We show instead that Kss1 is the principal target of Ste7 in the invasive-growth response in both haploids and diploids. We demonstrate further that Kss1 in its inactive form is a potent negative regulator of invasive growth. Ste7 acts to relieve this negative regulation by switching Kss1 from an inhibitor to an activator. These results indicate that this MAPK has a physiologically important function in its unactivated state. Comparison of normal and MAPK-deficient cells indicates that nitrogen starvation and activated Ras stimulate filamentous growth through both MAPK-independent and MAPK-dependent means.

To examine the role of Ste7, Kss1 and Fus3 in invasive growth, we analysed a set of eight haploid strains in the $\Sigma 1278b$ background, because this lineage readily displays invasive growth when stimulated by the appropriate medium^{5,6,10}. These strains represent all possible combinations of wild-type and null (deletion) alleles of *STE7*, *KSS1* and *FUS3*, but are otherwise isogenic (Fig. 1a). All of the strains grew equally vigorously (Fig. 1b). As expected, the only strains able to mate were those containing an intact MAPK cascade: JCY100 (*STE7⁺ KSS1⁺ FUS3⁺*), JCY110 (*STE7⁺ kss1 Δ FUS3⁺*), and JCY120 (*STE7⁺ KSS1⁺ fus3 Δ*) (Fig. 1c).

The standard agar penetration assay was used to score for invasive growth (Fig. 1d). We found, in agreement with a previous report⁵, that a strain lacking *KSS1* (JCY110) was unable to invade agar medium, whereas a strain (JCY120) lacking *FUS3* appeared to invade better than wild-type cells (JCY100). As also reported previously⁵, a strain (JCY107) lacking *STE7* or a strain (JCY127) lacking both *STE7* and *FUS3* were unable to invade agar, whereas a strain (JCY130) lacking both *KSS1* and *FUS3* invaded at least as well as wild-type cells. These data had been interpreted⁵ as suggesting that some Ste7-dependent (but MAPK-independent) process promotes invasive growth. To test this notion directly, we examined a *ste7 Δ kss1 Δ fus3 Δ* triple-mutant strain (JCY137), which had not previously been constructed. Remarkably, this triple mutant invaded agar medium just as well as its wild-type parent. Hence, the inability of a Ste7-deficient strain to invade agar medium is completely reversed when both Kss1 and Fus3 are also removed (Fig. 1d; compare strains JCY107 and JCY137), indicating that the MAPKs, especially Kss1, act to repress haploid invasive growth, and that the principal function of Ste7 is to overcome this repression. Indeed, by examining a *ste7 Δ kss1 Δ* double mutant, which had also not been tested previously, we found that the inability of a Ste7-deficient cell to invade agar could be suppressed by removing just *KSS1* (Fig. 1d; compare JCY117 and JCY107).

Transcription from filamentous response elements (FREs), which contain the binding sites for two transcription factors (Ste12 and Tec1) required for invasive growth^{5,6,11}, has been shown to correlate with Ras- and MAPK cascade-dependent signalling in this pathway^{12,13}. Quantitative measurement of the expression of an *FRE-lacZ* reporter gene in our set of eight isogenic strains (Fig. 1e) confirmed the results obtained using the agar penetration assay (Fig. 1d). For example, compared with the *ste7 Δ* single mutant (JCY107), full FRE-dependent transcription was restored in the *ste7 Δ kss1 Δ fus3 Δ* triple mutant (JCY137). Deletion of *STE12* in the *ste7 Δ kss1 Δ fus3 Δ* background (YLB637) eliminated both FRE-dependent transcription (Fig. 1e) and abolished invasive growth (data not shown), demonstrating that the MAPK-independent activity of Ste12 can promote FRE-dependent expression.

To determine whether Ste11, the MAPKK kinase that phosphorylates and activates Ste7 (ref. 14), contributes to the invasive growth observed in the *ste7 Δ kss1 Δ fus3 Δ* triple mutant, we generated a *ste11 Δ ste7 Δ kss1 Δ fus3 Δ* quadruple mutant. We found that both *FRE-lacZ* expression and invasive growth were undiminished (data not shown). Thus the entire MAPK cascade previously thought to be necessary for invasive growth is, in a formal sense, dispensable for this developmental pathway.

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