MYC suppresses cancer metastasis by direct transcriptional silencing of $\alpha_v$ and $\beta_3$ integrin subunits

Hong Liu$^{1,4,5}$, Derek C. Radisky$^{2,5,6,7}$, Dun Yang$^1$, Ren Xu$^3$, Evette S. Radisky$^2$, Mina J. Bissell$^{3,6}$ and J. Michael Bishop$^{1,6}$

Overexpression of MYC transforms cells in culture, elicits malignant tumours in experimental animals and is found in many human tumours. We now report the paradoxical finding that this powerful oncogene can also act as a suppressor of cell motility, invasiveness and metastasis. Overexpression of MYC stimulated proliferation of breast cancer cells both in culture and in vivo as expected, but inhibited motility and invasiveness in culture, and lung and liver metastases in xenografted tumours. We show further that MYC represses transcription of both subunits of $\alpha_v\beta_3$ integrin, and that exogenous expression of $\beta_3$ integrin in human breast cancer cells that do not express this integrin rescues invasiveness and migration when MYC is downregulated. These data uncover an unexpected function of MYC, provide an explanation for the hitherto puzzling literature on the relationship between MYC and metastasis, and reveal a variable that could influence the development of therapies that target MYC.

The proto-oncogene MYC encodes an exceptionally pleiotropic transcription factor (MYC) that participates in the control of a wide variety of genes$^{1-3}$. Included among these genes are functions vital to regulation of the cell cycle, cell growth, apoptosis, cell adhesion and genomic stability$^{4-9}$. Overexpression of MYC transforms cells in culture$^6$, elicits tumours in experimental animals$^7$ and is found in as many as 50% of all human cancers and 25% of human breast cancers$^8-13$. Paradoxically, such overexpression is on occasion dissociated from the propensity to invade and metastasize$^{10,13-18}$. These observations raise the possibility that MYC may inhibit cellular properties such as motility and invasion that are essential to metastasis. Consistent with this possibility, overexpression of MYC in mouse skin causes a severe impairment in wound healing and in keratinocyte migration, whereas deletion of MYC causes increased migration of keratinocytes$^{19,20}$.

Cellular invasion and migration are governed by extracellular and intracellular signals, and depend on the interaction of the cell with ligand molecules in the extracellular matrix$^{21-23}$ (ECM). The principal ECM receptors are the integrins, and altered expression of integrins is associated with tumour growth and metastasis$^{24,25}$. Overexpression of MYC was found to inhibit the spreading and adhesion of primary keratinocytes, which exhibited decreased expression of $\alpha_5$, $\beta_1$ and $\beta_4$ integrins in response to MYC expression$^{26}$. When MYC was overexpressed in cells from neuroblastomas and sarcomas, the levels of $\alpha_5$ and $\beta_1$ integrins were downmodulated$^{27,28}$, whereas genetic ablation of MYC in haematopoietic stem cells stimulated the expression of $\alpha_5$, $\alpha_6$, and $\beta_1$ integrins, and other ECM proteins$^{29}$. In addition, MYC binds to the E-box sequence of the promoters of the $\alpha_5$ and $\alpha_6$ integrin genes and silences their transcription in mouse myoblast and human sarcoma cells$^{27,30}$.

We report here that elevated expression of MYC reduced the motility and invasiveness of breast cancer cells in vitro, their capacity for local invasion and their ability to seed distant metastases. Concomitantly, MYC overexpression inhibited the formation of stress fibres and focal adhesions. These effects of MYC could be attributed to the decreased expression of $\alpha_5$ and $\beta_1$ integrins, mediated by repression of transcription from the corresponding genes. Our results provide an explanation for the dissociation between overexpression of MYC and metastasis, point to $\alpha_5$ and $\beta_1$ integrins as crucial elements in the metastasis of malignant cells and uncover a variable that may be important in the development of therapeutics that target MYC.

RESULTS

MYC inhibits invasion and metastasis

We explored the role of MYC in metastasis by manipulating expression of the gene in four established cell lines of human breast cancer, two of which express little MYC (MDA-MB-231 and BD549) and two of which express MYC at high levels (MCF-7 and T47D; Fig. 1a), and in human retinal pigment epithelium (RPE) cells, a cell line that has been used previously for investigating the effects of MYC on induction of proliferation and apoptosis.

1G.W. Hooper Foundation, University of California at San Francisco, San Francisco, California 94143, USA. 2Mayo Clinic Cancer Center, Jacksonville, Florida 32224, USA. 3Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. 4Present address: Eureka Therapeutics Inc. 5858 Horton Street, Suite 362, Emeryville, California 94608, USA. 5These authors contributed equally to this work. 6Co-contributing laboratories.

Correspondence should be addressed to D.C.R. (e-mail: radisky.derek@mayo.edu)

Received 23 February 2011; accepted 29 March 2012; published online 13 May 2012; DOI: 10.1038/ncb2491
We found that MDA-MB-231 cells overexpressing MYC showed while inhibiting invasion, both in culture and in vivo. Cells that did metastasize resulted in larger tumours (Fig. 2c). To assess tumour growth and metastasis from the orthotopic site, MDA-MB-231, BT549 and RPE cells were transduced with luciferase-expressing virus (Fig. 2d) and injected into the inguinal mammary gland. We found that the MYC-expressing tumours, as expected, grew more rapidly at the orthotopic site (Fig. 2e–g). However, isolation and quantification of luminescence of lungs (Fig. 2h,i) and livers (Fig. 2m,n) revealed decreased metastatic burden at 6 weeks following injection, although the difference was not statistically significant. Metastatic burden is a combination of both number of metastases and the size of the metastatic tumours; we found that expression of MYC significantly reduced the number of metastases but led to increased size of individual metastases in both lung (Fig. 2j–l) and liver (Fig. 2o–q). We conclude that overexpression of MYC can stimulate cell proliferation, producing larger tumours that have reduced invasiveness and metastatic potential. These findings are consistent with recent indications that dissemination of tumour cells may occur early during tumour progression and is not necessarily linked to primary tumour size31.

**MYC modulates cell interaction with the ECM**

Motility is a dynamic process associated with major changes in cellular phenotype including spreading, actin polymerization, formation of actin-rich protrusions at the leading edge of migrating cells, and creation and dissolution of focal adhesions during cell translocation32,33. We found that exogenous overexpression of MYC in both MDA-MB-231 and RPE cells (Fig. 3a,b) reduced cell spreading, decreased stress fibre and focal adhesion formation, and increased cortical actin (Fig. 3c,d). As formation of focal adhesions requires the interaction of cell surface integrins with their ligands in the ECM, we reasoned that the effect of overexpression of MYC on focal adhesion might reflect a defect in the interaction of cells with specific ECM ligands. To test this hypothesis, we assessed cell attachment to purified human ECM ligands and found that overexpression of MYC in MDA-MB-231 or RPE cells reduced their attachment to vitronectin (Fig. 3e,f). Such defects could in turn impair cellular motility. To explore this possibility, we measured chemotaxis towards purified vitronectin in Boyden chamber assays.

**Figure 1** Elevated MYC expression impedes the invasiveness of human breast cancer cells. (a) MYC expression levels in four breast cancer cell lines. The endogenous MYC expression levels of MDA-MB-231, BT549, MCF-7 and T47D cells were measured by western blots. (b,c) Ectopic overexpression of MYC increases proliferation but does not affect apoptosis. Cells were labelled with BrdU on tissue culture plastic for 30 min at 37°C (b, n = 2) or incubated with annexin V and propidium iodide (PI) for 5 min at room temperature (c, n = 2). Both positive and total nuclei were counted. (d) MYC overexpression inhibits cell migration. Migration of MDA-MB-231, BT549 and RPE cells was measured in Boyden chamber assays (n = 3 for each experiment). The cells were transfected with either vector control or a MYC construct. (e) The invasiveness of breast cancer cells is inhibited by MYC overexpression. Cell invasion through Matrigel-coated transwells was measured for MDA-MB-231 (n = 3) and BT549 (n = 3) cells stably transduced with vector control or exogenous MYC. (f) High level of MYC expression abrogates the invasive phenotype of breast cancer cells grown in 3D Matrigel. Results are shown for day 6. Scale bars, 50 μm. (g,h) MYC overexpression enhances tumour growth but reduces tumour invasion into nearby tissues. MDA-MB-231 cells stably expressing vector or exogenous MYC were inoculated subcutaneously into nude mice. The tumours were collected 4 weeks after injection, sectioned and stained with haematoxilin and eosin (H&E) and assessed for size (h, n = 5 for each group, P < 0.01). Scale bar, 200 μm. Results are expressed as mean ± s.d. *P < 0.05; **P < 0.01.

© 2012 Macmillan Publishers Limited. All rights reserved.
To explore the mechanism by which overexpression of MYC significantly decreases lung metastases of breast cancer cells, MDA-MB-231 cells stably expressing vector or ectopic MYC were injected into nude mice through the tail vein and lung metastases were assessed 6 weeks after injection. Sections of the lungs were stained with antibody against human vimentin (a) and quantification revealed that MYC significantly reduced lung metastasis (b, *P < 0.0049). Assessment of Ki-67 (c) revealed increased proliferation in the MYC-expressing cells. Scale bars, 200 μm. (d) Luminescence detection shows comparable luminescence per cell for both vector- and MYC-overexpressing cells. (e) MYC-expressing cells show more rapid growth at the primary tumour site, assessed by quantitative in vivo imaging (n = 5 for each group). (f) Sample images from in vivo imaging of primary tumours for vector and MYC (week 6). (g) Increased size of primary tumours from MYC-expressing cells (week 6; n = 5 for each group). (h,i) Decreased metastatic burden in lungs of mice implanted with MYC-expressing cells, assessed by luminescence (week 6); h, sample images, scale bar, 1 cm; i, quantification of lung luminescence; n = 5 for each group; differences between cells expressing vector alone and those expressing MYC were not statistically significant. (j) Images of lung metastases, stained for human cytokeratins (top), and with haematoxilin and eosin (H&E; bottom). Scale bar, 200 μm. (k,l) Quantification of number (k) and size (l) of lung metastases indicates that MYC cells form much fewer metastases but grow to a larger size (n = 5 for each group). (m,n) Decreased metastatic burden in livers of mice implanted with MYC-expressing cells (week 6; m, sample images, scale bar, 1 cm; n, quantification of liver luminescence; differences between conditions were not statistically significant; n = 6 for vector, n = 5 for MYC). (o) Images of liver metastases, stained for human cytokeratins (top) and with haematoxilin and eosin (bottom). Scale bar, 200 μm. (p,q) Quantification of number (p) and size (q) of liver metastases indicates that MYC-expressing cells form fewer metastases that grow to a larger size (n = 5 for each group). Results are expressed as mean ± s.e.m. *P < 0.05; ***P < 0.005.

Overexpression of MYC in MDA-MB-231 cells caused a reduction of DNA for both αv and β3 (Fig. 4b,c). A canonical E-box binding site for MYC is located upstream of the transcription initiation sites for both αv (CACATG) and β3 (CACGTG) integrins in human DNA (Fig. 4d). Quantitative chromatin immunoprecipitation (ChIP) assays showed that MYC bound directly to the E-box region of both these genes in MDA-MB-231 cells (Fig. 4e,f) and in RPE cells (not shown), but not to nonspecific sequences in the same domain. Although MYC was originally viewed as a transcriptional activator, it can also serve as a transcriptional repressor54. Our results show that overexpression of MYC represses transcription of integrin genes involved in migration and metastasis.

**Inhibition of invasion and metastasis by MYC depends on αv and β3 integrin modulation**

We found that the invasive and migratory phenotype of MDA-MB-231 and RPE cells was dependent on αvβ3 integrin (Fig. 5a,b, and data not shown). We then investigated whether the invasive and migratory phenotype was dependent on αvβ3 integrin (Fig. 5a,b, and data not shown). We then investigated whether the invasive and migratory phenotype was dependent on αvβ3 integrin (Fig. 5a,b, and data not shown).
We generated MYC-expressing MDA-MB-231 cells that also expressed MYC overexpression of MYC (Fig. 5h). When these cells were implanted orthotopically, we noticed that depletion of either \( \alpha_v \) and \( \beta_3 \) integrins in MDA-MB-231 (Fig. 5d) increased invasiveness (Fig. 6d,j) as well as cell spreading, focal adhesion and stress fibre formation in RPE cells were rescued as well (Fig. 5g). To evaluate whether exogenous overexpression of \( \alpha_v \) and \( \beta_3 \) integrins was sufficient to reconstitute metastatic capability in cells also overexpressing MYC, we generated MYC-expressing MDA-MB-231 cells that also expressed integrin \( \alpha_v \) and integrin \( \beta_3 \) using exogenous promoters unaffected by MYC (Fig. 5h). When these cells were implanted orthotopically, ectopic expression of integrins \( \alpha_v \) and \( \beta_3 \) substantially increased lung metastasis (Fig. 5i,j). These results indicate that suppression of integrin \( \alpha_v/\beta_3 \) expression is the key mechanism by which MYC inhibits breast cancer cell metastasis.

We noticed that depletion of either \( \alpha_v \) or \( \beta_3 \) integrin resulted in a parallel reduction of the other (Fig. 5a,b); conversely, exogenous expression of \( \beta_3 \) integrin increased the amount of \( \alpha_v \) integrin (see below, Fig. 6c,e and Supplementary Figs S1 and S2). Moreover, exogenous expression of either integrin at least partially rescued the phenotype suppressed by MYC (Fig. 5d–g). These findings could be explained by mass action, wherein an increase in either integrin alone can augment the formation of heterodimers and stabilize both components. We explored this possibility by co-immunoprecipitation to detect the formation of heterodimers. We found that transfecion of \( \alpha_v \) and \( \beta_3 \), either individually or together, substantially increased the amount of heterodimers in extracts of MDA-MB-231 cells (Supplementary Fig. S1). The relative amounts of heterodimer correlated with the extent to which exogenous expression of the integrins rescued a defect in invasion caused by MYC (compare Fig. 5d with Supplementary Fig. S1). We conclude that the defects in motility and invasion elicited by overexpression of MYC are due to reduced expression of the \( \alpha_v \) and \( \beta_3 \) integrin subunits, which in turn reduces formation of the heterodimer.

**MYC prevents \( \beta_3 \)-integrin-induced cell invasion**

We also explored the effect of depleting the high level of MYC in MCF-7 cells with RNA interference (Fig. 6a). Depletion of MYC significantly reduced cellular proliferation as expected (data not shown), whereas it increased cell adhesion to vitronectin and fibronectin (Fig. 6b), as well as cell spreading, focal adhesion and stress fibre formation (Fig. 6g). However, knockdown of MYC in these cells neither increased cell motility (data not shown) nor invasiveness (Fig. 6i), implicating the absence of \( \beta_3 \) integrin in MCF-7 cells and the consequent inability to form \( \alpha_v/\beta_3 \) heterodimers. Accordingly, exogenous expression of \( \beta_3 \) integrin in MCF-7 cells (Fig. 6c,i) increased invasiveness (Fig. 6d,j) as well as cell spreading (Fig. 6h); similar effects were seen with exogenous expression of \( \beta_3 \) integrin in T47D cells (Fig. 6e,fand data not shown).

MCF-7 cells grow in a cuboidal morphology with tight cell–cell junctions that restrict individual cell motility. To isolate the inhibitory effect of MYC on cell motility, we simultaneously knocked down E-cadherin and MYC, and expressed exogenous \( \beta_3 \) integrin in MCF-7 cells (Fig. 6l). This combination greatly increased invasion (Fig. 6j). We conclude that when cell–cell interactions are abrogated, inhibition of \( \alpha_v \) and \( \beta_3 \)-integrin expression is the primary barrier to cell invasion.
invasion in MCF-7 and possibly other human breast cancer cells that overexpress MYC.

**DISCUSSION**

MYC has been implicated extensively in tumour growth and cell transformation through studies in cultured cells, targeted expression in mice, and retrospective analyses of MYC expression in human tumours. MYC is highly pleiotropic and plays multiple biological roles in driving tumorigenesis. Examples include the ability to initiate tumorigenesis in mice and to inhibit metastasis through inhibition of transcription of an integrin gene involved in metastasis.

The possibility that MYC could act to inhibit metastasis could have been surmised by the observations that overexpression of the gene is sometimes dissociated from the tendency of tumours to metastasize. Why this may be the case, however, had not been explored. In the case of human breast cancer, distal metastases may express MYC at the level of cognate normal cells, even though the gene is overexpressed in the primary tumour. Similarly, mammary carcinoma and other tumours induced by MYC in mice frequently fail to metastasize. Examples include murine mammary cancer elicited by MYC under the control of the mouse mammary tumour virus promoter and aggressive breast tumours elicited by retroviral transduction of both MET and MYC into normal breast epithelial cells.

Here we explored the impact of MYC on metastasis by using cell lines derived from carcinomas of the human breast. Two of these lines express low levels of MYC and display a metastatic phenotype, whereas the other two overexpress the gene but do not metastasize. Irrespective of the reason for this variation, the differences in MYC expression allowed reciprocal studies on induction or repression of the metastatic phenotype by manipulation of MYC expression and its downstream targets. Clearly, human breast cancers that overexpress MYC may still metastasize if other factors override its function. In fact our in vivo assays demonstrated that expression of MYC will support increased growth of those few metastatic cells that escape the inhibitory function of MYC by means of other mutations or by changes in gene expression (Fig. 2m,r).

Given this complexity, we sought direct experimental demonstration that MYC can indeed inhibit metastasis. Using MDA-MB-231 cells, the human cell line that expresses only low levels of MYC and is used extensively for metastatic studies, we confirmed their highly metastatic potential, by both tail vein injection (Fig. 2c,d) and orthotopic implantation (Fig. 2i–r) in mouse models. However, when exogenous MYC was overexpressed in these cells, their ability to metastasize was greatly reduced. Furthermore, the behaviour of breast cancer cell lines was remarkably malleable when the level of MYC was modulated: it was possible to reduce the metastatic properties by overexpressing MYC, or to augment those properties by reducing MYC levels. At the same time, MYC could increase proliferation even in overtly malignant cells (Fig. 1b). We reported previously an apparent dichotomy between stimulation of proliferation and inhibition of other malignant properties of breast cancer cells also for the oncogene AKT1 (protein kinase B1; refs 42–44).

Our finding that MYC overexpression leads to inhibition of cell motility and invasiveness through direct downmodulation of αv and β3 integrin subunits supports reports that integrins with these subunits are involved in cellular motility, invasiveness, adhesion and transmigration through endothelium, and are associated with metastasis. There are also reports that MYC may be involved in controlling the expression of integrin genes using cells from different tissues. However, none of these reports has linked the effect of MYC on integrin expression to cellular components of metastasis, and none has implicated the αvβ3 heterodimer directly in the cellular changes elicited by MYC. Our results demonstrate that overexpression of MYC represses transcription from the promoters of the αv and β3 integrin genes, which in turn reduces properties that are essential for metastasis. These data, however, do not mean that MYC affects invasion and metastasis only through regulation of integrins αv and β3, nor that MYC is the only regulator of integrin αvβ3 function in invasion and metastasis. The fact that MYC siRNA increases invasion when MCF-7 cells are forced to have high levels of integrins αv and β3 (Fig. 6j) indicates that there may be mechanism(s) other than integrin αvβ3 by which MYC can inhibit invasion and metastasis. Nevertheless, as exogenous expression of these integrins is sufficient to bypass the repressive effect of MYC.

![Figure 4 MYC downregulates the expression of αv and β3 integrin genes through binding to their proximal promoters.](image)
Figure 5  MYC affects breast cancer cell invasiveness by suppressing integrin αv and β3 subunits. (a,b) Knockdown of αv or β3 integrin inhibited invasion (a) and migration (b) in a Boyden chamber assay, n = 3. Knockdown of the integrins by siRNA was confirmed by western blots Ctl, control. (c) Knockdown of αv or β3 integrin inhibits the invasiveness of MDA-MB-231 cells grown in a 3D Matrigel assay for 6 days; n = 3. Scale bar, 50 μm. (d,e) αv and β3 integrin rescues the compromised migration (d) and invasiveness (e) elicited by high MYC expression; n = 3 for each. MDA-MB-231 and RPE cells overexpressing MYC were transiently transfected with vector, αv, β3 or β3 integrin constructs. Cell invasiveness and migration were assessed by Boyden chamber assay. (f) Inhibition of cancer cell invasiveness by MYC overexpression can be rescued by exogenous expression of αv and β3 integrin subunits in BT549 cells (n = 3). (g) Expression of exogenous αv and β3 integrin partially rescued actin cytoskeleton, focal adhesion formation of RPE cells grown on 2D tissue culture plastic dishes, and the compromised invasiveness of MDA-MB-231 cells in a 3D Matrigel assay. RPE or MDA-MB-231 cells, stably expressing the indicated constructs, were plated on cell culture dishes for 24 h or in 3D Matrigel for 6 days. RPE cells were then stained with anti-vinculin antibody (green) and Texas Red-conjugated phalloidin (red). Images of 3D Matrigel culture were obtained by phase-contrast microscopy. Scale bars, 50 μm for phase-contrast and 5 μm for immunofluorescence-staining images. (h) Quantification of ITGAV and ITGB3 transcripts by quantitative PCR in MDA/MYC and MDA/MYC/αv/β3 cells; n = 3. (i) Quantification of increased lung metastases in mice orthotopically implanted with MDA/MYC/αv/β3 cells as compared with MDA/MYC cells, n = 5 for each. (j) Images of lungs of mice orthotopically implanted with MDA/MYC/αv/β3 or MDA/MYC cells. The images on the right show a magnified view of the area outlined in the left images. Results are expressed as mean ± s.d. *P < 0.05. Uncropped images of blots are shown in Supplementary Fig. S3.

invasion (Fig. 6d,f,j) and metastasis (Fig. 5h,i,j), it is apparent that a primary effect of MYC on invasion in breast cancer cells is mediated through its inhibition of integrins αv and β3.

Tumour progression often culminates in metastatic disease, indicating that there must be selection for antidotes to the MYC inhibition of the metastatic phenotype. One obvious possibility would be selection against overexpression of MYC during the course of tumour progression. Indeed, the two breast cancer cell lines used in the present study that express relatively low levels of MYC both originated from metastatic tumours: MDA-MB-231 from pleural effusion and BT549 from an invasive ductal tumour in regional lymph nodes. Other possibilities include loss of β3 integrin functions, as in the case of MCF-7 and T47D cell lines. Constitutive signalling from active Ras or overexpression of Bcl-XL can also override the inhibition of metastasis by MYC (unpublished results of H.L and D.Y). These complexities might account for the fact that in some experimental circumstances, MYC may seem to favour metastasis. The findings reported here prompt a cautionary note about therapeutic strategies involving MYC. The frequency with which MYC is overexpressed, and the variety of tumours in which that overexpression occurs, have made MYC a seemingly advantageous therapeutic target. Our findings raise the possibility that inhibition of MYC in human tumours might at times be contraindicated because its suppression may indeed promote metastasis.

METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

ACKNOWLEDGEMENTS

We thank D. Cheres, F. G. Giancotti, A. Goga and D. Sheppard for providing DNA constructs and cell lines. We are grateful to C.-Y. Chen, L. Prentice and B. Edenfeld for help with histology and imaging. We thank D. Khauv for work with the ChIP
**AUTHOR CONTRIBUTIONS**

H.L., D.C.R., J.M.B. designed the research; H.L., D.C.R., D.Y., E.S.R., M.J.B. and J.M.B. wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at www.nature.com/naturecellbiology

Reprints and permissions information is available online at www.nature.com/reprints

1. Grandori, C., Cowley, S. M., James, L. P. & Eisenman, R. N. The Myc/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.* 16, 653–699 (2000).

2. Pelengaris, S., Khan, M. & Evan, G. c-MYC: more than just a matter of life and death. *Nat. Rev. Cancer* 2, 764–776 (2002).

3. Dang, C. V. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell Biol.* 18, 1–11 (1999).

4. Kuttler, F. & Mai, S. c-Myc, genomic instability and disease. *Genome Dyn. I*, 171–190 (2006).

5. Oster, S. K., Ho, C. S., Soucie, E. L. & Penn, L. Z. The myc oncogene: MarvellousY Complex. *Adv. Cancer Res.* 84, 81–154 (2002).

6. Adhikary, S. & Eilers, M. Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* 6, 635–645 (2005).

7. Meyer, N. & Penn, L. Z. Reflecting on 25 years with MYC. *Nat. Rev. Cancer* 8, 976–990 (2008).

8. Nesbit, C. E., Gardner, C., Li, E. R., Arnold, I. & Watt, F. M. Evidence that Myc activation by c-Myc and E-cadherin by siRNA increased the invasiveness of MCF-7 cells only when β3 integrin was expressed. The depletion of MYC and E-Cadherin was assessed by western blot (I) and invasiveness was assessed by Boyden chamber assays (j; n = 3). Results are expressed as mean ± s.d. Uncropped images of blots are shown in Supplementary Fig. S3.

**Figure 6** MYC and E-cadherin can prevent β3-integrin-induced invasion. (a) Suppression of MYC expression by siRNA in MCF-7 cells assessed by western blot. (b) Suppression of MYC by siRNA augments cell adhesion to vitronectin and fibronectin. The cells in serum-free medium were plated into 96-well plates coated with purified matrix proteins (VN, vitronectin; FN, fibronectin; COL, collagen I; LN, laminin). After 45 min, adhered cells were counted in five fields, n = 2. (c-f) Ectopic expression of β3 integrin in MCF-7 (g) and T47D (e) cells, assessed by western blot, enhanced invasiveness (d,f; n = 3), as assessed by Boyden chamber assay. (g,b) Cell spreading, stress fibre and focal adhesion formation are enhanced in MCF-7 cells when MYC is depleted by siRNA (g) or when β3 integrin is exogenously expressed (h). Changes in cell shape, actin cytoskeleton and focal adhesion formation were demonstrated by phase-contrast microscopy (top), and staining with anti-vinculin (green) and Texas red phalloidin (bottom). Scale bar, 20 μm for phase-contrast and 5 μm for immunofluorescence-staining images. (i,j) Decreased expression of MYC and E-cadherin by siRNA increased the invasiveness of MCF-7 cells only when β3 integrin was expressed. The depletion of MYC and E-Cadherin was assessed by western blot (I) and invasiveness was assessed by Boyden chamber assays (j; n = 3). Results are expressed as mean ± s.d. Uncropped images of blots are shown in Supplementary Fig. S3.

© 2012 Macmillan Publishers Limited. All rights reserved.
ARTICLES

20. Waikel, R. L., Kawachi, Y., Waikel, P. A., Wang, X. J. & Roop, D. R. Deregulated expression of c-Myc depletes epidermal stem cells. Nat. Genet. 28, 165–168 (2001).

21. Guo, W. & Giancotti, F. G. Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr. Opin. Cell Biol. 15, 547–556 (2003).

22. Hood, J. D. & Cheresh, D. A. Role of integrins in cell invasion and migration. Nat. Rev. Mol. Cell Biol. 5, 816–826 (2004).

23. Lu, P., Weaver, V. M. & Werb, Z. The extracellular matrix: A dynamic niche in cancer progression. J. Cell Biol. 196, 395–406 (2012).

24. Felding-Habermann, B. Integrin adhesion receptors in tumor metastasis. Trends Cell Biol. 13, 146–150 (2003).

25. Mizejewski, G. J. Role of integrins in cancer: survey of expression patterns. Proc. Soc. Exp. Biol. Med. 222, 124–138 (1999).

26. Gebhardt, A. et al. Myc regulates keratinocyte adhesion and differentiation via integrin expression during muscle differentiation. Genes Dev. 16, 362–374 (2002).

27. De Nigris, F. et al. Cooperation between Myc and YY1 provides novel silencing transcriptional targets of αvβ3-integrin in tumour cells. Oncogene 26, 382–394 (2007).

28. Van Golen, C. M., Soules, M. E., Grauman, A. R. & Feldman, E. L. N-Myc overexpression leads to decreased β1 integrin expression and increased apoptosis in human neuroblastoma cells. Oncogene 22, 2664–2673 (2003).

29. Wilson, A. et al. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev. 18, 2747–2761 (2004).

30. Xiao, J., Jethanandani, P., Zieber, B. L. & Kramer, R. H. Regulation of αv integrin expression during muscle differentiation. J. Biol. Chem. 278, 49780–49788 (2003).

31. Husemann, Y. et al. Systemic spread is an early step in breast cancer. Cancer Cell 13, 58–68 (2008).

32. Ridley, A. J. et al. Cell migration: integrating signals from front to back. Science 302, 1704–1709 (2003).

33. Friedl, P. & Wolf, K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat. Rev. Cancer 3, 362–374 (2003).

34. Carman, C. V. & Springer, T. A. Integrin avidity regulation: are changes in affinity and conformation underemphasized? Curr. Opin. Cell Biol. 15, 547–556 (2003).

35. Liu, H., Radisky, D. C. & Bissell, M. J. Polarity and proliferation in breast cancer: unlocking the Gordian knot. Cell Cycle 4, 646–649 (2005).

36. Liu, H., Radisky, D. C., Wang, F. & Bissell, M. J. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. J. Cell Biol. 164, 603–612 (2004).

37. Small, M. B., Hay, N., Schwab, M. & Bishop, J. M. Neoplastic transformation by the human gene N-myc. Mol. Cell. Biol. 7, 1638–1645 (1987).

38. Elwood-Yen, K. et al. Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 4, 223–238 (2003).

39. Lu, P., Weaver, V. M. & Werb, Z. The extracellular matrix: A dynamic niche in cancer progression. J. Cell Biol. 196, 395–406 (2012).

40. Gebhardt, A. et al. Myc regulates keratinocyte adhesion and differentiation via integrin expression during muscle differentiation. Genes Dev. 16, 362–374 (2002).

41. Rapp, U. R. et al. Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. Proc. Natl Acad. Sci. USA 103, 4134–4139 (2006).

42. Arvanitis, C. & Felsher, D. W. Conditional transgenic models define how MYC initiates and maintains tumorigenesis. Semin. Cancer Biol. 16, 313–317 (2006).

43. Rapp, U. R. et al. Mechanism of Akt1 inhibition of breast cancer cell invasion reveals a protumorigenic role for TSC2. Proc. Natl Acad. Sci. USA 103, 4134–4139 (2006).

44. Liapis, H., Flath, A. & Kitazawa, S. Integrin expression and increased apoptosis of bone-residing breast cancer metastases. Diagn. Mol. Pathol. 5, 127–135 (1996).

45. Desgroiselles, J. S. et al. An integrin αvβ3-c-Src oncogenic unit promotes anchorage-independence and tumor progression. Nat. Med. 15, 1163–1169 (2009).

46. Ma, L., et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and breast cancer metastasis. Oncogene 29, 3133–317 (2010).

47. O’Donnell, S., et al. Systemic spread is an early step in breast cancer. Cancer Cell 13, 58–68 (2008).

48. Rapp, U. R. et al. MYC is a metastasis gene for non-small-cell lung cancer. PLoS One 4, e6029 (2009).

49. Wolfer, A. et al. MYC regulation of a ‘poor-prognosis’ metastatic cancer cell state. Proc. Natl Acad. Sci. USA 107, 3698–3703 (2010).

50. Vita, M. & Henriksson, M. The Myc oncprotein as a therapeutic target for human cancer. Semin. Cancer Biol. 16, 318–330 (2006).
METHODS

Cell lines, ECM proteins, DNA constructs and antibodies. Human breast cancer cell lines (MDA-MB-231, BT549, MCF-7 and T47D) were obtained from American Type Cell Collection. MDA-MB-231, human RPE cell line and MCF-7 transfection of PT67 packaging cells (Clontech), production of retroviral stock and viral infection were performed according to the standard protocols. The stably transfected cells were selected in the presence of neomycin (500-1,000 \( \mu \)g ml\(^{-1} \)) or hygromycin B (50 \( \mu \)g ml\(^{-1} \)) or puromycin (2-4 \( \mu \)g ml\(^{-1} \)) for 2 weeks, and surviving clones were pooled.

Growth-factor-reduced Matrigel, purified human vitronectin, fibronectin, collagen I and laminin 1 were purchased from BD Biosciences. Antibodies against the following proteins were obtained as indicated: \( \alpha \) integrin (catalogue no. 611012, clone no. 21, 1:1,000 dilution for western blot), \( \beta \) integrin (catalogue no. 610647, no. 18, 1:1,000 dilution for western blot), \( \beta \) integrin (catalogue no. 610444, clone no. 42, 1:1,000 dilution for western blot) from BD Biosciences; Myc (catalogue no. sc764, 1:500 dilution for western blot), \( \alpha \) integrin (catalogue no. sc76199, clone no. C9, 1:500 dilution for western blot) and \( \beta \) integrin (catalogue no. sc401, 1:500 dilution for western blot) from Santa Cruz Biotechnology; \( \alpha \), \( \beta \) integrin (catalogue no. C8LS44, clone 23C6, 0.1 \( \mu \)g antibody \(^{-1} \) total lysate for immunoprecipitation) from Chemicon; \( \beta \)-actin (catalogue no. A1978, clone no. AC15, 1:5,000 dilution for western blot) and vinculin (catalogue no. V9193, clone no. hVIN-1, 1:400 dilution for immunofluorescence analysis) from Sigma.

Human MVA/psCV/pymycin vector was a kind gift from A. Goga, and \( \beta \) integrin and \( \beta \) integrin in the pBabe/puromycin vector were kindly provided by D. Sheppard, both from University of California, San Francisco, USA. \( \alpha \) integrin in pCDM8 vector was a kind gift from D. Cheresh, University of California, San Diego, and was subcloned into pCDNA3.1 (Invitrogen). \( \beta \) integrin in the pRS-S vector was the generous gift of F. G. Giancotti, Memorial Sloan-Kettering Cancer Center, New York, USA. For reconstitution of \( \alpha \) and \( \beta \) integrin in MDA/Myc cells, the genes were subcloned into pLenti/V5-DEST and pLent63/5'V5-DEST (respectively; both from Invitrogen) using Gateway LR recombination (Invitrogen).

siRNA oligonucleotides targeting \( \alpha \) integrin, \( \beta \) integrin, E-cadherin and Myc were purchased from Ambion or Dharmaco.

For knockdown of ITGB3, \( \alpha \) integrin in MCF-7 and T47D cells, the unattached cells were removed by quickly decanting the plates and washing three times with PBS. The attached cells were fixed with 4% formaldehyde and stained with 0.3% crystal violet. Each condition was quadruplicated and attached cells were counted in five fields under \( \times 400 \) magnification. The experiments were repeated at least twice.

PCR with reverse transcription and ChIP assay. RNA quantification was performed in triplicate using TaqMan assays (Applied Biosystems, Life Technologies): \( \alpha \) integrin, Hs00233808_s1; \( \beta \) integrin, Hs00101649_s1. The ChIP assay was performed according to the Active Motif ChIP-IT Express Enzymatic manufacturer’s suggested protocol with a few modifications. Cellular components were crosslinked by adding methanol-free formaldehyde (2806, Sigma) to a final concentration of 1% and incubated at room temperature for 10 min. Immunoprecipitation was performed using 25 
\( \mu \)l of the enzymatically sheared chromatin and purified with an antibody for Myc (N262, Santa Cruz Biotechnology) at a concentration of 1 \( \mu \)g ml\(^{-1} \). The isolated DNA was then analysed by quantitative PCR using custom TaqMan assays (Applied Biosystems): \( \alpha \) forward primer, 5'-GTC CAC ACA ATG CAC TTA CAA T-3'; reverse primer, 5'-CAA AGT GGC CAG AGA AGT GAC T-3'; probe, 5'-GTG TAG ATG AAA CAC-3'. Control reactions were from Applied Biosystems (for \( \alpha \); ltgav_s1, Hs01999927_s1; for \( \beta \); lbh3_s1, Hs03674646_s1).

Invasion and migration assays. For the invasion assay, cell culture inserts (8 \( \mu \)m, 24-well format, Becton Dickinson Labware) were evenly coated with diluted Matrigel (1:5 dilution with blank medium). Cells (1 \( \times 10^5 \)) were added to the upper chamber and the lower chamber was filled with 300 \( \mu \)l medium containing 10% FBS. The culture was maintained for 24 h for MDA-MB-231 and BT549 cells, and 48 h for MCF-7 and T47D cells. The cell migration assay was similar to the invasion assay, except that inserts were not coated with Matrigel and the culture was maintained for 24 h. For the assay of cell migration on purified matrix proteins, cell culture inserts (8 \( \mu \)m, 24-well format, Becton Dickinson Labware) were first coated with the indicated matrix proteins (1 \( \mu \)g ml\(^{-1} \)) at 4 \( ^\circ \)C overnight and then blocked with 2% BSA in PBS at 37 \( ^\circ \)C for 2 h. Cells (1 \( \times 10^5 \)) in serum-free medium were added to the upper chamber and the lower chamber was filled with 300 \( \mu \)l of the same medium. The culture was maintained for 3 h. Cells were then fixed with 4% formaldehyde for 10 min and stained with 0.5% crystal violet for 10 min. The cells on the upper side of the filters were removed with cotton-tipped swabs. The cells on the underside of the filters were counted under \( \times 20 \) objective lens in five randomly chosen fields. The results are presented as the fold change when compared with vector control cells.

Metastasis assays. Female nude mice (Foxn1-nu), 6–7 weeks of age, were purchased from Harlan. MDA-MB-231 cells expressing MYC or vector alone were propagated as monolayers and trypsinized. For tail inoculation, cells were resuspended in DMEM at a concentration of \( 2 \times 10^5 \) cells ml\(^{-1} \). Cell suspension (0.1 ml; 2 \( \times 10^5 \) cells) was injected into tail veins of nude mice. All of the mice were killed by CO\(_2\), 6 weeks after inoculation. The lungs were removed, fixed in 4% formaldehyde, paraffin-embedded and sectioned (3 \( \mu \)m). Serial sections were stained with anti-human vimentin and all the nodules on both lungs were counted using \( \times 20 \) objective lens. With subcutaneous inoculation, cells were resuspended in DMEM medium with 50% Matrigel at a concentration of \( 5 \times 10^5 \) cells ml\(^{-1} \). Cells (0.1 ml; 5 \( \times 10^5 \) cells) were injected into the right flank of female nude mice. The tumours were measured weekly and the tumour volume was calculated according to the formula \( \text{length} \times \text{width}^2/2 \). The mice were killed 4 weeks after the inoculation. For orthotopic metastasis assays, cells were transduced with p53-Luc and 1 \( \times 10^5 \) cells were mixed with growth-factor-reduced Matrigel and injected into the inguinal mammary gland in 7-week-old NOD/SCID mice. For weekly imaging, mice were anesthetized with -chloral hydrate and imaged using the IVIS200 "Living Image" system. The images were acquired with a Xenogen IVIS200 system (Xenogen).
were injected i.p. with 150 mg kg⁻¹ body weight α-luciferin solution (Xenogen), anaesthetized with isofluorane and imaged using a bioluminescence imaging system (IVIS Imaging Spectrum System). Bioluminescence was calculated using IVIS Imaging Spectrum Software. Tissues were fixed and sections were stained for human cytokeratin; stained sections were scanned using the T2 ScanScope console (Aperio Technologies) and images were captured using Aperio ImageScope software. Tumour number and size assessments were performed by an investigator blinded to the treatment status of the sample.

Statistics. All data analysis was performed using Excel. Bar graphs represent means ±s.d. or s.e.m., as indicated. Statistical significance was assessed using the Student t-test.
Supplementary figure 1. Transfection of integrin αv and β3 subunits augments formation of αvβ3 integrin heterodimers.

**Figure S1** Transfection of integrin αv and β3 subunits augments formation of αvβ3 integrin heterodimers.
Supplementary figure 2. Exogenous expression of integrin β3 in MCF7 cells leads to increased generation of αvβ3 heterodimers even if MYC is not knocked down.

**IP:αVβ3 heterodimers**

| MYC siRNA | MCF-7-vector | MCF-7-β3 ITG |
|-----------|--------------|--------------|
| Ctrl #1   | #2           |              |
| Blot: β3 ITG |              |              |
| Blot: αV ITG |              |              |

**IP:αV integrin**

| MYC siRNA | MCF-7-vector | MCF-7-β3 ITG |
|-----------|--------------|--------------|
| Ctrl #1   | #2           |              |
| Blot: β3 ITG |              |              |
| Blot: αV ITG |              |              |

**Total lysates**

| MYC siRNA | MCF-7-vector | MCF-7-β3 ITG |
|-----------|--------------|--------------|
| Ctrl #1   | #2           |              |
| β3 ITG |              |              |
| αV ITG |              |              |
| MYC |              |              |
| Actin |              |              |

Figure S2 Exogenous expression of integrin β3 in MCF7 cells leads to increased generation of αvβ3 heterodimers even if MYC is not knocked down.
**Figure 4a**

| MDA-Mb-231 | BT-549 |
|-----------|--------|
| 150 KD | 150 KD |
| 100 KD | 100 KD |
| 75 KD | 75 KD |
| 50 KD | 50 KD |

**Figure 5**

| MDA-Mb-231 |
|-----------|
| Av ITG siRNA |
| B3 ITG siRNA |

**Figure S1**

- IP: αv ITG
- β3 ITG

**Figure S3**

Full scans of key western blots.

**Figure 6**

- MCF-7-
- MCF-7-B3
- C-MYC siRNA-
- E-Cad siRNA -

© 2012 Macmillan Publishers Limited. All rights reserved.